Aminopeptidases of mechanically strained and normal rat gingiva, with special reference to aminopeptidase B

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Aminopeptidase activity of normal and mechanically strained rat gingiva was studied using various *N*-L-aminoacyl-2-naphthylamine derivatives as substrates. Mechanical occlusal strain was directed to the first and second molar. The healing was studied 1, 3, 8 and 12 hours after the treatment. The mechanical treatment caused the disappearance of a gingival aminopeptidase which was inhibited by 0.2 M NaCl. This enzyme reappeared in the eight hour samples. All tissue samples (of the strained and control tissue) contained an enzyme so closely related to the rat liver aminopeptidase B (APB) that the presence of a true APB in rat gingiva was considered to be very likely. The activity of APB increased during the first hours, but this took place almost equally in both control and strained tissue, an indication that the strong mechanical treatment had also affected the control tissue of the test animals. The mechanical treatment used led to disturbances in the content of the studied enzymes in gingiva. The healing of the tissue, however, restored the initial situation within eight or twelve hours.

Key-words: Aminopeptidases; gingiva; tissue injury

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Aminopeptidase B (APB) of rat liver (and other closely related enzymes in other animal tissues) has been suggested to be active in certain pathological conditions only (*c.f. Mäkinen & Paunio*, 1972; *Mäkinen*, 1974). Before any further assumptions about the role of this enzyme can be provided, however, it is necessary to obtain additional proof on its involvement in pathological conditions. Recent evidence on the existence of APB in the condition mentioned includes its demonstration in crevical exudate during reduced oral hygiene (*Paunio, Mäkinen & Scheinin*, 1971, 1973) and in oral abscesses and cyst fluid (*Mäkinen & Oksala*, 1974). Other papers have dealt with related enzyme activity in human and swine dental pulp (*Mäkinen, Brummer & Scheinin*, 1970), human gingiva (*Paunio & Mäkinen*, 1970; *Larmas, Mäkinen & Paunio*, 1973; *Mäkinen & Paunio*, 1972), human periodontal ligament (*Paunio & Mäkinen*, 1969; *Mäkinen & Paunio*, 1970; *Knuuttila & Mäkinen*, 1971), etc. This paper provides evidence on an APB-like activity in the gingival tissue of the rat.

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MATERIAL AND METHODS

All the chemicals and chemical methods used in the study were the same as those

referred to in earlier papers (*Mäkinen*, 1968; *Mäkinen & Oksala*, 1974). Aminopeptidase activity was assayed using different *N*-L-aminoacyl-2-naphthylamines as substrate. The materials and methods used in the preparation of the tissue samples have also been described elsewhere (*Virtanen*, 1973). The following details, however, may be mentioned. The test animals were 6 month old female Long Evans rats weighing on average 280 g. Altogether twentyfive rats were divided into four groups according to the time of killing after causing the mechanical strain, to be described later. The healing times and number of test animals in the various groups were as follows: 1 hour (5 animals), 3 hours (5), 8 hours (5) and 12 hours (10). The doubling of the number of test animals in the 12 hour test was because enough material was obtained from these ten test animals to carry out the biochemical experiments described later. After twelve hours the aminopeptidase pattern had returned to normal values.

The mechanical stress on the tooth was created by an electronic foil condenser (Electro-Mallet, R. C. McSchirley, Glendale, Calif., U.S.A.) at the maximal intensity and frequency. The stress was applied to the occlusal surfaces of the first and second molars of the lower jaw for three minutes in each case. In each group of animals, the stress was applied to the teeth of the right half of the jaw. The left half surved as an untreated control.

After the decapitation of the test animals specimens from the gingival margin were immediately taken on the buccal side of the lower molars from the stressed area and from the contralateral side. The gingiva was detached with surgical instruments starting from the bottom of the gingival pocket. The specimens were kept on a piece of solid carbon dioxide until all the animals of the same time group had been treated (approximately 30 minutes).

The specimens in each time group were pooled and homogenized in a cooled porcelain mortar by hand using glass wool to achieve more effective homogenization. The sample and glass wool were ground for three minutes with a porcelain pestle in 0.5 ml of 0.05 M phosphate buffer, pH 7.2 at +4°C. The resulting homogenate was centrifuged for 15 minutes at 14000 rpm (23500 × g) in cold (+4° C).

