The Nature of the Collagenolytic Cathepsin of Rat Liver and its Distribution in other Rat Tissues

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1. An enzyme present in rat liver extracts degraded insoluble collagen maximally at pH 3.5. Collagenolytic activity was more abundant in kidney, spleen and bone marrow and was also present in decreasing concentrations in ileum, lung, heart, skin and muscle. 2. The crude collagenolytic cathepsin was activated by cysteine and dithiothreitol, but not by 2-mercaptoethanol. Iodoacetamide, p-chloromercuribenzoate and 7-amino-1-chloro-3-L-tosylamidoheptan-2-one hydrochloride inhibited the enzyme. Zn^{2+}, Fe^{3+} and Hg^{2+} ions were strongly inhibitory, but Ca^{2+}, Co^{2+}, Mg^{2+} and Fe^{2+} ions had little or no effect. EDTA was an activator of the enzyme. Inhibitors of cathepsin B were found to enhance collagenolysis, but phenylpyruvic acid, a cathepsin D inhibitor, inhibited the enzyme. Di-isopropyl phosphorofluoridate had no effect. 3. Collagenolysis at pH 3.5 and 28°C was restricted to cleavage of the telopeptide region in insoluble collagen, and the material that was solubilized consisted mostly of α-chains. 4. The collagenolytic cathepsin was separated from cathepsins B2 and D by fractionation on Sephadex G-100 and a partial separation from cathepsin B1 was obtained by chromatography on DEAE-Sephadex. 5. The function of the collagenolytic cathepsin in the catabolism of collagen is discussed in relation to the action of the other lysosomal proteinases and the neutral collagenase.

Measurement of turnover rates for collagen has shown that this protein is relatively inert metabolically and is particularly resistant to proteolytic degradation (Neuberger & Slack, 1953). However, certain physiological changes, e.g. the post-partum involution of the uterus, are accompanied by a very rapid removal of collagen (Harkness & Morallee, 1956). In all those tissues where the collagen is being rapidly resorbed, the use of tissue-culture techniques has revealed the presence of a specific neutral collagenase, that can cleave all three chains in the collagen triple helix (for review see Gross, 1970). Eisen *et al.* (1971) have shown that a collagenase is a normal constituent in human skin, but that it is usually present in an inactivated form because of complex-formation with specific serum proteins. This suggests that the catabolism of collagen is under a dual control, first by control of the rate of synthesis of the collagenase and secondly from the subsequent action of serum inhibitors on the active enzyme.

Electron-microscope studies on the involuting mouse uterus have revealed the presence of macrophages that contain secondary lysosomes laden with collagenous material (Parakkal, 1969). It would thus appear that the uterine neutral collagenase does not completely solubilize the collagen in the extracellular space, but instead causes fragmentation of the collagen fibrils. This restricted action may be due to the rapid removal of collagenase by serum inhibitors.

However, Leibovich & Weiss (1971) have shown that insoluble collagen is resistant to cleavage by rheumatoid synovium collagenase. The macrophage lysosomes provide a very efficient means for the second-stage, intracellular digestion of collagen, as indicated by the rapid depletion of these digestive vacuoles. A two-stage mechanism for the breakdown of cartilage was suggested by Fell (1969), and such a mechanism may operate for other structural collagenous components.

Collagenolytic activity in the acid pH-range has been demonstrated previously by several groups of workers (Frankland & Wynn, 1962; Woessner & Brewer, 1963; Schaub, 1964; Bazin & Delaunay, 1966; Anderson, 1969; Hirayama *et al.*, 1969). Frankland & Wynn (1962) were the first to demonstrate the lysosomal origin of the enzyme and Anderson (1969), who used a preparation of insoluble collagen as the substrate, found that collagenolysis was restricted to near pH 3.5. Much of the other published data have been collected in studies with the more labile acid-soluble collagen as the substrate. With such a preparation, Bazin & Delaunay (1966) have demonstrated up to three different acid pH optima for collagen breakdown for both normal liver and granuloma extracts. They observed maximal degradation at pH 4.6.

The present work was undertaken to characterize further the nature of the collagenolytic cathepsin,

with insoluble collagen from bovine tendons as the substrate.

