From:
The Institute of Dentistry,
University of Turku,
Finland

STUDIES ON HYDROLYTIC ENZYME ACTIVITY IN THE CONNECTIVE TISSUE OF THE HUMAN PERIODONTAL LIGAMENT

OBSERVATIONS APART FROM AREAS OF INFLAMMATION

by

K. U. PAUNIO

K. K. MÄKINEN

INTRODUCTION

There are only few reports in the literature on the biochemistry of connective tissue apart from the inflammatory focus but still under its chemical influence. *Fullmer* (1961) showed that the collagen fibres away from the inflammatory focus in the periodontium were altered, and two different layers could be observed: a degradative layer and a zone of repair. The material collected for the present study has been tested biochemically with the following results:

1) The structure of the collagen was altered in the diseased material (*Paunio*, 1965, 1966). The contraction temperature of collagen was thus lower in the effected material than in the control material. The solubility of the collagen in the diseased material was increased as compared with that of the control material.

2) The content of DNA, RNA and chondroitin sulphates was also increased in the diseased material as compared with control material (*Paunio*, 1969).

This paper will provide information about the existence in human peroidontal ligament of hydrolytic enzymes which are able to alter the structure of collagen fibres isolated from the same tissue. The main task was to study

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enzyme activities of the diseased periodontal ligament, but the same experiments were carried out simultaneously on normal or »healthy» material used as a control. Similar enzyme activities have already been described, for example, by *Gross* and his coworkers in tadpole experiments *Gross & Lapiere*, 1962; *Lapiere & Gross*, 1962) *Nagai, Lapiere & Gross*, 1964; (*Nagai*, 1965; although these studies did not include investigating the effect created by the proximity of the inflammatory focus.

MATERIALS AND METHODS

All of the enzyme substrates were obtained from the same sources as mentioned earlier (*Mäkinen*, 1966 a, b).

ENZYME PREPARATIONS

For each series of experiments two pools, representing control tissue and diseased periodontal ligament, were collected. The material was obtained from human teeth extracted at the Department of Oral Surgery. The pools were, however, equivalent with regard to the age and sex distribution. The teeth were placed immediately after the extraction to a temperature of —20°C for not more than 21 days. The teeth were thereafter divided into two groups representing diseased or control tissue. The dividing was made on basis of the location of the epithelial attachment. If the epithelial attachment of the tooth was located apically from the cementoenamel junction, the material was classified as diseased. Only distinct cases were accepted. The teeth with normal epithelial attachment were used as control material (in these cases the attachment was located at its normal place). Even here only distinct cases were accepted. Teeth with severe migration of the epithelial attachment down to being closer to the apex than to the cementoenamel junction were discarded, as well as teeth with periapical lesions. The periodontal ligament on the surfaces of the tooth was scaled from the middle region of the ligament. The material near the epithelial attachment and the apical part of the root were carefully avoided. In order to avoid also the inclusion of inorganic material the periodontal ligament was removed as gently as possible. The collection was carried out at room temperature (20°C).

Approximately 2 mg of ligament material could be obtained from every root surface. The collected fibrous material was freeze-dried and exactly the same amount of both kinds of dry material was weighed for further treatment. This procedure gave very finely divided material. All together, eight separate pools were prepared, four from diseased material and four from

control material. In total, over 1000 teeth were used in the experiments. The pools consisted in the first case of 150 mg each of control and diseased dry ligament material (obtained from about 40 persons with »healthy» and 40 persons with diseased periodontal ligament), in the second case of 900 mg (obtained from about 100+100 persons), and in the third and fourth cases of 80 mg (obtained from about 20+20 persons). Consequently, 100 mg dry ligament material corresponds to about 40 teeth. 2 ml of cold water (4°) per 150 mg of freeze-dried material was added to the pools, and the mixtures were shaken in test tubes for 2 hr at 1°. After centrifuging in rotor SS-34 of the Sorvall Superspeed RC-2B centrifuge (this rotor and centrifuge was used throughout the work) at 10 000 rev/min (12 100 g) for 10 min at 4° (throughout the work the centrifugal forces quoted are average). The supernatant solutions were used as enzyme preparations in the determination of the collagenase-like enzyme activity. The four enzyme solutions from the diseased material contained constantly 3.0, 3.5, 3.1 and 3.9 mg protein per ml as estimated by the Folin Ciocalteu method (*Layne*, 1963). The corresponding values for the enzyme preparations of the control material were 2.4, 2.8, 2.5 and 3.0 mg/ml, respectively. In each case the protein concentration was about 25 % higher in the diseased material, although the same amount of dried control and diseased tissue was originally weighed. The latter therefore contained more water soluble proteins.

Attention was paid to handling both kind of tissue samples exactly in the same way. The obtained crude enzyme solutions were diluted 15-fold with cold (about 4°) water just before use. Consequently, the preparing of the enzyme preparations involved only conducting an extraction with water, followed by centrifugation.

Estimation of aminopeptidase and esterase activity

The hydrolysis of the derivatives of 2-naphthylamide and 1- and 2-naphthols was estimated as described elsewhere (*Mäkinen*, 1966 a; 1969).

Estimation of collagenase-like activity

In order to elucidate the ability of enzyme extracts from both the control material and the diseased periodontal ligament to degradate insoluble collagen from the same periodontal membrane, as well as to study the size of the liberated hydroxyproline containing products of reactions, the following procedures were carried out. Insoluble collagen from periodontal ligament was collected from extracted teeth representing »healthy» or control material.

