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The Purification of Bovine Cathepsin B1 and its Mode of Action on Bovine Collagens

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1. Cathepsin B1 was isolated from bovine spleen by autolysis, $(NH_4)_2SO_4$ fractionation and chromatography on Amberlite IRC-50. Two isoenzyme forms were purified to homogeneity by chromatography on CM-cellulose and DEAE-Sephadex. 2. A collagenolytic cathepsin was separated from cathepsin B1 during purification. The remaining collagenolytic activity of the purified cathepsin-B1 isoenzymes was no greater than 0.3 unit/unit of cathepsin B1 compared with about 5.0 unit/unit of cathepsin B1 in the autolysed spleen extracts. 3. The cathepsin B1 isoenzymes lowered the viscosity of gelatin at 37°C. Optimum activity was at pH4–5. 4. At 28°C the interchain cross-links in native tropocollagen were cleaved most effectively at pH4–5. Insoluble tendon collagen was digested at pH3.5 and 28°C to yield mainly α-chain components, with the loss of a short *N*-terminal sequence. 5. Electron-microscope studies of collagen fibrils showed that cathepsin B1 caused longitudinal splitting and dissociation of the protofilaments. The effect was not general but occurred at selected sites. 6. The isoenzymes of cathepsin B1 cleaved the telopeptide region of calf skin tropocollagen between the lysine-derived cross-link and the triple helix. The CB1 peptide fragments obtained from enzyme-degraded α1 chains were hydrolysed at Gly_{12}-Ile_{13} and Ser_{14}-Val_{15}. The residual α2 CB1 peptides were hydrolysed at Ala_8-Asp_9 and Asp_9-Phe_{10}.

Most proteinases have little action on the native structure of collagen, but two different types of tissue enzyme are now known that can effectively degrade this protein. These are the collagenases and the collagenolytic cathepsins.

The collagenases, either mammalian or amphibian, will at neutral pH values cleave all three chains in the helical region of the tropocollagen molecule and at an initial locus 25% from the C-terminal end of the molecule (Gross, 1970).

The collagenolytic cathepsin system, which is located in the tissue lysosomes (Frankland & Wynn, 1962; Milsom *et al.*, 1972), forms a second enzymic mechanism for the dissolution of collagen. In native collagen cleavage is restricted to the non-helical regions or telopeptides, which carry the interchain cross-links (Etherington, 1972). Optimum activity is at pH3.5–4.0 for insoluble collagen (Frankland & Wynn, 1962; Anderson, 1969; Etherington, 1972, 1973) and for the more labile acid-soluble collagen as substrate an optimum at pH4.6 has been found by Bazin & Delaunay (1966).

There are a number of physiological events that are associated with an extensive tissue remodelling. These changes necessitate a rapid dissolution of collagen as part of the process, and the most drastic decrease occurs in the uterus at post-partum involution (Harkness & Moralee, 1956). Histological examination has revealed that the collagen is first fragmented and then degraded intracellularly within the digestive vacoules of macrophages (Parakkal, 1969, 1972; Brandes & Anton, 1969). The collagenolytic activity during this change follows the same pattern as that of the other lysosomal enzymes in the uterus, but shows a more rapid return to the non-gravid value (Etherington, 1973).

The collagenolytic activity is sensitive to the same thiol activators and inhibitors as cathepsin B1, although certain other cathepsin B1 inhibitors do not decrease collagenolysis *in vitro*, suggesting that cathepsin B1 is not the only lysosomal enzyme to degrade collagen (Etherington, 1972). Burleigh (1973) has shown that pure human cathepsin B1 will degrade collagen either in solution or as reconstituted fibrils.

In the present investigation cathepsin B1 of bovine spleen has been separated from another collagenolytic cathepsin and purified to homogeneity. Its action on different collagens has been studied by both chemical and physical means.

Experimental

Materials

Bovine spleens were collected immediately after slaughter and stored at −20°C until required.

Insoluble bovine tendon collagen was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Calf skin tropocollagen was prepared

as described by Jackson & Cleary (1967) and dissolved in 0.01M-acetic acid at a concentration of 5mg/ml. Gelatin was prepared from this tropocollagen solution by heating to 45°C for 30min and then storing as a freeze-dried powder.

Methods

Extraction of cathepsin B1. The extraction procedure was based on the methods of Otto (1967) and McDonald *et al.* (1970). Spleens were partially thawed, freed of fascia and coarse blood vessels and then passed once through a meat mincer. About 1kg of mince was added to 2vol. of ice-cold 3% (w/v) NaCl in 15mM-HCl containing 1mM-EDTA and 0.5mM-dithiothreitol and homogenized with a Polytron (Kinematica G.m.b.H., Lucerne, Switzerland). The homogenate was stirred for 2h at 2°C, adjusted to pH3.5 with 2M-HCl and then allowed to autolyse at 37°C for 18h with continuous stirring.

Insoluble debris was removed by centrifugation for 45min at 2800*g* in an MSE Mistral 6L centrifuge. Solid $(NH_4)_2SO_4$ was dissolved in the supernatant to 40% saturation, the solution stirred for 2h at 2°C and then clarified by centrifugation at 2800*g*. Further $(NH_4)_2SO_4$ was added to 70% saturation and after stirring for 2h the precipitate containing cathepsin B1 was recovered by centrifugation at 2800*g* for 30min. The pellet was resuspended and dialysed against 0.09M-sodium citrate buffer, pH5.3, containing 1mM-EDTA and 0.5mM-dithiothreitol. Insoluble material was removed by centrifugation and the clear-brown supernatant used for the purification of cathepsin B1.

Column chromatography. Chromatography on Amberlite IRC-50 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) was performed by the method of Otto (1967) but with buffer containing 1mM-EDTA and 0.5mM-dithiothreitol (Fig. 1). The unadsorbed protein, containing all the cathepsin B1, was precipitated by $(NH_4)_2SO_4$ at 90% saturation. A second collagenolytic enzyme could be eluted from the column with 0.5M-Tris–HCl buffer, pH8.0, containing 1mM-EDTA and 0.5mM-dithiothreitol.

The concentrated preparation of crude cathepsin B1 from the Amberlite IRC-50 column was dialysed against 20mM-sodium acetate buffer, pH5.0, containing 1mM-EDTA and 0.5mM-dithiothreitol and then chromatographed on CM-cellulose (Whatman CM-52; Reeve Angel Scientific Ltd., London EC4V 6AY, U.K.) equilibrated with the same buffer (Fig. 2).