Experimental

Preparation of tissue extracts

Wistar rats (Carworth CFE strain, 4–6 months old) were stunned, decapitated and bled.The organs were rapidly excised and were rinsed thoroughly in ice-cold 0.9% NaCl. Each organ was homogenized in 9 vol. of 0.1 M-sodium acetate buffer, pH 5.0, containing 0.1% (v/v) Triton X-100. The homogenates were prepared on ice by using a Polytron (Kinematica G.m.b.H., Lucerne, Switzerland) operated at full speed for several short periods with intermittent cooling. Bone marrows were extruded from the long bones of pairs of rats by compressed N_2, and were combined and homogenized in a motor-driven Potter–Elvehjem-type homogenizer. Each extract was stirred in the cold for 2 h and then centrifuged at 12000g for 15 min.

For some experiments the enzyme in the liver extracts was concentrated by precipitation with $(NH_4)_2SO_4$. The protein that precipitated between 40% and 70% saturation was collected by centrifugation at 12000g for 15 min. The crude precipitated enzyme was resuspended in water and then exhaustively dialysed against large volumes of water. Any undissolved protein was removed by centrifugation at 12000g and the solution was then adjusted to 1 ml/g of the original liver. The enzymic material for the chromatographic separations was obtained in a highly active form by autolysing extracts of liver at pH 3.5 (McDonald et al., 1970). The material that precipitated with $(NH_4)_2SO_4$ between 40% and 70% saturation was collected by centrifugation at 12000g for 15 min, dialysed thoroughly against several large volumes of water and freeze-dried.

Determination of collagenolytic activity

The method was a modification of that described by Anderson (1969). Bovine tendon collagen (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was suspended at 10 mg/ml in a solution of 0.35% acetic acid in 25% (v/v) glycerol. The collagen was dispersed with the Polytron homogenizer as described for the tissue extracts. This fine suspension, with a pH of approx. 3.5, remained stable for at least 2 weeks. The collagenolytic cathepsin was assayed at pH 3.5 in 15 ml conical centrifuge tubes. Each tube contained 0.4 ml of the collagen suspension, 0.6 ml of 0.2 M-sodium formate buffer containing 10 mM-cysteine and 0.2 ml of the enzyme sample. The tubes were incubated at 37°C for 3 or 16 h depending on the enzyme activity. At the end of the incubation period the tubes were centrifuged at 2900g for 10 min

to remove the residual collagen. Blank readings were obtained from assay mixtures that had been prepared on ice and then centrifuged without prior incubation.

The amount of collagen degraded was calculated from the concentration of hydroxyproline in solution, which was assumed to be 14% (w/w) of the collagen. A sample (0.2 ml) of the supernatant was transferred to a 14 ml McCartney bottle and 0.2 ml of conc. HCl was added. The bottles were then sealed with Teflon-lined metal screw caps, and the hydrolysis was performed at 110°C for 16 h. After cooling, 9.6 ml of 0.04 M-citric acid–0.32 M-NaOH was added to each bottle. A small amount of Methyl Red in this alkali diluent indicated that the final pH was between 5 and 6. Hydroxyproline was determined by the method of Grant (1964) by using the Technicon AutoAnalyzer.

The stability of the collagen substrate was tested by incubating the buffered collagen for 16 h at 37°C and then adjusting to pH 7.5–8 with 1 ml of 2% (w/v) NaHCO₃. Trypsin (0.2 ml; 1 mg/ml) was then added and the collagen was further incubated at 37°C for 2 h. The amount of collagen solubilized did not exceed 1% (w/w) of the total.

The solubilization of collagen by the liver enzyme was linear up to 1 μg of hydroxyproline/ml in the
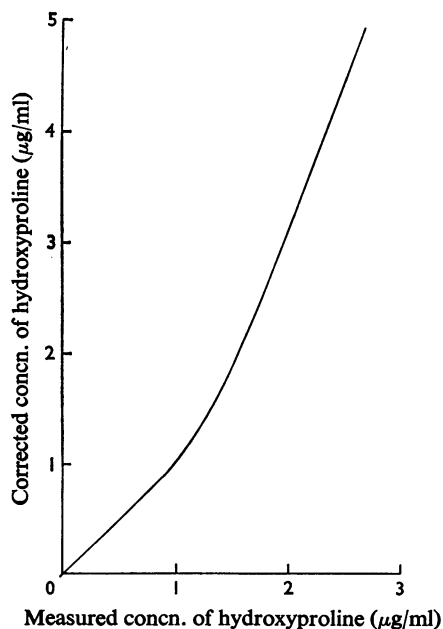

Fig. 1. *Calibration curve for correcting the measured concentration of hydroxyproline to a scale that is linear to the enzyme concentration*

Experimental details are given in the text.