The amount of collagen used in these experiments was obtained from about 250 extracted teeth, taken equally from healthy and diseased connective tissue. The teeth were stored for 3 weeks at —20° before preparation. They were first washed by shaking in 50 ml erlenmeyer flasks with cold 0.5 M EDTA solution (pH was adjusted to 7.0 with KOH) for 3 hr at 1° in order to help the later detaching of the periodontal membrane. The shaking was carried out using the Edmund Bühler shaker, Typ Sm2 at 130 rev/min (Tübingen, Germany). The material was erased carefully avoiding mixing of the periodontal membrane with the material from the apical and coronary area of the teeth.

The detached bulk was homogenized for 60 sec. in 4 ml of cold (4°) 0.5 M acetic acid using the Ultra-Turrax top drive homogenizer. The resultant mixture was then agitated by shaking for 15 hr at 1° (as above). The pellet obtained after centrifuging at 14 000 rev/min (23 500 g) for 15 min at 4° consisted of so-called insoluble collagen (that is insoluble in 0.5 M acetic acid) and was washed three times with cold (4°) water in the centrifuge tube. The purity of this collagen was tested by estimating the hydroxyproline content of the preparate. According to this the obtained material was constantly over 90 % pure collagen.

200 mg (wet weight) of this insoluble collagen from the bottom of the centrifuge tube was incubated with 0.5 ml of the enzyme solution (undiluted) from the control tissue or diseased periodontal ligament in 4.5 ml of 0.1 M tris-HCl buffer (pH 7.0) for 20 hr at 35°. The reactions were carried out in 10 ml centrifuge tubes of glass sealed with Parafilm. The enzyme reactions were not stopped by any special reagent, but the mixtures were rapidly centrifuged at 10 000 rev/min (12 100 g) for 10 min at 4°. The sediment was discarded and the supernatant fluid was immediately freeze-dried. This was done to reduce the volume of the solution. The remaining material was dissolved in 0.7 ml of 0.1 M tris-HCl buffer (pH 7.0). 0.5 ml of this solution, now containing products of the possible breakdown of collagen fibres or molecules of the periodontal ligament, was fractionated through Sephadex® G-10 and G-200 columns. The instructions of the manufacturer of the gels (Pharmacia, Uppsala, Sweden) were followed in packing, elution and handling. The following bland mixtures were also prepared and passed through the same columns:

1. Enzyme preparations from diseased (0.5 ml) and control (0.5 ml) material together in 0.1 M tris-HCl buffer (4.0 ml, pH 7.0). This mixture was incubated and centrifuged as above. 2. The same mixture as above, but this time not incubated. 3. Substrate blank: this was prepared by suspending the 200 mg of wet collagen in 5.0 ml of 0.1 M tris-HCl buffer (pH 7.0). In

each reaction mixture the volume was thus 5.0 ml. The volume of the 200 mg of collagen was neglected. All of the mixtures were then incubated, centrifuged and freeze-dried as above. Great attention was paid in preparing, fractionating and general handling of the samples in exactly the same way. The same Sephadex G-200 and Sephadex G-10 columns were used throughout for every filtration, washing the gel between different samples thoroughly wit the elution buffer. The fractions from the gel filtrations were analyzed for their protein concentration. The fractions from all filtrations were then pooled to form two different solutions as given in Results. The solutions were freeze-dried. The remaining material was dissolved in 1.5 ml water and analyzed for hydroxyproline content. The separate fractions from the gel filtrations were separated into two pools because the hydroxyproline content of the fractions was too low to permit any reliable determination.

Estimation of hydroxyproline

The concentration of hydroxyproline was estimated by the method of *Stegeman* (1958) and *Woessner* (1961). Pig-skin collagen donated by Dr. Eastoe was used as a hydroxyproline standard.

RESULTS

1. *Hydrolysis of the derivatives of 2-naphthylamine and 1- and 2-naphthol*

The results are given in Table I only for pH 7.0 where the rates of hydrolysis were calculated as liberated μmoles of 2-naphthylamide or 1- and 2-naphthol per min and per mg protein. Results were of quite the same nature at each pH value studied. As to the naphthylamides, the substrates generally most rapidly hydrolyzed were the 2-naphthylamides of alanine, arginine, leucine, lysine and methionine. The most noticeable result was the much higher enzyme activity of the control material as compared with the diseased periodontal membrane. The differences between the two preparations were very clear when the enzyme activities were calculated as for Table I, but a significant difference (about 20—30 %) was also seen when the enzyme activities were calculated versus the original weight of the dry lyophilized material and not per mg of enzyme protein in the reaction mixture as in Table I. All these results were most easily seen at pH 7.0 and 8.0, where the activities were higher and almost equal. At pH 5.8, where the activities were lower, these differences were not so easily detectable. Of the derivatives of 1- and 2-naphthol, those of acetic acid were hydrolyzed most quickly by both enzyme preparations (from diseased and control material) at all

Table I.

Ability of the crude enzyme preparations from diseased and healthy periodontal membrane to hydrolyze 2-naphthylamides (2-NA) of various amino acids and derivates of 1- and 2-naphthol in 0.025 M phosphate buffer (pH 7.0). In the case of 2-naphthylamides the rate of hydrolysis is given in liberated μmoles of 2-naphthylamine per min and per mg protein ($\times 10^3$). In the case of the derivates of the naphthols the hydrolysis is given in liberated μmoles of the corresponding naphthol per min and per mg protein ($\times 10^3$). Table gives mean values from four experiments. Substrate concentration: 0.166×10^{-3} M. The zeros mean that no measurable hydrolysis of the substrates involved was observed under the conditions employed.