In accordance with the aim of the study (to show the possible presence of an APB-like enzyme in the tissues concerned) the following separate experiments were carried out on the final supernatant solutions:

1. Determination of the specific activity of the supernatant fluid of the tissue homogenates. When the enzyme assay is carried out in the presence of 0.2 M NaCl and in the absence of added salt, the difference between the rates attained can be allotted to represent the involvement of APB (or of a closely related enzyme). True APB (the rat liver enzyme is here called true APB) is revealed by the rate-increasing effect of 0.2 M NaCl.

2. Fractionation of the supernatant fluids on Sephadex G-100 Superfine columns under standardized conditions. True APB is fractionated in a characteristic elution volume (*Mäkinen*, 1972).

3. Determination of the properties of the resulting enzyme preparations after the molecular permeation chromatography. True APB can be characterized, for example, by the following tests: mixed substrate experiment, effect of *p*-chloromercuribenzoate, determination of

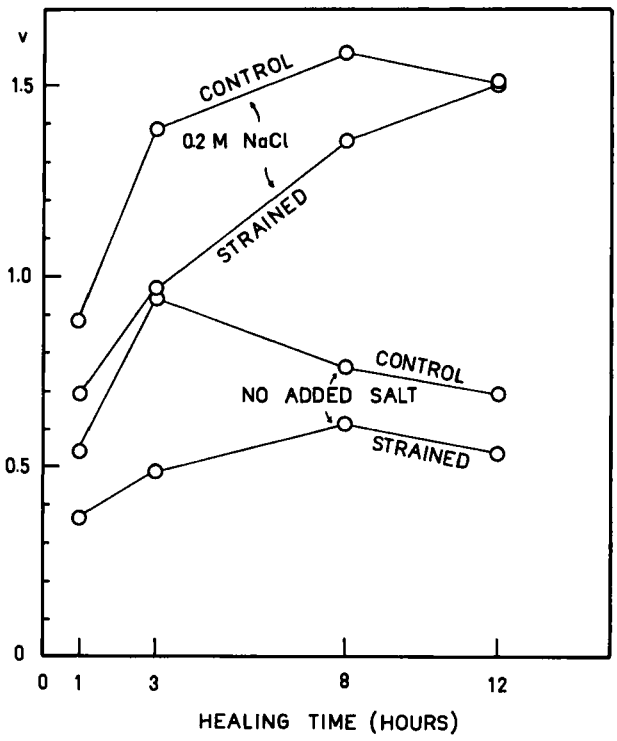

Fig. 1. A plot of the total specific aminopeptidase activity (in μmoles of liberated 2-NA per min and mg protein $\times$ 10^3) of untreated and mechanically strained gingiva against the healing time. The rate of the reaction was determined in the presence of 0.2 M NaCl and without added salt to show the possible involvement of an APB-like enzyme.

the substrate specificity of the enzyme, effect of temperature on the stability of the enzyme, and the determination of the molecular weight.

The wet weight of the tissue pools (representing the number of animals mentioned above) was approximately 0.2 g (five test animals) or 0.4 g (10 animals). Fig. 3 gives the exact weight of the samples.

RESULTS

1. *Total specific activity of rat gingival aminopeptidases*

Fig. 1. shows the specific activity of rat gingival aminopeptidases acting on *N*-L-arginyl-2-naphthylamine in the presence of 0.2 M NaCl and in the absence of added salt. These results were obtained with the mechanically strained and control tissue. One of the aims of this experiment was to show that the presence of 0.2 M NaCl in the reaction mixture leads to a higher rate of the hydrolysis of the substrate used. The figure shows that NaCl had caused a strong rate-increment in the hydrolysis between the first and the eighth hour after the treatment of the tissue (unpublished results indicate that in a 16 hour test the increment was less). The first tissue samples were removed 1 hour after the treatment. Fig. 1 also shows that, somewhat unexpectedly, the specific activities also changed as a function of time in the control side as well.