Table 1. *Distribution of cathepsin D and collagenolytic cathepsin in different rat organs*

Six rats, 4–5 months old, were used and the results are expressed as the mean activity±s.e.m. Experimental details are given in the text.

Organ	Protein (mg/ml)	Acid proteinase activity (μg of tyrosine released/h per mg)	Collagenolytic cathepsin activity (μg of hydroxyproline released/h per mg)
Liver	7.69±0.27	62± 4.7	8.7 ±1.2
Kidney	6.46±0.36	167± 18	27.2 ±1.4
Spleen	7.13±0.21	675± 39	23.9 ±1.9
Bone marrow*	7.34±0.62	492±110	15.0 ±2.7
Ileum	2.58±0.39	194± 24	15.3 ±4.7
Lung	9.80±0.78	62± 5.9	3.7 ±0.3
Heart	4.64±0.48	83± 8.7	2.6 ±0.1
Skin	1.67±0.29	126± 22	2.4 ±0.1
Muscle	3.85±0.36	29± 3.3	0.48±0.05

* Results obtained for five pairs of rats.

diluted hydrolysate (11 % of the collagen solubilized). To adjust for this loss of linearity a correction curve was constructed from different concentrations of an autolysed liver preparation.

Determination of protein

The Technicon AutoAnalyzer was adapted for the method of Lowry *et al.* (1951) and bovine serum albumin was used as the standard. The effluent from the chromatography columns was monitored at 280nm with an LKB Uvicord II spectrophotometer.

Effect of pH and buffer ions on collagenolytic activity

A pH–activity curve was constructed from the crude 40–70%-satd. $(NH_4)_2SO_4$ fraction of rat liver; 0.2M-sodium formate buffers containing 10mM-cysteine were used throughout. The pH values were determined from replicate assay mixtures that had been incubated at 37°C. The effect of different buffer ions on the activity of the enzyme was investigated at pH3.5.

Inhibitor and activator studies

The compounds were incorporated directly into formate buffers. Because of the importance of cysteine as an activator of the enzyme, most of these compounds were also tested in combination with this activator. Normally the crude $(NH_4)_2SO_4$ fraction was not preincubated with the test compounds. Di-isopropyl phosphorofluoridate and p-chloromercuri-benzoate were exceptions and with these the procedure was as follows: the enzyme was preincubated for 30min at room temperature in 0.1 M-potassium phosphate buffer, pH7.5, with 2mM-di-isopropyl phosphorofluoridate dissolved in propan-2-ol. A

separate control with propan-2-ol was treated similarly. An aqueous solution of p-chloromercuribenzoate (1.7mM) was tested under the same preincubation conditions. The assays for enzyme activity were then performed by the standard method.

Investigation of the action of the crude enzyme on bovine collagens

Calf skin tropocollagen was prepared as described by Jackson & Cleary (1967) and was dissolved in 0.01M-acetic acid. Replicate 3ml reaction mixtures were prepared, each containing 10mg of either insoluble collagen or tropocollagen in the standard assay buffer. The incubations were performed at 28°C for various times up to 19h. Calf skin tropocollagen and the supernatant material from the tendon collagen incubation mixtures were freed of enzymic protein (Drake *et al.*, 1966) and then analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis by the method of Sykes & Bailey (1971).

Column chromatography

Exclusion chromatography was performed on a column (1.8cm × 65cm) of Sephadex G-100 [Pharmacia (G.B.) Ltd., London, W.13, U.K.] with a similar buffer system to that used in the preparation of cathepsin B (Otto, 1971): 0.15M-sodium acetate buffer, pH4.6, containing 0.1M-NaCl, 4mM-2-mercaptoethanol and 1mM-EDTA. A portion (30mg) of the freeze-dried rat liver autolysate, dissolved in 2ml of the same buffer, was applied to the column, which was then developed at a flow-rate of 6ml/h. Fractions (2ml) were collected.

