Changes in aminopeptidase activity in the palatal mucosa and gingiva of the rat following tooth extractions

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Huusko, P. J. Changes in aminopeptidase activity in the palatal mucosa and gingiva of the rat following tooth extractions. *Acta Odont. Scand.* 32, 269—280, 1974.

This study was carried out in order to observe the changes in aminopeptidase activity which might occur in the palatal mucosa and gingiva of the rat in the initial phase of healing after tooth extractions. The material consisted of 115 male Sprague-Dawley rats. Aminopeptidase activity was studied at time intervals of 30 min., 1, 2, 4, 8, 16 hours and 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days after the extractions. The azocoupling principle was used for the histochemical demonstration of enzyme activity. However, the incubation solution was in gel form. A semipermeable membrane was placed between the tissue sections and the incubation medium in order to prevent enzyme diffusion and dissolving of enzymes into the incubation medium. The substrates used were *N*-aminoacyl 2-naphthylamines of L-leucine and L-arginine. Histological investigations were carried out simultaneously with the histochemical study. The principal increase in aminopeptidase activity occurred relatively late after the tooth extractions. The most intense staining was observed in 4- to 7-day wounds. During the same period the most active fibroblastic proliferation was observed histologically. The changes were demonstrable using both of the substrates. However, the staining was more intense when *N*-L-leucyl-2-naphthylamine was used as the substrate. By using *N*-L-arginyl-2-naphthylamine as the substrate, chloride ions caused a marked increase in staining intensity. It was thus assumed that aminopeptidase B would also be activated during the healing.

Key-words: Tooth extraction; wound healing; aminopeptidase; histochemistry

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About 50 years ago *Euler* (1923) carried out histological studies on the healing process in tooth extraction wounds in dogs. His study may be regarded as the first in a series of systematic studies on the healing of tooth extraction wounds. After *Euler* many authors have performed histological studies on this subject by using different animals (*Meyer*, 1935; *Claflin*, 1936; *Mangos*, 1941; *Glickman, Pruzansky & Ostrach*, 1947; *Huebsch et al.*, 1952; *Pietrokowsky & Massler*, 1967; *Schüle & Ludwig*, 1968; *Todo*, 1968) as well as human material (*Amler, Johnson & Salman*, 1960). The studies carried out by the authors mentioned above support

Received for publication, February 28, 1974.

each other in their principal features. Thus the histological processes during healing in tooth extraction wounds are well known. Enzyme histochemical studies on extraction wounds are very few (*Amler, Johnson & Salman*, 1960; *Todo*, 1968; *Plagman & Lange*, 1970). However, it is well known that changes in the activity of several enzymes can be demonstrated histochemically in very early phases of healing in various tissue wounds (*Raekallio*, 1960, 1970). Most of the histological studies on extraction wound-healing have been started on the second day after the extractions, or even later than this.

This study is an attempt to demonstrate by a histochemical method those changes which possibly occur in aminopeptidase activity after tooth extractions in the palatal mucosa and gingiva of the rat in the initial phase of healing.

MATERIAL AND METHODS

The material consisted of 115 male Sprague-Dawley rats. The age of the animals at the beginning of the study was 55 days and their average weight was 212 g. The rats received normal laboratory food pellets (Hankkija, Finland). After the tooth extractions they were kept without food for 4 hours, but they did receive water ad libitum. The circumstances of the animals in the cages were standardized as far as possible.

The extractions were carried out under slight ether anaesthesia. The palatal gingiva was first loosened very carefully from all molars on the left side with a sharp instrument. The tooth was then carefully rotated with modified forceps and it was extracted with a strong vertical pull. Root fractures were uncommon and, if they were observed, the rat was discarded. The total extraction time was never more than one minute per rat. Immediately after the extractions the rats were conscious and in good condition.