Substrate	Rate of hydrolysis	
	Diseased	Control
L-Alanyl-2-NA	7.2	10.2
L-Arginyl-2-NA	6.6	9.0
L-Asparagyl-2-NA	0	0
L-α-Aspartyl-2-NA	0	0
L-β-Aspartyl-2-NA	0	0
L-Cystine-di-2-NA	0	0
L-γ-Glutamyl-2-NA	0.42	0.60
Glycyl-2-NA	0.60	0.90
L-Histidyl-2-NA	0.60	0.90
L-Hydroxyprolyl-2-NA	0	0
L-Isoleucyl-2-NA	0.60	0.90
L-Leucyl-2-NA	6.0	9.0
L-Lysyl-2-NA	7.2	10.2
L-Methionyl-2-NA	11.4	15.4
N-α-Benzoyl-DL-arginyl-2-NA	0.30	0.60
L-Ornityl-2-NA	0.9	1.5
L-Phenylalanyl-2-NA	5.4	7.2
L-Prolyl-2-NA	0.18	0.30
L-Seryl-2-NA	0	0
L-Threonyl-2-NA	0	0.18
L-N-α-Tosyl-L-arginyl-2-NA	0	0.12
L-Tryptophyl-2-NA	0	0.12
L-Tyrocyl-2-NA	0	0.18
L-Valyl-2-NA	0.6	0.90
6-Bromo-2-naphthyl-α-D-glucoside	0	0
6-Bromo-2-naphthyl-β-D-glucuronide	0.36	0.54
6-Bromo-2-naphthyl carbonaphthoxycholine iodide	0.36	0.54
6-Bromo-2-naphthyl sulphate (potassium salt)	0.48	0.66
1-Naphthyl phosphate (calcium salt)	1.80	2.7
1-Naphthyl phosphate (sodium salt)	4.7	6.6
1-Naphthyl acetate	3.6	4.8
2-Naphthyl acetate	3.6	4.8
1-Naphthyl propionate	3.0	3.5
1-Naphthyl valerate	1.2	1.8
1-Naphthyl laurate	0.48	0.60
2-Naphthyl laurate	0.24	0.36
2-Naphthyl palmitate	0.24	0.28
1-Naphthyl myristate	0.12	0.24

pH values tested. At pH 7.0 and 8.0 the breakdown of these two esters was nearly the same. The lowest activity was obtained with insoluble substrates. 1-naphthyl phosphate and 1-naphthol derivatives of propionic, n-butyric and n-valeric acids were hydrolyzed by the healthy control preparation from 10 to 50 % more rapidly than by the diseased enzyme preparation. At pH 5.8 the enzyme activities were low, as they were with 2-naphthylamides. As seen in Table I, many of the substrates were hydrolyzed at all by either enzyme preparation. Fig. 1 gives some of the results in the form of progress curves of the hydrolysis. The higher proteolytic enzyme activity of the control material is clearly seen.

When it was observed that the enzyme preparations contained very small amounts of blood which could in part or *in toto* be responsible for the hydrolytic activity, the four series of experiments in this investigation were planned to consider this possibility. In the preparation of the third and fourth pairs of enzyme solutions, the teeth were washed quite thoroughly for removing all of the traces of blood, unlike the preparation of the first two pairs. These solutions therefore contained very little, if any, of contaiminating blood proteins, and the washing was conducted purposely, even at the expense of losing some periodontal protein. No differences were, however, noted in the rates of hydrolysis caused by the compared preparations. Therefore, the higher enzyme activity in »healthy» preparations cannot be due to the traces of blood often present in them. The enzyme blank of the »healthy» preparation (not thoroughly washed) had constantly an extinction of 0.020—0.30, and the enzyme blank from the »diseased» material an extinction of 0.005—0.015. The more thoroughly washed teeth gave enzyme preparations which gave no extinction. Consequently, the observed differences in activity are indeed due to the qualitatively or quantitatively different enzyme content of the connective tissue cells.

It was noted that the protein concentration of the extracts of diseased periodontal membrane was constantly from 20 to 30 % higher than in the extracts from control membrane material. Inasmuch as the extractions with water were conducted precisely in the same way each time, the results clearly show that diseased connective tissue contained higher amounts of water soluble protein than did control tissue. Nevertheless, the enzyme activities studied were clearly higher in the control material. This indicates that inflammatory processes remote from the connective tissue studied cause alterations in its enzyme pattern.