DEAE-Sephadex A-50 was used for ion-exchange chromatography. A column (2.1cm × 24cm) was equilibrated with 25mM-sodium phosphate buffer,

pH 7.4, containing 4 mM-2-mercaptoethanol and 1 mM-EDTA. Then 50 mg of the freeze-dried rat liver autolysate, dissolved in the same buffer, was loaded on the column. A linear salt gradient was established between a mixing flask containing 200 ml of the initial buffer and a reservoir containing 200 ml of 0.4 M-NaCl in the buffer. A flow rate of 14 ml/h was maintained and fractions (4.4 ml) were collected.

Enzyme assays

Acid proteinase (cathepsin D) was determined by using a 2% (w/v) solution of haemoglobin (Worthington) in 0.2 M-sodium formate buffer, pH 3.5. The enzyme sample (0.2 ml) was incubated in 2 ml of reaction mixture at 45°C. The reaction was stopped by the addition of 2 ml of 10% (w/v) trichloroacetic acid
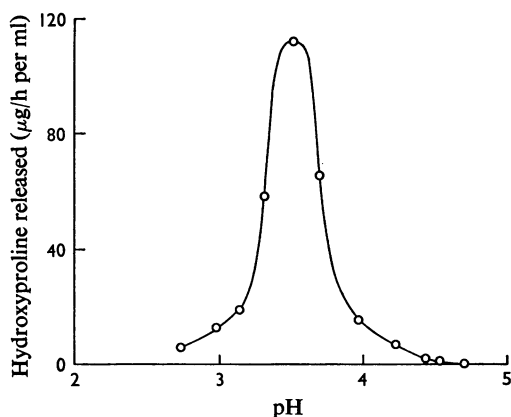
and soluble tyrosine was determined in the filtrate by the method of Hanley et al. (1966).

Cathepsin B activity was determined with α-N-benzoyl-L-arginine amide by the method of Otto & Schepers (1967). α-N-Benzoyl-DL-arginine-β-naphthylamide hydrochloride was used for the determination of cathepsin B1 (Otto, 1971) by the method of Ali et al. (1967).

Concentration of sodium

NaCl in the effluent fractions was determined with a Unicam SP.90 atomic-absorption spectrophotometer.

Results

Distribution of the collagenolytic cathepsin

The assay system for the determination of collagenolysis proceeded at a linear rate up to 11% of the total. Fig. 1 gives the correction curve that was used for higher values.

Table 1 shows the concentrations of the collagenolytic cathepsin and acid proteinase activity in nine different rat organs, with the respective protein concentrations. The greatest amounts of collagenolytic activity were located in the kidney and spleen, with lesser concentrations in the ileum, bone marrow, liver and lungs. The lowest concentrations were found in muscle, skin and heart. Acid proteinase activities followed the same general pattern, but clearly the relative concentrations in the kidney and spleen extracts were different from the respective concentrations of the collagenolytic cathepsin.

The liver contained only moderate collagenolytic activity, but in terms of total activity it presented the richest and most convenient single source from which to prepare sufficient quantities of the enzyme.


Fig. 2. *Effect of pH on the solubilization of insoluble collagen by the crude 40–70%-satd. $(NH_4)_2SO_4$ fraction of the rat liver extract*

The incubation mixtures were buffered with sodium formate at a final concentration of 0.1 M and included 5 mM-cysteine as activator.

Properties of the enzyme

The pH–activity curve, constructed by using formate buffers containing 5 mM-cysteine, showed a sharp

Table 2. *Effect of different buffer ions on collagenolysis*

The 40–70%-satd. $(NH_4)_2SO_4$ fraction of the rat liver extract was used. Cysteine was included to 5 mM final concentration. Experimental details are given in the text.

