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Cathepsin B1

A LYSOSOMAL ENZYME THAT DEGRADES NATIVE COLLAGEN

By MARY C. BURLEIGH, ALAN J. BARRETT and GERALD S. LAZARUS*
Tissue Physiology Department, Strangeways Research Laboratory, Cambridge CB1 4RN, U.K.

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1. Experiments were made to determine whether the purified lysosomal proteinases, cathepsins B1 and D, degrade acid-soluble collagen in solution, reconstituted collagen fibrils, insoluble collagen or gelatin. 2. At acid pH values cathepsin B1 released ^{14}C-labelled peptides from collagen fibrils reconstituted at neutral pH from soluble collagen. The purified enzyme required activation by cysteine and EDTA and was inhibited by 4-chloromercuribenzoate, by the chloromethyl ketones derived from tosyl-lysine and acetyltetra-alanine and by human α_2-macroglobulin. 3. Cathepsin B1 degraded collagen in solution, the pH optimum being pH4.5–5.0. The initial action was cleavage of the non-helical region containing the cross-link; this was seen as a decrease in viscosity with no change in optical rotation. The enzyme also attacked the helical region of collagen by a mechanism different from that of mammalian neutral collagenase. No discrete intermediate products of a specific size were observed in segment-long-spacing crystalloids (measured as native collagen molecules aligned with *N*-termini together along the long axis) or as separate peaks on gel filtration chromatography. This suggests that once an α-chain was attacked it was rapidly degraded to low-molecular-weight peptides. 4. Cathepsin B1 degraded insoluble collagen with a pH optimum below 4; this value is lower than that found for the soluble substrate, and a possible explanation is given. 5. The lysosomal carboxyl proteinase, cathepsin D, had no action on collagen or gelatin at pH3.0. Neither cathepsin B1 nor D cleaved Pz-Pro-Leu-Gly-Pro-D-Arg. 6. Cathepsin B1 activity was shown to be essential for the degradation of collagen by lysosomal extracts. 7. Cathepsin B1 may provide an alternative route for collagen breakdown in physiological and pathological situations.

The only purified mammalian enzymes that have been shown to attack the triple helix of native collagen are the collagenases (reviewed by Eisen *et al.*, 1970; Evanson, 1971; Lazarus, 1973). The 'specific' collagenases have been well characterized (Gross, 1970); their initial action produces a cleavage across all three polypeptide chains at a point three-quarters of the way from the *N*-terminal end of the collagen molecule. Proteolytic enzymes of comparatively broad specificity, such as pepsin and trypsin, attack only the non-helical peptides at either end of native collagen, although they degrade the heat-denatured form, gelatin (Rubin *et al.*, 1963; Steven, 1965; Drake *et al.*, 1966; Zimmermann *et al.*, 1970). Since the intramolecular cross-links in soluble collagen are largely confined to the non-helical regions, this type of attack can lead to separation of the individual α chains.

There have been several reports of the breakdown of both gelatin (Morrione & Seifter, 1962) and native collagen under acid pH conditions by crude enzyme preparations from rat liver (Frankland & Wynn, 1962; Bazin & Delaunay, 1964), bone (Woods & Nicholls, 1965), post-partum uterus of rat (Woessner, 1962; Schaub, 1963) and human (Woessner & Brewer, 1963) and rat granulation tissue (Bazin & Delaunay, 1966). The activity against both acid-soluble and insoluble collagen has been demonstrated in lysosomal preparations from rat liver (Frankland & Wynn, 1962; Wynn, 1967; Anderson, 1969; Etherington, 1972). There is evidence from electron-microscopic studies for the presence of portions of collagen-like material in the secondary lysosomes of fibroblasts of embryonic chick limb-bones cultured in the presence of sucrose (Glauert *et al.*, 1969), macrophages in mouse hair sheath (Parakkal, 1969*a*), involuting post-partum uterus in mouse (Parakkal, 1969*b*) and rat (Brandes & Anton, 1969) and in cells of carrageenin granulomas in rabbit and guinea pig (Perez-Tamao, 1970). It has been suggested that the carboxyl proteinase cathepsin D might be responsible for the collagen-degrading activity (Woessner, 1968, 1970; Anderson, 1969).

The present studies were designed to investigate whether either of the human cathepsins, B1 and D, in a pure form, have any action on native acid-soluble

* Present address: Montefiore Hospital and Medical Centre, 111 East 210th Street, Bronx, N.Y. 10467, U.S.A.

or insoluble collagen. A preliminary account of part of this work has been presented (Burleigh, 1973).

Materials

Bovine trypsin (twice crystallized), papain (twice crystallized), Fast Garnet GBC and phenolphthalein solution (1mg/ml), were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Bz-DL-Arg-$NHC_{10}H_7$ (α-*N*-benzoyl-DL-arginine 2-naphthylamide hydrochloride) was purchased from Bachem, Liestal, Switzerland.

The sodium salt of 4-chloromercuribenzoic acid was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Sephadex G-10 was purchased from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. and CM-cellulose (CM-52) was from Reeve Angel Scientific Ltd., London EC4V 6AY, U.K. Bio-Gel A-0.5 (200–400 mesh) and the anion-exchange resin AG-1 (X8; 200–400 mesh) were obtained from Bio-Rad Laboratories, St. Albans, Herts., U.K.

The sources of all other chemicals and biochemicals were as described by Werb & Burleigh (1974).

Female Hooded rats were from the Strangeways Research Laboratory animal colony.

Methods

Purification and assay of cathepsins B1 and D

The purifications of cathepsins B1 and D from human liver have been described by Barrett (1970, 1973). Cathepsin B1 preparations were stored in 50mM-sodium acetate buffer, pH5.0, and diluted and assayed by the method of Barrett (1972) by using Bz-DL-Arg-$NHC_{10}H_7$ as substrate and measuring the release of 2-naphthylamine by a colorimetric method after coupling with Fast Garnet GBC (diazotized *o*-aminoazotoluene). One unit of enzymic activity hydrolysed 1μmol of substrate/min at 40°C under the reaction conditions. Cathepsin D was assayed by its activity against denatured haemoglobin as described by Barrett (1970).

Preparation of rat liver extracts

Rats were anaesthetized with ether and the livers rapidly perfused via the portal vein with a solution of 0.9% NaCl to remove as much blood from the tissue as possible and thus lower the concentration of the plasma inhibitor of cathepsin B1, α_2-macroglobulin (Starkey & Barrett, 1973). The animals were decapitated and the livers removed and rinsed thoroughly in ice-cold homogenizing medium. All subsequent operations were carried out at 4°C. Livers were homogenized in 4 vol. of 0.25M-sucrose in buffer A [10mM-sodium–potassium phosphate buffer, pH6.0, containing 1mM-EDTA (disodium salt)] in the automatic homogenizer described by Dingle & Barrett (1969). The homogenates were fractionated by a method based on that of de Duve *et al.* (1955). The heavy mitochondrial fraction was collected by centrifugation of the supernatant from the nuclear fraction at 5000*g* for 6min, and the supernatant was centrifuged at 10000*g* for 25min to pellet the light mitochondrial fraction. The microsomal fraction was the pellet obtained after further centrifugation at 100000*g* for 1h. Fractions were resuspended in homogenizing medium and the membrane-bound enzymes were released by disruption of the organelles with an Ultra–Turrax homogenizer for two bursts of 1–2s with cooling in ice. The sucrose concentration of the fractions was lowered by an 18h dialysis against buffer A.