The rats were killed under ether anaesthesia by cutting the aorta; in the 5-rat groups 0.5, 1, 2, 4, 8 and 16 hours after the extractions and, in the 8-rat groups 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days after the extractions. Three individuals from each of the larger groups were used exclusively for the histological study. Five rats were killed without any extractions. The reason for cutting the aorta was to diminish bleeding during the taking of the tissue specimen and bleeding was, indeed, minimal. After the killing of the rat, two slits were made with a surgical knife across the palate. One was made mesial to the first molar and theo the rdistal to the third molar. The palatal mucosa between the two slits was then removed and quickly frozen on dry ice. The tissue specimen was then frozen on the specimen holder of the cryostat (Ames, model 4552) with distilled water. Serial sections at 16 microns were cut in the direction from the extraction side to the control side. For the more accurate histological study on gingival healing in 1 to 10 day-old wounds, that half of the upper jaw where the teeth had been extracted, was removed from three rats. The specimen consisted of the alveolar sockets, a half of the hard palate and the soft tissues of these areas. The specimen were fixed for 2 days in 10 % neutral formalin, decalcified for one week in 10 % EDTA and embedded in paraffin. The sections of 7 microns were stained by using the haematoxylin-eosin and van Gieson techniques.

For the histochemical demonstration of aminopeptidase activity, a semipermeable membrane — gel combination was developed for the incubation of the sections. The principle was similar to that described

by *Meijer* (1972) for improving the histochemical demonstration of acid phosphatases. The composition of the incubation gel was modified from the method described by *Nachlas, Crawford & Seligman* (1957) and was as follows:

0.05 M phosphate buffer, pH 7.2	8 ml
Substrate, dissolved in 1 ml aq. dest.	9 mg
Fast blue B salt in 1 ml aq. dest.	36 mg
NaCl in 2 ml aq. dest. to the concentr. of	0.2 M
2 % Agar solution	12 ml

The substrates used were *N*-L-arginyl-2-naphthylamine and *N*-L-leucyl-2-naphthylamine obtained from Mann Research Laboratories Inc., New York. Fast blue B salt was obtained from Curr Ltd., London, and Agar from Difco, Detroit, U.S.A. In the present study 72 ml incubation gel was needed at every time interval.

Visking dialysis tubing (5-24/32", Medicell Internat., London) was cut into 5 × 5 cm pieces. The pieces were prepared in EDTA solution as described by *Meijer* (1972). Incubation vessels were made by cutting up lengths of glass tubing into sections 2.5 cm in length and 2 cm in diameter. The membrane piece was stretched over one end of the vessel and fastened with elastic bands. The vessels with the membranes were then placed on a moist glass plate, the membrane side placed towards the glass. The substrate and the other reagents were mixed together and the agar solution was added. The vessels were filled with the warm incubation medium and left to solidify at room temperature for 15 minutes. For each rat three vessels were prepared. The sections were mounted from the cryostat knife onto the membranes by gently but quickly pushing the membrane side of the vessels onto the sections. Before mounting the membrane was dried thoroughly. 6—8 sections were mounted on each membrane. After mounting, the vessel was immediately placed for incubation into a temperature of 32° C. The incubation time with both substrates was 1 hour. After the incubation the vessels with membranes and gels were placed into formaldehyde vapour for 15 minutes. The membranes were then loosened from the vessels and the part containing the sections was separated with scissors, washed in distilled water, kept in 0.1 M cupric sulphate solution for 2 minutes, dehydrated in alcohol, cleared in xylol and mounted on glass slides in canada balsam and overlaid with coverslips. For the histological study a portion of the sections was mounted in the cryostat on glass slides, dried at room temperature, fixed in 10 % formalin for 30 minutes and stained by using the van Gieson technique. Control sections, to check that the staining was in fact caused by diazonium salt coupled with free 2-naphthylamine, were prepared as follows: (a) with the normal gel composition after boiling in water for 15 minutes, (b) after fixation for 15 minutes in formaldehyde vapour, (c) with the gel, from which the substrate was omitted and (d) with the gel, from which Fast blue B salt was omitted. For the demonstration of the effect of chloride ions on the enzyme activity, a portion of the sections was incubated with the gel, from which NaCl was omitted. The purpose of this procedure was to discover whether *N*-L-arginyl-2-naphthylamine was specifically cloven by aminopeptidase B, which is known to be strongly activated by chloride ions (*Hopsu, Mäkinen & Glenner*, 1966).