Chromatography on DEAE-Sephadex [Pharmacia (G.B.) Ltd., London W5 5SS, U.K.] was used as the final purification step for the individual cathepsin B1 isoenzymes (Fig. 3). Each isoenzyme was concentrated by pressure dialysis and then dialysed against several changes of the starting buffer.

Analytical isoelectric focusing. The purification stages in the isolation of the cathepsin B1 isoenzymes were checked by the analytical isoelectric-focusing method of Barrett (1970). The carrier ampholyte (LKB, South Croydon CR2 8YD, U.K.) had a pH range of 3–10 and was present at a final concentration of 1% (w/v). Samples containing approximately 25–200μg of protein were incorporated into each gel.

Assay of cathepsin B1. The method of Otto (1967) was used with α-*N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride [Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames KT2 7BH, U.K.]. A unit of enzyme activity is defined as the amount of enzyme to release 1μmol of *p*-nitroaniline in 1min at 37°C.

Assay of collagenolytic activity. A dispersion of insoluble native collagen was used as the substrate and the release of soluble hydroxyproline determined as described by Etherington (1972). A unit of enzyme activity is defined as the amount of enzyme to release 1μmol of hydroxyproline in 1min at 37°C.

Determination of protein. The method of Lowry *et al.* (1951) was used with bovine serum albumin (puriss, Koch–Light, Colnbrook, Bucks., U.K.) as standard. As a routine the effluent from the chromatography columns was monitored at 280nm with a Uvicord II spectrophotometer (LKB). Collagen chains were located in the CM-cellulose chromatograms from their absorption at 230nm.

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

Viscometry. A 4% (w/v) gelatin solution was prepared in water from the freeze-dried preparation as required. A sample (80ml) of this solution was diluted to 130ml with 0.4M-Na_2HPO_4 containing 1mM-EDTA and 0.5mM-dithiothreitol. Samples (10ml) of this solution were adjusted to the desired pH value with an equimolar mixture of formic acid and acetic acid and then diluted to a constant volume (14.3ml). Samples (6.5ml) of the buffered gelatin were transferred to each of two Ostwald viscometers (water outflow times about 0.5min) and equilibrated to 37°C. At zero time 0.5ml of enzyme sample was added, the contents were rapidly mixed and the first timings made. The concentration of gelatin in the reaction mixture was 1.6% (w/v) and the concentration of phosphate 0.1M. Control determinations were subsequently made in each viscometer with water in place of enzyme. Outflow times were recorded at frequent intervals for up to 1h. At the end of each experiment the pH values of the reaction mixtures were checked.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Calf skin tropocollagen was incubated with pure cathepsin B1 isoenzymes for up to 22h at 28°C and at pH values between 3.0 and 7.0. The

reaction mixtures consisted of 1.2mg of tropocollagen/ml in 0.1M-formic acid containing 5mM-cysteine, which had been adjusted to the desired pH value with an equimolar mixture of sodium acetate and Na_2HPO_4. At specific times the incubations were terminated, the collagen products freed of enzyme (Drake *et al.*, 1966) and then portions (50–100μg of sample protein in 30μl of solution) analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis by the method of Sykes & Bailey (1971).

Insoluble collagen was incubated with the cathepsin B1 isoenzyme at pH3.5 and 28°C (Etherington, 1972). The solubilized collagenous material was similarly purified before electrophoresis.

Electron-microscope studies. Insoluble collagen fibrils were incubated at 28°C and pH3.5 with pure cathepsin B1 isoenzymes. The fibrils were then washed several times with 0.1M-acetic acid and a drop was placed on a carbon–collodion-coated copper grid. The fibrils were negatively stained with sodium silicotungstate, pH7.8, and examined in an AEI EM6B electron microscope by using an accelerating voltage of 60kV.

Investigation of the enzyme-sensitive region in native tropocollagen. Portions (100mg) of calf skin tropocollagen in 75ml of 0.1M-sodium formate buffer, pH4.0, containing 1mM-EDTA and 0.5mM-dithiothreitol were digested with the pure cathepsin B1 isoenzymes for 18h at 28°C. The digests were then freed of enzyme by salt fractionation (Drake *et al.*, 1966), exhaustively dialysed against 0.1M-acetic acid and freeze-dried. The α1 and α2 chains were separated by chromatography on CM-cellulose (Bellamy & Bornstein, 1971), dialysed against 0.1M-acetic acid and freeze-dried. Portions of each chain were then digested with CNBr (Bornstein & Piez, 1966) and freeze-dried.

The enzymically shortened CNBr-derived peptides derived from peptides α1 CB1 and α2 CB1 of the telopeptide region (Traub & Piez, 1971) and containing the new *N*-terminal residues were separated by exclusion chromatography on Bio-Gel P2 (Bio-Rad Laboratories Ltd., St. Albans, Herts., U.K.). Samples of each digest containing 10–20mg of collagen peptides were dissolved in 1.5ml of 10% (v/v) acetic acid. The retarded, low-molecular-weight peptides were located by the cadmium acetate–ninhydrin stain of Atfield & Morris (1961) after spotting from each tube on filter paper and drying. The fractions were pooled and concentrated by freeze-drying. The *N*-terminal peptides were then purified by high-voltage electrophoresis at 4kV on Whatman no. 3 paper in a Camag electrophoresis unit (Griffin and George Ltd., Wembley HA0 1HJ, U.K.). The buffer, pH1.9, was composed of formic acid (2.5%, v/v) and acetic acid (8.7%, v/v). The peptides were eluted from the paper with 10% (v/v) acetic acid by the technique of Blackburn (1965). The *N*-terminal residues were dansylated as described by Gros & Labouesse (1969) and identified by t.l.c. on polyamide sheets (Woods & Wang, 1967) after hydrolysis in 5.7M-HCl for 16h.

An amino acid analysis was obtained for each peptide with a Locarte analyser after hydrolysis of a portion for 24h in 5.7M-HCl.