Fig. 2 shows the results of Fig. 1 expressed as differences of the specific activities between the strained and control tissue. The zero level on the y-axis represents the activity of the control. It is evident that there was an initial drop in the

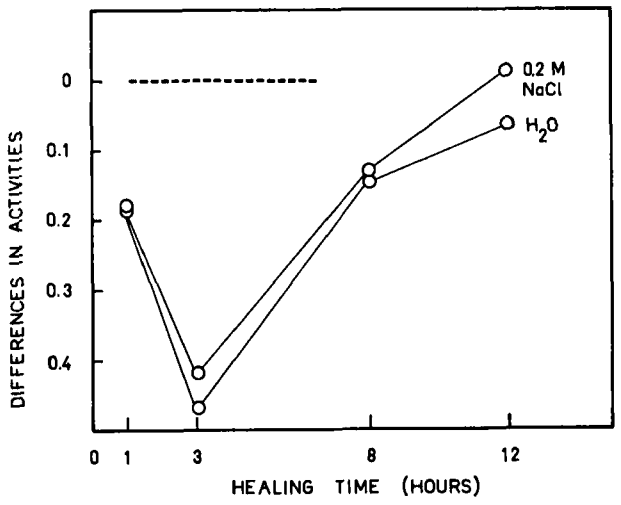

Fig. 2. The relationship of the specific activity of the injured tissue to that of untreated control tissue (the zero line) during the healing of the gingiva. The differences in activities were obtained by substracting the specific activities of the treated samples from those of the control samples. The effect of 0.2 M NaCl is also indicated.

specific activity when compared to the control. At the 12th hour the activities reached the normal (control tissue) level. The rate-increasing effect of NaCl is also to be seen in Fig. 2.

When studying arylaminopeptidase activity in the tissues used in the present investigation, it was possible to assume that related enzymes derived from serum or blood cells would affect the results, and that specific injured (or wound) tissue aminopeptidases would not be involved. These enzyme sources may confuse the treatment of the results in spite of the use of control tissue. Therefore, the amount of hemoglobin in the supernatant fluids of the gingival homogenates was determined in all test groups and the results were compared with 1) the fresh weight of the tissue samples and 2) the changes in the specific enzyme activity of the supernatant fluids. The determination of hemoglobin (spectrophotometrically at 430 nm) was considered to reflect the contribution of blood to the tissue samples.

Results in Fig. 3 show that, excluding the first hour samples, the amount of hemoglobin and the fresh weight of the

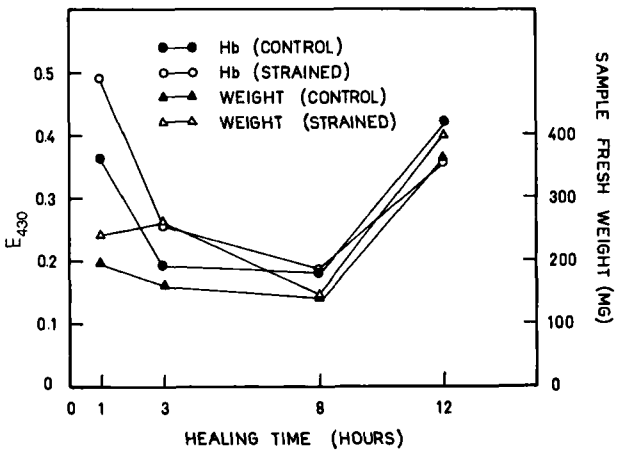

Fig. 3. The relationship between the fresh weight of the samples and the relative content of hemoglobin in the supernatant fluid of the tissue homogenates.

samples were correlated. However, it was precisely at the first and third hour when the strongest drop in the comparative specific aminopeptidase activity had taken place (Fig. 2). The amount of hemoglobin in the eight hour samples was approximately the same as in the third hour samples, but the enzyme activity was, regardless of this, clearly increased eight hours after causing the injury. This was considered to lend support to the idea of the involvement of specific injured tissue aminopeptidases in the present study.