RESULTS

Histological findings

The palatal mucosa was keratinized up to the top of the free gingiva. The epithelium

of the gingival sulcus was not keratinized, but sometimes a parakeratotic layer was observed. Immediately under the epithelium the connective tissue was dense and its fibres ran horizontally parallel to the epithelium across the whole palatal mucosa. Dense connective tissue was also found within the area of the gingival pocket bottom and towards the periosteum of the hard palate. The blood vessels and capillaries were located in looser connective tissue. Around capillaries and blood vessels cell accumulations were observed where the cells were mainly lymphocytes and fibroblasts. There were very few polymorphonuclear leucocytes. In the areas where the connective tissue was dense, fibroblasts and fibrocytes were numerous. Near the gingival sulcus and the gingival pocket bottom also mononuclear leucocytes were abundant (see Fig. 1).

In the histological study it could be seen that it was possible to perform the extractions without much damage to gingival tissue. Slight oedema could be seen in the palatal mucosa a few hours after the extractions, increasing somewhat within the first 24 hours. The first signs of increase in the number of cells were observed 4 hours after the extractions. This increase occurred around the capillaries and blood vessels in the palatal mucosa. The infiltrating cells were mainly polymorphonuclear leucocytes but an increase of lymphocytes was also noted. The number of leucocytes increased evenly in the wounds up to two days, when they seemed to be at the maximum level, and the cellular infiltration was spread over the whole palatal mucosa on the extraction side. In the 1 day-old wounds and in older ones, eosinophilic granulocytes were observed in the vicinity of the capillaries and the blood vessels in the palatal mucosa but never in the gingival tissue at the wound edge. The fibroblastic proliferation began in 1 day-old wounds and continued strongly up to the 7 day-old wounds. Five days after the extractions, fibroblasts seemed to be more numerous than leucocytes. In older wounds polymorphonuclear leucocytes were located near the wound edge, whereas fibroblasts and mononuclear leucocytes were mainly located more centrally in the palatal mucosa. The cellular infiltration can be seen in Fig. 1. The epithelial proliferation at the wound edge started on the fourth day after the extractions. The formation of new connective tissue at the wound edge was observed from the sixth day onwards. Only the most distal and mesial parts of the extraction wounds were covered by a thin epithelial cell layer in 10 day-old wounds.

Histochemical findings

The active sites in the sections stained blue or reddish blue. In rats on which no extractions were performed, the most active areas were found around the capillaries and blood vessels. They were the same areas where histologically cell accumulations were observed. There was also intense staining in the vicinity of the gingival sulcus and in the vicinity of the gingival pocket bottom. With both substrates the basal cell layer of the epithelium stained very slightly. When *N*-L-arginyl-2-naphthylamine was used as the substrate, granular staining also appeared in other layers of the epithelium, with the exception of the keratotic layer, which stained yellowish red with both substrates, but this was not attributed to enzyme activity. The staining in the sections was more intense when *N*-L-leucyl-2-naphthylamine was used as the substrate. From the

Fig. 1. The palatal mucosa and gingiva on the extraction side (1 a) 7 days after the extraction (rat No. 3). Fig. 1 b is the control side of the same section. The strong cellular infiltration is spread over the whole of the palatal mucosa on the extraction side. The most abundant cell accumulation is seen in the area where the gingival pocket bottom was located. Van Gieson-staining (195 ×).

