Peptide Hydrolases in Mammalian Connective Tissue. I. Survey of Activities and Preliminary Characterization of Certain Peptidases*

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ABSTRACT: A survey of the total peptide hydrolase activity of bovine fibroblasts (derived from dental pulp) allows classifications with respect to substrate specificity, chromatographic behavior, and ion dependence. Identifications are by comparison with properties of known peptidases from other tissues. Approximate molecular weights of several peptide hydrolases are reported. Experiments with connective tissue slices indicate that these enzymes (in contrast to cathepsins) are probably active in this tissue at all times and that they degrade endogenous as well as exogenous peptides very readily.

The ability of connective tissue to repair damaged tissues and to remodel tissue interfaces may reasonably be accredited to a highly active system for protein synthesis and degradation, *i.e.*, with peptide hydrolases. It seems important to determine whether fibroblasts have special peptidases and how these enzymes are related to the remodeling function of connective tissue.

A variety of peptide hydrolases from hog kidney and intestinal mucosa have been examined by Smith (1951; Smith and Spackman, 1955) and Fruton *et al.* (1948). Histochemical studies have since revealed (Mottet, 1961) the presence of peptide hydrolase activity in fibroblasts situated adjacent to invasive tumors.

Fibroblast peptidases have not been isolated and purified due to the difficulty involved in obtaining samples of cellular connective tissue in sufficient amounts. Bovine dental pulp, a pure cellular connective tissue, however, does not suffer from these deficiencies (Schwabe and Kalnitsky, 1965).

This paper constitutes a survey to precede a specific study of the various enzymes.

Materials and Methods

Bovine dental pulp was obtained from the Wilson Packing Co. of Cedar Rapids, Iowa. The tissue was frozen (Dry Ice) upon removal from the animals and lyophilized without thawing. The dry pulp was degraded in a Waring Blendor and Wiley mill to a 60-mesh powder at room temperature. The enzymatic activity of the powder remained constant for at least 1 year when stored at −20°. Peptides purchased from Cyclo Chemical Co., Mann Biochemicals, and Sigma Chemical Co. were shown (by quantitative amino acid analysis) to contain less than 5% impurity. Peptides prepared in our laboratory by the *N*-carboxyanhydride method of Denkewalter *et al.* (1966) were purified by chromatography on Bio-Gel P-2 and by recrystallization. Their structure was verified by amino acid analysis.

The dipeptidase, tripeptidase, and aminoacylase activities were assayed as described by Matheson and Tattri (1964).

Crude pulp extract (1-mg/ml final concentration) and substrate, usually 5 mmoles, were incubated together at 40° in a water bath. At designated intervals three samples, 25 μl each, of the reaction mixture were pipetted into three tubes containing 500 μl of 0.1 N acetic acid. After the last interval 1 ml of citrate (pH 5.0) buffer and 1 ml of the ninhydrin–methyl Cellosolve–cyanide reagent[1] were added to all tubes in sequence from automatic syringe burets. The time difference between additions to the tubes had no effect on the final color intensity of a sample, as long as all tubes were kept in ice during this procedure. The contents of the test tubes were vigorously boiled for exactly 8.5 min and transferred to an ice bath; 2 ml of 50% isopropyl alcohol was added. After rapidly mixing on a test tube shaker the absorbance at 570 mμ was read on a Beckman DK-2A spectrophotometer within 1 hr. Conversion to micromoles of substrate consumed was accomplished with previously determined ε values of reactants and products. Protein concentrations were measured assuming that a milligram of extract per milliliter gives an optical density of 1.0 at 280 mμ. To assay the effluent from analytical columns the method was modified. To test a column effluent with five substrates, a set of five 25-μl samples were pipetted as well

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[1] This reagent is stable for 1 month if kept at −20°. The amount needed is prepared just prior to its use by dilution of this reagent 1:1 with 50% methyl Cellosolve.

as one enzyme blank from every second fraction. The substrates (5 mmoles) were rapidly added to each member of a given set using a 1-ml Hamilton syringe in a dispensing head (20 μl/injection). Since the column effluent and the substrates were buffered, pH checks were not necessary. The tubes were incubated at 40° for 10 min and then 0.5 ml of 0.1 N acetic acid was added from a syringe buret to stop the reaction. The color was developed as described above. The absorbance difference between the protein blanks and the corresponding experimental tubes was plotted as a function of tube number.

To ascertain the susceptible bond of tripeptides, thin-layer chromatography was employed. In a typical experiment 50 μl of pulp extract (2–4 mg/ml) and 50 μl of peptide (5 mmoles) were mixed in a test tube and 10 μl of aliquots was spotted on the silica gel plate at 10-min intervals. The plates were developed either in butanol–acetic acid–water (60:20:20, w/w) or in 75% phenol. After evaporation of the water the developed plates were sprayed with a cadmium–ninhydrin–Cellosolve spray (50 mg of cadmium acetate, 1 ml of glacial acetic acid, 5 ml of distilled water, 40 ml of acetone, and 10 ml of 5% ninhydrin in methyl Cellosolve).

The peptidase activity in pulp tissue slices was measured manometrically, utilizing L-ophioamino acid oxidase. Approximately 2 g of wet connective tissue slices (prepared on a Stadi-Rigg slicer) were placed into a 5-ml double side arm Warburg flask in pH 7.2 phosphate buffer. One side arm contained 200 μl (0.2%) of snake venom (Ross Allan's Snake Farm, Fla.) and the other (in some experiments) contained 300 μl of Leu-Tyr (2×10^{-2} M). The O_2 uptake was measured in a differential manometer (Roger Gilmont, N. J.), adapted to fit a Precision Scientific water bath. The differential manometer system was equilibrated (37°) until the monometer fluid level remained unchanged for 15 min. Snake venom was added first to the slices in the main compartment in order to eliminate endogenous free amino acids prior to addition of peptides. Controls did not contain amino acid oxidase or contained boiled tissue slices and amino acid oxidase. The system was tested by the addition of leucine (1 μmole) which, in the presence of L-ophioamino acid oxidase, gave rise to the theoretical O_2 uptake.

DEAE-cellulose Type 70 and CM-cellulose (Schleicher & Schüll) were washed with 0.1 M NaOH and 0.1 M HCl prior to equilibration with buffer. Columns (2 × 20 cm) were packed and equilibrated for 24 hr with slow-flowing Tris buffer.

Exclusion chromatography was performed on Bio-Gel A 0.5 m (spherical agar (100–200 mesh) exclusion limit 500,000 molecular weight, Bio-Rad).

Hydroxylapatite used for column chromatography was prepared according to Jenkins (1962). Urea (Mann, Ultra Pure) was used without pretreatment.

Experimental Section and Results

Extraction of Enzyme. Pulp powder (50 g) was extracted in 500 ml of distilled water for 1 hr at room temperature on a magnetic stirrer. The suspension was then centrifuged in a Sorvall high-speed centrifuge at 8000 rpm for 15 min at 4°. The supernatant was collected and the pellet was reextracted. The combined supernatants (~900 ml) were dialyzed for 24 hr at 4° against two changes each of 6 l. of distilled water containing 1 g of free Tris base. The extract contained more than 90% of the extractable activity (tested by further extractions) and 50% of the original mass.[2] After lyophilization and storage at −20° for up to 1 year less than 5% of the enzymatic activities was lost.

