formate buffers containing 10 mM cysteine were used throughout. The pH values were determined from duplicate assay mixtures that had been incubated at 37 °C.

Results and discussion. The pH dependence of the activity of collagenolytic cathepsin is illustrated in the Figure. A sharp maximum of activity is observed at pH 3.5. This result is in keeping with the finding of ANDERSON[12], who found the optimum activity near pH 3.5 in rat liver extract, and also with the findings of ETHERINGTON[4], who found the same optimum pH in liver extract and in extracts from the involuting uterus[13]. BAZIN and DELAUNAY investigated collagenolytic cathepsin in turpentine granuloma with very labile acid soluble collagen as substrate and located maximal activity at pH 4.6, with smaller peaks at pH 3.2 and 5.6. We could not find any activity at pH 4.6 when using insoluble collagen as substrate. The collagenolytic activity of the granuloma extracts was strongly inhibited by iodoacetamide and *p*-chloromercuribenzoate.

The activities of the enzymes investigated in relation to the age of the granuloma are summarized in the Table. The activity of cathepsin D reached maximum values in 10-day-old granuloma, while activities of collagenolytic cathepsin and Pz-peptidase have their maximum in 14-day-old granuloma. It is interesting that both Pz-peptidase and collagenolytic cathepsin, which are closely correlated with the degradation of collagen, reach their maximum at the same time.

The collagen concentration in turpentine granuloma declines slowly after 7 days, which is associated with the absorbtion of the granuloma tissue[14]. It is probable that high activities of collagenolytic enzymes in this stage reflect the increased degradation of the collagen component of granuloma tissue.

Zusammenfassung. Die Kathepsin D-Aktivität des kollagenolytischen Kathepsins und der Pz-Peptidase wurden während der Bildung des mittels s.c. Injektion von Terpentinol hervorgerufenen Granuloms bei Ratten verfolgt. Die Kathepsin D-Aktivität erreichte am 10. Tag ihren Höhepunkt, diejenige des kollagenolytischen Katheppsins und der Pz-Peptidase erst am 14. Tag. In dieser Phase ist die Kollagenkonzentration herabgesetzt, während sich eine ausgeprägte Resorption des Granuloms zeigt.

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9 November 1973

[12] A. J. ANDERSON, Biochem. *J. 113*, 457 (1969).
[13] D. J. ETHERINGTON, Eur. J. Biochem. *32*, 126 (1973).
[14] K. TRNAVSKÝ, Z. TRNAVSKÁ and V. LAPÁROVÁ, Experientia *17*, 320 (1961).

Proteinase Inhibitor Activity in Connective Tissues

Many proteinase inhibitors have now been identified in tissues and body fluids of many species[1]. Naturally occurring proteinase inhibitors generally are polypeptides or glycoproteins of varying molecular weights and can be anionic or cationic. In man, at least 7 proteinase inhibitors have been found in blood[2]. We have recently observed that cartilage contains a cationic trypsin inhibitor[3], and now report that other connective tissues contain a similar inhibitor.

The method for the demonstration of the trypsin inhibitor was as follows: weighed aliquots of fresh diced tissue were stirred in a ratio of 1:10 (w/v) overnight at room temperature in 3 M guanidinium chloride (GuCl) buffered with 0.02 M N-morpholinoethane sulfonic acid, pH 6. The extract was decanted, dialyzed against deionized water, and any precipitate which formed was removed by centrifugation. The clear supernate was lyophilized and redissolved in distilled water to a volume 1/10th that of the original extract and used to assay for the presence of trypsin inhibitor. Plates containing denatured fibrinogen dissolved in agar yield a cloudy semi-opaque gel when the agar solidifies. If trypsin is placed into a well in the gel, it diffuses into the agar, digests the fibrinogen, and leaves a clear zone whose radius is exponentially related to the trypsin concentration[4]. If a solution containing a trypsin inhibitor is placed into wells opposite those containing trypsin the inhibitor also diffuses into the agar. At the interface between enzyme and inhibitor the circular ring of clearing is interrupted and a straight line of inhibition forms[5].