2. *Fractionation of reaction products*

Fig. 2 gives the results from the Sephadex G-200 and G-10 filtrations with

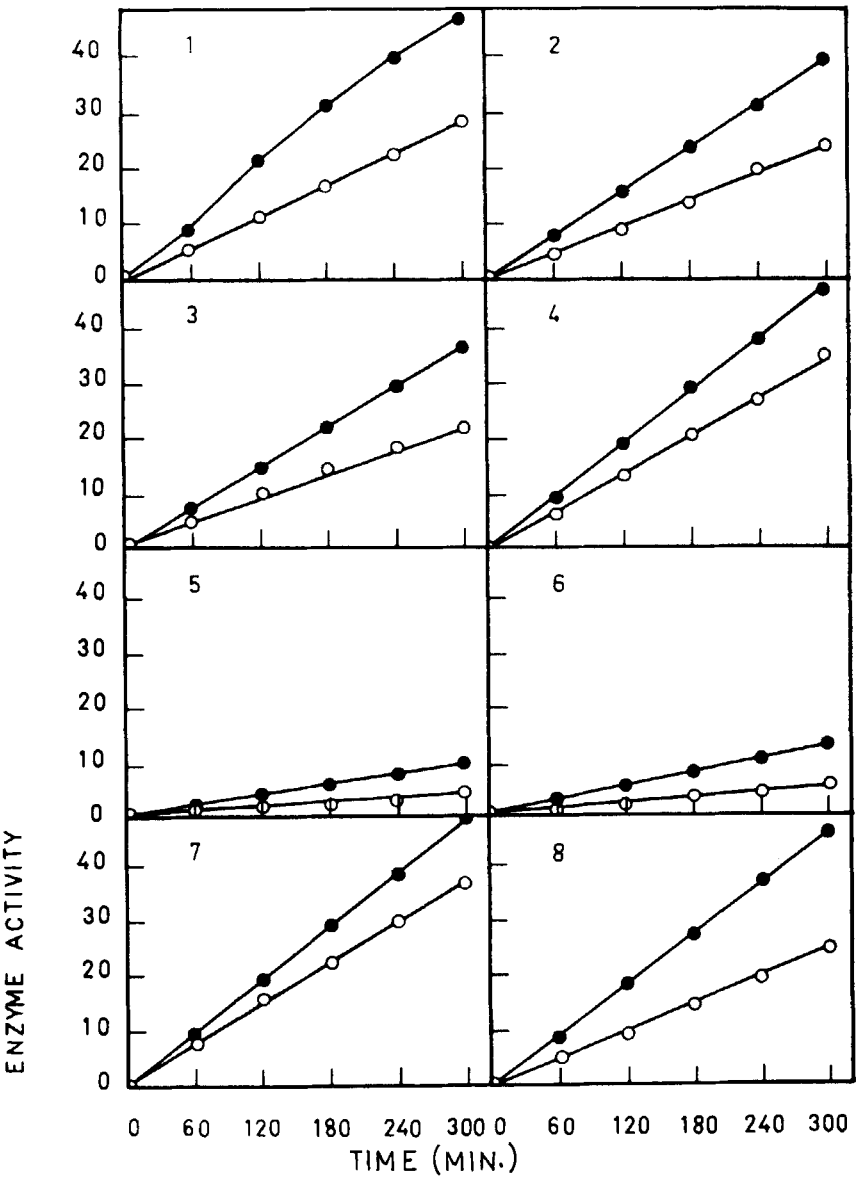


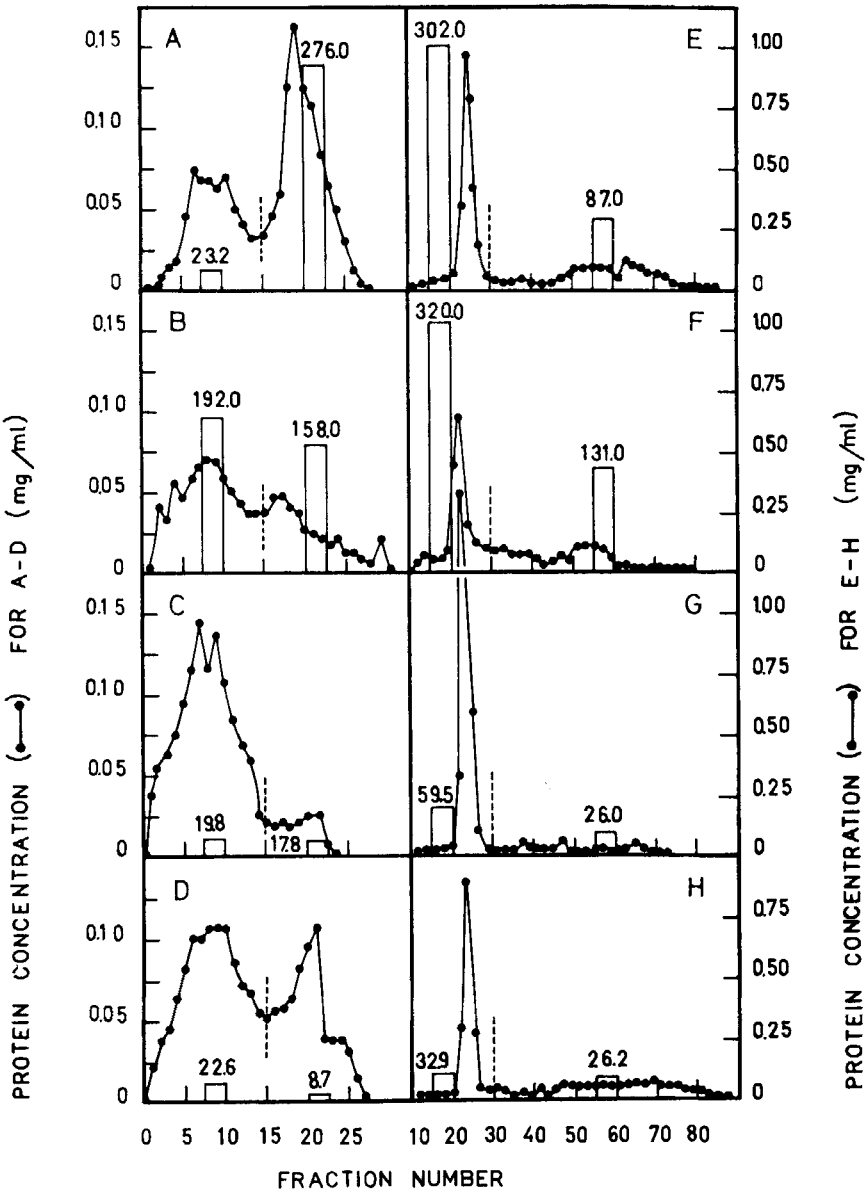
Fig. 1. Progress curves for hydrolysis of four 2-naphthylamides and four 1-naphthylesters by enzyme preparations from control tissue and diseased tissue. 1. L-Alanyl-2-naphthylamide. 2. L-Arginyl-2-naphthylamide. 3. L-Lysyl-2-naphthylamide. 4. L-Methionyl-2-naphthylamide. 5. 1-Naphthyl phosphate (calcium salt). 6. 1-Naphthyl phosphate (sodium salt). 7. 1-Naphthyl acetate. 8. 1-Naphthyl propionate. ●: Control tissue. o: Diseased tissue. Enzyme activity is expressed as liberated μmoles of 2-naphthylamine or 1-naphthol per min and per mg protein ($\times 10^3$).