Buffer	Concentration (M)	Soluble hydroxyproline (μg/h per ml)
Sodium formate	0.025	342
	0.05	322
	0.10	293
	0.25	57
Sodium acetate	0.10	179
Ammonium formate	0.10	232
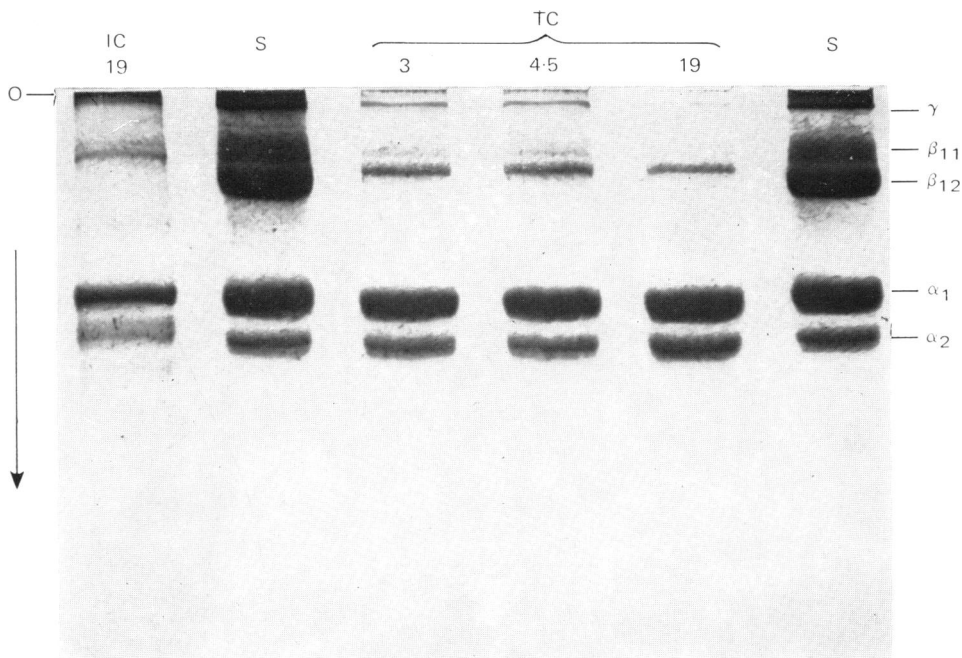
EXPLANATION OF PLATE I

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of enzymic digests of calf skin tropocollagen and of the material enzymically solubilized from insoluble bovine tendon collagen

The 40–70%-satd. $(NH_4)_2SO_4$ fraction of the rat liver extract was used as the source of the enzyme. Untreated calf skin tropocollagen was used as the standard (S). The incubations were performed at 28°C and for 3, 4.5 or 19h for calf skin tropocollagen (TC); insoluble bovine tendon collagen (IC) was incubated for 19h only. The collagen consisted mostly of α-chains after treatment with the crude liver enzyme. O denotes the origin.

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Table 3. *Effect of various compounds on collagenolysis*

The 40–70%-satd. $(NH_4)_2SO_4$ fraction of the rat liver extract was used as the enzyme. The results are expressed as percentages of the activity of the control sample assayed in the presence of 5mM-cysteine. Experimental details are given in the text.

Compound	Concn. (mM)	Concn. of cysteine (mM) ...	Activity (% of control)	
			0	5
No addition	—		34	—
Cysteine	5		—	100
Dithiothreitol	2		47	—
2-Mercaptoethanol	5		30	72
Iodoacetamide	12.5		0	0
p-Chloromercuribenzoate	1.7*		8	—
7-Amino-1-chloro-3-L-tosylamido- heptan-2-one hydrochloride (TLCK)	2		—	4
Mercuric chloride	2.5		15	—
Calcium chloride	5		35	86
Magnesium acetate	5		33	104
Cobalt chloride	5		35	83
Zinc acetate	5		32	37
Ferrous sulphate	5		21	75
Ferric chloride	5		1	—
EDTA	5		33	125
Phenylpyruvic acid	4		27	47
	10		25	44
6-Aminohexanoic acid	10		33	103
	50		33	129
Chloroquine	20		56	141
Arginine	10		38	118
	50		41	156
Di-isopropyl phosphorofluoridate	2*		—	96

* Concentration of the compound in the preincubation buffer.

maximum at pH3.5. No activity was detectable at pH4.5 (Fig. 2). The influence of different buffers on the activity of the enzyme at pH3.5 is shown in Table 2.

Table 3 records the effects of different compounds on the activity of the collagenolytic cathepsin in the crude liver preparation. The strong activating effect of cysteine could not be repeated with 2-mercapto-ethanol; dithiothreitol was only partially effective. Iodoacetamide and p-chloromercuribenzoate were strongly inhibitory. Hg^{2+} and Fe^{3+} abolished all activity, whereas Fe^{2+} and Zn^{2+} gave partial inhibition. Arginine, chloroquine and 6-aminohexanoic acid all enhanced activity.