A crude $(NH_4)_2SO_4$ preparation of the liver enzyme was obtained from the homogenate by the method of Etherington (1972). The enzyme protein was precipitated between 40 and 70% saturation with $(NH_4)_2SO_4$ and collected by centrifugation at 12000*g* for 15min. The protein was resuspended in water and dialysed overnight against three changes (300vol) of water containing 1mM-EDTA (disodium salt). The preparation was centrifuged at 12000*g* for 15min to remove any undissolved protein and the volume of the supernatant adjusted to 1ml/g of the original liver.

Release of ^{14}C-labelled material from reconstituted collagen fibrils

Acid-soluble rat skin collagen labelled with [^{14}C]-glycine was prepared as described by Werb & Burleigh (1974); it had radioactivity of 10140d.p.m./mg. Fibrils were reconstituted by heating 2mg/ml solutions of the collagen containing 50mM-Tris–HCl buffer, pH7.2 (at 4°C), with 0.2M-NaCl at 35°C overnight. Buffer containing EDTA (disodium salt) and cysteine (each 1.5mM) was added with enzyme in a total volume of 200μl, the fibrils were resuspended in the buffer by shaking and the reaction mixtures were incubated at 35°C for the time-periods indicated in the text. Reactions were terminated and the radioactivity released was measured as described by Werb & Burleigh (1974). Reconstituted fibrils were also incubated with trypsin to check that the collagen had not been denatured during preparation and with clostridial collagenase to measure total fibril lysis (Werb & Burleigh, 1974). Control incubation mixtures contained 50mM-sodium acetate buffer in place of the cathepsin B1. The pH values of the final incubation mixtures were measured by use of a capillary electrode (Pye Unicam) after removal of unchanged collagen at the end of the incubation period. Results are expressed as a percentage of total

fibril lysis after subtraction of the buffer control values.

Assay for the degradation of collagen in solution

The ^{14}C-labelled acid-soluble collagen was dissolved in 0.1M-acetic acid at a concentration of 5mg/ml and solutions at different pH values were prepared by dialysis overnight against 100mM-sodium acetate or formate buffers at the appropriate pH value. The collagen solutions were centrifuged at 20000*g* for 1h at 4°C to remove insoluble aggregates before use. Incubation mixtures for the detection of the action of cathepsin B1 on collagen contained collagen (2.5mg/ml), 1mM-cysteine, 1mM-EDTA (disodium salt), 50mM buffer and cathepsin B1 as described in the text. Assays for cathepsin D activity did not contain cysteine or EDTA and were carried out in the presence of 100mM-sodium formate buffer, pH3.0.

Changes in viscosity at 24°C of 2.0ml reaction mixtures were followed in Ostwald micro-viscometers [type UM-4; Camlab (Glass) Ltd., Cambridge CB4 1TH, U.K.] having a flow-time for water of 25–28s. Optical rotation changes were monitored at 590nm in a Thorn Bendix–N.P.L. Automatic Polarimeter type 143 equipped with a water-jacketed cell.

Characterization of the reaction products

Segment-long-spacing aggregates were prepared by the method of Harris *et al.* (1969) as described by Werb & Burleigh (1974).

Incubations of collagen with cathepsin B1 were terminated by the inactivation of the enzyme with Tos-LysCH_2Cl or Ac-[Ala]$_3$-AlaCH_2Cl at a final concentration of 0.1mM. The reaction products were denatured by heating in 8M-urea at 50°C for 30min and separated by disc-gel electrophoresis by a method based on that of Nagai *et al.* (1964) (Werb & Burleigh, 1974). Products of the digestion of gelatin were examined in the same way.

Reaction products were further examined by gel filtration on a column (1.5cm×85cm; 150cm^3) of Bio-Gel A-0.5, equilibrated with 1M-$CaCl_2$ in 50mM-Tris–HCl buffer, pH7.4, at room temperature. The pH of the samples was adjusted by the addition of 1M-Tris before application to the column. The columns were eluted with the same buffer at a flow rate of 14ml/h. Eluents were monitored at 230 and 222nm. Collagen α- and β-chain markers were prepared from heat-denatured acid-soluble rat skin by the method of Piez (1968) with a column (2.2cm× 15cm; 57cm^3) of CM-cellulose at 40°C. The column was equilibrated with 60mM-sodium acetate buffer, pH4.7, and eluted at 120ml/h with a linear gradient (800ml) of 0–0.1M-NaCl in the acetate buffer. Cyanogen bromide peptide markers from the α1 chain of rat skin collagen, as characterized by Butler *et al.* (1967), α1-CB3 (mol.wt. 13600), α1-CB6 (mol.wt. 18000) and α1-CB7 (mol.wt. 24500), were kindly given by Dr. A. Nicholls, National Institutes of Health, Bethesda, Md., U.S.A.

Assay of 'gelatinase' activity

Gelatin was prepared and assayed as described by Werb & Burleigh (1974) with the buffers and enzyme given in the Results section. Harris & Krane (1972) demonstrated that fragments of gelatin of mol.wt. 5000 or less remain in solution in 15% trichloroacetic acid under the conditions used.

Measurement of the degradation of insoluble collagen by cathepsin B1

Incubation mixtures contained 4mg of bovine tendon collagen in 0.1M-sodium formate buffer with 1mM-cysteine and 1mM-EDTA (disodium salt) and 0.6 unit of cathepsin B1 in a total volume of 1.0ml. Tubes were rotated in a roller rack at 37°C. After incubation periods of up to 24h, the collagen was removed by filtration with Whatman no. 1 paper, as this was found to be more reproducible than centrifugation. Samples of the filtrate were hydrolysed in 6M-HCl for 20h at 105°C in sealed glass ampules before analysis for hydroxyproline by a method based on those of Woessner (1961) and M. J. Crossley, D. E. Woolley & J. M. Evanson (unpublished work). Samples were cooled and clarified by the addition of charcoal resin decolorizer (prepared from equal weights of activated charcoal and AG-1 X8 anion-exchange resin), which was then removed by filtration. The filtrates were neutralized by the addition of NaOH with 100μl of phenolphthalein solution (1mg/ml) as internal indicator and made up to a total volume of 2.0ml. Three portions (0.5ml) were taken from each sample; one was assayed directly, another served as a non-oxidized control and 5μg of hydroxyproline was added to the third as an internal standard. An equal volume of half-saturated NaCl was added to each tube followed by 1.0ml of freshly-prepared chloramine-T reagent. The chloramine-T reagent consisted of chloramine-T (0.353g), methoxyethanol (30ml), water (20ml) and 50ml of a buffer containing citric acid (2.5g), acetic acid (0.6ml), anhydrous sodium acetate (3.575g) and NaOH (1.7g) adjusted to pH6.0. After exactly 4min at room temperature, 1.0ml of colour reagent containing equal volumes of aq. 32% (w/v) $HClO_4$ and 5% (w/v) *p*-dimethylaminobenzaldehyde in propan-1-ol was added to the oxidized mixtures. The colour reagent was added to the non-oxidized controls before the addition of the chloramine-T reagent. All tubes were incubated at 65°C for 12min and the E_{560} read after cooling. After