Survey of the Hydrolytic Capacity of Fibroblasts. In order to ascertain the general type of peptide hydrolase activity present in mammalian fibroblasts, a wide range of substrates have been subjected to degradation by this connective tissue extract. The rate of hydrolysis of most substrates was linear for 10–60 min. Table I gives zero-order proteolytic coefficients[3] which reflect the relative capacity of the extract to hydrolyze the different peptides. Values from Dixon and Webb (1964) and Smith (1951) are given for comparison. If connective tissue enzymes are similar to those of the intestinal mucosa, most of the activity appears to be of an aminopeptidase type. In contrast, carboxypeptidase activity is absent or not detectable, but a glycylglycine dipeptidase, an imidodipeptidase, an iminodipeptidase, an aminotripeptidase, and an acylase appear to be present. In addition a trace amount of Leu-β-napthylamide hydrolyzing activity (arylpeptidase) could be demonstrated by thin-layer chromatography.

In order to identify various activities as distinct enzymes it is necessary to attempt separations based on several different principles. Charge (DEAE ion exchanger), adsorption (hydroxylapatite), and molecular size (Agarose) will be the properties used for analytical type separations in the following sections of this paper.

Chromatography on DEAE-cellulose. Preliminary experiments had shown that DEAE is most suited for group separation of enzymes contained in connective tissue extract. The conditions determined to be best for our purpose were standardized so that column experiments could be accurately reproduced. The results of DEAE chromatography are shown in Figures 1 and 2.

The first protein fraction (not numbered in Figures 1 and 2) was not adsorbed on the DEAE-cellulose at the initial buffer strength ($\mu = 0.03$). The fractions I, II, and III, henceforth referred to as DEAE I, II, and III, were eluted at $\mu = 0.1$, 0.2, and 0.4, respectively. The various activities were detected by the column effluent assay described in the Materials and Methods section.

Leucylglycylglycine (Leu-Gly-Gly) was hydrolyzed by DEAE I and II, leucylleucine (Leu-Leu) and leucinamide ($LeuNH_2$) only by DEAE II (Figure 2). Since Leu-Gly-Gly is a substrate for both the aminotripepti-

[2] Assuming that a protein concentration of 1 mg/ml has an absorbance of 1.0 when measured at 280 mμ in a 1-cm cuvet.

[3] $C_0 = k_0/E$, E = mg of protein/ml. The nitrogen content of this tissue extract is ~12–14%. Our values should be multiplied by 10 for comparison with Smith *et al.* (1955).

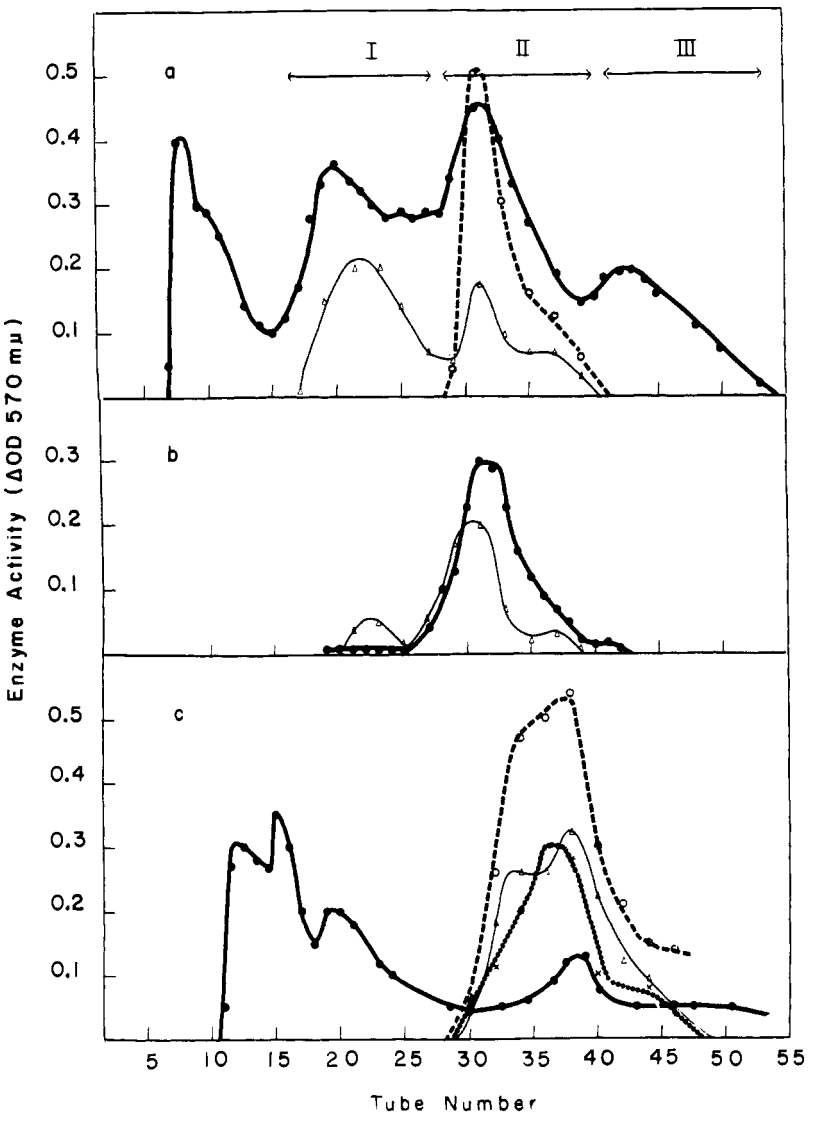

FIGURE 1: Chromatography studies. (a) The effluent record of a fibroblast extract chromatographed at 4° on a DEAE-cellulose column. A nearly linear gradient (μ = 0.03–0.4) was employed. The flow rate was adjusted to 0.25 ml/min. A 2 × 20 cm DEAE column was equilibrated at 4° with 0.05 M Tris-HCl buffer at pH 8.0, 0.01 M in NaCl. Lyophilized extract (300 mg) was dissolved in 5 ml of distilled water, and applied to the column, and washed into the cellulose with 2 ml of initial buffer. Elution was started using 250 ml of the initial buffer in chamber 1 (500-ml erlenmeyer), 250 ml of same buffer but 0.2 M in NaCl in chamber 2, and 400 ml of 0.05 M Tris, 0.5 M in NaCl in chamber 3. The first two chambers (stirred magnetically) were air tight to keep a constant volume. Buffers were pumped by means of a micropump (Buchler) at 0.25 ml/min. The gradient produces a nearly linear increase in ionic strength from 0.03 to about 0.4. Protein was measured at 280 mμ; activity as described under Methods. (b) Rechromatography of fraction II (DEAE II) under identical conditions. (c) Chromatography of a rat kidney extract on the same column. (●—●) Protein (280 mμ), (○---○) Leu-Leu, (Δ—Δ) Leu-Gly-Gly, and (X···X) leucinamide.