Results

Purification of cathepsin B1

The extraction procedure of Otto (1967) was found to be improved by allowing the homogenate to autolyse at 37°C as described by McDonald *et al.* (1970). However, the Amberlite IRC-50 column separation used by Otto (1967) was retained, since this step gave a complete separation of cathepsin B1 from another collagenolytic enzyme. The chromatogram of an extract containing 6.77units of cathepsin B1 is shown in Fig. 1. After cathepsin B1 had been collected in the breakthrough peak the collagenolytic cathepsin was eluted with 0.5M-Tris–HCl buffer, pH8.0, and found to be free of cathepsin B1 activity. The protein in this second peak was stored as an $(NH_4)_2SO_4$ suspension for further examination.

The crude cathepsin B1 (6.5 units) was next separated into two main isoenzyme fractions, I and II, together with a third minor fraction by chromatography on CM-cellulose (Fig. 2). Final purification of the separated isoenzymes, I (1.88 units) and II (1.63 units), was performed on a column of DEAE-Sephadex (A-50) (Fig. 3). The isoenzymes were eluted at different positions in the chromatograms and in the reverse order to their appearance from the CM-cellulose column. However, owing to the high capacity and good flow properties of this latter exchanger, its usefulness was more valuable at an earlier stage in the purification sequence.

Each step in the purification of the cathepsin B1 isoenzymes was checked by analytical isoelectric focusing in a pH gradient of 3–10. The isoenzymes obtained from the CM-cellulose column appeared to be nearly pure and exhibited differences in their isoelectric points. When the isoenzymes were rerun on DEAE-Sephadex only a single electrophoretic band was detected for each fraction. The difference in isoelectric point was confirmed by focusing a mixture of the two enzymes fractions (Plate 1).

The recovery of total cathepsin B1 was of the order 20–25% of the activity originally present in the supernatant from the autolysed homogenate. The ultimate purification factors were 387-fold for fraction I and 332-fold for fraction II. The results are presented in detail in Table 1. The corresponding specific-activity determinations for collagen digestion show that increases of only 23-fold for fraction I and 15-fold for

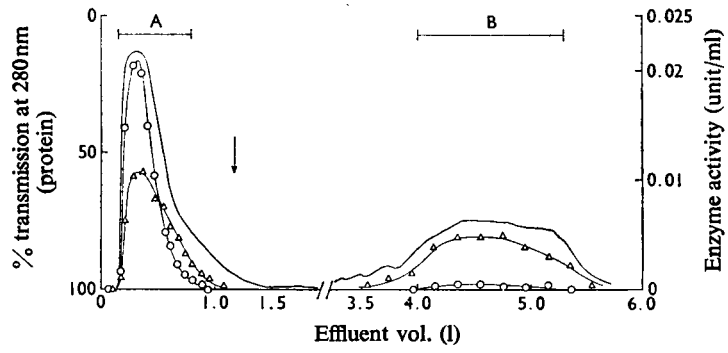


Fig. 1. *Chromatography of cathepsin B1 on Amberlite IRC-50*

Cathepsin B1 (6.77 units) was eluted from a column (4.5cm × 25cm) in the breakthrough peak A with 0.09M-sodium citrate buffer, pH5.3, containing 1mM-EDTA and 0.5mM-dithiothreitol. Collagenolytic cathepsin peak B was subsequently eluted with 0.5M-Tris–HCl, pH8.0, containing 1mM-EDTA and 0.5mM-dithiothreitol. A flow rate of 4ml/min was maintained throughout. ——, % transmission at 280nm (protein); ○, cathepsin B1 (units/ml); △, collagenolytic activity (units/ml). The arrow indicates the point at which the second buffer was applied to the column.

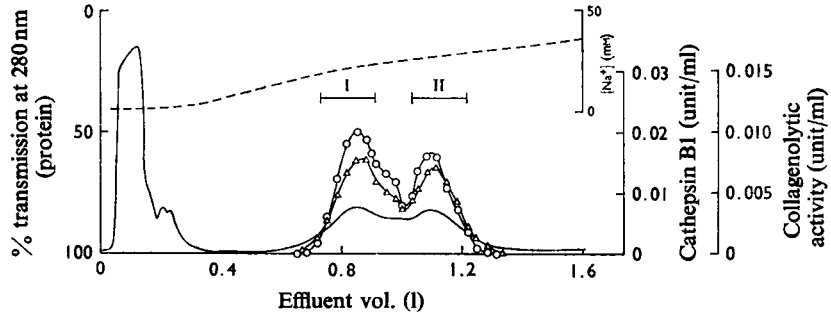


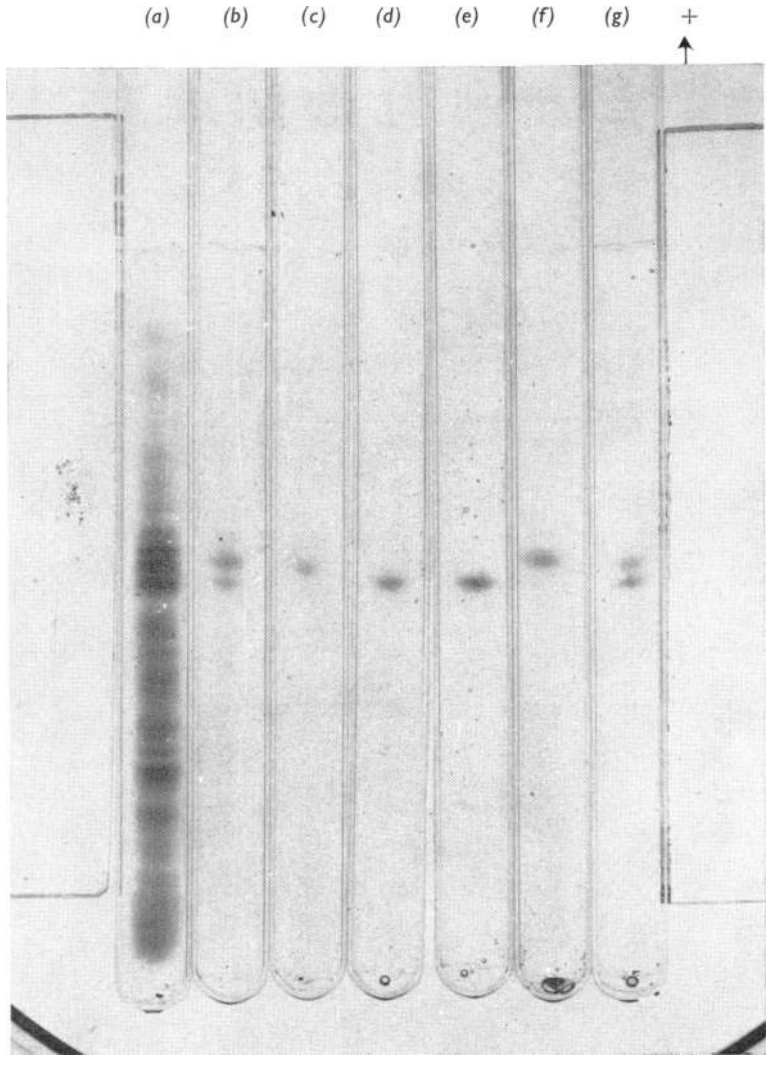
Fig. 2. *Chromatography of cathepsin B1 from the Amberlite IRC-50 column on CM-cellulose*