correction of all readings for the non-oxidized control, the observed increase in extinction owing to the internal hydroxyproline standard was used to correct the experimental hydroxyproline measurements. The extinction was linear with concentration up to an E_{560} of 0.8. The concentration of hydroxyproline in the hydrolysis of the original reaction mixture was 15% of the total collagen, i.e. 600μg/ml.

Results

Release of radioactive peptides from reconstituted collagen fibrils by cathepsin B1

Effect of pH, time and enzyme concentration. Fibrils were reconstituted from ^{14}C-labelled collagen at pH7.2 and incubated with enzyme over the pH range 5.6–7.4. The fibrils were reconstituted at pH7.2 because fibrils formed under acid conditions have different characteristics (Martin *et al.*, 1961). The results shown in Fig. 1 indicate that cathepsin B1 caused significant release of ^{14}C-labelled peptides from the reconstituted fibrils at pH values between 5.6 and 7.0, the activity increasing towards acid pH. It was not possible to study fibril lysis at lower pH values, since acid solubilization of the collagen gave rise to excessively high control values; for instance 30% of the collagen was solubilized in the buffer control incubation at pH5.5. Low activity above pH7 may be partly attributable to irreversible inactivation of the enzyme (Barrett, 1973). A similar extent of fibril lysis was observed at pH5.6 when the Tris–maleate buffer was replaced by 33mM- sodium acetate buffer.

The degradation of collagen fibrils by 0.5 unit of cathepsin B1 as a function of time is shown in Fig. 2(*a*). For each time-period, results were corrected by using buffer control values obtained by incubating buffer in place of enzyme.The results shown in Fig. 2(*b*) indicate that release of radioactivity increased with enzyme concentration up to 40% lysis. The upper limit varied slightly between preparations of fibrils.

Effects of inhibitors. The effects of a selection of inhibitors of cathepsin B1 on the degradation of collagen fibrils and the effect of omitting the activators, EDTA (disodium salt) and cysteine, were investigated. The results are presented in Table 1; buffer controls were incubated for each inhibitor in case the inhibitor altered the amount of fibrils solubilized. The thiol-blocking agent, 4-chloromercuribenzoate, and Tos-LysCH_2Cl and Ac-[Ala]$_3$-AlaCH_2Cl were all inhibitory; the absence of EDTA and cysteine greatly decreased the action of the enzyme on collagen. The inhibition of cathepsin

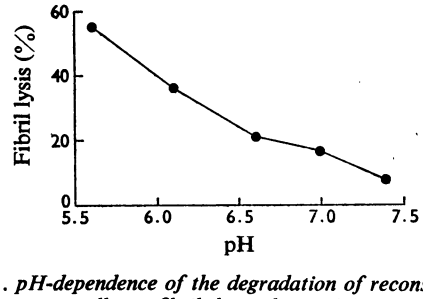


Fig. 1. *pH-dependence of the degradation of reconstituted collagen fibrils by cathepsin B1*

The extent of fibril lysis was measured after incubation of reconstituted collagen fibrils with 0.4 unit of cathepsin B1 in the presence of 1mM-EDTA (disodium salt), 1mM-cysteine and 33mM-Tris–maleic acid buffer for 20h at 35°C as described in the Methods section. Assays were carried out in triplicate and buffer control values subtracted for each pH value.

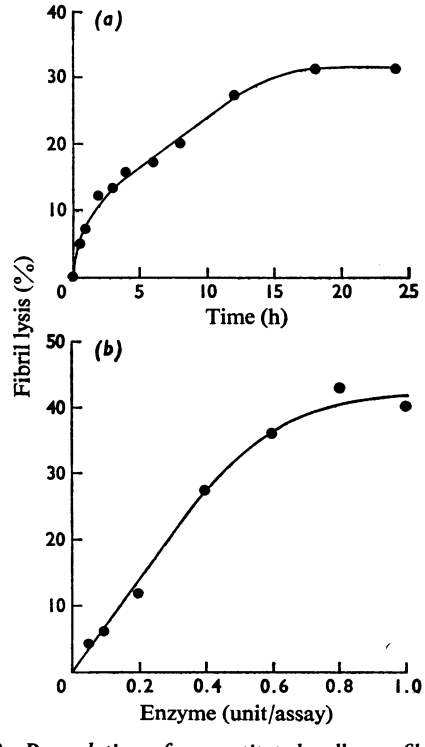


Fig. 2. *Degradation of reconstituted collagen fibrils by cathepsin B1*

(*a*) Effect of incubation time in the presence of 0.5 unit of cathepsin B1 and (*b*) effect of the amount of enzyme, over an 8h time-period. Incubation mixtures in 33mM-sodium acetate buffer, pH5.6, were as described in the Methods section. Buffer control values for each time-period were subtracted from the experimental results.

Table 1. *Effect of inhibitors on the degradation of reconstituted collagen fibrils by cathepsin B1*

Cathepsin B1 (0.4 unit for an 8h incubation, or 0.14 unit for 20h with α_2-macroglobulin) was premixed with the inhibitor and added to the assay mixture which contained 33mM-sodium acetate buffer, pH5.6. Results are expressed as the mean values from triplicate assays after subtraction of the appropriate inhibitor control values. In the absence of inhibitor, the extent of fibril lysis in this experiment was 42%.

Compound	Final concn. (mM)	Activity (% of control)
4-Chloromercuribenzoate	2.0	9
Tos-LysCH_2Cl	0.5	2
Ac-[Ala]$_3$-AlaCH_2Cl	0.1	0
α_2-Macroglobulin	5.8×10^{-3}	0
No EDTA or cysteine	—	21

B1 by α_2-macroglobulin has been described in detail by Starkey & Barrett (1973); in the present work, this naturally occurring inhibitor, in 1.9-fold molar excess, completely prevented the action of the enzyme on collagen.

Characterization of the products. Assays with reconstituted collagen fibrils were normally carried out at 35°C to avoid the risk of accidental denaturation of the substrate (the mid-point 'melting' temperature of the acid-soluble rat skin collagen being 39°C, as reported below). Assays were made with 0.5 unit of cathepsin B1 during 8h at 35° and 37°C, with the following results. At 35°C, 32% of the radioactivity was released from the collagen, of which 73% was soluble in 15% (w/v) trichloroacetic acid. At 37°C, 46% lysis occurred and 95% of the radioactivity was soluble in 15% (w/v) trichloroacetic acid.