2. *Molecular permeation chromatography*

The determination of the specific activity in the presence and absence of sodium chloride does not alone provide sufficient evidence on the involvement of APB or other closely related enzymes in the tissues studied. True rat liver APB is fractionated on Sephadex G-100 Superfine gel in a characteristic way. Fig. 4 shows a series of chromatograms representing molecular permeation chromatography of the gingival tissue samples. All chromatograms displayed heterogeneity. The following comments can be made:

— In the mechanically strained samples, representing the first and third hour, there was no clear arginine aminopeptidase peak in fractions 290—310. In the eight hour sample this peak was revealed. This peak is, however, to be seen in chromatograms of the control tissue. In contrast to aminopeptidase B it was inhibited by NaCl. This enzyme preparation was called Pool I.

— 0.2 M NaCl had caused the appearance of an APB-like enzyme most clearly in the 12th hour samples (as evidenced by the higher enzyme activity in fractions 320—340, when 0.2 M NaCl was added into the reaction mixture). The active fractions were pooled (Pool II was obtained).

— Eight hours after the treatment of the tissue, according to the chromatograms, the situation was nearly normalized as indicated by the similar chromatographic pattern. Molecular weight determinations on Sephadex G-100 Superfine gel gave the following results: Pool I, 95000; Pool II (the APB-like enzyme), 65000; Pool III, 57500. This estimate was based on the use of following proteins as reference: myoglobin, chymotrypsinogen, ovalbumin, serum albumin and γ-globulin.

3. *Characterization of the enzymes*

In order to show the possible involvement of an APB-like enzyme in the tissues studied, the active fractions indicated in the chromatograms of Fig. 4 were pooled and tested as shown below.

The effect of sodium chloride and potassium fluoride on the rate of the hydrolysis of *N*-L-arginyl-2-naphthylamine, catalyzed by enzyme pools representing the third and eighth hour of the experiment, is shown in Fig. 5. With enzyme pools II and III all the curves of rate versus the concentration of sodium chloride were of exactly the same appearance as usually obtained with true APB. Enzyme pool I behaved constantly in the opposite way: 0.2 M NaCl inhibited the reaction. The Fig. also shows that fluoride ions did not affect the rate of the hydrolysis to any noticeable extent; there was a low maximum and at lower fluoride concentrations the rate was gradually reduced. This result is exactly as earlier obtained with true APB.