Cathepsin B1 isoenzymes (6.5 units) were fractionated on a column (2.2cm × 35cm) equilibrated to 20mM-sodium acetate buffer, pH5.0, containing 1mM-EDTA and 0.5mM-dithiothreitol. A Na^+ concentration gradient (-----) of 0–50mM-NaCl was established by using two 800ml volumes of buffer and pumped at a flow rate of 1.2ml/min. ——, % transmission at 280nm (protein); ○, cathepsin B1 (units/ml); △, collagenolytic activity (units/ml). For details of fractions I and II see the text.

fraction II were obtained during the purification of cathepsin B1. The ratio of collagenolytic activity units/cathepsin B1 units shows a sharp decrease with both the $(NH_4)_2SO_4$ precipitation stage and with the chromatographic separation on Amberlite IRC-50. The collagen-degrading activity of the pure cathepsin B1 isoenzymes is therefore very much decreased as compared with the crude starting material.

Stability of cathepsin B1 preparations

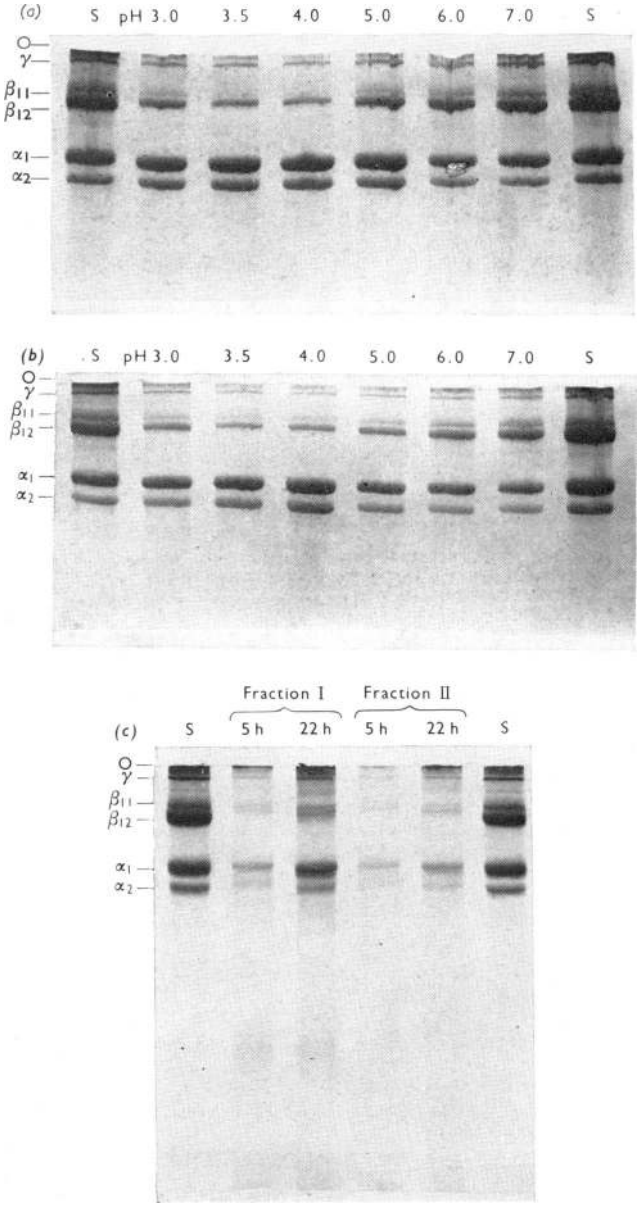
In the earlier preparations, the purified cathepsin B1 isoenzymes were dialysed against a solution of 1mM-EDTA and 0.5mM-dithiothreitol or against dilute acetate buffers of pH4 or 5 and containing the same concentration of activators. Storage of these dialysed enzyme preparations at −20°C resulted in an appreciable loss of activity after 2 or 3 weeks. Barrett (1973) reported that human cathepsin B1 is stable in buffers below pH7 but very unstable above neutrality. The most recent preparations of bovine cathepsin B1 have been dialysed against EDTA (disodium salt) and dithiothreitol and then diluted with 0.5vol. of glycerol before freezing to −25°C. No significant loss of activity has been observed after 6 weeks of storage.



EXPLANATION OF PLATE 1

Isoelectric focusing in polyacrylamide gels with a pH gradient of 3–10 *of fractions obtained during the purification of cathepsin B*1