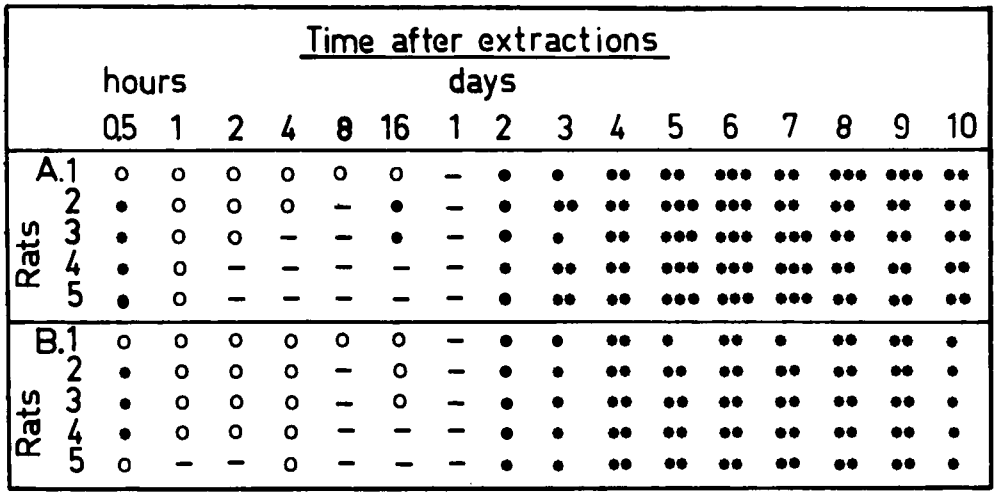


Fig. 2. A symbolic presentation of the changes in staining intensity in the palatal mucosa and gingiva of each rat, following tooth extraction at various time intervals as compared with the control side of the same animal. A. Substrate *N*-L-leucyl-2-naphthylamine. B. Substrate *N*-L-arginyl-2-naphthylamine. O = no visible difference, — = slight decrease, * = slight increase, ** = moderate increase, *** = strong increase.

control sections prepared as described in Material and Methods, groups a, c and d were negative. In the group d, only weak staining appeared. By omitting the chloride ions from the incubation gel, the staining was strongly diminished when *N*-L-arginyl-2-naphthylamine was used as the substrate. The effect of the chloride ions was only weak, when the substrate was *N*-L-leucyl-2-naphthylamine.

The changes in staining intensity on the extraction side in the palatal mucosa and gingiva at different time intervals are presented in Fig. 2. The control side of the same animal was used for comparison. The changes can be seen separately in each rat. As Fig. 2 shows, the main intensification in staining occurred from the two day-old wounds onwards. An initial increase was observed in 30 min. wounds. Two rats showed a slight increase at 16 hr., when *N*-L-leucyl-2-naphthylamine was used as the substrate. At every other time interval during the first 24 hours a slight decrease or no changes at all could be observed. A slight difference appeared between the members of the groups in the first hours of healing, but with both of the substrates, in 1 day-old wounds, in all rats a slight decrease in the activity had occurred. The slight decrease in a tissue section can be seen in Fig. 3. A strong increase in the staining intensity was observed in 5-, 6-, and 7-day wounds, when *N*-L-leucyl-2-naphthylamine was used as the substrate. A similar increase was never found with *N*-L-arginyl-2-naphthylamine; a moderate increase appeared in 4- to 9-day wounds. The increased staining in tissue sections can be seen in Figs. 4 & 5.

The sites where changes in the staining intensity were found were the same with both of the substrates. The initial increase at 30 min. was seen in the connective tissue near the gingival pocket bottom. The slight decrease which appeared during the first day of healing took place both in the