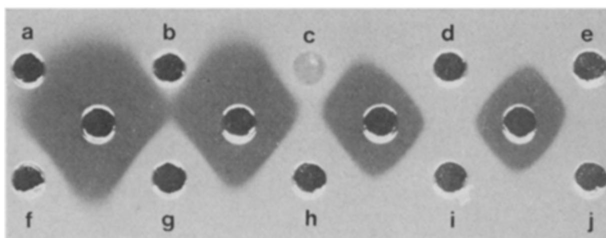

Fig. 1. Agar diffusion method for the detection of trypsin inhibitor. The central row of wells contains trypsin (40 µg/ml). Well a) contains 0.15 M NaCl; well b) dialyzed epiphyseal cartilage extract; well c) dialyzed (concentrated 1:10) epiphyseal cartilage extract; well d) dialyzed (concentrated 1:10) articular cartilage extract; well e) dialyzed (concentrated 1:10) aorta extract; wells f) to j) Trasylol[8] solutions containing 1, 5, 25, 100 and 250 µg/ml respectively. In the Table an inhibitory activity equivalent to that of 100 to 250 µg/ml Trasylol was designated as ++++ and on inhibitory activity equivalent to that of 5 to 25 µg/ml Trasylol was designated as +++.

10 µl of a 40 µg/ml trypsin solution were placed into one well and 10 µl of the test sample into opposite wells to test for trypsin inhibition (Figure 1). This method gives a semi-quantitative estimation of inhibitory activity.

To determine relative charge, an electrophoretic sandwich technique similar to that described for the detection of small amounts of lysozyme was used[6,7]. A cellulose acetate membrane was soaked in barbital

[1] Proceedings of the International Research Conference on Proteinase Inhibitors, Munich, November 1970 (Eds. H. FRITZ and H. TSCHESCHE; Walter de Gruyter, New York, Berlin 1971).
[2] E. WERLE and G. ZICKGRAF-RÜDEL, Z. klin. Chem. klin. Biochem. *10*, 139 (1972).
[3] K. E. KUETTNER, in *The Comparative Molecular Biology of Extracellular Matrices* (Ed. H. C. SLAVKIN; Academic Press, New York, London 1972), p. 358.
[4] G. F. B. SCHUMACHER, J. Reprod. Med. *5*, 13 (1970).
[5] L. CARLSSON and B. KARLSSON, Experientia *28*, 990 (1972).
[6] K. E. KUETTNER, H. L. GUENTHER, R. D. RAY and G. F. B. SCHUMACHER, Calc. Tissue Res. *1*, 298 (1968).
[7] C. ARSENIS, R. EISENSTEIN, L. W. SOBLE and K. E. KUETTNER, J. Cell Biol. *49*, 459 (1971).

buffer, pH 8.6, and up to 2.5 μl of the test sample were applied to the membrane and electrophoresed (20 min, 250 volts). The membrane was then placed face down on a fibrin-containing agar plate and incubated for 30 min at 37 °C. During this time material from the electrophoretic strip diffuses into the agar. A new cellulose membrane previously soaked in a 1 μg/ml trypsin solution was placed on the agar and incubated at 37 °C for 10 min (Figure 2). The membrane was removed and the plate incubated at 37 °C for 5 h. The agar plate was photographed after removing the membrane. The trypsin cleared the agar but where a trypsin inhibitor was present fibrin was not lysed and the agar remained opaque (Figure 3).

Results of this semi-quantitative assay for trypsin inhibition on several tissues is listed in the Table. The results are expressed in an arbitrary grading system of 1–4 and compared with standard solutions of Trasylol[8]. The extracts contain tissue components, including enzymes and proteins, which interfere with the reactions and make precise quantitation difficult. It is of interest, that dentin, an acellular tissue, has inhibitory activity thus suggesting that the inhibitor may be extra-cellular in location. Complete extraction of the tissue proteinase inhibitor requires salt solutions of rather high molarity, perhaps because it is bound to some other material in connective tissues.

Since plasmin is present in endothelial cells[9], and inhibited by Trasylol[8], the distribution of natural proteinase inhibitor activity in aortic wall was assessed. Bovine aorta segments were frozen on a cryostat with the intima