When tropocollagen was digested with the crude liver enzyme most of the β-chains disappeared and there was a corresponding increase in the amounts of the α-chains as observed by electrophoresis in sodium dodecyl sulphate – polyacrylamide gels (Plate

1). These derived α-chains in the enzyme-treated tropocollagen moved slightly ahead of the standard. The material solubilized from tendon collagen consisted mostly of α-chains, but a small quantity of the β component was also released into solution.

Chromatographic studies

The autolysed rat liver preparation was used for chromatography as it contained considerably less non-enzymic protein than that obtained by direct $(NH_4)_2SO_4$ fractionation of the liver extract. Fig. 3 shows the separation of cathepsins B1, B2, D and the collagenolytic cathepsin on Sephadex G-100. When the rat liver autolysate was fractionated on DEAE-Sephadex the collagenolytic cathepsin was again eluted in a wide peak, but with maximal activity preceding the peak for cathepsin B1 (Fig. 4).
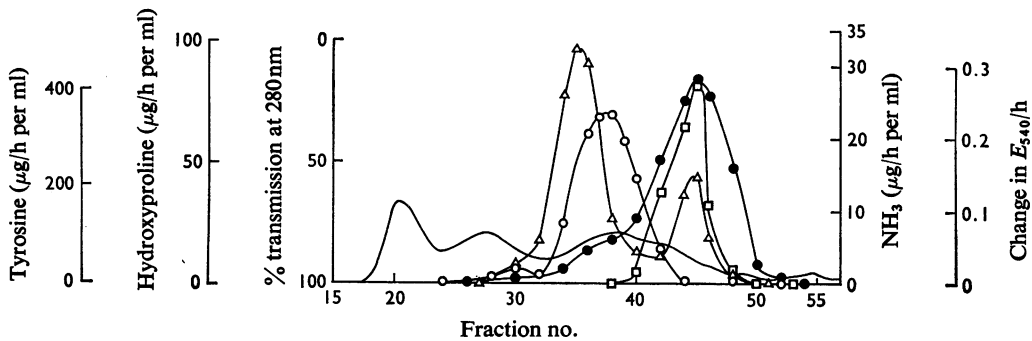
Fig. 3. *Sephadex G-100 chromatography of an autolysed rat liver preparation*

The autolysed rat liver preparation (30mg) was chromatographed on a column (1.8cm×65cm) of Sephadex G-100, which was equilibrated with a buffer consisting of 0.15M-sodium acetate, containing 0.1M-sodium chloride, 4mM-2-mercaptoethanol and 1mM-EDTA, pH4.6. Fractions (2ml) were collected at a flow rate of 6ml/h. ——, % Transmission at 280nm (protein); ○, μg of tyrosine/h per ml (cathepsin D); △, μg of NH_3/h per ml (cathepsins B1 and B2, with α-N-benzoyl-L-arginine amide as the substrate); □, ΔE_{540}/h (cathepsin B1, with α-N-benzoyl-DL-arginine-β-naphthylamide hydrochloride as the substrate); ●, μg of hydroxyproline/h per ml (collagenolytic cathepsin).
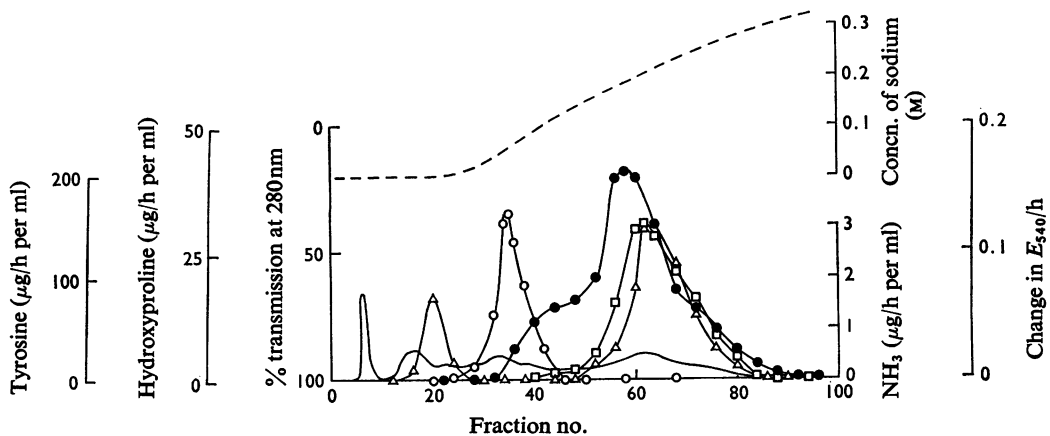