The products released from the fibrils during assays at both temperatures were denatured by heating in 8M-urea as described in the Methods section and studied by disc-gel electrophoresis (Plate 1*a*). The pattern obtained with untreated collagen shows the α doublet, the β doublet (comprised of cross-linked pairs of α chains) and higher aggregates (γ-components) of still lower mobility (Werb & Burleigh, 1974). The collagen solubilized in the absence of enzyme at each temperature showed small amounts of β and α components. The products resulting from the incubation of fibrils with cathepsin B1 show a marked loss of β components and the presence of α components. Although the α bands appear less intense at 37°C than at 35°C, presumably because of further degradation, no new bands appeared. The absence of such new bands is taken to mean that once degradation of an α chain has been initiated, the chain is rapidly converted into low-molecular-weight products soluble in 12–15% (w/v) trichloroacetic acid, and therefore undetected in the gels. From the work of Harris & Krane (1972) it would be concluded that these products were of less than 5000 molecular weight.

Degradation of collagen in solution by cathepsin B1

Results of disc-gel electrophoresis. The effect of cathepsin B1 on collagen in solution was investigated over the pH range 3.7–6.3. An incubation temperature of 24°C was chosen, since the products of digestion of collagen by neutral collagenase, collagen A and B pieces, have 'melting' temperatures of 33° and 28°C respectively (Sakai & Gross, 1967; Evanson *et al.*, 1968), and it was possible that the action of cathepsin B1 might also give products with relatively low denaturation temperatures. The results of disc-gel electrophoresis of the urea-denatured reaction products are shown in Plate 1(*b*). Buffer control assays were run at each pH value, but all gave similar results, so that only one is shown in Plate 1(*b*). The pattern of the control collagen shows the α and β doublets together with the higher aggregates, which did not run far into the gel. Below pH6, incubation with cathepsin B1 resulted in conversion of β into α components, which indicated a cleavage of the non-helical peptide region containing the intramolecular cross-link. In addition, there was a series of bands which ran faster than the α doublet, and which probably represent products of further degradation.

Viscosity, optical rotation and segment-long-spacing aggregates. The effects of cathepsin B1 on collagen in solution at pH4.7 were investigated further by monitoring changes in viscosity and optical rotation in identical reaction mixtures at 24°C. Results of one such experiment are shown in Fig. 3. Cathepsin B1 caused a fall in viscosity from 3500 to 1400ml/g, a decrease of almost 60%. Since no significant change in optical rotation was observed, it can be concluded that the helical conformation of the reaction products had been maintained. At various time-intervals during the above experiment, samples of the reaction mixture were removed for examination by the preparation of segment-long-spacing aggregates. These are crystalloids in which the collagen molecules line up beside one another with all the *N*-terminal or A-ends in register, under the influence of a polyanion such as ATP. No discrete aggregates of shorter length were found, so that there was no indication of the existence of a specific cleavage region of the collagen molecule.

'Melting' curves of collagen before and after reaction with cathepsin B1. The 'melting' profiles of soluble collagen, and of the products of its degradation by cathepsin B1, were monitored by changes in optical rotation, at pH4.7. Three series of experiments gave similar 'melting' curves, one of which is shown in Fig. 4. Incubation with the enzyme caused

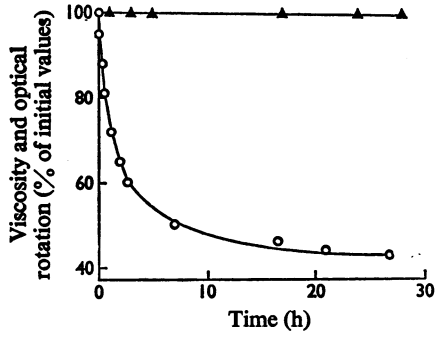


Fig. 3. *Changes in optical rotation and viscosity during incubation of cathepsin B1 with acid-soluble collagen in solution at* 24°C

Incubations were set up as described in the Methods section, in 50mM-sodium acetate buffer, pH4.7, with 0.3 unit of enzyme/ml. ▲, Optical rotation; ○, viscosity.

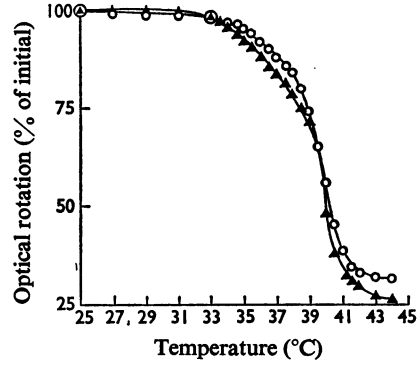


Fig. 4. *'Melting' profiles for collagen before and after incubation with cathepsin B1 at* 24°C

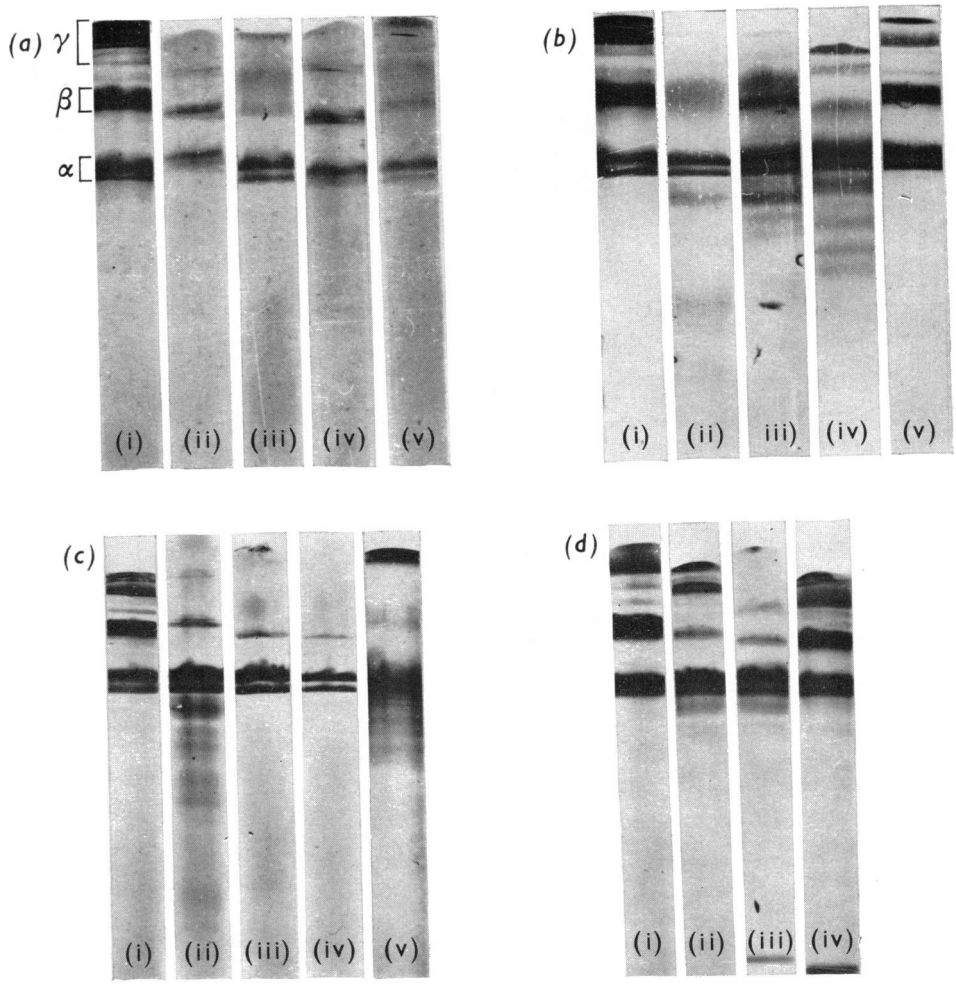
The reaction mixtures used were those described in Fig. 3. The temperature of the water jacket was raised by 1°C every 30min. ○, Control incubation in the absence of enzyme; ▲, material after digestion by cathepsin B1.