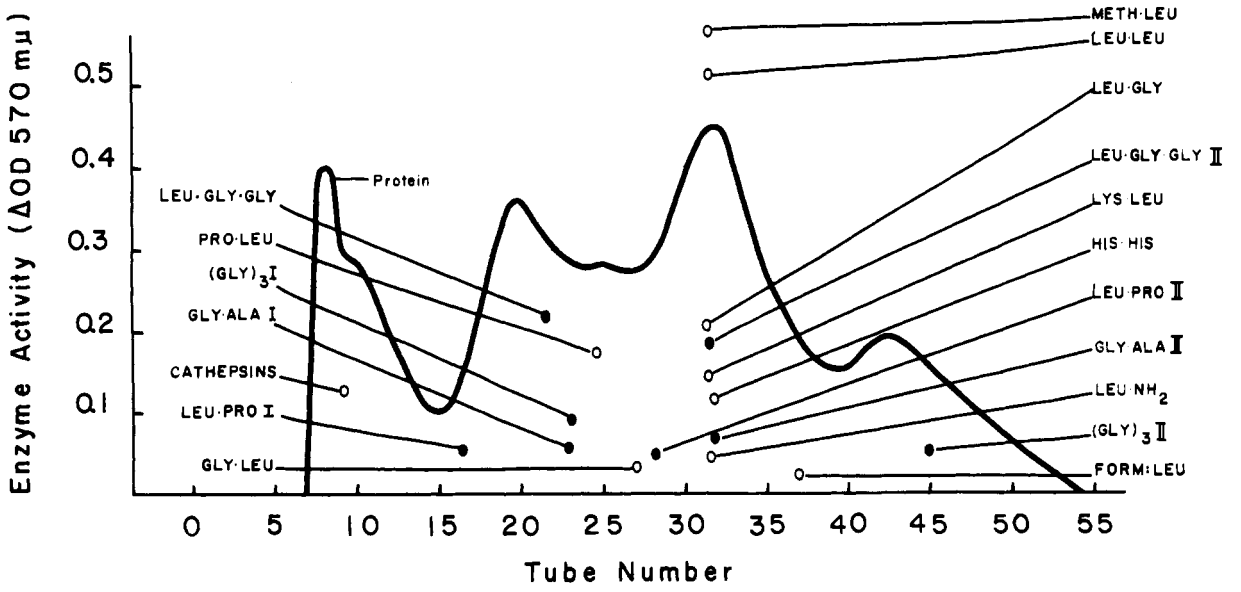

FIGURE 2: The "enzyme map" of bovine connective tissue as obtained by column chromatography on DEAE. Only the peaks (in proportional height) of the various activities are shown. The information summarized in this figure was plotted from curves such as shown in Figure 1a. Activities occurring in two regions of the column effluent are drawn as solid dots, single activities as open dots.

TABLE I: The Hydrolytic Capacity of a Crude Bovine Connective Tissue Extract.[a]

Substrate [S]	Concn of [S] (μmoles/ml)	C_0 ($\times 10^2$)	Enzyme Assignment[b]					
			Leucine Aminopeptidase	Amino Tripeptidase	Gly-Gly Dipeptidase	Imino Dipeptidase	Imido Dipeptidase	Carboxy-peptidase
Gly-Gly	2.7	1.70		+	++++			
Gly-Ala	2.5	3.00	+					
Gly-Leu	2.9	6.20	++					
Gly-Tyr	2.5	0.80	+					
Gly-Asp	10.6	0.50						
Gly-D-Asp	10.4	0						
Gly-Pro	4.0	Trace				++	+++	
Gly-Hyp	4.5	Trace					++	
Gly-Gly EtOH	9.8	Trace						
$(Gly)_3$	3.2	0.20		+++				
$(Gly)_4$	2.4	Trace		+				
Gly-Leu-Tyr	5.1	.15		+				+
Bz-Gly								
$LeuNH_2$	5.0	0.15	++					
Leu-Gly	10.5	3.30	++++	+				
Leu-Ala	9.6	4.50	++++					
Leu-Leu	9.8	4.30	++++					
Leu-Tyr	7.4	3.70	+					
Leu-Met	5.6	0.20					++	
Leu-Pro	8.9	0.70						
Leu-D-Ala	9.8	0	+					
D-Leu-Tyr	7.3	0						
Leu-D-Phe	8.0	0						
Nor-Leu-Tyr	9.2	2.00	+++					
$Leu\text{-}TyrNH_2$	8.7							
Leu-Gly-Gly	6.3	3.00	+++	+++				
Leu-β-naphthylamide		Trace	+?	+?				
N-Formyl-Leu		Trace						
Leu-β-Ala	8.2	1.00	+	+				
Ala-β-Ala	7.0	Trace	+					
β-Ala-Ala	6.3	0	+					
His-Ala	4.8	2.90						
His-His	4.2	3.00						
γ-Glu-Ala		0						+
γ-Glu-Cys-Gly		0						
Bz-Ala		0						

β-Ala-Leu	3.9	0			
Lys-Leu	9.6	1.50	+		
Ser-Leu	7.9	3.80			
Pro-Leu	4.2	6.50			
Met-Leu	10.2	11.00		++	
Phe-Leu	3.1	3.20			
Tyr-Tyr		Trace			
Tyr-Glu	9.8	0.60			
Pro-Pro	10.7	Trace		+	
Pro-Gly	8.4	Trace		+	
Hyp-Gly	6.3	0		+	
Cbz-Gly-Tyr		0			+++
Cbz-Gly-Phe		0			+++
Poly-Leu		0	+		
Poly-Glu		0			
Poly-Pro		0			

[a] Initial velocities were obtained by the ninhydrin procedure described under Methods. Trace activities were usually detected by thin-layer chromatography. Gly-Gly EtOH is split at the ester bond. The N-terminal residues of Gly-Leu-Tyr, Leu-TyrNH$_2$, and Leu-Gly-Gly are removed first by the corresponding enzyme(s). [b] Dixon and Webb (1965).

dase and leucine aminopeptidase, and LeuNH$_2$, His-His, Lys-Leu are substrates for leucine aminopeptidase only, it seems reasonable to assume that DEAE II contains leucine aminopeptidase[4] and DEAE I an aminotripeptidase. (In either fraction other dipeptide hydrolases are present as well.) Figure 1b shows that DEAE II is not an artifact of the isolation procedure. Here only the relevant Leu-Gly-Gly activity is shown since it is the substrate for both enzymes. Figure 1c shows that the kidney leucine aminopeptidase elutes at a higher salt concentration and that aminotripeptidase and leucine aminopeptidase activity cannot be separated on this column under the conditions employed. The data presented in Figure 2 are the results of five columns run under identical conditions, each being assayed with all the substrates shown in the figure. Since the pattern is very reproducible, we believe that double peaks of certain activities are real and that differences in two or three tubes between the peaks of various substrates indicate that different enzymes are responsible for their degradation. Thus Pro-Leu (iminopeptidase) and Leu-Pro (imidopeptidase) occur in DEAE I but are well separated. Similarly Leu-Gly-Gly, Pro-Leu, and Gly-Leu hydrolyzing activities are separated, while this is not clear for Leu-Gly-Gly and (Gly)$_3$ I. Gly-Ala is hydrolyzed by a fraction which contains mainly aminotripeptidase. Since Gly-Ala is not a substrate for the tripeptidase it must be assumed that a Gly-Ala dipeptidase elutes together with the tripeptidase from the DEAE column. The Leu-Pro activity could conceivably be attributed to the Gly-Leu dipeptidase, and Gly-Ala II perhaps to the leucine aminopeptidase activity. The (Gly)$_3$ II and the formylleucine hydrolysis are due to distinctly different enzymes.