The effect of *p*-chloromercuribenzoate was studied with Pools I (inhibited by

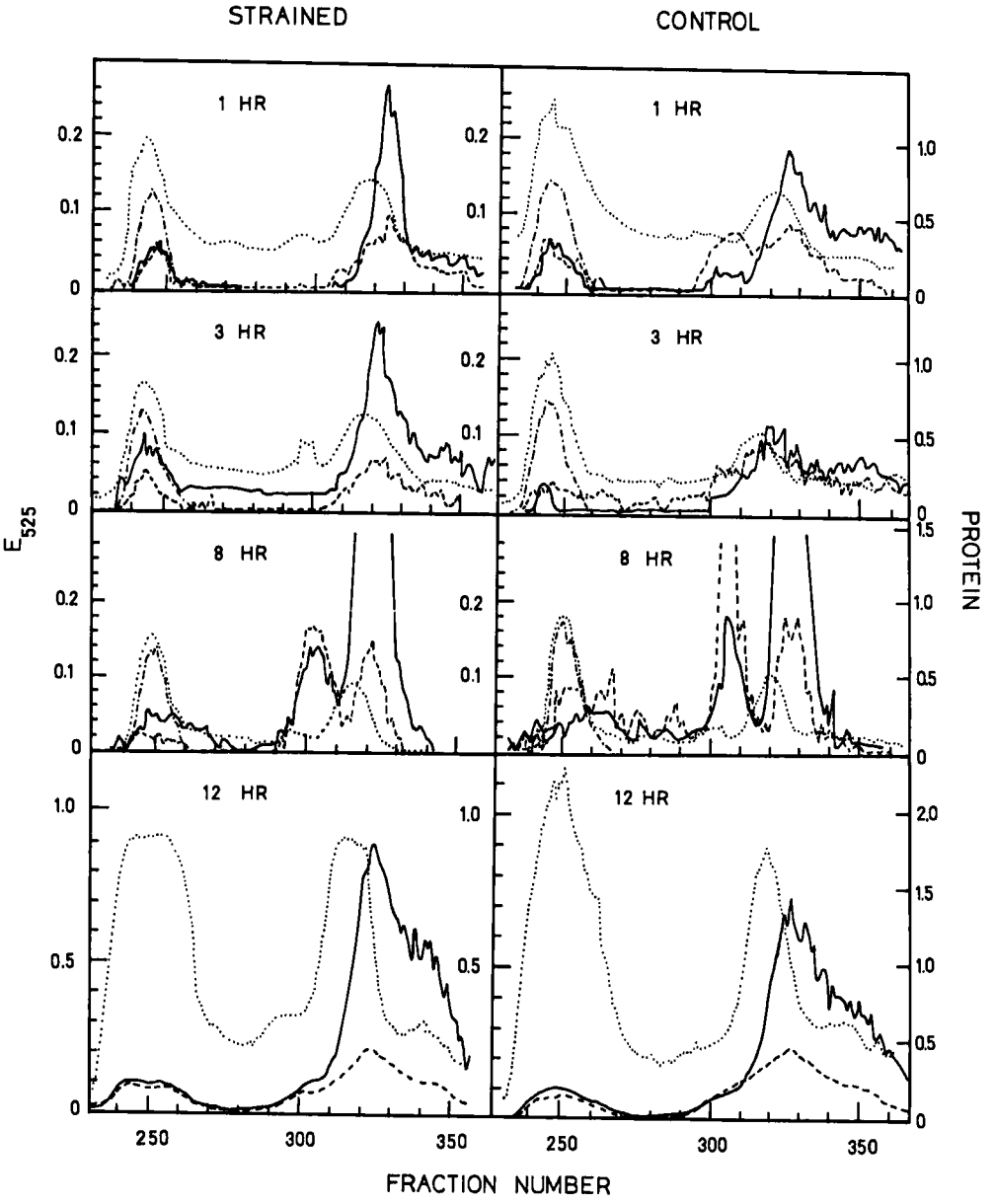

Fig. 4. Molecular permeation chromatography of the supernatant fluids of gingival homogenates representing untreated and mechanically strained tissues. Columns: Sephadex G-100 Superfine, 1,8 × 140 cm; elution: 0.01 M β,β-dimethylglutarate buffer, pH 7.2; flow rate: 0.1 ml/min.; temperature +2°C; samples: 1.2 ml of the above mentioned supernatant fluids (in the 12 hour experiment 1.9 ml was applied), mixed with 0.25 ml Blue Dextran. ——, enzyme activity (E_{525}) against *N*-L-arginyl-2-naphthylamine, tested in the presence of 0.2 M NaCl; - - - -, as above, but tested without added salt; . . . ., protein (E_{500}; using the Folin-Ciocalteu reagens). Pool I = fractions 290—310; Pool II = fractions 320—340; Pool III = fractions 341—360. The left side chromatograms of the 1st and 3rd hour samples show the disappearance of Pool I. The eight hour's chromatogram shows the appearance of it. Enzyme peaks in the twelve hour chromatograms were overlapping due to application of a greater sample volume than in other cases.