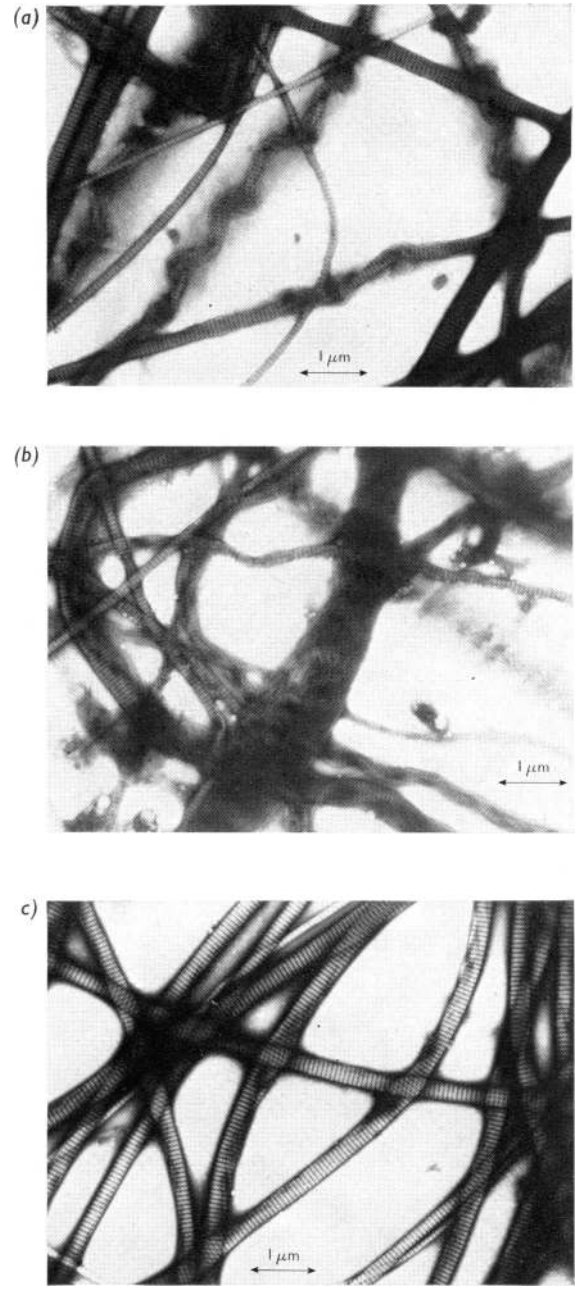
(*a*) 40–70%-satd.-$(NH_4)_2SO_4$ fraction; (*b*) protein not adsorbed on Amberlite IRC-50; (*c*) CM-cellulose isoenzyme fraction I; (*d*) CM-cellulose isoenzyme fraction II; (*e*) isoenzyme fraction II after chromatography on DEAE-Sephadex; (*f*) isoenzyme fraction I after chromatography on DEAE-Sephadex; (*g*) mixture of (*e*) and (*f*).



EXPLANATION OF PLATE 2

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of collagen chains after digestion by pure cathepsin B1 isoenzymes

(*a*) Digestion of tropocollagen at 28°C for 3h and at pH values between 3 and 7 by cathepsin B1 fraction I (0.0033 unit/ml). Untreated collagen (S) was used as the standard. o denotes the origin. (*b*) Digestion of tropocollagen at 28°C for 3h by cathepsin B1 fraction II (0.002 unit/ml). (*c*) Digestion of insoluble bovine tendon collagen at 28°C and pH 3.5 by cathepsin B1 isoenzymes I and II for 5 and 22h.



EXPLANATION OF PLATE 3

Effect of cathepsin B1 fraction I (0.032 *unit/ml*) *on native collagen fibrils at pH* 3.5 *and* 28°*C*: *electron micrographs of washed fibrils*

(*a*) 5h digest; (*b*) 16h digest; (*c*) water control after 16h incubation. Digestion with fraction II (0.032 unit/ml) produced the same effects as fraction I.

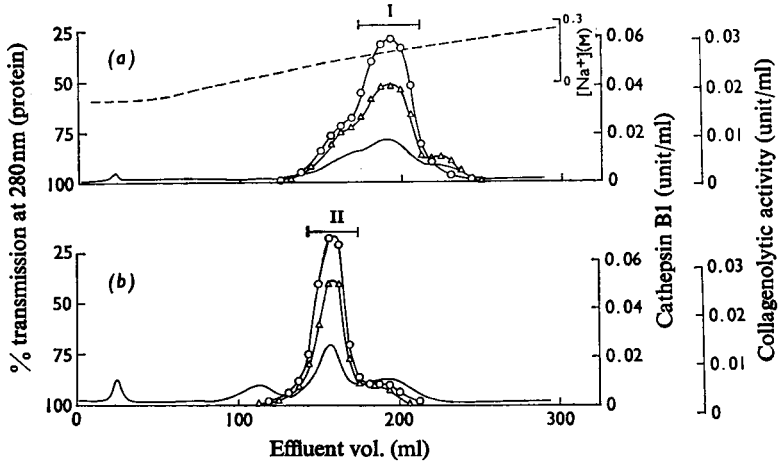


Fig. 3. *Chromatography of cathepsin B1 isoenzymes on DEAE-Sephadex*

Each column (2.2cm × 20cm) was equilibrated to 10mM-sodium phosphate buffer, pH6.5, containing 1mM-EDTA and 0.5mM-dithiothreitol. The isoenzymes were eluted by a gradient of 0–0.3M-NaCl (----) prepared from two 150ml volumes of buffer and with a flow rate of 0.23ml/min. (*a*) CM-cellulose fraction I (1.88 units); (*b*) CM-cellulose fraction II (1.63 units); ——, % transmission at 280nm (protein); ○, cathepsin B1 (units/ml); △, collagenolytic activity (units/ml).

Digestion of gelatin

The decrease in viscosity of gelatin from digestion by the pure cathepsin B1 isoenzymes was investigated over the pH range 3–7. Each determination was made with 0.0017 unit of fraction I or 0.001 unit of fraction II in 7ml of buffered gelatin, which had an initial specific viscosity of 1.4–1.6. The fall in viscosity was most rapid between pH4 and 5 for both isoenzymes and appreciable activity was still exhibited at pH6 (Fig. 4). Hydrolysis of peptide bonds in the gelatin substrate was confirmed by reaction of samples of the incubation mixtures with ninhydrin (Moore & Stein, 1954). At the pH optimum for digestion up to 15% of the total peptide bonds in gelatin were found to be hydrolysed with DL-leucine (BDH Chemicals Ltd., Poole, Dorset, U.K.) as the standard. The viscosity of the control mixtures remained unchanged.

Digestion of tropocollagen and insoluble collagen

The effect of pH on the digestion of soluble native collagen was observed from the conversion of the cross-linked β and γ components into single α chains by molecular-sieve electrophoresis.

The tropocollagen digests contained 0.0033 unit of fraction I/ml or 0.002 unit of fraction II/ml. Electrophoresis of the 3h digests in polyacrylamide gels containing sodium dodecyl sulphate gave the separations shown in Plates 2(*a*) and 2(*b*). The removal of the cross-linking telopeptide region was most apparent in the pH4 digest. Analyses of 5 and 22h digests gave similar results but with an increased production of α chains in all except the pH7 digests. Precipitation of the digested collagen with NaCl left the telopeptide fragments in solution. The existence of any additional hydroxyproline-containing peptides with these telopeptides was not determined.