As a result of this experiment it appears likely that the following enzymes or activities are present: aminotripeptidase, Gly-Ala-dipeptidase, imino- and imidopeptidase, (Gly)$_3$ tripeptidase, aminoacylase, and a leucine aminopeptidase. While Gly-Gly peptidase activity could be demonstrated in the original extract, none was detected after chromatography, probably due to the extreme lability of the enzyme (Smith, 1951).

Chromatography on Hydroxylapatite. In order to test whether the hydrolysis of peptides by DEAE fraction II was due to one or more enzymes, this portion of the DEAE column effluent was subjected to adsorption chromatography on hydroxylapatite. This adsorbent has proven useful at occasions where other fractionation methods failed (Schwabe and Kalnitsky, 1966). A 2 × 10 cm column was packed with a hydroxylapatite–cellulose mixture[5] (1:1, w/w) (wet slurry) and equilibrated at 4° with 0.05 M Tris, pH 8.0, 0.02 M in Na$_2$SO$_4$. A linear Na$_2$SO$_4$ gradient was used instead of the usual phosphate buffer since phosphate inhibits some of the enzymes present.

A comparison of Figures 2 and 3 reveals that one enzyme is responsible for the hydrolysis of such widely

[4] Leucine aminopeptidase defined by its action on leucinamide and its Mn^{2+} dependence.

[5] Cellulose improves the flow rate.

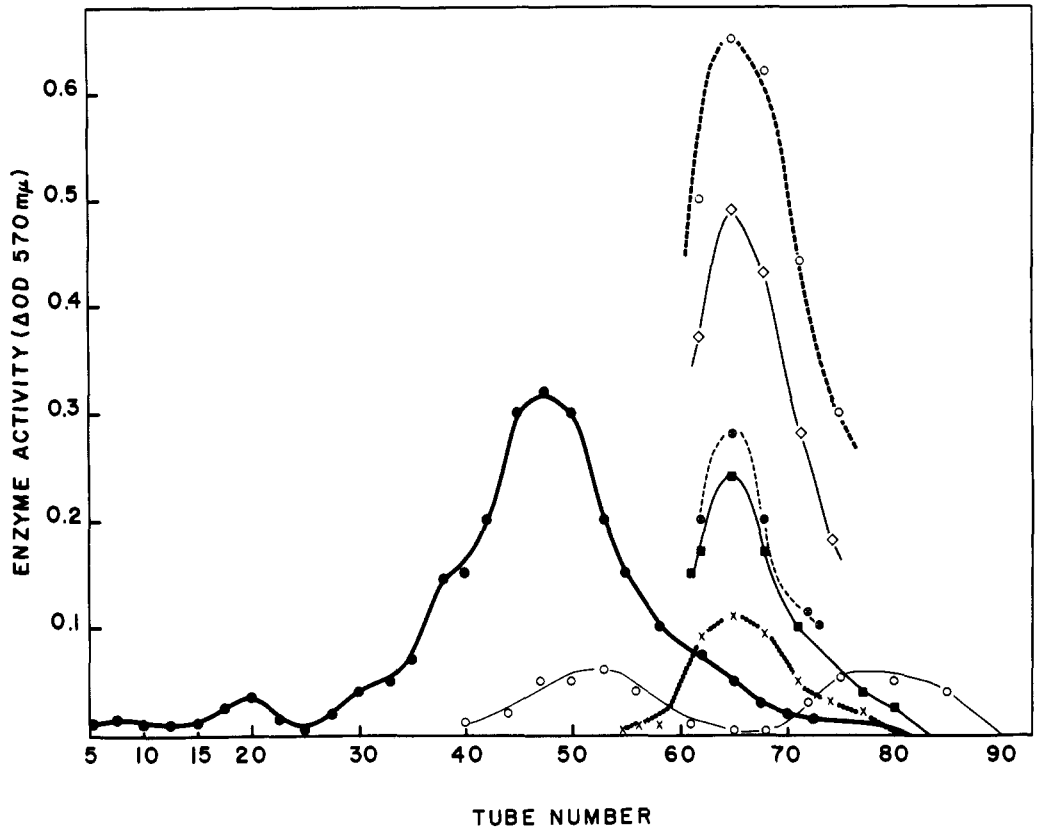

FIGURE 3: Distribution of activities in the effluent of a hydroxylapatite column. Hydroxylapatite slurry (50 g) was suspended in 100 ml of 50 mM Tris buffer at pH 8.0. To this suspension 50 g of a cellulose slurry (Whatman cellulose powder) was added under stirring. The mixture was permitted to settle and the supernate decanted. Sufficient buffer was added to make a slurry convenient for column packing. A 2 × 10 cm column was used for this experiment. A linear gradient in Na_2SO_4 was generated as follows: vessel I (constant volume), 200 ml of 0.05 M Tris, pH 8.0, 0.02 M in Na_2SO_4; vessel II, 400 ml of 0.05 M Tris, pH 8.0, 0.5 M in Na_2SO_4. The buffer was pumped onto the column with a Buchler micropump at about 0.1 ml/min. During an experiment the column was cooled to 4°. At the end of a run the column was washed with 100 ml of 1 M Na_2SO_4 followed by regeneration with initial buffer. Washing is best done at room temperature to prevent crystallization of the sodium sulfate. (◇—◇) Met-Leu, (■—■) His-His, (○---○) Lys-Leu, and (○—○) Gly-Leu.

differing peptides as His-His, Leu-Leu, Lys-Leu, and Leu-Gly. Leucinamide is hydrolyzed by the same enzyme at about 10% of the rate of dipeptides as is typical for leucine aminopeptidase. The Gly-Leu activity has been resolved into two peaks on the hydroxylapatite column.

Agarose Column Chromatography. Adsorption chromatography did not subdivide the DEAE fraction II into different active components. Through separation, based on molecular size, we hoped to supplement the data obtained from DEAE and hydroxylapatite columns sufficiently to permit identification of the most prominent peptide hydrolyzing enzymes in the connective tissue extract. Since the enzymes are detected by their activity an estimate of their molecular size can simultaneously be obtained independent of other proteins present. A 0.9 × 120 cm Agarose column with an exclusion limit of 500,000 molecular weight was used for this purpose. Effluent records (Figure 4a,b) depict the typical activities and the protein. Detailed information is given in Table II. The most prominent first activity in Figure 4a represents Leu-Gly-Gly and $LeuNH_2$ activity (leucine aminopeptidase). The second peak represents aminotripeptidase hydrolyzing Leu-Gly-Gly only. This peak is a contamination from DEAE fraction I. Fractions I and II overlap somewhat on the DEAE column (Figure 1a). DEAE I (Figure 4b) contains mainly the aminotripeptidase and only a trace of leucine aminopeptidase.