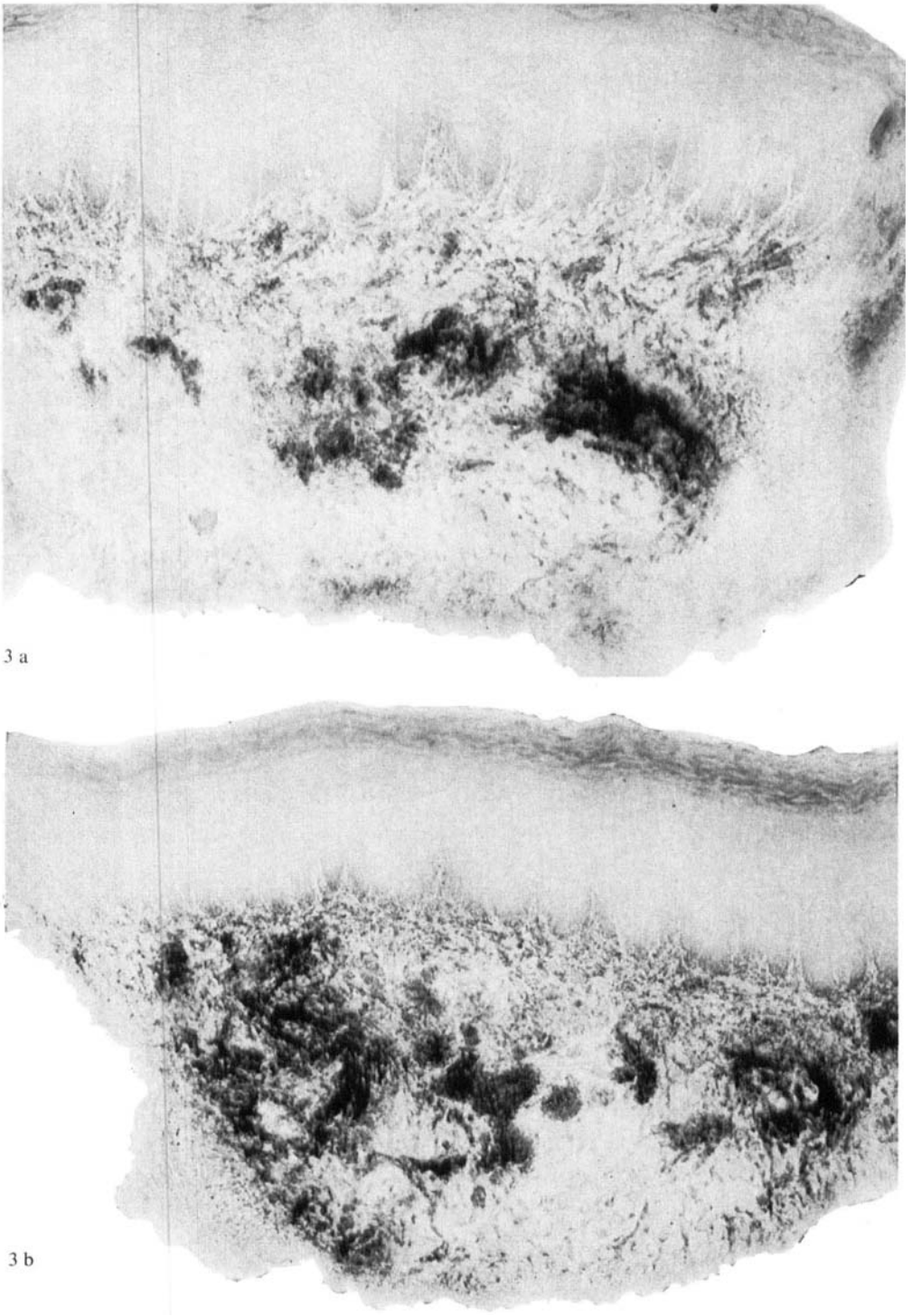
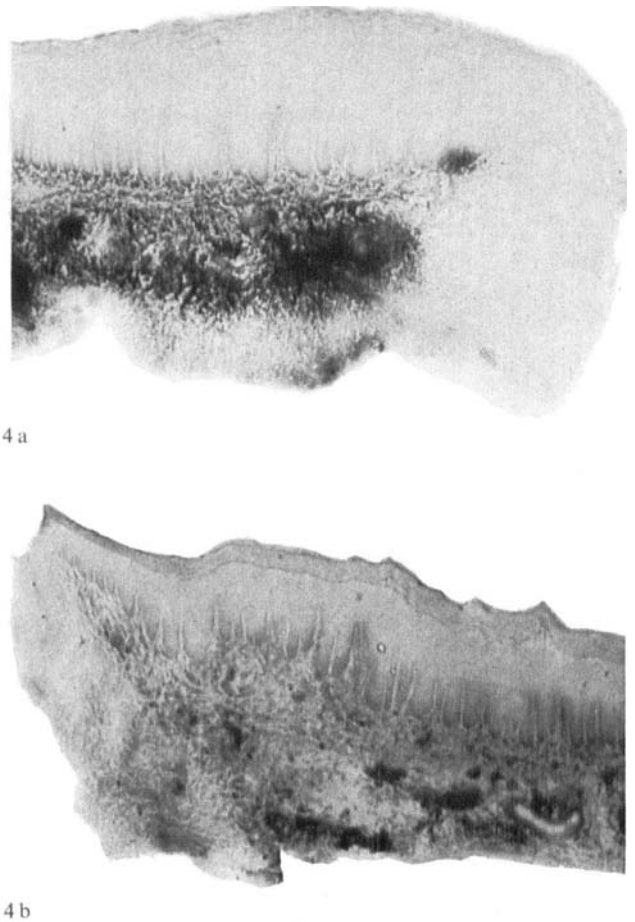