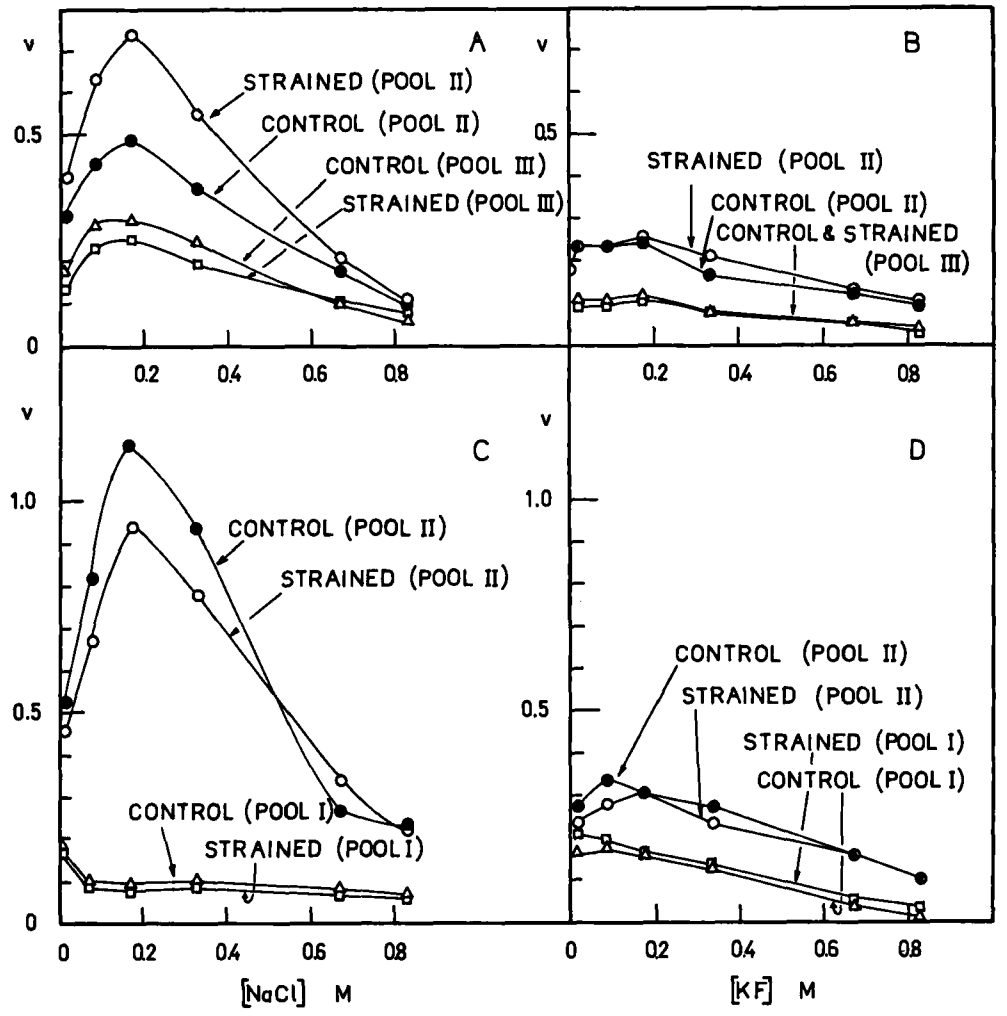

Fig. 5. The effect of sodium chloride and potassium fluoride on the rate of the hydrolysis of *N*-L-arginyl-2-naphthylamine, catalyzed by different enzyme pools of the third (A, B) and eighth (C, D) hour samples. Samples obtained from other time groups behavied in a similar way.

0.2 M NaCl) and Pool II (the APB-like enzyme, being activated by 0.2 M NaCl). The results are shown in Table I. The experiments were carried out with three different substrates (the *N*-L-aminoacyl-2-naphthylamines of arginine, alanine and methionine), of which the arginine derivative has been considered a substrate for true APB (in addition to the lysine derivative). The enzyme pools indicated in Table I did not represent thoroughly purified preparations. In spite of this, it is evident that Pool II represented an APB-like enzyme, whereas Pool I contained a different peptidase. True APB has been shown to be very sensitive to *p*-chloromercuribenzoate and this was also shown to be the case for the gingival enzyme. Pool I was not so strongly inhibited by this reagent. Pool III, which it was possible to separate in fractionations representing the third hour, behaved like true

Table I. *Inhibition by p-chloromercuribenzoate of the hydrolysis of three N-L-aminoacyl-2-naphthylamines (-2-NA), catalyzed by enzyme preparations obtained after eight hour healing. The results are given in percentages of inhibition. Pool II was considered to represent an APB-like enzyme*

		Alanyl-2-NA	Arginyl-2-NA	Methionyl-2-NA
Strained	Pool I	56.5	30.2	54.5
	Pool II	9.1	91.0	75.0
Control	Pool I	61.0	20.9	41.0
	Pool II	60.0	93.0	*

* Pool II of the control tissue did not hydrolyze this substrate at a measurable rate

APB, an indication that different molecular forms of APB were revealed in Pool III, or, more likely, that Pool III represented a peptidase with low activity and which was strongly contaminated by Pool II (true APB-like enzyme). The enzyme preparations obtained from different time groups behaved in similar way in the presence of *p*-chloromercuribenzoate, although only the results of the 8th hour experiment are shown.