no change in the mid-point 'melting' temperature, or T_m, of the rat skin collagen, though the shoulder of the curve was less sharp for the collagen that had been incubated with the enzyme. The shallow slope may reflect a polydisperse size distribution among the reaction products.

Products of degradation of collagen in solution at 37°*C*. The melting profile of collagen after incubation with cathepsin B1 indicated that the digestion products were not denatured below 37°C; therefore in this experiment the products of the reaction at 24°C were compared with those at 37°C. A reaction mixture of total volume 20ml was made up as described in Fig. 3 and incubated at 24°C for 24h, by which time a 60% fall in viscosity had occurred. One 5ml sample was frozen, two were further incubated at 37°C for 4 and 8h respectively and the fourth was heated at 37°C for 4h in the presence of a final concentration of 0.1mM-Ac-[Ala]$_3$-AlaCH$_2$Cl, sufficient to inhibit the enzyme totally.

The gel-electrophoresis patterns of the denatured products of the last three reaction mixtures are presented in Plate 1(*c*); the incubation mixture that was not heated above 24°C (not shown) gave a similar pattern to that incubated at 37°C in the presence of inhibitor. In both cases the proportion of radioactivity from the undenatured reaction products soluble in 15% (w/v) trichloroacetic acid was 15%. It is concluded that heating to 37°C in the absence of further enzymic activity did not cause release of small peptides which might have been held together by non-covalent interactions. Heating the mixture to 37°C while the enzyme was still active caused a further loss of β components and total loss of higher aggregates together with a decrease in intensity of the α bands which was more pronounced in the 8h incubation products. There were no bands of higher mobility than the α doublet in either of these two reaction mixtures. The proportion of products soluble in 15% trichloroacetic acid increased to 55% after 4h and to 83% after 8h at 37°C. It seemed probable that most of the material precipitated by trichloroacetic acid at this stage consisted of α chains.

Separation of the reaction products by gel filtration. An attempt was made to characterize further the products of the action of cathepsin B1 on collagen in solution by separation on a column of Bio-Gel A-0.5. Peptides obtained by the cyanogen bromide cleavage of rat skin collagen together with α and β chains prepared from rat skin collagen were used as markers, since polypeptides derived from collagen chromatograph differently from globular proteins of similar molecular weight (Piez, 1968). Fig. 5 shows the elution profiles of the reaction products obtained from the experiments described in the previous section. It is of interest to compare the elution profiles with the patterns found by disc-gel electrophoresis (Plate 1*c*). The β components and higher aggregates were excluded by the Bio-Gel A-0.5, and the α chains were eluted soon afterwards; these were the only peaks observed in the control incubation mixture. The peak of excluded material was entirely absent from the three reaction mixtures shown, indicating a loss of β and larger components at the end of the incubation at 24°C. The products of further enzymic degradation at 37°C for 4h showed a decrease in the amount of α chains with a corresponding appearance of included material. The elution profile of the material incubated for a further 4h gave the same two main peaks, with a decrease in the height of the peak corresponding to



EXPLANATION OF PLATE I

Disc-gel electrophoresis of denatured products of digestion of collagen

For all gels migration was downward, towards the cathode. (*a*) Products of digestion of collagen fibrils by cathepsin B1. (i) Untreated collagen fibril control; (ii) collagen solubilized at 35°C in the absence of enzyme; (iii) products solubilized by cathepsin B1 at 35°C; (iv), as (ii), but at 37°C; (v), as (iii), but at 37°C. (*b*) Effect of pH on digestion of collagen in solution at 24°C by cathepsin B1. Incubation mixtures contained 500 μg of collagen, 50mM-sodium acetate (pH3.7, 4.6 and 5.5) or phosphate buffer (pH6.3), 0.3 unit of cathepsin B1 and 1mM-EDTA and cysteine in a total volume of 200 μl. (i) Control without enzyme, pH4.6; (ii) with enzyme, pH3.7; (iii) pH4.6, (iv) pH5.5 and (v) pH6.3. (*c*) Products of the further incubation of soluble collagen with cathepsin B1 at 37°C after treatment at 24°C, and incubation with papain at 24°C. Incubation mixtures were as described in the text. (i) Control collagen incubated at 37°C in the absence of enzyme, (ii) further incubation with cathepsin B1 at 37°C for 4h in the presence of 0.1mM-Ac-$[Ala]_3$-$AlaCH_2Cl$, (iii) further incubation at 37°C for 4h, (iv) further incubation at 37°C for 8h, (v) incubation with 50 μg of papain at pH4.3 at 24°C for 24h. (*d*) Effect of a rat liver lysosomal preparation on collagen in solution. Incubation mixtures contained 200 μg of collagen, 100mM-sodium acetate buffer, pH4.0, 380 μg of lysosomal protein plus potential inhibitors or activators in a total volume of 90 μl. (i) Control incubated in the absence of lysosomal protein, (ii) lysosomal protein with no inhibitors, (iii) with 1mM-EDTA (disodium salt) and 1mM-cysteine, (iv) with 0.1mM-Ac-$[Ala]_3$-$AlaCH_2Cl$.