Fig. 3. Aminopeptidase activity in the palatal mucosa and gingiva of the rat on the extraction side (a), and on the control side (b), 8 hours after the extraction (rat No. 2). Slight decrease in staining on the extraction side in the vicinity of the gingival sulcus epithelium and in the area of the palatal mucosa. Substrate *N*-L-leucyl-2-naphthylamine. (195 ×)



4a

4b

Fig. 4. Aminopeptidase activity in the palatal mucosa and gingiva of the rat on the extraction side (a), and on the control side (b), 7 days after the extraction (rat No. 3). Strong increase in staining on the extraction side in the area of the palatal mucosa. Substrate *N*-L-leucyl-2-naphthylamine. (120 ×)

gingival tissue and in the palatal mucosa. The increase in the older wounds began in the tissue close to the palatal blood vessels and capillaries within the same areas where, histologically, the first signs of the increase in the number of cells were seen. In 4-day wounds and in older ones intensified staining appeared in the whole palatal mucosa on the extraction side, especially in the areas where the cellular proliferation was most abundant. The proliferating connective tissue at the wound edge stained very weakly, in spite of the abundance of polymorphonuclear leucocytes.

DISCUSSION

In previous studies, e.g. on skin wounds, leucocytic infiltration has also been found 4—6 hr. after wounding (*Raekallio*, 1961; *Ross & Benditt*, 1961). Studies with tritiated thymidine on gingival wounds (*Tonna, Stahl & Weiss*, 1969), and on tooth extraction wounds (*Todo*, 1968), have shown that the most intense labelling of fibroblasts appears in 4- to 6-day wounds. In the present study this same period of healing showed the most active phase of fibroblastic proliferation. According to *Ross* (1968), fibroblasts can be seen in skin wounds during the first