the reaction products after incubating the collagenous substrate with the enzyme preparations from diseased and control material. As to G-200 filtration, the two upper chromatograms in the left side of Fig. 2 (chromatograms A and B) are to be compared with each other. It is seen, first, that the used enzyme preparations are able to breakdown collagen fibres (those insoluble in 0.5 M acetic acid), and, second, that the enzyme preparations from the control material exhibit higher enzyme activity towards the collagenous substrate than that from the diseased material. These results are seen from the concentration of hydroxyproline determined from the two pools from each filtration. The first pool was formed by protein molecules of high molecular weight (about 200.000 and more, as based on the known exclusion limit of Sephadex G-200 gel) and was fractionated out of the column in the void volume of the gel column. The second pool consisted of molecules with lower molecular weight than approximately 200.000. The first chromatogram shows that the enzyme preparation from healthy material altered the structure of collagen in the reaction mixture, forming smaller collagen units. This is evidenced by the large amount of hydroxyproline in the second pool. The enzyme preparation from diseased material, on the contrary, was able to produce only about half as many smaller collagen units, as evidenced by the lower concentration of hydroxyproline of the second pool in chromatogram B.

With Sephadex G-10 no such large differences between the two enzyme preparations were found as with Sephadex G-200. This is evidently due to the fact that these two gels divide the sample applied on them in different way (based on the known exlusion limit of the gels). However, the first two Sephadex G-10 chromatograms (E and F) show that during the reaction both enzyme preparations liberated from the substrate peptides rich in hydroxyproline. The molecular weight of these peptides may be about 700 and more (suggested by the known exclusion limit of Sephadex G-10 gel). Consequently, the »collagenolytic» enzyme activity of the periodontal membrane is clearly evidenced by the Sephadex G-10 chromatography. It is to be noted that each of the enzyme blank mixtures applied on the G-200 or G-10 columns, whether incubated (chromatograms C and G) or not (chromatograms D and H), contained only traces of hydroxyproline, and that incubation had no effect on the occurrence of hydroxyproline in each of the pools.

3. *Fractionation of the enzymes*

In order to get the enzyme activities studied separated to some extent, the enzyme preparations from control tissue and diseased connective tissue were


A
B
C
D
E
F
G
H
276.0
23.2
192.0
158.0
19.8
17.8
22.6
8.7
302.0
87.0
320.0
131.0
59.5
26.0
32.9
26.2
PROTEIN CONCENTRATION (—●—) FOR A-D (mg/ml)
PROTEIN CONCENTRATION (—●—) FOR E-H (mg/ml)
FRACTION NUMBER

fractionated through DEAE-cellulose columns. Exactly the same volume of both protein solutions were applied on two different columns in a simultaneous chromatography to obtain higher comparability between the results. The columns were packed from one and the same material, and the fractionations were conducted identically. Results are given in Fig. 3. No ideal separation was achieved, although quite the same fractionation procedure used for other enzyme samples had already been proved in this laboratory to be successful. In spite of similarities in the absorption qualities of the enzymes involved, two main peaks hydrolyzing 2-naphthylamides of L-arginine and L-alanine were observed in both fractionations (Fig. 3).

It is possible that the use of these substrates revealed the fractionation of aminopeptidase- like enzymes. The first enzyme peak (fractions 42—50) is obviously responsible for the higher enzyme activity of the control material. The enzyme activities in the second peak (fractions 58—65) are higher with the preparation from the diseased membrane material. The results suggest that the connective tissue cells of the periodontal membrane contain in diseased condition (that is, where inflammation occurs remote from the cells) enzyme activities that differ at least quantitatively from those of the control tissue.