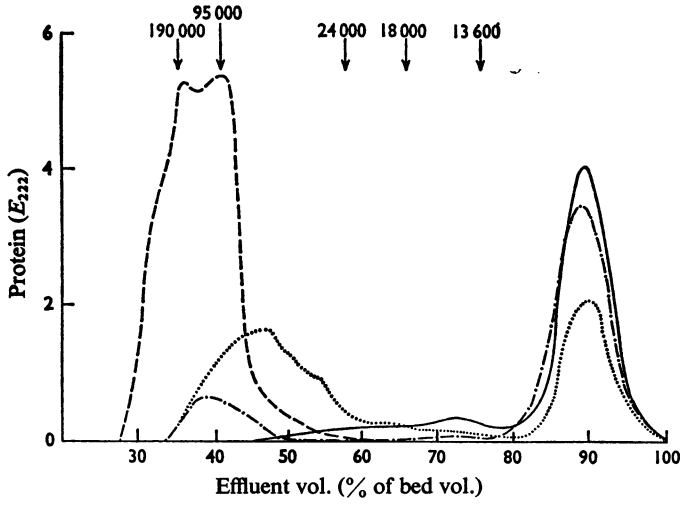


Fig. 5. *Elution profile of the products from the digestion of collagen in solution by cathepsin B1*

Reaction mixtures were those described in Fig. 3 and the text. The reaction mixture (4ml) was applied to each column after adjustment of pH and $CaCl_2$ concentration as described in the Methods section. ----, Control, consisting of collagen incubated at 37°C in the absence of enzyme and heat-denatured before application to the column; -·-·, reaction mixture incubated at 24°C for 24h, then at 37°C for 4h; ——, reaction mixture incubated at 24°C for 24h, then at 37°C for 8h; ····, reaction mixture incubated at 24°C for 24h then heated to 37°C for 4h in the presence of Ac-$(Ala)_3$-$AlaCH_2Cl$. The molecular-weight markers were α1 chains (mol.wt. 95000), α1-CB7 (mol.wt. 24500), α1-CB6 (mol.wt. 18000), α1-CB3 (mol.wt. 13600).

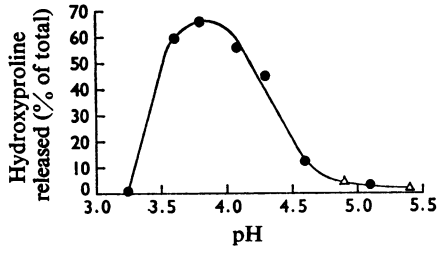


Fig. 6. *Effect of pH on the degradation of bovine achilles-tendon collagen by cathepsin B1*

Reaction mixtures containing 0.3 unit of cathepsin B1, 2mg of collagen, EDTA and cysteine (1mM each) and 80mM-sodium formate (●) or acetate (△) buffer in a total volume of 0.5ml were incubated at 37°C for 24h. The pH of each incubation mixture was measured after the removal of the undegraded collagen.

α chains and a corresponding increase in the size of the peak of the included products. The fast-moving bands observed in the gel-electrophoresis pattern of Plate 1(*c*) gel (ii) did not correspond to a series of discrete peaks in the elution profile; instead, they appeared as a 'tail' from the α chain peak, the position of which indicated a molecular-weight range of 25000–95000.

Effects of cathepsin B1 on insoluble collagen

The results of incubating cathepsin B1 with insoluble collagen from bovine achilles tendon in buffers over a range of pH values are shown in Fig. 6 as the percentage of hydroxyproline released into the medium. Up to 67% of the total hydroxyproline was released over the 24h assay period. In a separate experiment, with sodium formate buffer at pH3.5, it was found that 39% of the total hydroxyproline was released after 2h and 51% after 4h. Samples of the reaction products were mixed with 0.5vol. of 45% (w/v) trichloroacetic acid, as described in the Methods section for the gelatinase assay. The material soluble in 15% trichloroacetic acid was analysed for hydroxyproline content; on average, 75% of the hydroxyproline released from the collagen was soluble in 15% (w/v) trichloroacetic acid and was thus shown to be in peptides of mol.wt. 5000 or less. The proportion remained constant over incubation times ranging from 2 to 24h. When the material released from the tendon collagen by cathepsin B1 was denatured by heating in urea and examined by disc-gel electrophoresis, the only products stained with Coomassie

Table 2. *Reaction products from the incubation of cathepsins B1 and D with gelatin and collagen*

Incubation mixtures contained 100μg of ^{14}C-labelled gelatin in 100mM-sodium acetate buffer, pH4.8, EDTA and cysteine and 0.03 unit of cathepsin B1. With cathepsin D, 100mM-sodium formate buffer, pH3.0, was used. Assays were made at 37°C for 4 or 20h and terminated by the addition of trichloroacetic acid to 15% (w/v). The amount of products soluble in the trichloroacetic acid (mean value from triplicate assay) is quoted as 'acid-soluble (%)'.

Enzyme	Amount (units)	Substrate	Ratio of $\alpha/(\beta+\gamma)$ components		Acid-soluble (%)		
			Control	Enzyme (20h)	Control	Enzyme (4h)	Enzyme (20h)
Cathepsin D	15	Gelatin	1.41	1.43	2	0	0
	4	Collagen	1.40	1.38	0	0	0
Cathepsin B1	0.03	Gelatin	1.23	(Complete conversion)	3	89	100
	0.06	Collagen	1.26	3.93*	0	55	100

* After 4h incubation; after 20h, there was no $(\beta+\gamma)$ component left.

Brilliant Blue formed a diffuse zone of protein running in the region of the α bands.

Action of cathepsin B1 on gelatin and Pz-Pro-Leu-Gly-Pro-D-Arg

The effect of cathepsin B1 on gelatin at pH4.8 was investigated and the results are shown in Table 2. Identical assays were set up in which the reaction products were heated in 8M-urea and examined by polyacrylamide-disc-gel electrophoresis. The radioactivity solubilized from slices of the gels was used to calculate the ratio of α components to $(\beta+\gamma)$ components, and these ratios are presented in Table 2. For comparison, the effects of the enzymes on collagen in solution under similar reaction conditions are also represented.

Cathepsin B1 degraded gelatin to peptides of less than 5000mol.wt. The reaction had nearly reached completion after 4h. It was impossible to give a ratio of α to $(\beta+\gamma)$ components at this stage, since there were no components remaining larger in size than α chains.

In view of the ability of cathepsin B1 to attack the helical region of the collagen molecule, its activity against Pz-Pro-Leu-Gly-Pro-D-Arg was tested. Clostridial collagenase shows both activities (Wünsch & Heidrich, 1963), and the synthetic peptide is also cleaved by endopeptidases from tadpole (Harper & Gross, 1969) and human synovium (Harris & Krane, 1972). Assays were made as described by Werb & Burleigh (1974). No activity was detected with up to 0.1 unit of cathepsin B1, in the presence of EDTA and cysteine at pH6, during 1h at 37°C.

Action of papain on collagen in solution

Assays were made on collagen in solution in 50mM-sodium acetate buffers of pH4.3 and 4.8, at 24°C for 24h. The reaction mixtures (200μl) contained 500μg of collagen and 1mM-EDTA and cysteine. The amounts of papain used were 10, 20 or 50μg (i.e. 0.05, 0.10 and 0.25 unit in the assay described in the Methods section for cathepsin B1). Polyacrylamide-gel electrophoresis of the reaction products (Plate 1*c*) showed complete loss of the higher aggregates, a large or almost complete depletion of the β components, and the appearance of many bands of greater mobility than the α components. Thus the products appeared closely similar to those produced at the same temperature (24°C) by 0.30 unit of cathepsin B1 [Plate 1*c* gel (ii)].