It appears (Table II) that leucine aminopeptidase of fibroblasts is larger than the corresponding enzyme from hog kidney. Additionally, every experiment revealed a second peak of the same activity (see shoulder on peak I). Its position on the column suggested a molecular weight of about 200,000 for this smaller fraction. This phenomenon was further examined and the results are reported in a subsequent paper (Schwabe, 1969). A minor fraction of Gly-Ala dipeptidase eluted together with the smaller leucine aminopeptidase component. The major Gly-Ala activity eluted at a position indicative of a lower molecular weight (DEAE I) suggesting the possibility of a subunit structure for this enzyme or the existence of two enzymes. Importantly the $(Gly)_3$ activity is clearly differentiated from the aminotripeptidase (DEAE I). The DEAE column already separated the two fractions, although insuf-

TABLE II: Gel Chromatography of Connective Tissue Enzymes.[a]

	V_e/V_0	Mol Wt (g)	Substrates Hydrolyzed	Remarks
Blue Dextran	1.00	>1,000,000		Standards
Catalase	1.15	230,000		
Lactate dehydrogenase	1.38	125,000		
Bovine serum albumin	1.50	70,000		
Carbonic anhydrase	1.80	34,000		
Lysozyme	2.10	17,000		
		>500,000		
DEAE II	a. 1.05	400,000–500,000	1–6	Major
	b. 1.22	200,000	1–6,9	Minor
	c. 1.27	150,000	7	Single
	d. 1.42	100,000	3	Minor
	e. 1.70	50,000	9	Major
DEAE I	a. 1.05	400,000–500,000	1–3	Trace
	b. 1.20	250,000	10, 8	Single
	c. 1.42	100,000	3	Major
	d. 1.70	50,000	9	Minor

[a] A Bio-Gel A 0.5-m column (0.9 × 120 cm) was equilibrated at 4° with 0.05 M Tris buffer at pH 8.0 (0.2 M in NaCl). Various samples (0.3 ml) were applied with the aid of a Technicon sample injector in 10% sucrose. Flow rate was 0.1 ml/min. The effluent protein was monitored by an ultraviolet scan III (Buchler) and collected in 0.5-ml fractions. The assays were performed as described under Methods. See also Figure 3. Code for substrates: (1) $LeuNH_2$, (2) Leu-Leu, (3) Leu-Gly-Gly, (4) Leu-Gly, (5) Lys-Leu, (6) His-His, (7) Leu-Pro, (8) Pro-Leu, (9) Gly-Ala, and (10) $(Gly)_3$.

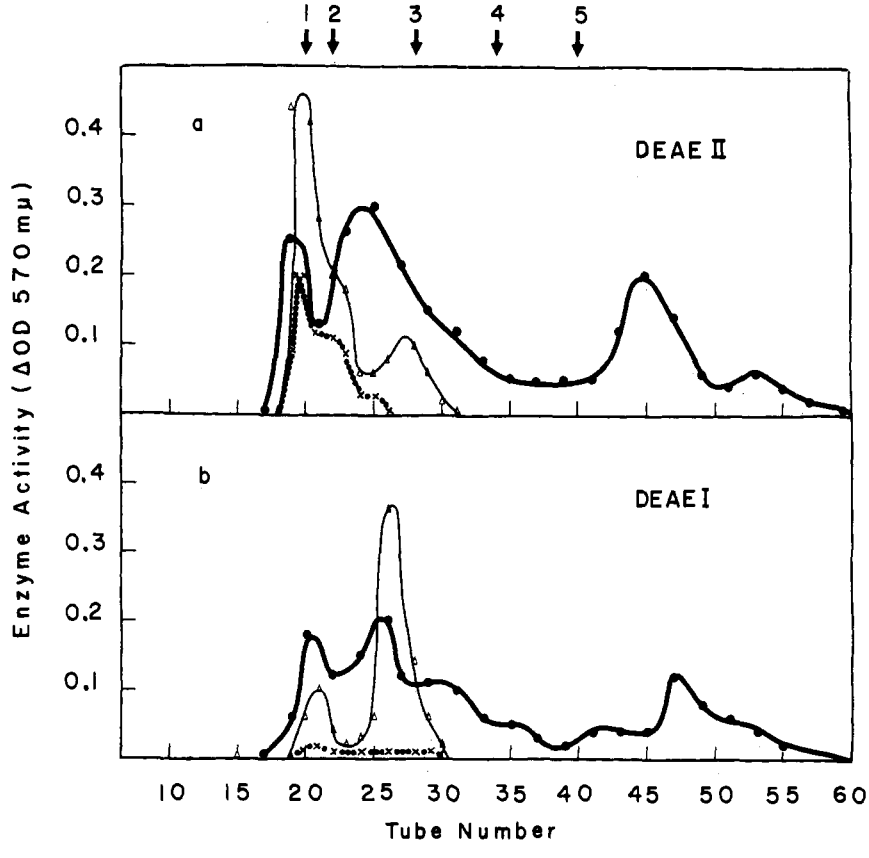

FIGURE 4: Bio-Gel A 0.5-m (agar) chromatography of a bovine connective tissue preparation (DEAE fraction II and fraction I). An Agarose column (Bio-Gel A 0.5 m) 0.9 × 120 cm was equilibrated with 0.05 M Tris buffer pH 8.0, 0.1 M in NaCl. Thereafter a Technicon amino acid analyzer pump was used at a flow rate of 0.1 ml/min. Using a Technicon sample injector, 100 mg of either DEAE I or DEAE II was applied in 300 μl of 0.1 M Tris buffer in 10% sucrose. The absorbance was at 280 mμ. (●—●) Protein, (△—△) Leu-Gly-Gly, and (X···X) $LeuNH_2$ activity are plotted. All runs are performed on the same column with frequent standardization. The position of the standards is indicated by the number in the upper part of the figure (1, dextran; 2, catalase; 3, bovine serum albumin; 4, carbonic anhydrase; and 5, lysozyme).