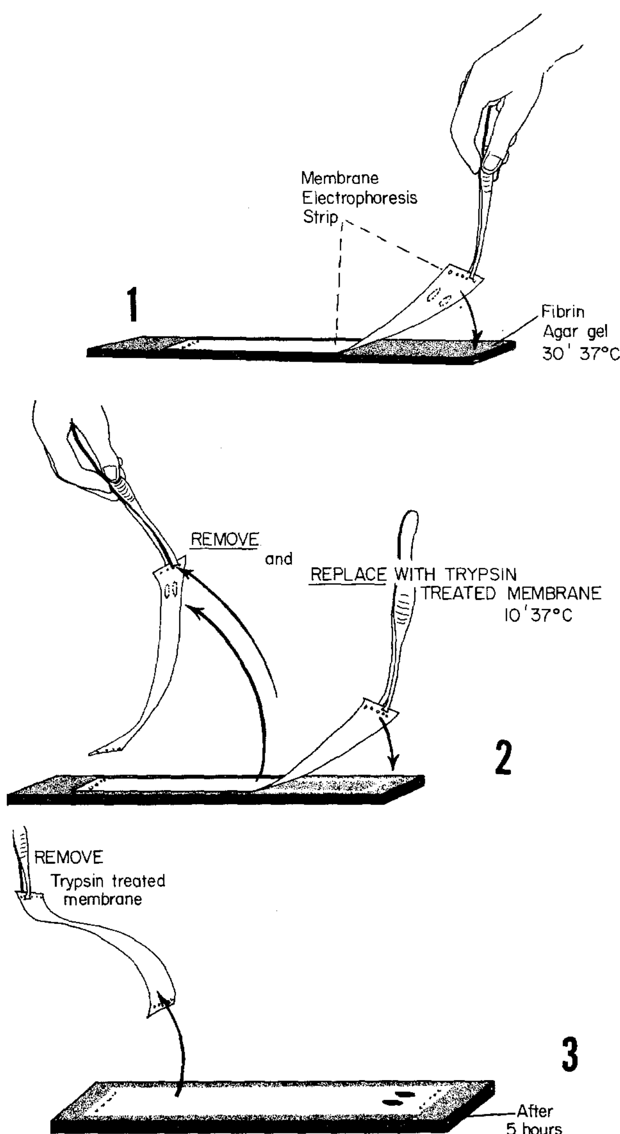

Fig. 2. 'Sandwich' method for the demonstration of trypsin inhibitory activity by microzone electrophoresis. After the sample is electrophoresed, the membrane is placed upsidedown on the surface of an agar gel containing fibrinogen for 30 min at 37 °C. The membrane is then removed, replaced with a new membrane which has been soaked with trypsin, and incubated for 10 min at 37 °C. The membrane is then removed and the agar plate incubated at 37 °C for 5 h. The fibrinogen in the agar is digested and the agar becomes clear except where the inhibitor has migrated.

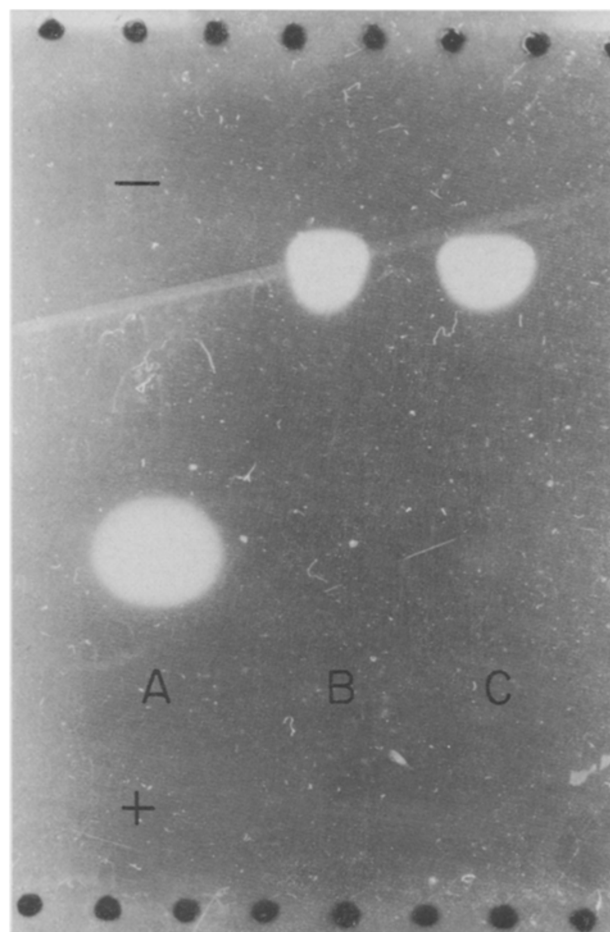

Fig. 3. A photograph of a 'sandwich' for the detection of trypsin inhibitor. The samples were applied at the center of the membrane. Sample A) is soybean trypsin inhibitor; B) Trasylol; and C) cartilage extract. Note that the cartilage inhibitor has an electrophoretic mobility similar to that of Trasylol, which is also a relatively small cationic material.

[8] Trasylol is the trade name for a cationic protease inhibitor obtained from beef lung. This material was a gift of Doctor G. F. B. SCHUMACHER.