Fig. 4. *DEAE-Sephadex chromatography of an autolysed rat liver preparation*

The autolysed rat liver preparation (50mg) was chromatographed on a column (2.1cm×24cm) of DEAE-Sephadex A-50, equilibrated with 25mM-sodium phosphate buffer, pH7.4, containing 4mM-2-mercaptoethanol and 1mM-EDTA. The column was developed with a linear salt gradient to 0.4M-NaCl in the same buffer. Fractions (4.4ml) were collected at a flow rate of 14ml/h. ----, Concn. of Na^+; ——, % transmission at 280nm (protein); ○, μg of tyrosine/h per ml (cathepsin D); △, μg of NH_3/h per ml (cathepsins B1 and B2, with α-N-benzoyl-L-arginine amide as the substrate); □, ΔE_{540}/h (cathepsin B1, with α-N-benzoyl-DL-arginine-β-naphthylamide hydrochloride as the substrate); ●, μg of hydroxyproline/h per ml (collagenolytic cathepsin).

Discussion

The rat collagenolytic cathepsin was present in all tissues investigated and it was most abundant in those tissues which were also rich in the acid proteinases, of which the predominant enzyme is cathepsin D.

The low pH range of activity would prevent it from acting freely outside the cell, as has been postulated for the neutral collagenase (Eisen *et al.*, 1970; Gross, 1970). However, for the attack on fibrin fibrils by neutrophils, these cells first attach themselves to the

fibrils (Riddle & Barnhart, 1964). This suggested that an acidic medium could be generated within the restricted intervening space, which enabled breakdown of the substrate to occur by secreted lysosomal proteinases (Riddle & Barnhart, 1964; Woessner, 1965). If this does represent an important extracellular function for lysosomal proteinases, no such mechanism has been shown to occur for the breakdown of collagen fibrils. The increasing evidence for the production of a neutral specific collagenase to initiate the process of collagenolysis favours the argument that the lysosomal acid proteinases complete the digestion intracellularly. However, lysosomal enzymes, and particularly cathepsin D, have been implicated in the extracellular breakdown of the protein–polysaccharide component of cartilage, particularly in arthritic conditions (Barrett, 1968), but for this material the protein moiety is most sensitive to hydrolysis at pH5 (Dingle et al., 1971). If the collagenolytic cathepsin is secreted into the extracellular space, then its action would be very restricted in view of the enzyme's requirement for a lower pH range for activity.

The collagenolytic cathepsin displayed maximal activity at pH3.5 and had no action on insoluble collagen between pH4.5 and 5, in contrast with the collagen-degrading enzyme investigated by Bazin & Delaunay (1966, 1971). These authors, using acid-soluble collagen as the substrate, located maximal activity in rat liver and granuloma extracts at pH4.6, with smaller peaks at pH3.2 and 5.6. Only the activity at pH4.6 was investigated in detail. It cleaved collagen primarily at the telopeptide and in its other properties it appeared to be similar to the enzyme of the present study (Bazin & Delaunay, 1971). The smaller peak at pH3.2 has not been investigated, but from its similar pH optimum it may be identical with the enzyme that degraded insoluble collagen. Anderson (1969), who devised an assay system with insoluble collagen, but in the absence of any added cysteine to activate the enzyme, obtained a similarly shaped curve exhibiting maximal activity also at pH3.5.

The behaviour of the crude cathepsin with activators and inhibitors produced some surprising results. Cysteine was a very efficient activator, but 2-mercaptoethanol had no effect. This suggested that cysteine may be acting as a chelator of metal ions (Dixon & Webb, 1964) rather than as a reducing agent, a possibility that was supported by the activating effect of EDTA. The special importance of cysteine made it necessary to re-check most of the compounds in combination with this activator. The enzyme was strongly inhibited by iodoacetamide and p-chloromercuribenzoate, but the chemical basis of the inactivation was not investigated. In the chromatographic separations, the inclusion of EDTA and 2-mercaptoethanol in the buffers had a marked protective action on the enzyme. Whether this effect

was derived from one or both of these additives was not determined.