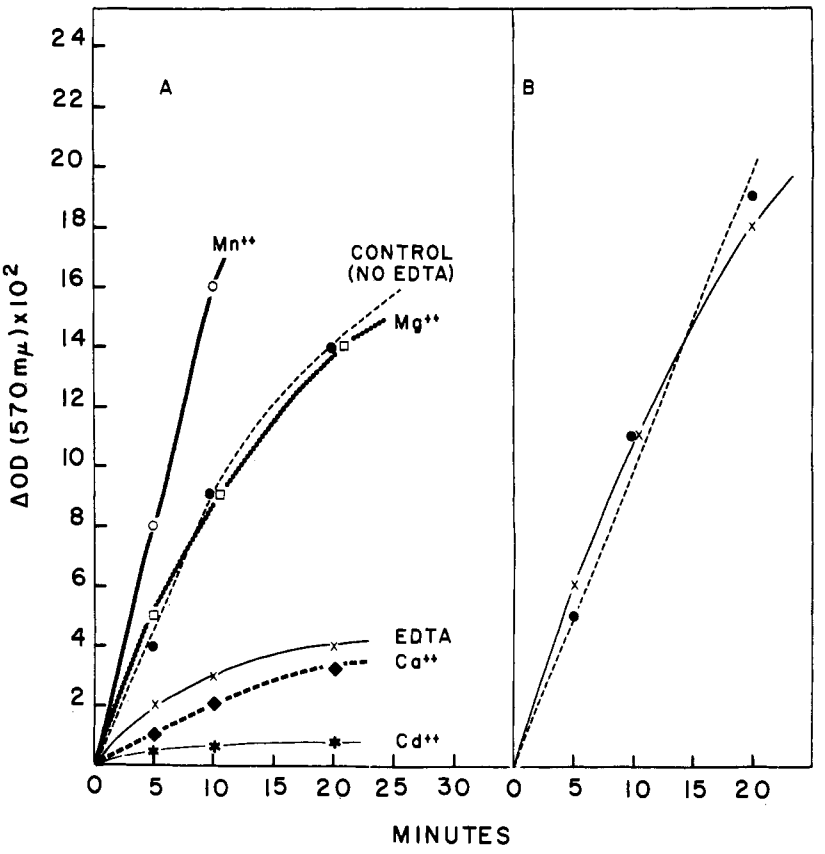

FIGURE 5: EDTA studies. (A) The activity of the connective tissue peptidase toward Leu-Leu in the presence of various ions after 30-min preincubation with 10^{-2} M EDTA at 40°. (B) The effect of EDTA (30 min) at room temperature without ions. (●---●) EDTA and no (X—X) EDTA.

ficiently for identification. Neither iminopeptidase, imidopeptidase, nor triglycylpeptidase has been purified or sufficiently examined regarding molecular size to permit comparison with literature values.

The Effect of Ions and Buffers. While most of the peptide hydrolases have been shown to depend upon divalent ions for activity, the effects of buffer ions have not often been considered, although their influence can be significant. After these effects have been considered in isolation, they may interact so that a given divalent ion which inhibits an enzyme in Tris buffer may have no influence or may, in an extreme case, activate when the buffer is changed to phosphate. The effect of inhibitors and activators (mostly divalent ions) on the connective tissue enzyme (Table III) are compared with published values for peptidases from other tissues. Differences between the enzymes from fibroblasts and intestinal mucosa or uterus are observed in several instances. The glycylleucine dipeptidase of pulp is inhibited by Zn^+ in phosphate as well as in Tris, while the uterus enzyme is strongly activated by this ion. Notable also is the inhibition and activation of prolinase by Zn^{2+} in the presence of Tris or phosphate buffer, respectively.

The slight stimulation of the leucine aminopeptidase by NaCN might be attributed to the binding of traces of heavy metal by the zyanide. Manganous ions appear to be the specific activator for the aminopeptidase activity in mammalian connective tissue. Unlike with hog kidney enzyme, Mg^{2+} cannot substitute for Mn^{2+} in the case of fibroblast leucine aminopeptidase.

The response to incubation with EDTA suggests very tight bonding of the activating ion. Subjecting the connective tissue peptidase to 10^{-2} M EDTA for 30 min at room temperature and at 40°, respectively, leads to inhibition (>60%) at the elevated temperature only (Figure 5A,B). At the same time the figure shows that Mn^{2+} stimulates, Mg^{2+} protects, while Ca^{2+} and Cd^{2+} (all at 10^{-3} M concentration) have no activity-enhancing effect. Manganous ion shows an increase over the control (which contains probably residual Mn^{2+}). The preferential role of Mn^{2+} became more apparent when the enzyme was inactivated with EDTA at 40° followed by reactivation with various ions (Figure 6).

Further evidence regarding the effect of Mn^{2+} is derived from the following observation. Copper ions at 10^{-4} M strongly inhibit the pulp leucine aminopeptidase. This effect can be overcome by an equimolar amount of manganous ion. In Table IV the activity of the connective tissue leucine aminopeptidase toward Leu-Leu in the presence of copper and manganous chloride is shown. The partial protection by Mg^{2+} is nonspecific since Ca^{2+}, for example, has the same effect.

The copper ion behaves like a competitive inhibitor of the connective tissue leucine aminopeptidase. The K_M obtained with Leu-Tyr as substrate (pH 8.0) is 8×10^{-4} M and the inhibitor constant (with 5×10^{-4} M copper) is 5×10^{-3} M (Figure 7).

The pH Optimum of Connective Tissue Leucine Aminopeptidase. For this experiment lyophilized DEAE II samples were dissolved in Tris buffers ranging in pH

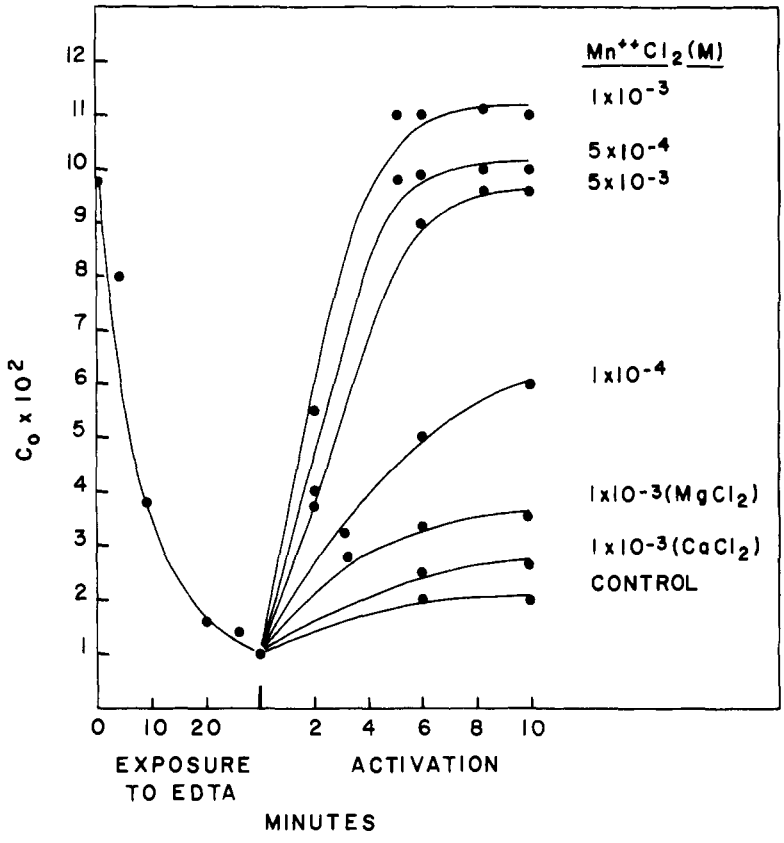

FIGURE 6: Inactivation of Leu-Leu activity from bovine fibroblasts and reactivation by various $MnCl_2$ concentrations as well as $MgCl_2$ and $CaCl_2$, all at 40°.

from 6.0 to 10.0. The substrates were also dissolved in buffer and the pH was accurately adjusted with small amounts of 1 N NaOH or HCl. Substrate concentrations were varied from 0.4 to 7 mM. The sensitivity of the ninhydrin method employed here permits good data to be obtained at low substrate concentrations. (The method of Grassmann and Hyde (1929) requires 50 mM substrate. Accordingly the pH optimum of leucine aminopeptidase is given to be 9.1 or 9.3, a nonphysiological pH.) Connective tissue leucine aminopeptidase (Figures 8 and 9) has the greatest affinity for the substrate at pH 7.5. Peptide concentration in tissues can safely be assumed to be lower than 0.4 mM, so that under physiological conditions, V_{max} lies in the range of the tissue pH. It also lies in the range of the pK value of the amino groups of most peptides (7.1–7.8). The break in the curve (Figure 9, pK_M *vs.* pH) at pH 7.6 designates a pK of a group either on the substrate or the enzyme, a function which is probably important for substrate binding. The pK at pH 8.0 indicates an ionizing group on the enzyme–substrate complex, while a slope of one suggests that a unit change in net charge occurs during substrate binding (Dixon and Webb, 1964).