Fig. 2. Determination of breakdown of »healthy» and diseased periodontal collagen. Filtration of the supernatant fluids of reaction mixtures through Sephadex G-200 gel (chromatograms A, B, C and D) and Sephadex G-10 gel (chromatograms E, F, G and H). Sephadex G-200 fractionations: column: 45×1.6 cm; sample: 0.5 ml, whose preparation is given in text; elution: 100 ml of 0.1 M tris-HCl buffer, pH 7.0; flow rate: 4 ml/60min; hydrostatic pressure: 5—10 cm; temperature: 0°; fraction volume: 2 ml. Fractions were pooled to form two pools as indicated by dotted lines. The diagrams and the adjacent figures indicate the amount of hydroxyproline estimated in the pools after first determining the protein concentration from each fraction. A. Enzyme reaction with the preparation from healthy tissues. B. Enzyme reaction with preparation from diseased tissues. C. Blank mixture without the collagenous substrate, but with both diseased and control enzyme preparations (incubated). D. The same as C, but not incubated. The substrate blanks (supernatant fluid mixtures containing only collagen in 5 ml of 0.1 M tris-HCl buffer, pH 7.0) contained only traces of protein and hydroxyproline. These would not be detectable in chromatograms if drawn in the same scale as for other filtrations. Sephadex G-10 fractionations: column: 24×2.6 cm; sample: 0.5 ml, whose preparation is given in text; elution: 200 ml 0.1 M tris-HCl buffer, pH 7.0; flow rate: 0.5 ml/min; hydrostatic pressure: 15 cm; temperature: 0°; fraction volume 1.8 ml. Fractions were pooled to form two pools as indicated by dotted lines; the columns and the adjacent figures indicate the amount of hydroxyproline estimated in the pools after first determining the protein concentration for each fraction. E. Enzyme reaction with the preparation from control tissue. F. Enzyme reaction with preparation from diseased tissue. G. Blank mixture without the collagenous substrate but with both diseased and control enzyme preparations (incubated). H. The same as C, but not incubated. The substrate blank gave the same results as in Sephadex G-200 filtration.

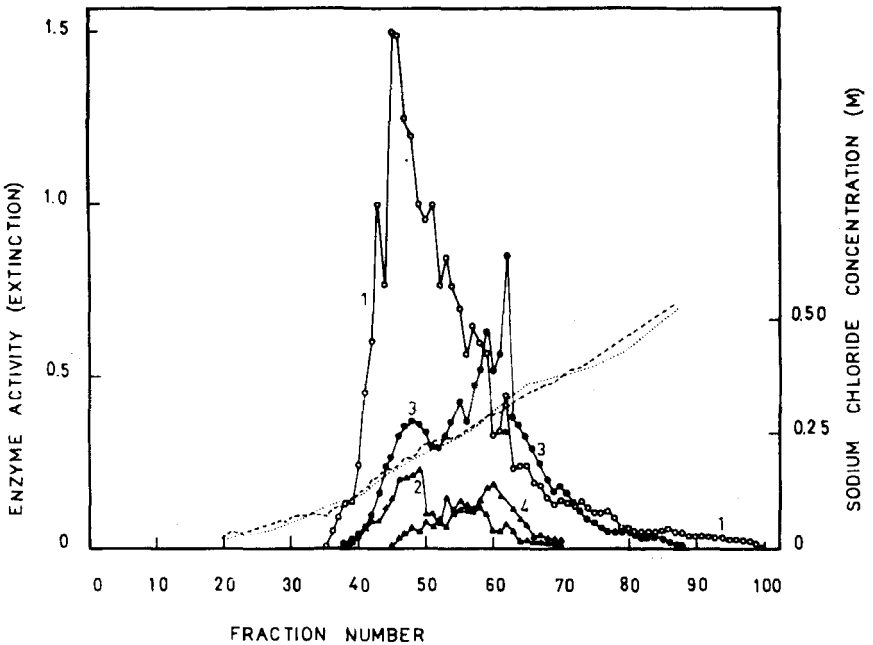


Fig. 3. Fractionation of aminopeptidase-like enzymes of »healthy» and diseased periodontal membrane on DEAE-cellulose (Schleicher & Schüll, Kr. Einbeck, Germany; a fraction of 100—140 mesh was sieved from the commercial preparation). The two fractionations are drawn in the same figure and for both the same conditions apply. Column: 16.5×1.6 cm; sample: 1 ml of enzyme extract from control material or diseased membrane material, the preparation of which is given in Methods; elution: 0.1 M of tris-HCl buffer (pH 7.0) comprising a linear sodium chloride gradient from 0 to 1.0 M; flow rate: 0.3 ml/min; mixing volume: 125+125 ml; hydrostatic pressure: 100 cm (implies also packing with the above buffer); temperature: 0°; fraction volume: 1.5 ml. Enzyme activities and sodium chloride gradient were determined for each fraction. The form of the gradient was determined by measuring the sodium content of the fractions with Perkin-Elmer atomic absorption spectrophotometer Model 303, 1 = control, L-alanyl-2-naphthylamide; 2 = control, L-arginyl-2-naphthylamide; 3 = diseased membrane, L-alanyl-2-naphthylamide; 4 = diseased membrane, L-arginyl-2-naphthylamide. Sodium chloride gradients: - - - -, in the fractionation of diseased material; . . . . . , in the fractionation of control material.

DISCUSSION

The so-called collagenolytic enzyme activity in animal tissues has been studied by *Gross* and *Nagai* (1965) with tadpole collagenolytic enzyme; by *Wiley* and *Peacock* (1966) in various human tissues; by *Goldstein et al.* (1964) in intact and necrotic connective tissue; by *Fullmer et al.* (1966) in certain pathological conditions of the human skin; and by *Bazin* and *Delaunay* (1965) in inflamed tissues of the rat. In the last mentioned case collageno-

lytic activity was found in both the diseased and the healthy tissue — the former displaying, however, higher enzyme activity: that is, as a contrast to results presented in this paper. Collagenolytic activity has been detected also in inflamed human gingiva while healthy gingival tissue was shown to possess no such activity (*Bennick & Hunt*, 1967). Consequently, although reports on so-called collagenolytic activity have so far dealt with normal tissue or the inflamed tissue itself, there seems to have been no study concerning the biochemistry of connective tissue remote from the inflammatory area.