Assays of cathepsin D activity against collagen, gelatin and Pz-Pro-Leu-Gly-Pro-D-Arg

Assays were made to test for any activity of purified human cathepsin D against soluble collagen and reconstituted fibrils. At pH5.6, 4 units of enzyme/ml caused less than 3% lysis of the reconstituted fibrils above the buffer control values; this was lower than the lysis obtained in control incubations with trypsin. The results suggest that fibrils were not solubilized by cathepsin D under these conditions; a lower pH value could not be used in these experiments because of the spontaneous solubilization of the fibrils.

To study the action of cathepsin D on collagen at a pH value nearer the optimum for its activity on haemoglobin, up to 4 units of purified enzyme were incubated with soluble collagen at pH3. The results in Table 2 indicate that cathepsin D had no action on soluble collagen; neither a conversion of β into α components nor an appearance of peptide material soluble in 15% (w/v) trichloroacetic acid was observed. Similarly, there was no evidence of degradation of gelatin incubated with up to 15 units of cathepsin D, at pH3.2. These results are in direct contrast with those obtained with cathepsin B1.

Table 3. *Specific activities of cathepsins B1 and D in subcellular fractions and a partially purified preparation from rat liver*

Subcellular fractions were prepared and assayed for cathepsin B1 and D activities as described in the Methods section; the protein concentrations were determined as described in the text.

Fraction	$10^3 \times$ Specific activity of cathepsin B1 (units/mg)	Specific activity of cathepsin D (units/mg)
Homogenate	3.8	0.27
Nuclei plus cell debris	6.4	0.28
Heavy mitochondria	10.9	0.36
Light mitochondria	16.4	0.53
Microsomal fraction	4.2	0.24
Cytosol	0	0.17
$(NH_4)_2SO_4$ fraction	25.6	0.92

Cathepsin D (9 units), like cathepsin B1, failed to cause hydrolysis of Pz-Pro-Leu-Gly-Pro-D-Arg in a 1h assay at 37°C, at pH3.2.

Degradation of collagen by extracts from rat liver

In order to test whether cathepsin B1 contributes to the breakdown of collagen by lysosomal fractions obtained from rat liver (Frankland & Wynn, 1962; Anderson, 1969) or an $(NH_4)_2SO_4$ fraction from the same source (Etherington, 1972), the appropriate crude enzyme preparations were incubated with collagen in solution in the presence of various potential inhibitors and activators.

Subcellular fractions were prepared from rat liver as described in the Methods section and each was incubated with collagen in solution at pH4.0 in 100mM-sodium acetate buffer for 24h at 24°C. The amount of each subcellular fraction included in the 90μl reaction volume was 740μg (nuclei plus cell debris), 390μg (heavy mitochondria), 380μg (light mitochondria), 150μg (microsomal fraction), 190μg (cytosol); the amount of each fraction is expressed as μg of protein determined by the method of Lowry *et al.* (1951), bovine serum albumin being used as standard. The reaction products were denatured by heating in urea and examined as usual, by disc-gel electrophoresis. The light mitochondrial fraction showed marked production of degradation products of collagen, whereas the heavy mitochondria and nuclei plus cell-debris fractions were less active even at greater protein concentrations. Assays for cathepsins B1 and D in these fractions (Table 3) showed that the activity against collagen was correlated with the activity of these lysosomal enzymes. It is well known that lysosomes are present in the light-mitochondrial fraction in this fractionation scheme (de Duve *et al.*, 1955).

Further assays were made with selective inhibitors of the lysosomal proteinases in an attempt to establish which was active against collagen (Plate 1*d*). The pattern obtained without added factors [Plate 1*d* gel (ii)] showed a decrease in β components relative to α, and the appearance of faster moving bands. An identical result was obtained in the presence of pepstatin (25μg/ml), so that a role of cathepsin D or any other carboxyl proteinase may be excluded (Barrett & Dingle, 1972). Since the fractions were isolated in the presence of EDTA, and the addition of cysteine marginally increased degradation, metallo-proteinases can also be excluded. In contrast, degradation was completely inhibited by 0.1mM-Ac-$[Ala]_3$-$AlaCH_2Cl$, a finding that is consistent with the action of cathepsin B1. The $(NH_4)_2SO_4$ fraction from rat liver (130μg of protein/assay) was incubated with collagen in solution at pH3.5, 4.0 or 5.0 in the presence of the same factors, and essentially similar results were obtained at all three pH values. In addition, no activity was observed in the presence of two other inhibitors of cathepsin B1, 0.5mM-Tos-$LysCH_2Cl$ and 1mM-4-chloromercuribenzoate. Incubation of collagen in solution with an amount of purified cathepsin B1 corresponding to the activity present in the lysosomal fraction (0.07unit/ml) showed degradation products similar to those represented in Plate 1(*d*) gel (iii) (although larger amounts of cathepsin B1 were used in many of our experiments with the purified enzyme). Thus all of the activity in the lysosomal preparation could be accounted for by cathepsin B1.

Discussion

Our results indicate that cathepsin B1 degrades collagen in solution, reconstituted collagen fibrils and insoluble collagen. The digestion process was most readily characterized by examination of the reaction products obtained from collagen in solution. The first action of cathepsin B1 was seen as a loss of β components and higher aggregates and a build-up of α chains. We interpret this as an attack on the non-helical peptide regions whereby the cross-links were eliminated. The loss of intermolecular cross-links would explain the large fall in viscosity with no concomitant change in optical rotation, since these cross-links give rise to dimeric collagen molecules in solution (Davison & Drake, 1966). Cleavage of the dimers by ultrasonic irradiation has been shown to produce a fall in viscosity of a similar magnitude to that which we observed, with no change in optical rotation. The solubilization of polymeric collagen by a lysosomal 'depolymerase' reported by Milsom *et al.* (1972) might well be explained by cleavage of the *N*-terminal region, resulting in loss of intermolecular cross-links. Since this activity was inhibited by iodoacetamide and stimulated by cysteine, it may well

have been due to cathepsin B1, but since cysteine was not used in those experiments for which the reaction products were characterized, these authors did not observe the more complete degradation reported in the present paper.

Cathepsin B1 differs from most serine and carboxyl proteinases, e.g. chymotrypsin and pepsin, in that it causes degradation of the α chains in the helical region of the molecule. This effect was seen most clearly at the higher enzyme concentrations, and was more extensive at 37°C than 24°C with soluble collagen. Since the products failed to form segment-long-spacing aggregates under conditions in which the undegraded molecules formed aggregates, it seems probable that they were not predominantly of any one size. This finding contrasts clearly with that for specific collagenase, which gives rise to three-quarters- and one-quarter-length fragments (Gross & Nagai, 1965).