This leads to the question of physiological significance of the neutral peptide hydrolases in connective tissue. It would be of interest to know how much enzyme is present in relation to the endogenous substrates available and whether the enzyme is present in its active form in the tissue rather than activated during extraction and purification by removal of inhibitors.

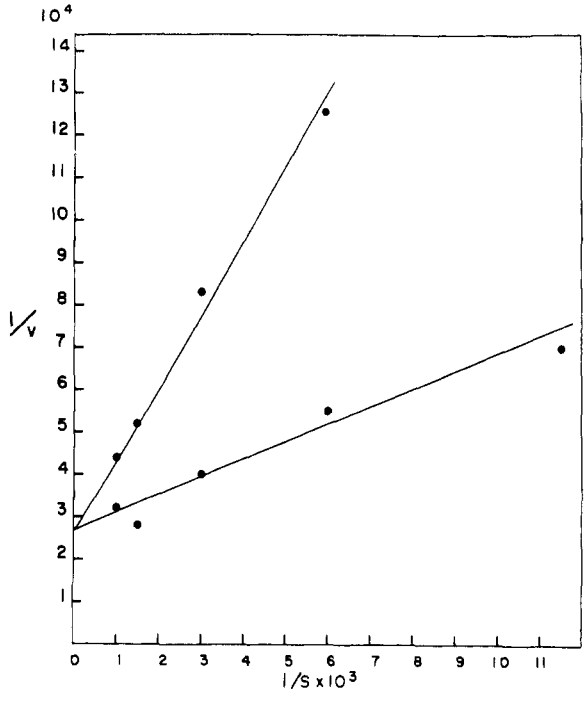

FIGURE 7: Inhibition of connective tissue aminopeptidase in the presence of 5×10^{-3} M copper chloride. The upper line represents the inhibited reaction, the lower is the control without copper.

TABLE III: The Effect of Various Ions on the Peptide Hydrolases from Bovine Connective Tissue.[a]

Activity	Tissue	Stimulators, Inhibitors										
		Mn^{2+}	Mg^{2+}	Co^{2+}	Ca^{2+}	Cd^{2+}	Zn^{2+}	Cu^{2+}	CN^-	CysSH	EDTA	PP_i 10^{-2} M
Leucine aminopeptidase	Pulp	+++	0	0	0	−	−	−−	+0	0	−−−[b]	−
	Intestine	+++	+++				−	−−	−−		−−−	
Gly-Leu dipeptidase	Pulp	0	0	0	T−	0	T−−−		0	0	−−−	−−−
					P−−		P−−					
	Intestine	+++			−−							
	Uterus				−−		P+++					
Prolidase [imidodipeptidase]	Pulp	TO	0	0	0	0	T−−−		0	0	0	
		P++					P++					
	Intestine	+++	0	−	−		−					
Prolinase [iminodipeptidase]	Pulp	T++				−			0		−−−	−−−
	Intestine	+				+						−
Aminotripeptidase	Pulp	−	0		0	−−	0	0	+		++	0
	Bovine muscle	0						0	0	−−		−−
	Intestine	0						0	0	−−		−−

[a] Stimulation (+) and inhibition (−) are given in three degrees of intensity. Tris buffer, T, or phosphate buffer, P, are listed wherever the effect of a particular ion was buffer dependent. Values from other tissues given for comparison are taken from Smith (1951). [b] Only when incubated at 40°. No or little effect at room temperature (see Figure 5).

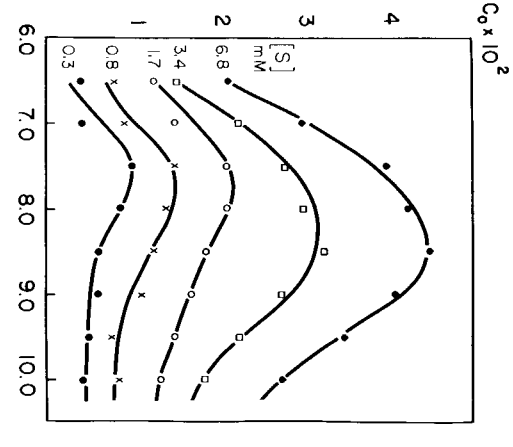

FIGURE 8: Hydrolysis of leucyltyrosine by connective tissue leucine aminopeptidase as a function of pH and substrate concentration. Initial velocities at each pH and substrate concentration (triplicates) were obtained for each point on the graph. The maximal velocity increases with substrate concentration. The highest substrate concentration is only one-eighth the concentration used generally for the assay of leucine aminopeptidase. Under those conditions a pH optimum of 9.1–9.3 has been reported.

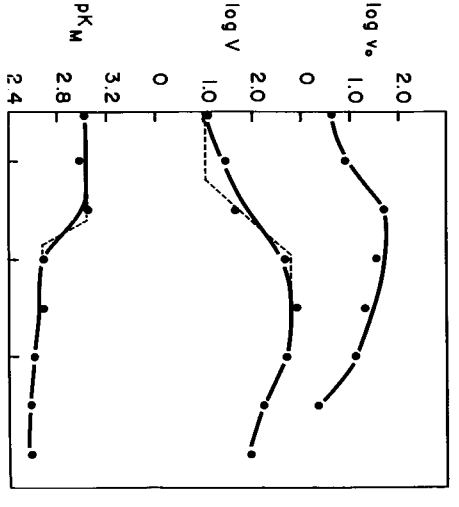

FIGURE 9: pK_M ($-\log K_M$), $\log V_0$, and $\log V_{max}$ were calculated from the data in Figure 8 and plotted against pH. These curves strongly suggest that groups with a pK of 7.6 and 7.9–8.0 are involved in substrate binding and catalysis, respectively. The pK of the substrate used for this experiment (Leu-Tyr) is 7.6 (amino function).

TABLE IV: Connective Tissue Aminopeptidase Activity in the Presence of Cu^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} Ions.[a]

	% Act.
Control	100
5×10^{-3} M Cu^{2+}	10
1×10^{-3} M Mn^{2+} + 5×10^{-3} M Cu^{2+}	10
1×10^{-2} M Mn^{2+} + 5×10^{-3} M Cu^{2+}	80
1×10^{-2} M Mg^{2+} + 5×10^{-3} M Cu^{2+}	40
1×10^{-2} M Ca^{2+} + 5×10^{-2} M Cu^{2+}	30

[a] Ions were added simultaneously and incubated with the enzyme for 15 min at 40° prior to the addition of substrate.