Digestion of insoluble collagen by crude enzyme preparations is optimum at pH3.5 (Anderson, 1969; Etherington, 1972, 1973). The pure cathepsin B1 isoenzymes were allowed to digest insoluble collagen at this pH for 5 and 22h. The solubilized collagen was subsequently analysed by electrophoresis and consisted principally of α chains, but some β and γ components were also apparent (Plate 2*c*). The electrophoresis pattern was very similar to that obtained for crude rat liver extracts (Etherington, 1972).

Electron-microscope studies

The digests of insoluble collagen fibrils each contained 0.0032 unit of pure cathepsin B1 isoenzyme/ml. Fibrils were examined after 5h and found to be considerably damaged (Plate 3). The effect was variable and some fibrils appeared to be still intact. The damage produced by both isoenzymes was seen as regions of longitudinal splitting with separation of the protofilaments. Affected fibrils became swollen and less dense in appearance. Frequently the splitting

Table 1. *Purification of cathepsin B1 isoenzymes from 585g of minced bovine spleen: summary of data*

For experimental details see the text.

		Collagenolytic activity					Cathepsin B1				
	Protein (mg)	Activity (units)	10^{-3} × Specific activity (units/mg of protein)	Purification	Yield (%)		Activity (units)	10^{-3} × Specific activity (units/mg of protein)	Purification	Yield (%)		Units of collagenolytic activity/unit of cathepsin B1
Autolysed spleen extract	27599	43.2	1.57	(1)	(100)		8.53	0.31	(1)	(100)		5.06
40–70%-satd.-$(NH_4)_2SO_4$ fraction	1632	8.52	5.22	3.32	19.7		6.77	4.15	13.4	79.4		1.26
Protein not absorbed on Amberlite IRC-50	673	3.12	4.64	2.96	7.2		6.50	9.66	31.2	76.2		0.48
CM-cellulose fraction I	48.4	0.725	15.0	9.55	1.68	3.38	1.88	38.8	125	22.0	41.1	0.39
CM-cellulose fraction II	63.2	0.733	11.6	7.39	1.70		1.63	25.8	83.2	19.1		0.45
Fraction I DEAE-Sephadex	9.9	0.352	35.6	22.7	0.81	1.32	1.19	120	387	14.0	25.5	0.30
Fraction II DEAE-Sephadex	9.6	0.222	23.1	14.7	0.51		0.985	103	332	11.5		0.23

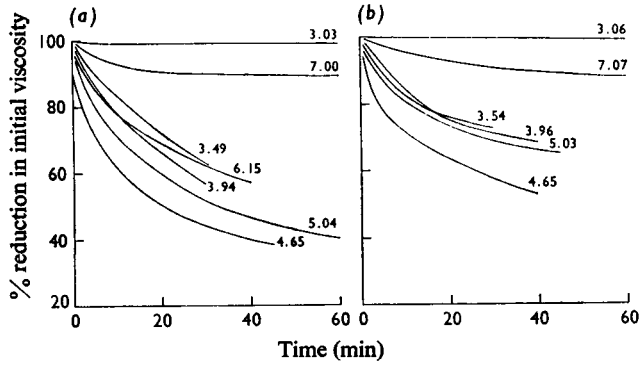


Fig. 4. *Decrease in viscosity of calf skin gelatin by pure cathepsin B1 isoenzymes*

Determinations were made between pH3 and 7 at 37°C. Concentration of gelatin was 1.6% (w/v). The pH values of each reaction mixture are indicated on the curves. (*a*) Digestion by fraction I (0.0017 unit/ml); (*b*) digestion by fraction II (0.001 unit/ml).

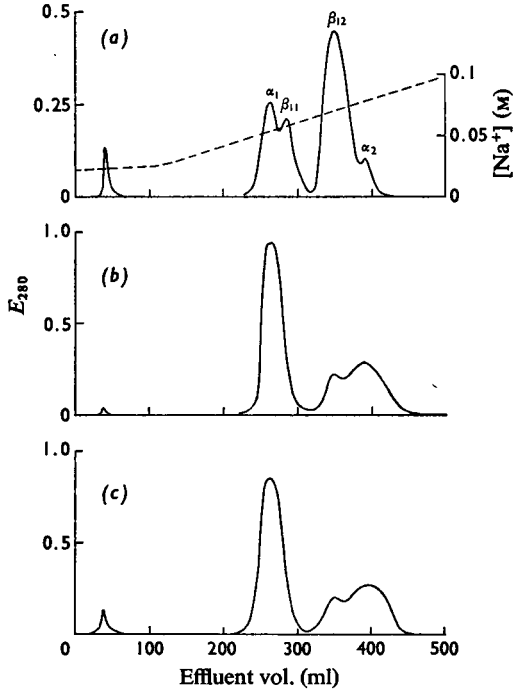


Fig. 5. *Chromatography of collagen chains on CM-cellulose*

Collagen chains were separated on a column (1.6cm × 16cm) at 42°C by using a 500ml linear gradient of 0.02–0.1M-NaCl (-----) in 0.04M-sodium acetate buffer, pH4.8, containing 4M-urea. ——, E_{230} (peptide bond); (*a*) 20mg of untreated calf skin tropocollagen; (*b*) 30mg of tropocollagen digested at 28°C and pH4 for 18h by cathepsin B1 fraction I (0.083 unit); (*c*) 30mg of tropocollagen digested by fraction II (0.083 unit) under the same conditions. For details of α and β components see the text.

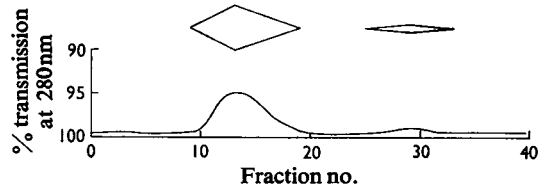


Fig. 6. *Chromatography of CNBr digest of collagen α chain on Bio-Gel P2*

A 15mg sample of α1 chain, degraded by cathepsin B1 fraction I, was eluted from a column (1.2cm × 50cm) with 0.1M-acetic acid. Peptide peaks were located by the intensity of ninhydrin colour (shown by diamond-shaped symbols) after spotting on paper and indicated by the height of these symbols. Fractions (1.3ml) were collected at a flow rate of 0.32ml/min. ——, % transmission at 280nm. Similar chromatograms were obtained with the remaining CNBr digests of the enzyme-degraded chains.

was restricted to a number of short regions in a single fibril. After 16h of incubation at 28°C the damage caused by each isoenzyme was more extensive. Many fibrils were considerably swollen and some were becoming very diffuse as the collagen dissolved. At this stage a number of highly organized and essentially intact fibrils could still be seen. Controls containing water in place of enzyme were shown to remain intact after 16h of incubation.