Generally, most studies concerning so-called collagenolytic enzyme activity have been carried out between 20° and 37°. However, when keeping the temperature of the reaction mixture, say, at 20°, one cannot be sure that quite the same kind of enzyme protein is involved as, for example, at 35°. In order to imitate as much as possible the *in vivo* conditions, a temperature of 35° was used in demonstrating the enzymic degradation of collagen in this study. On other hand, the temperature was not raised above 35° because of the lability of collagen near 37° and above. In this investigation the term collagenolytic activity has not been used because it is possible that it is not at all any classical collagenase, for example, *Clostridium histolyticum* which degrades the connective tissue collagen of the periodontal ligament, but merely proteolytic enzymes in general being able to hydrolyze protein molecules of that kind present in the ligament.

One would expect, on the basis of studies concerning proteolytic activities of inflamed tissues, that the enzyme preparations obtained from the diseased connective tissue would be able to break down more rapidly the collagenous substrate from the periodontal ligament than do those from the control material. The higher protein concentration of the former would support this expection. Yet no clear differences could be observed between the enzyme preparations in this respect. The results showed, however, that the used collagenous substrate is degraded by enzymes in the periodontal ligament. It is possible, although by no means proven, that the enzymes involved are in general those responsible for the normal turnover of collagen in periodontal ligament. The situation was quite the reverse when the incubations were carried out with synthetic substrates. There seem to be at least two possibilities for explaining the decreased hydrolytic activity of the enzyme preparations obtained from diseased material. First, the action of the enzyme involved may have been inhibited in some manner. Second, the biosynthesis of the enzyme proteins involved may have been blocked at some stage. In both cases the inhibition may be due to certain chemical products of the metabolism of the oral micro-organisms, cabable of diffusion to the site of

the enzyme action or biosynthesis. The possibility that the biosynthesis itself is blocked is perhaps more easy to explain according to what is generally known about the inhibition of protein biosynthesis by certain end products of microbial metabolism. Many compounds possessing antimetabolic activity could be responsible for the observed effects, and several oral micro-organisms are also known to form and liberate various toxins which may interfere with, for example, enzyme synthesis. However, the synthesis of all proteins in the diseased connective tissue cells studied in this investigation is certainly not, inhibited. For example, it has been found, in connection with the present studies, that the amount of RNA in the diseased connective tissue cells of the periodontal ligament is markedly increased as compared to the RNA content of the control material, this apparently displaying a general increase in the rate of protein biosynthesis in the diseased tissue (*Paunio*, 1969). Therefore it is possible that the inhibition of protein synthesis may be confined, for example, only to enzyme proteins of the kind hydrolyzing the synthetic substrates given.

The nature of the biochemical events of the diseased connective tissue studied in this paper may be compared to those occurring generally in wound healing, where the amount of RNA content of the wound tissue cells first decreases, but then markedly increases (*Tsanev*, 1963; *Tashiro & Inouye*, 1959). According to Tsanev, ribonucleoproteins themselves are very sensitive to all injuries occurring in the protoplasma, and it is the reactions of the ribonucleoproteins in the injured cells which start the healing process. Thus diseased connective tissue cells of the periodontal ligament may be considered as being in a state of continuous healing and under a continuously increased RNA turnover caused, for example, by the influence of a nearby inflammation. Because of the special nature of the environment of the diseased periodontal ligament (the proximity of the inflammatory focus at a few millimeters distance), this healing process practically never reaches the same final stage as in usual wound healing. By still comparing the diseased connective tissue used in this study to tissue in the ordinary wound healing process, it may be stated that the relative quantity of histochemically detectable nonspecific esterases in the mammalian epithelium decreases during wound healing (*Nowinsky*, 1960). This decrease would correspond to the lower enzyme activity of diseased connective tissue on the naphthyl esters used in this study.

The results presented in this paper indicate that the decreased proteolytic and esterolytic enzyme activity of the diseased connective tissue can to an extent, be compared with a certain phase of usual wound healing. It may be concluded that enzymic hydrolysis of collagen molecules isolated

from periodontal ligament has in fact been demonstrated. The enzyme preparation of the periodontal ligament was able to form hydroxyproline containing peptides of higher or lower molecular weight.

ADDENDUM

While this paper was in press, certain experiments carried out in this and other laboratories (*Mäkinen, K. K.* and *Mäkinen, P-L.*, data to be published) revealed that the lower enzyme activity of the inflamed material on amino acid 2-naphthylamides and 1- and 2-naphthol esters found in the present paper could also be due to rapid chemical reaction between the diazotized 4-amino-1,3′-dimethylazobenzene and certain tissue fluid proteins. These proteins are hemoglobin, globin, serum albumin and other proteins in which globin occurs. The presence of these proteins in large amounts in the extracts of the inflamed material is explained by the generally higher solubility of proteins found with this kind of tissue, as evidenced in this paper.

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SUMMARY

Large numbers of synthetic aminopeptidase, esterase, and other substrates were used to study enzyme activity in human periodontal connective tissue affected by inflammatory processes, but separated from their foci. Insoluble collagen isolated from the same connective tissue was also used as a substrate for the diseased connective tissue enzymes. As a control, enzyme preparations obtained from corresponding »healthy» connective tissue were used. The synthetic substrates most rapidly hydrolyzed were the 2-naphthylamides of L-alanine, L-arginine, L-lysine, L-leucine and L-methionine and 1- and 2-naphthyl acetate. Enzyme preparations from the control material hydrolyzed nearly all substrates significantly more rapidly than did those from the diseased material. When insoluble collagen was used as a substrate, enzyme preparations from both the diseased and the control material were able to release peptides rich in hydroxyproline, but no clear differences between the enzyme preparations were observed. When enzyme preparations

were fractionated by DEAE-cellulose, a quantitatively different elution pattern was obtained using certain amino acid 2-naphthylamides, the control material displaying higher enzyme activity. The results suggest that the enzymes involved with connective tissue need not necessarily be collagenase-like, but merely enzymes generally able to cleave substrates of that kind used in the studies. It is also suggested that the inflammatory focus apart from the connective tissue studied affects the biochemical events of the latter by interfering with its enzyme synthesis or by producing diffusable chemical compounds which inhibit enzyme reactions. The usual wound healing process is then compared with enzyme reactions of the connective tissue involved.