Peptide Hydrolase Activity in Connective Tissue Slices. To determine the activity of neutral peptide hydrolases *in situ*, bovine dental pulp slices (0.5 mm thick) were prepared and suspended in Ringer's solution at pH 7.5 in a Warburg apparatus. The production of free amino acids from peptides became measurable through (oxygen uptake) the addition of L-ophioamino acid oxidase (Meister, 1957). The Warburg flasks were kept at 37° and gas exchange measurements were made every 30 min. Additions during the run were made from side arms (Methods). Results of this experiment are depicted in Figure 10. The oxygen uptake in the control and experimental vessels during the first hour was due to free amino acids in the tissue. Here the boiled and fresh tissues do not vary. The O_2 uptake in the experimental vessels during the next 6 hr must be attributed to a constant, slow release of amino acids from peptides and might well be an indication of protein turnover. The burst of O_2 uptake upon addition of either Leu-Tyr, Leu-Gly-Gly, Leu-Trp, or Leu-Gly after 6 hr shows that aminopeptidases are active in connective tissue at all times and that the amount of enzyme is not a rate-limiting factor in the breakdown of endogenous peptides.

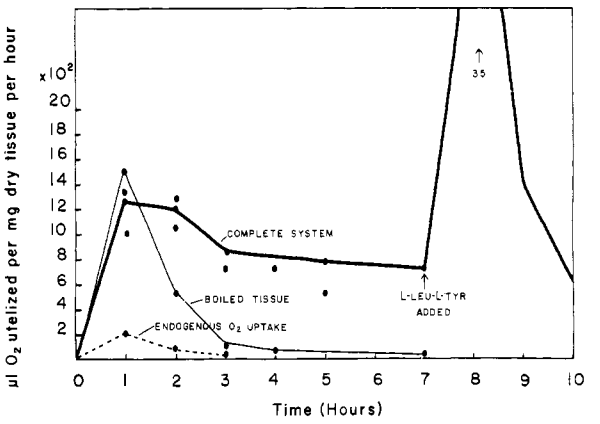

FIGURE 10: The oxygen uptake of bovine dental pulp slices in the presence and absence of L-ophioamino acid oxidase. Endogenous peptides are slowly but constantly produced and converted to amino acids. Exogenous peptides are very rapidly hydrolyzed, indicating that the amount of peptidase is not a limiting factor. The activity was measured in differential manometers (see Methods) at 37°.

Discussion

The aminopeptidase-like activity in connective tissue is 50–80% that of a kidney extract per milligram of protein. By comparing oxygen quotients, Q_{O_2} (an expression of the general metabolic activity of a tissue), the following discrepancy becomes evident. The Q_{O_2} of dental pulp is approximately 1.5 (Fisher and Schwabe, 1961) compared with 20–25 for kidney. A 10–15-fold greater respiratory activity is accompanied by only a 1.5–2-fold or less increase in peptide hydrolase activity. Considering the relative scantness of cells within the ground substance of dental pulp (Schwabe and Kalnitsky, 1965) (in comparison with kidney) a similar conclusion is reached. Kidney cells are densely packed while connective tissue cells are widely separated by a collagenous matrix. Consequently, 1 g of kidney contains more cells than 1 g of dental pulp and the amount of leucine aminopeptidase extracted from pulp must be the product of fewer cells. Therefore it must be concluded that a fibroblast contains more neutral peptide hydrolase activity than kidney cells. From this study it is apparent that neutral peptide hydrolases are a major constituent of mammalian fibroblasts.

The connective tissue extract contains nearly all known peptide hydrolases. (Occasionally an enzyme occurs in two chromatographically distinguishable forms.) Consequently very few, if any, peptides will be released by the connective tissue without being hydrolyzed. As a notable exception peptides, possessing hydroxyproline in the N-terminal position, are very slowly hydrolyzed by connective tissue extract. This is commensurate with the occurrence of urinary hydroxyproline peptides during periods of massive bone resorption, for example.

The ion dependence of the connective tissue enzymes varies slightly from enzymes derived from kidney. Considering the fact that differentiation of glandular tissue (ectoderm) and connective tissue (mesoderm) has occurred so early in the development of an organism, the differences between the peptidases are surprisingly small. A discussion of leucine aminopeptidase, appearing to be the most notably different enzyme, will be found in the next report (Schwabe, 1969).

The Gly-Leu dipeptidase has higher activity in phosphate than in Tris buffer at the same pH. It has been reported (Smith, 1951) that Ca^{2+} is an inhibitor and that phosphate buffer enhances the enzymatic activity. We observed that Ca^{2+} in phosphate inhibits more than in Tris, which would point to a direct effect of phosphate at least in addition to the Ca^{2+} effect. An even more striking example of buffer effects is given by the imidopeptidase activity and its response to Zn^{2+} in Tris *vs.* phosphate buffer of equal ionic strength and pH (Table V).

The molecular weight of the connective tissue peptidases varies from 400,000 for leucine aminopeptidase to 50,000 for a Gly-Ala dipeptidase. This finding is not significantly different from estimates reported in the literature (Dixon and Webb, 1964).

TABLE V: Imidodipeptidase Activity in the Presence of Tris and Phosphate Buffer and Zinc and Manganous Ions.[a]

Buffer (pH 8.0)	Ion (10^{-3} M)	μmoles Hydrolyzed min^{-1} mg^{-1} ml^{-1}
Tris	None	7.3×10^{-5}
	Zn^{2+}	1.0×10^{-6}
	Mn^{2+}	7.0×10^{-3}
Phosphate	None	1.5×10^{-3}
	Zn^{2+}	5.4×10^{-3}
	Mn^{2+}	5.4×10^{-3}

[a] The data obtained by the ninhydrin method were verified by thin-layer chromatography (see Methods).

TABLE VI: Distribution of Peptide Hydrolases in Bovine Fibroblasts, Intestinal Mucosa (Hog), and Rat Kidney.[a]

	Rel Rate		
Substrate	Bovine Fibroblast	Intestine[b] Mucosa	Rat Kidney
Leu-Gly-Gly	1.00	1.00	1.00
Leu-Gly	1.00	0.56	2.00
$LeuNH_2$	0.10	0.20	0.40
Leu-Leu	1.30		2.00
Leu-β-Ala	0.33	0.40	0.01
$GlyNH_2$	0	0.01	
Gly-Leu	2.00	0.50	2.00
Gly-Pro	0.01	0.14	
Gly-Ala	1.00	0.51	
Gly-Gly	0.50	0.22	
Pro-Gly	0.01	0.50	
Pro-Leu	2.00		0.80

[a] The Leu-Gly-Gly hydrolyzing activity was taken as 1 in the three tissues. For assay conditions see Methods section. [b] Estimated from Smith (1951).

A comparison of the distribution of peptidases in various tissues is given in Table VI. Of the first six peptides listed, only Leu-Gly-Gly is degraded by both the aminotripeptidase and the aminopeptidase. The capability of the three tissues to degrade certain peptides varies by more than one order of magnitude in some cases.

By using bovine dental pulp slices it could be clearly shown that the neutral peptide hydrolases are not inhibited *in situ* in contrast to cathepsins, which only become activated at low pH values. The addition of exogenous peptides did further reveal that enzyme concentration is not rate limiting for the degradation of peptides in this tissue. While the peptidases are most probably the catalysts of the terminal chain of events in protein turnover, it appears that enough enzyme is present for additional tasks. The repair and remodeling function of the connective tissue could conceivably rely largely upon the presence of neutral peptide hydrolases.

Acknowledgments

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