Chromatography of collagen chains

CM-cellulose chromatography in the presence of 4M-urea has provided an efficient means for resolving the α, β and γ components of collagen. Fig. 5(*a*)

Table 2. *Analysis of the CNBr-derived fragments from the telopeptide region of cathepsin B1-digested calf skin tropocollagen*

The sequences of the complete peptides are included for comparison. α1 chain CB1 (Rauterberg *et al.*, 1972): □Glu-Leu-Ser-Tyr-Gly-Tyr-Asp-Glu-Lys*-Ser-Thr-Gly-Ile-Ser-Val-Pro-Gly-Pro-Hse. α2 chain CB1† (Piez, 1972): □Glu-Tyr-Asp Ser-Gly-Lys*-Ala-Asp-Phe-Gly-Glx-Gly-Pro-Hse.

α1 CB1 Fragments	Composition	*N*-Terminal residue	Sequence
Fraction I digest	Gly, Hse, Ile, Pro, Ser, Val	Ile, Val	Ile_{13}–Hse_{19} and Val_{15}–Hse_{19}
Fraction II digest	Gly, Hse, Ile, Pro, Ser, Val	Ile, Val	Ile_{13}–Hse_{19} and Val_{15}–Hse_{19}
α2 CB1 Fragments			
Fraction I digest	Asp, Glx, Gly, Hse, Phe, Pro	Asp, Phe	Asp_{9}–Hse_{15} and Phe_{10}–Hse_{15}†
Fraction II digest	Asp, Glx, Gly, Hse, Phe, Pro	Asp, Phe	Asp_{9}–Hse_{15} and Phe_{10}–Hse_{15}†

* Cross-link derived from lysyl residues.
† Sequence determined by homology.

shows the separation obtained for 20mg of calf skin tropocollagen. Enzyme digests composed of 100mg of tropocollagen with 0.28 unit of pure cathepsin B1 isoenzyme were similarly fractionated after 18h of incubation at 28°C. The chromatograms reproduced in Figs. 5(*b*) and 5(*c*) were each obtained with 30mg of cathepsin B1-digested tropocollagen. Both fractions I and II have decreased the amount of cross-linked components to a low value under the conditions employed. The derived α chains chromatographed similarly to the intact, undigested α chains in the control preparation. Fractions in each peak were checked for purity by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and then pooled.

Analysis of CNBr digests

Cleavage of the collagen α chains by CNBr results in the release of the telopeptides as small fragments designated α1 CB1 and α2 CB1, consisting of 19 (Rauterberg & Kühn, 1971) and 14 residues (Fietzek *et al.*, 1970) respectively. Enzyme digestion by cathepsin B1 isoenzymes removed the interchain cross-link and thus shortened these CNBr-derived peptides. The enzyme-degraded peptides were conveniently separated from the larger CNBr-derived peptides by exclusion chromatography on Bio-Gel P2. All four chromatograms were essentially the same and that for the CNBr-cleaved α1 chain from the fraction I digest is shown in Fig. 6. All the large peptides were found in the breakthrough peak. The ninhydrin stain located the retarded material in fractions 26–32, which were then pooled.

The Bio-Gel-retarded peptide fractions were each purified by high-voltage electrophoresis. For the CNBr digests of the α1 chain the fragment of the CB1 peptide in each preparation was freed of a small amount of contaminating amino acids which migrated ahead of the peptide. The CNBr digests of the α2 chains each contained the tripeptide CB0 consisting of glycine, leucine and homoserine (Fietzek *et al.*, 1970). This also moved towards the cathode during electrophoresis, whereas the α2 CB1 fragment remained near the origin.

The analytical results obtained for these peptide fragments are presented in Table 2, together with the published structures of peptide α1 CB1 (Rauterberg *et al.*, 1972) and α2 CB1 (Piez, 1972). The α1 CB1 peptide fragment produced by cathepsin B1 fraction I consisted of Ile_{13}–Hse_{19}. The smaller peptide Val_{15}–Hse_{19} appeared to be present also, since valine was detected with isoleucine in the *N*-terminal analyses. Similar results were obtained in the peptide analysis for fraction II, showing an identical specificity for these two isoenzymes on the α1 chain.

The α2 chain was also cleaved in a similar manner by both isoenzymes. The remaining fragment consisted of Asp_{9}–Hse_{15}. The presence of Dns-Phe together with Dns–Asp in the *N*-terminal analyses suggested that the terminal bond at Asp_{9}–Phe_{10} was also partially cleaved by both isoenzymes.

Discussion

Otto (1967) first demonstrated that cathepsin B could be separated into two distinct enzymes, B1 and B2. Subsequent examination (Otto, 1971) revealed that cathepsin B1 could be detected in a variety of different tissues in the rat. The purified enzyme hydrolysed both protein and peptide substrates whereas cathepsin B2 could act only as a peptidase. When the B chain of oxidized insulin was investigated as a test substrate cathepsin B1 was found to exhibit a wide specificity (Otto, 1971; Keilová, 1971), which resembled that of papain (Keilová, 1971).

Cathepsin B1 has been shown to exist in multiple forms in various tissues (Suominen & Hopsu-Havu, 1971; Franklin & Metrione, 1972; Keilová &

Tomášek, 1973). Barrett (1973), using a 'post-coupling' method for detecting proteolytic zones in polyacrylamide gels, has located at least six isoenzymes in extracts of human liver.

The purification procedure employed in the present work enabled a complete separation of two major isoenzymes of cathepsin B1 from bovine spleen. A minor third component observed in the CM-cellulose chromatograms was not examined further. The two major forms were designated fraction I and fraction II and correlated with the two enzyme peaks obtained by Franklin & Metrione (1972) using CM-cellulose. The relationship to the isoenzyme forms separated by Keilová & Tomášek (1973) is uncertain.