RÉSUMÉ

ÉTUDES SUR L'ACTIVITÉ ENZYMATIQUE HYDROLYTIQUE DANS LE TISSU CONJONCTIF DU DESMODONTE À DISTANCE DES FOYERS INFLAMMATOIRES

Un grand nombre de substrats synthétiques, aminopeptidase et estérase entre autres, ont été utilisés pour étudier l'activité enzymatique dans le tissu conjonctif desmodontal humain atteint par un processus inflammatoire, mais à distance des foyers. On a aussi utilisé le collagène insoluble isolé à partir du même tissu conjonctif comme substrat pour les enzyme du tissu conjonctif enflammé. A titre de témoins, on a utilisé des préparations enzymatiques provenant de tissu conjonctif «sain» correspondant. Les substrats synthétiques hydrolysés le plus rapidement ont été les 2-naphtylamides de L-alanine, L-arginine, L-lysine, L-leucine et L-méthionine, et l'acétate de 1-naphtyl et de 2-naphtyl. Les préparations enzymatiques provenant du matériel témoin hydrolysaient presque tous les substrats d'une manière significativement plus rapide que celles qui provenaient du matériel enflammé. Lorsqu'on utilisait le collagène insoluble comme substrat, les préparations enzymatiques provenant du matériel enflammé ainsi que celles provenant du matériel témoin étaient capables de libérer des peptides riches en hydroxyproline, mais il n'existait pas de différence bien nette entre les préparations enzymatiques employées. Quand le fractionnement des préparations enzymatiques était fait au moyen de la DEAE-cellulose, l'élution se faisait de manière différente du point de vue quantitatif lors de l'emploi de certaines 2-naphtylamides d'aminoacides, le matériel témoin présentant une activité enzymatique plus élevée. Les résultats obtenus semblent indiquer que les enzymes entrant en jeu dans le tissu conjonctif ne sont pas nécessairement

du type collagénase, mais seraient simplement des enzymes généralement capables de dissocier les substrats de ce genre utilisés dans la présente étude. Il semblerait aussi que le foyer inflammatoire situé à distance du tissu conjonctif étudié interviendrait dans les processus biochimiques qui y ont lieu, soit en entravant la synthèse enzymatique, soit en produisant des composés chimiques capables de diffuser et inhibant les réactions enzymatiques du tissu conjonctif. Le processus habituel de cicatrisation des plaies est ensuite comparé aux réactions enzymatiques du tissu conjonctif en cause.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER HYDROLYTISCHE ENZYMAKTIVITÄTEN DES VOM ENTZÜNDUNGSZENTRUM GETRENNTEN BINDEGEWEBES IN DER MENSCHLICHEN PERIODONTALEN MEMBRAN

Mehrere synthetische Aminopeptidase- und Esterasesubstrate wurden zur Untersuchung der Enzymaktivität der in der Nähe des Entzündungszentrums gelegenen periodontalen Membran verwendet. Als Substrat wurde das aus derselben periodontalen Membran isolierte unlösliche Kollagen verwendet.

Zur Kontrollierung dienten Enzympräparate aus dem Bindegewebe einer normalen Membran. Unter den Substraten wurden 2-Naphthylamid von L-Alanin, L-Arginin, L-Lysin, L-Leucin und L-Methionin sowie 1- und 2-Naphthylacetat am schnellsten hydrolysiert. Die aus dem Kontrollmaterial entnommenen Enzympräparate hydrolysierten fast alle Substrate schneller als die aus dem »kranken» Gewebe. Die Enzympräparate sowohl aus dem Kontrollgewebe als aus den »kranken» waren imstande, aus dem unlöslichen Kollagen Peptide zu isolieren, die reichlich Hydroxyprolin enthielten; in dieser Hinsicht gab es aber zwischen verschiedenen Enzympräparaten keinen Unterschied.

Die mit der DEAE-Zellulosechromatographie gewonnenen Chromatogramme variierten bei dem Kontrollgewebe und dem »kranken» Gewebe quantitativ. Aus den Resultaten ergab sich, dass die Enzyme des Bindegewebes im Periodontium nicht unbedingt einer sog. Kollagenase zu gleichen brauchen, sondern sind eher Enzyme, die überhaupt Substrate wie hier hydrolysieren können. Mit den Resultaten wurde auch nachgewiesen, dass ein in einer bestimmten Entfernung von dem untersuchten Gewebe gelegenes Entzündungszentrum auf die biochemischen Vorgänge in dem untersuchten Gewebe einwirkt, indem es die Synthetisierungsgeschwindigkeit der

Enzyme verändert oder zu diffundierende chemische Bindungen bildet, die die Enzymreaktionen inhibieren.

Zum Schluss wurde der allgemeine Heilungsvorgang einer Wunde mit der Biochemie des untersuchten periodontalen Bindegewebes vergleichen.

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Address:
Institute of Dentistry,
University of Turku,
Lemminkäisenkatu 2,
Turku 3,
Finland