[9] G. A. PADGETT and J. G. HIRSCH, Aust. J. exp. Biol. Med. Sci. *45*, 569 (1967).

facing upward and serial frozen sections done. The sections were inserted into pre-weighed bottles containing 3 *M* GuCl. Each bottle contained 100 μm of thickness of the vascular wall. Large amounts of proteinase inhibitor were found in each of 10 such segments cut through the thickness of the arterial wall. Thus, unlike the plasminogen activator the proteinase inhibitor appears to be fairly uniformly distributed through the aortic wall.

Presence of trypsin inhibitory activity in selected canine connective tissues

Tissues	Trypsin Inhibitory activity in supernate of dialyzed extract
Pulmonary Vein	++++
Cornea	++++
Aorta	++++
Articular cartilage	++++
Epiphyseal cartilage	+++
Knee joint ligament	+++
Tooth pulp	+++
Iris	++
Vena cava	++
Femoral periosteum	++
Trachea	++
Dentin	++
Foot tendon	++
Fascia	+
Heart valves	+
Pulmonary artery	+
Sclera	+
Skin	±
Vitreous humor	±
Lens	±

Neither the relationships between the material assayed here and other tissue cationic proteinase inhibitors nor the spectrum of enzymes it inhibits is known. However, the observation that a cationic proteinase inhibitor is widely distributed in normal connective tissues is of interest. It suggests that this inhibitor may play a role in regulating the metabolism of normal and diseased connective tissues. Preliminary data indicate that this proteinase inhibitor can inhibit the proteolytic activity of buffy coats of blood leukocytes.

We have recently isolated this proteinase inhibitor from cartilage and aorta by affinity chromatography. Current experiments aim at characterizing the molecule and determining the spectrum of enzymes which it inhibits.

Zusammenfassung. Extrakte verschiedener Bindegewebe wurden auf ihre Proteasehemmung hin elektrophoretisch untersucht. Alle Gewebe zeigten kationisch wandernde Aktivität und ihre Anwesenheit im zellfreien Dentin lässt den Schluss zu, dass das Material extrazellulär lokalisiert ist.

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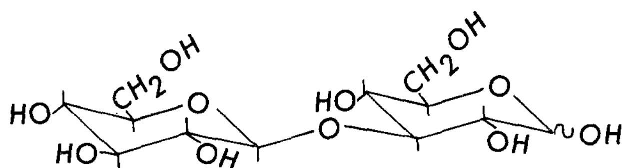
[10] We wish to thank Mrs. P. LISK and Mrs. H. E. GUENTHER for their skilled technical assistance. We are particularly grateful to Mrs. A. CROXEN for her assistance in the trypsin inhibitor assays, and to Mrs. M. GILBERT for preparation of the manuscript.

[11] This work was supported by the National Institute of Health, Grants No. AM-09132, HL-14968 and a Grant from the Chicago Heart Association.

Laminaranase Activity in the Crystalline Style of the Surf Clam (*Spissula solidissima*)

The crystalline style of the surf clam, *Spissula solidissima*, is an anatomical structure containing various enzymes[1] immobilized on a glycoprotein substrate. The function of the style, located in the stomach of bivalvia, is to triturate, with mortar and pestle-like action[1], particulate diatomaceous and algal food matter while at the same time initiating enzymic hydrolysis of carbohydrate polymers through both exo and endo action to ultimately produce glucose as an energy source. The term 'crystalline' is used to designate the occurrence of calcium oxalate crystals in the cortical layers of the style[2] which probably assist grinding of the food. The entire style may apparently dissolve and disappear under adverse conditions, such as inadequate food supply, only to again reform[3].



Laminarabiose

We have examined the stomach and intestines for various carbohydrase activities, and prepared an acetone precipitated powder, yield 0.7%, with the various carbohydrase activities shown in the Table. Afterwards we found that the style itself is the source of this activity, and could be prepared in 10% yield (60% of the style material on a dry basis), by triturating the style with a minimum of water in a mortar and pestle, centrifuging, and precipitating with an equal volume of cold acetone added to the supernatant. The acetone-dried friable, white powder, (N content 9.5%, glycose content 5.9% dry basis) was identical in turn-over number and activities to that shown for the previous material in the Table. Between 16 and 50 mg glucose can be generated from laminaran by one gram of the material per min, and we wish to announce that this seems to be the major carbohydrase activity of the style.

The preparation is stable indefinitely at 0° and is without loss of activity (turn-over numbers determined

[1] G. OWEN, in *Physiology of Mollusca*. II (Eds. K. M. WILBUR and C. M. YOUNGE (Academic Press, New York 1966).

[2] J. C. NELSON, Biol. Bull. *49*, 86 (1925).

[3] M. R. SCHMEER, Science *144*, 413 (1964).