Fractions I and II were each purified over 300-fold from the autolysed spleen extracts, and the final preparations were shown to be homogeneous by isoelectric focusing in polyacrylamide gels. Barrett (1973) obtained a similar improvement in the specific activity of human cathepsin B1 in the various purification steps after autolysis, although the overall yield of pure enzyme was lower. Concurrent analyses for collagenolytic activity demonstrated much lower increases in the specific activity during the purification sequence and a comparison of the ratio of collagenolytic units/cathepsin B1 units showed a fall from greater than 5.0 to no more than 0.3 for each pure cathepsin B1 isoenzyme. This decrease could be accounted for, in part at least, by the removal of a second collagenolytic cathepsin, which had no activity against the cathepsin B1 substrate. However, the existence of a co-operative or synergistic action between cathepsin B1 and another enzyme cannot be excluded on the present evidence.

Investigation of the mechanism by which collagen is degraded by cathepsin B1 has formed the major part of the present study. Cathepsin B1 is the first lysosomal enzyme with collagenolytic activity to be obtained in pure form. A role for cathepsin B in collagenolysis was first suggested from the experiments of Barsky & Farrison (1963) and Burleigh (1973) showed cathepsin B1 to be the enzyme responsible.

When collagen is heat-denatured it loses its helical structure and becomes susceptible to non-specific proteolytic digestion. The cathepsin B1 isoenzymes were each found to decrease rapidly the viscosity of gelatin prepared from pure calf skin tropocollagen. Maximum digestion occurred near pH4.5 and appreciable activity was still apparent at pH6. Cathepsin B1 degrades its synthetic substrate most efficiently at pH6.0-6.5, but for those non-collagen protein substrates which have been examined the preferred pH range is 4.0–5.0 (Otto, 1971).

Electrophoretic analysis of collagen digests showed that cathepsin B1 acted in a similar manner to a crude mixture of lysosomal enzymes obtained from rat liver (Etherington, 1972). Intramolecularly cross-linked tropocollagen was converted almost totally into α chains, and insoluble collagen yielded α chains together with a small amount of higher-molecular-weight material. Insoluble collagen is degraded optimally at pH3.4–3.5 and there is little solubilization above pH4.5 (Anderson, 1969; Etherington, 1972, 1973). Tropocollagen, however, was more susceptible to cleavage by cathepsin B1 at pH4 than at pH3.5. Bazin & Delaunay (1966) have clearly shown that acid-soluble collagen is preferentially degraded at pH4.6 by various tissue extracts.

The initial physical state of the collagen substrate has a considerable influence on the collagenolytic action of tissue proteinases. Collagen fibres swell considerably when the pH is lowered, and the effect is most pronounced below about pH4 (Gustavson, 1956). This suggests that the susceptible bonds in the telopeptide regions of the native fibrils are inaccessible to catheptic cleavage unless the structure is allowed to swell and only then can the enzyme gain access. However, owing to the lower pH value, the enzyme is acting with a diminished efficiency and measurable solubilization of insoluble collagen is exhibited over a narrow pH range only. Differences in the reported pH maxima for the digestion of collagen may therefore be interpreted as a result of variations in the conformational integrity of the substrate. The swelling of collagen fibrils at pH3.5 is a reversible process and does not cause denaturation. This was confirmed by showing the resistance of the fibrils to tryptic digestion after subsequent neutralization (Etherington, 1972).

Collagen fragments exhibiting an apparently normal periodicity have been demonstrated within uterine macrophages shortly after parturition (Parakkal, 1972). Whether these fragments can swell more easily than intact fibrils is not known, but it would appear from the present evidence that the lysosomal enzymes are hindered from cleaving the telopeptide region in unswollen fibrillar collagen.

Examination of the enzyme-degraded fibrils by electron microscopy revealed that cathepsin B1 caused the protofilaments to become dissociated in a random manner as though there were points of weakness along the fibril. A similar dissociating action of a complete lysosomal preparation on collagen fibrils has been described by Milsom *et al.* (1972). Wagner (1972) used immune precipitates to induce collagen damage in rabbit corneas, and has produced evidence to show that both splitting and fragmentation of collagen fibrils can occur and he attributes this process to the action of secreted lysosomal enzymes.

Pure bovine cathepsin B1 thus acted in an analogous manner to the complete mixture of lysosomal enzymes from other sources. The mode of action on collagen of the collagenolytic cathepsin has yet to be determined, but it seems unlikely that it can behave in a manner very different from that of cathepsin B1.

When cathepsin B1 removes the cross-link joining adjacent α chains in the native tropocollagen molecule it is restricted to those peptide bonds in the non-helical telopeptide region. Cleavage occurs therefore at one or more bonds between the lysine-derived cross-link and the first triplet sequence, Gly-Pro-X, which marks the beginning of the proteinase-resistant triple helix. This portion of the collagen structure involves eight bonds in the α1 chain of calf skin collagen (Rauterberg *et al.*, 1972) and six bonds in the α2 chain (Piez, 1972). The cathepsin B1 isoenzymes were shown to cleave the Gly-Ile bond and also the bond at Ser-Val in the α1 chain. In the α2 chain the bonds at Ala-Asp and Asp-Phe were broken. The specificity of action was therefore consistent with that shown earlier against the B chain of insulin (Otto, 1971; Keilová, 1971). This functionally important region of the telopeptide is totally resistant to the action of cathepsin D (Etherington, 1972; Burleigh, 1973).

The relationship of cathepsin B1 and the collagenolytic cathepsin to the neutral collagenase in the physiological removal or replacement of collagen is an area for speculation (Woessner, 1968; Evanson, 1971), which as yet appears to be far from gaining a clear answer. It is generally considered that the neutral collagenases act extracellularly and precede the lysosomal enzymes in digesting collagen (Eisen *et al.*, 1970); the lysosomal enzymes, in acting secondarily to the neutral collagenase, may not be restricted to an intracellular function, but also produce degradative changes in certain specific locations outside the cell (Woessner, 1968).

The naturally occurring mechanism for collagenolysis is undoubtedly a complex event. Considerable attention has been paid to the action of the pure neutral collagenase (Gross, 1970; Eisen *et al.*, 1970) on reconstituted fibrils prepared from tropocollagen solution, a substrate that is convenient to study, but which does not exist to any significant extent *in vivo*. An investigation of the action of pure cathepsin B1 on gelatin, tropocollagen and on fibrils of insoluble collagen has been accomplished. However, since it is normally participating with other enzymes, its true contribution to the collagenolytic process may not be identical with its action in isolation.

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