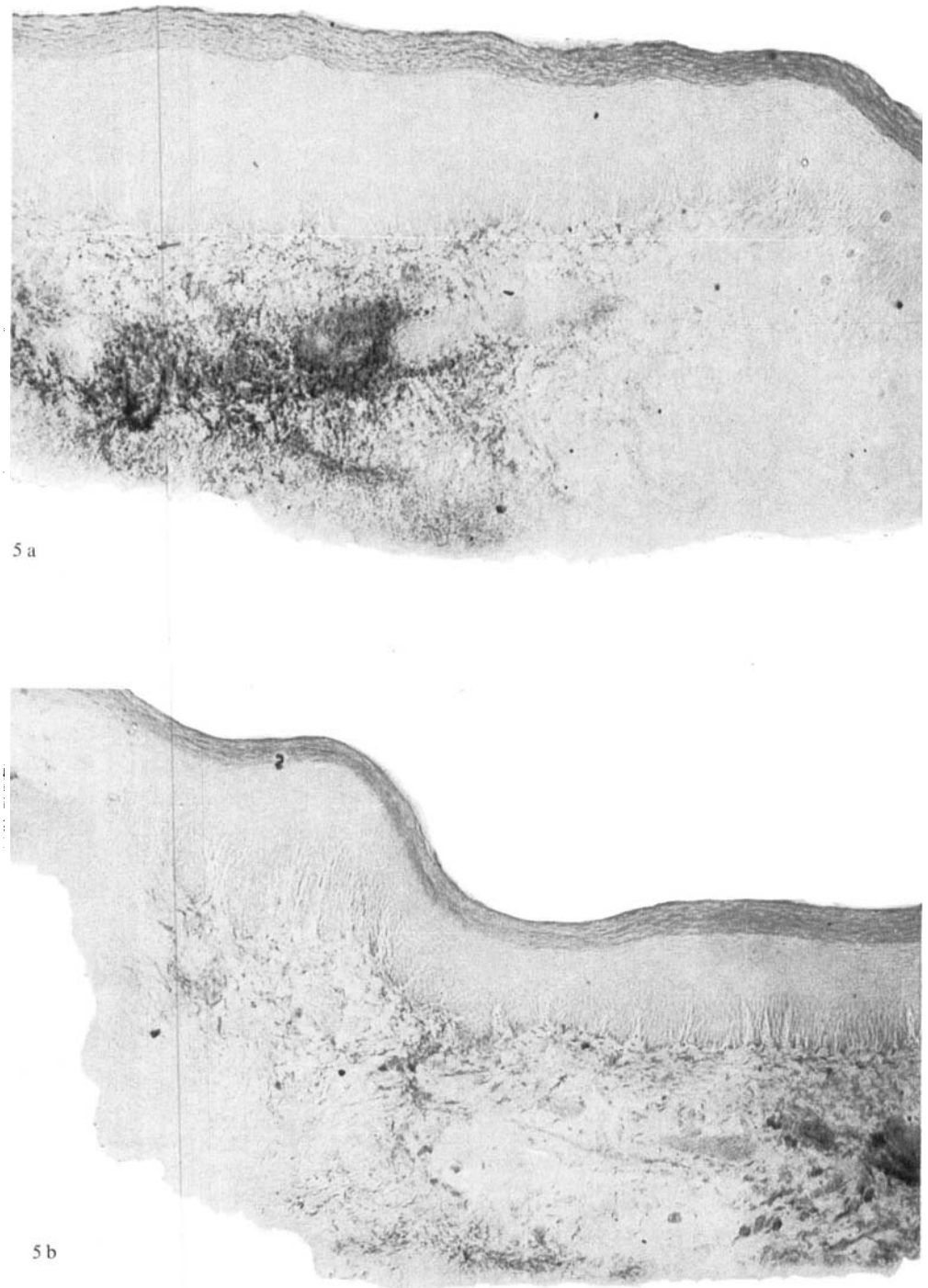


Fig. 5. Aminopeptidase activity in the palatal mucosa and gingiva of the rat on the extraction side (a), and on the control side (b), 7 days after the extraction (rat No. 3). Substrate *N*-L-arginyl-2-naphthylamine. Moderate increase in staining on the extraction side in the area of the palatal mucosa. (195 ×)

48—72 hr. In healing skin wounds *Ross* also found that the number of leucocytes reaches the maximum level within 2 or 3 days and then gradually decreases. The findings in the present study were similar as regards the area of the palatal mucosa, but at the wound edge leucocytes were still abundant in 10 day-old wounds. This was perhaps due to the fact that the healing was to a certain extent disturbed by food debris packed into the alveolar sockets.

In this study there was no intention of gauging to what extent the method used was better than the classical methods for the demonstration of aminopeptidase activity in tissue sections. However, in preliminary studies it was noted that the morphology of the sections was better preserved on the membranes than on the glass slides in the incubation solution. The histochemical picture was also more exact in detail. It is reasonable to expect that the advantages which *Meijer* (1972) has gained by developing a semipermeable membrane-gel combination for the histochemical demonstration of acid phosphatases, can also be gained by a similar method for aminopeptidases. The appropriate concentrations of the substrates and Fast blue B salt were determined in the preliminary studies.

In earlier studies aminopeptidase activity has been found in the oral epithelium and in subepithelial connective tissue. *Mori & Kishiro* (1961) observed significant activity in normal gingival and alveolar epithelium, especially in the basal cell layer. In their experiments the ground substance in the connective tissue was almost negative, while slight activity was found in fibrocytes and in infiltrating cells. Furthermore, they noted that in cronic inflamed human gingiva aminopeptidase activity increased markedly both in the ground substance and in inflammatory cells, especially in polymorphonuclear leucocytes and granulation tissue. In a comparative study of human alveolar and gingival mucosa *Kapur et al.* (1963) found aminopeptidase activity in the epithelium and in the connective tissue. They observed no difference between gingival tissue and alveolar mucosa. In the epithelium the activity appeared only in the basal cell layer. In the connective tissue activity was found in collagen fibre bundles and in the area of inflammatory cells.