In some chromatograms the above mentioned enzyme pools were to a certain extent overlapped by each other. It was, therefore, necessary to show that even in these cases the first active fractions (active towards the arginine derivative) represented an enzyme clearly different from true APB, and that Pool II really contained an APB-like enzyme. This was shown to be true when studying the effect of temperature on the stability of the preparations, as well as by carrying out mixed substrate experiments with the enzyme preparations involved. Earlier results have shown that true APB is a thermolabile enzyme. When Pools I and II were kept at 30, 50 and 65°C for 15 minutes, the activity of Pool II towards *N*-L-arginyl-2-naphthylamine was after treatment at 50° C only 18 % of that obtained at 30° C. At 65° C the activity was totally destroyed. Consequently, Pool II resembled in this sense true APB. The activity of Pool I was not noticeably reduced in this experiment.

DISCUSSION

It has been possible to show in several earlier papers the rate-enhancing effect of NaCl in the hydrolysis on *N*-L-arginyl-2-naphthylamine (for example, *Mäkinen*, 1969, 1974). This rate increment has been usually attributed to the involvement of APB. However, when examining the results of Fig. 1, it is evident that even at zero time-lapse there was a slight difference between the rates of the hydrolysis in favour of the presence of NaCl in the reaction mixture. This situation has been shown to be true in other experiments in this laboratory: the presence of NaCl always causes the reaction to proceed slightly more rapidly (although no tissue injury would be involved).

The results of the present paper also showed that rat gingival tissue contains an aminopeptidase identical or very closely related to true APB (i.e. the liver enzyme). This suggestion was based on results obtained with sodium chloride, *p*-chloromercuribenzoate, and potassium fluoride. The behaviour of rat liver APB in the presence of the above reagents and gel filtration has been described in earlier papers (*Mäkinen*, 1968, 1969, 1974).

The results also showed that the change in the specific aminopeptidase activity of the supernatant fluids of the tissue homogenates was actually due to a response of gingival enzymes to the

mechanical injury and was not dependent on a possible contribution of serum or blood cell enzymes to the results obtained. This view is also supported by chromatograms described earlier (Fig. 4), where it is evident that tissue injury caused the disappearance of a particular aminopeptidase activity in the chromatograms. This is in good agreement with results earlier obtained by *Raekallio* and *Mäkinen* (1967, 1969, 1971), who showed that wound tissue arylaminopeptidases (the term used by the authors for enzymes acting on *N*-L-aminoacyl-2-naphthylamines) were separate enzymes from those of serum, erythrocytes and leucocytes.

The finding that the specific activities also changed as a function of time in the control side as well, was considered to be due to the fact that the application of the mechanical strain also affected tissues elsewhere in the lower jaw. Preliminary experiments had shown that the treatment of the upper jaws was lethal to the animals.

Two kinds of aminopeptidase activity have to be considered in the present case: the increase in the absolute specific activity and the decrease in the comparative specific activity, as a function of healing time. Absolute specific activity (shown in Fig. 1) gives the rate of the reaction in liberated μmoles of 2-NA (min $\times$ mg protein),* whereas the comparative activity determines the difference in the absolute specific activities between the treated and untreated tissue (Fig. 2). The decrease in the comparative specific activity in the first and third hour samples was most likely (or in part) due to the disappearance of a particular aminopeptidase in the tissue [the very enzyme which is inhibited by 0.2 M NaCl (Pool I)]. Fig. 2 demonstrates the decrease of the activity and Fig. 4 shows the likely reason for this.

An alternative explanation for the disappearance of Peak I in the first and third hour samples could be a possible inhibitory effect of the extracellular sodium chloride on the enzyme involved. A sufficient amount of salt could diffuse into the injured tissue during the mechanical strain.

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* 2-NA = 2-naphthylamine

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