The enzyme was partially inhibited by phenylpyruvic acid, which has been shown to be an inhibitor of cathepsin D (Barrett, 1967). This finding could support a role for cathepsin D in the breakdown of insoluble collagen, but in the chromatographic separations, cathepsin D on its own was found to have no action. Bazin & Delaunay (1970) have also found that cathepsin D will not degrade acid-soluble collagen.

Of the metal ions tested, Fe^{3+}, Hg^{2+} and Zn^{2+} were inhibitory and Zn^{2+} ions appeared to block directly the activating effect by cysteine. Anderson (1969) showed that copper ions were also strongly inhibitory to the enzyme in his assay system. Chloroquine, 6-aminohexanoic acid and arginine have been found to inhibit cathepsin B activity in cartilage (Ali, 1964; Ali et al., 1967). The influence of these substances on collagenolysis generally gave a marked activation, which suggested that cathepsin B itself had no action upon collagen. However, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one hydrochloride, which is a potent inhibitor of cathepsin B1 (Otto, 1971; Keilova, 1971), was also shown to inhibit collagen breakdown strongly. The enzyme was not inhibited by di-isopropyl phosphorofluoridate.

The action of crude preparations of the collagenolytic cathepsin on bovine collagens was investigated by electrophoresis in sodium dodecyl sulphate-polyacrylamide gels. The formation of α-chains from β-chains in calf skin tropocollagen showed that the enzymic cleavage was limited to the cross-linked (telopeptide) end of the tropocollagen molecule. No breakages occurred in the helical region, and therefore the enzyme was quite distinct in its action from the true mammalian collagenases as defined by Gross (1970). On electrophoresis, the α-chains of this tropocollagen digest moved slightly ahead of the α-chains in the standard. The removal of the telopeptide was therefore just detectable from this small decrease in molecular weight. The material released from insoluble collagen consisted mostly of α-chains, with a small amount of the β-chain component, and thus the insoluble collagen was solubilized by removal of the intermolecular cross-links with these telopeptides.

The gel-fractionation studies with the liver preparations showed that the collagenolytic cathepsin was readily separated from cathepsins B2 and D. However, the collagenolytic activity was widely distributed and partially overlapped with the cathepsin D peak, at which point collagenolysis was slightly enhanced. This apparent synergistic effect was more obvious when an attempt was made to fractionate spleen autolysates (D. J. Etherington, unpublished work), in which the concentration of cathepsin D is proportionately higher than in the liver. Maximal collagen breakdown was located at the position of

cathepsin B1, which has a molecular weight of approx. 25000 (Otto, 1971). Because the peak for collagenolysis was more widely distributed, this enzyme activity was clearly not due to cathepsin B1. The chromatographic behaviour of the collagenolytic cathepsin suggested that it was interacting with the Sephadex, and previous attempts to use DEAE-cellulose had proved unsuccessful, as the enzyme was only partially desorbed from this material.

DEAE-Sephadex did not permit a good separation of the collagenolytic cathepsin. Although activity was distributed through many fractions, maximal collagenolysis was located immediately before maximal cathepsin B1 activity. The chromatogram therefore served to show that the two enzymes are different, although as yet it has not been possible to establish whether cathepsin B1 does contribute to collagenolysis by acting synergistically with the major collagen-degrading cathepsin. However, the activating effects of arginine, chloroquine and 6-amino-hexanoic acid do not support such a role for cathepsin B1.

At pH3.5 and 37°C the insoluble collagen was not spontaneously denatured, as confirmed by its subsequent resistance to trypsin hydrolysis. However, the material solubilized from collagen is spontaneously denatured under these conditions and would then be susceptible to the action of the full range of lysosomal proteinases. The fragments of collagen ingested by macrophages during tissue resorption therefore would not be immediately susceptible to the action of cathepsins generally. The collagenolytic cathepsin, by solubilizing this collagen, would be the more important enzyme during this second intracellular phase of digestion.

The specificity of the collagenolytic cathepsin is not known. It displayed negligible activity against haemoglobin, but whether it can degrade other proteins has yet to be determined.

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