The results of gel chromatography of the products of the action of cathepsin B1 on soluble collagen at 24° and 37°C confirmed their polydisperse size distribution. After incubation at 37°C only very small amounts of products of molecular weight greater than 13600 were detected by gel chromatography, and precipitation with trichloroacetic acid indicated that over half of the molecules were of less than 5000 daltons. Analysis of the soluble products of digestion of fibrils at 35°C indicated that these too were principally of low molecular weight. In the incubation mixtures with soluble or fibrillar collagen it was found that almost all of the material insoluble in 15% trichloroacetic acid was at least as large as α chains. It is concluded that once an α chain has been attacked by cathepsin B1, it is rapidly degraded to peptides of low molecular weight. Huisman *et al.* (1973) have reported that the degradation of several other proteins by thiol-dependent cathepsins is an all or none process.

Different pH optima were observed for the action of cathepsin B1 on soluble collagen and on insoluble collagen. The pH optimum for the purified enzyme has been found to vary with the substrate, approximate values being pH6.0 (Bz-DL-Arg-$NHC_{10}H_7$; Barrett, 1972), 5.0 (cartilage proteoglycan; Morrison *et al.*, 1973) or 4.0–4.5 (haemoglobin; Otto, 1971). Results obtained in the present work indicated that the highest activity against collagen in solution is obtained in the region of pH4.5–5.0. The finding that the release of soluble material from insoluble collagen was most rapid at pH3.5 may be explained by mechanical swelling of the collagen under acid conditions increasing the accessibility of susceptible bonds in the substrate to the enzyme. In agreement with the results obtained here for purified cathepsin B1, different pH optima have been reported for the action of lysosomal extracts on the two types of substrate. Thus several workers have found optimum activity near pH3.5 in degradation of insoluble collagen (Woessner & Brewer, 1963; Anderson, 1969; Etherington, 1972, 1973), whereas Bazin & Delaunay (1966) found three peaks of activity against acid-soluble collagen in solution, the highest at pH4.6.

The products resulting from the action of papain on collagen in solution closely resembled those produced by cathepsin B1, in gel electrophoresis. This suggests that the mechanism of degradation of collagen shown for cathepsin B1 may be shared by other thiol proteinases. Sheldon & Kimball (1962) showed fibrillar collagen within intracellular vacuoles of the cells of rabbit ear cartilage after intravenous injection of papain. These results can be interpreted in terms of extracellular degradation of insoluble collagen by the thiol proteinase, followed by a second stage of digestion within the cells.

It has been suggested that cathepsin D might be responsible for the degradation of collagen by rat liver and uterine lysosomal extracts (see the introduction), but evidence has been presented here that purified cathepsin D has no action on collagen. This finding agrees with the work of Etherington (1972), who reported the separation by gel filtration of collagen-degrading activity of rat liver from the activity against haemoglobin assayed in the absence of cysteine.

Harris *et al.* (1972) reported that a preparation of cathepsin D from bovine spleen caused conversion of β into α chains in gelatin. In our experiments no such action of purified human cathepsin D, at approximately 10-fold higher concentration, could be demonstrated, nor was there any formation of products soluble in 15% trichloroacetic acid.

The collagen substrates used in our experiments were extracted from rat skin under acidic conditions. Becker *et al.* (1972) and Stoltz *et al.* (1972) have shown that collagen extracted in this way from skin of calf and rat respectively loses a small portion of the non-helical peptide region at the carboxyl end, suggesting limited digestion by acid proteinases during extraction. Cathepsin D may have been responsible, and such an action would not have been detected in the present work. However, cleavage of such a short region of non-helical peptides would not be important in the degradation of skin collagen.

Evidence has been presented here that cathepsin B1 plays an essential part in the degradation of soluble collagen by lysosomal enzyme preparations *in vitro*; this has been shown with an extract of rat liver lysosomes, and with an enzyme preparation from rat liver made as described by Etherington (1972). Similar patterns of degradation products were observed in gel electrophoresis when collagen in solution was digested by purified cathepsin B1 or by either of the rat liver enzyme preparations. Activity of the $(NH_4)_2SO_4$ fraction was stimulated a little by

EDTA and cysteine and inhibited by 4-chloromercuribenzoate, suggesting the action of a thiol proteinase; total inhibition of the activity also occurred in the presence of low concentrations of the chloromethylketones derived from acetyltetra-alanine and tosyl-lysine, both known to inhibit cathepsin B1 (Barrett, 1973). Pepstatin and di-isopropyl phosphorofluoridate had no effect. Similar sensitivity to inhibitors has been reported for the enzyme extracted from involuting post-partum rat uterus, which degrades collagen (Etherington, 1973). Data in several earlier reports of collagen-degrading activities in lysosomal preparations have indicated that a thiol proteinase was involved (Woessner, 1968; Anderson, 1969; Bazin & Delaunay, 1970; Etherington, 1972). In the present work the importance of cathepsin B1 in the degradation of collagen was shown by the use of soluble collagen as substrate. The susceptibility of collagen, in this form, to enzymic degradation is such that any enzyme capable of acting on insoluble collagen should have been detected. Since cathepsin B1 seemed to be of primary importance for the degradation of soluble collagen by lysosomal enzymes, it is probably safe to assume that it plays a part in the breakdown of insoluble collagen within living cells. The possibility that other lysosomal enzymes contribute to the further degradation of the α chains has not been eliminated.

It is proposed that there are at least two independent ways in which the helical region of native collagen may be degraded under physiological conditions. One of these, perhaps the more important in the extracellular matrix, involves the action of a specific collagenase. The second, which may well be the major route of degradation of collagen taken into cells, is mediated by cathepsin B1. The two enzymes require very different conditions for activity: collagenase is most active at pH 7–8, in the presence of Ca^{2+}, and, being a metallo-proteinase, is inhibited by thiol and chelating agents (Evanson, 1971). Cathepsin B1, in contrast, is most active in the range pH 3–5, is unstable above pH 7, and is activated by thiol compounds. The action of both enzymes on collagen can be completely inhibited by the plasma protein α_2-macroglobulin (Werb, 1973; Barrett & Starkey, 1973).

The idea that a significant amount of collagen degradation *in vivo* may occur within cells is supported by previous work. The electron micrographs of Parakkal (1969*b*, 1972) and Brandes & Anton (1969) show collagen fibres within intracellular vacuoles of macrophages in involuting uterus, and similar results have been obtained by Perez-Tamao (1970) for cells of carrageenin granulomas. Since repeated attempts to demonstrate the presence of specific collagenase in rabbit alveolar macrophages were unsuccessful (M. C. Burleigh & G. S. Lazarus, unpublished work), it seems possible that cathepsin B1 is solely responsible for the initial attack on collagen by macrophages. Similar activity may be shown by cathepsin B1 in the lysosomal system of other cell types, particularly under conditions of rapid collagen breakdown.

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