Mäkinen & Paunio (1972) have demonstrated aminopeptidase B activity in human gingiva. They found activity in the basal cell layer and in the connective tissue. They also observed that the activity increased in the area of tissue injury. According to them, the mechanical injury can release aminopeptidase B into the tissue fluids at which time the enzyme would be activated by extracellular chloride ions. *Virtanen* (1973) carried out studies on enzymatic response to mechanical occlusal trauma in the gingiva of the rat. In the control sections the localization of aminopeptidases was similar to that found in the present study. He observed that in spite of no visible histological changes, an increase in aminopeptidase activity could be demonstrated histochemically 2 hours after the mechanical stress. The most pronounced staining was found at time intervals of 4—8 hours in the vicinity of the gingival pocket bottom.

Todo (1968), in his studies on healing extraction wounds in rats, found moderate activity in the connective tissue at the wound edge at an early stage of healing. In older wounds regenerating connective tissue showed no activity. Also in the present study no or only very slight activity was found in the proliferating connective tissue at the wound edge. This is a very interesting observation

because the wound edge was rich in inflammatory cells, especially in polymorphonuclear leucocytes.

If the present study is compared with those on the healing skin wounds, some discrepancies can be observed. *Raekallio* (1970), in his monograph dealing with enzyme histochemistry of wound healing, has described an increase in aminopeptidase activity in skin wounds as early as 2 hours after the injury. In addition, he has referred to the results of several other authors, according to which there is an increase in aminopeptidase activity during the first 2—4 hours. According to *Raekallio* the intensification of aminopeptidase activity in 4- to 7-hour skin wounds was principally caused by the immigrating polymorphonuclear leucocytes. In 16-hour wounds the reaction was at the maximum and very active mononuclear cells predominated. In the present study an initial increase could be seen in 30 min. wounds. This increase may have been caused by the trauma accompanying the loosening of the gingiva from the tooth. Thereafter, except for two rats in the group at 16 hours, no intensification in the staining was seen up to the 2 day-old wounds. This may be explained by two factors: firstly, there is normally an abundance of leucocytes in the palatal mucosa and gingiva. Similarly, aminopeptidase activity is moderately strong in these areas. Perhaps the intensification caused by the immigrating leucocytes is relatively so slight that it is difficult to demonstrate histochemically. Secondly, the tissue oedema may disguise the intensification. For the same reason the staining seemed still to be decreased in 1 day-old wounds. In the present study the most intense staining was found in the 5—7 day-old wounds. At this time fibroblasts were more numerous than leucocytes. The autoradiographic studies mentioned above (*Todo*, 1968; *Tonna, Stahl & Weiss*, 1969) have shown that the highest labelling of fibroblasts in gingival healing can be seen in 4—6 day-old wounds. It can thus be assumed that the fibroblasts are in their most active phase at that time interval and that the intensified staining is principally caused by aminopeptidase produced by fibroblasts. Also in skin wounds *Monis, Nachlas & Seligman* (1959) found strong aminopeptidase activity in proliferating fibroblasts. In the present study, due to the abundance of cells at the most intense stage of staining. it was impossible to establish for certain whether the activity was in leucocytes or in fibroblasts or in both.

The principal increase in aminopeptidase activity appears relatively late in the healing process after tooth extractions. The increase takes place within the area of the whole palatal mucosa on the extraction side. Thus the whole palatal mucosa on the extraction side participates in the completion of the gingival healing. The study demonstrates that aminopeptidase B is also activated in the healing process. This was confirmed by the distinct activating effect of chloride ions in the incubation gel when *N*-L-arginyl-2-naphthylamine was used as the substrate. Further studies are required for the elucidation of the origin and significance of aminopeptidases in the healing mechanism in the palatal mucosa after tooth extractions.

Acknowledgement. I am deeply grateful to Mr. Lauri Nieminen, M.Sc., for the proficient arrangements he provided in the pharmacological laboratory of Research Center / Lääke-Medipolar and to Miss Ulla Helinä Sammallahti and Mrs. Toini Tolvanen for their skilful technical assistance. I also wish to thank Mr. Jarmo Koskinen for preparing the photographs. This study was supported by grants from the Sigrid Juselius Foundation to the department of Forensic Medicine, University of Turku, Finland.

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