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The Degradation of Cartilage Matrix by an Intracellular Protease

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Papain has been shown by Thomas (1956) and Potter, McCluskey, Weissmann & Thomas (1960) to have a gross chondrolytic effect on rabbit ear cartilage *in vitro* and *in vivo*. It has been shown that the intravenous injection of the blood proenzyme plasminogen into rabbits during experimentally enhanced permeability conditions, which permit the diffusion and activation of the enzyme in cartilage, produces a sequence of events similar to that caused by papain, though to a comparatively smaller extent (Lack, Anderson & Ali, 1961; Lack, 1961).

It was noticed that rabbit ear cartilage incubated *in vitro* at neutral pH at 37° for up to 24 hr. frequently released mucopolysaccharide components into the buffer, particularly during the longer periods of incubation, even without the addition of any external proteolytic enzyme. This release was completely inhibited in the presence of ϵ -amino-hexanoic acid (ϵ -aminocaproic acid) which is known to be a potent inhibitor of plasminogen activation (Alkjaersig, Fletcher & Sherry, 1959).

As Fell & Dingle (1963) and Lucy, Dingle & Fell (1961) have shown that the effect of an excess of vitamin A on embryonic chick cartilage is due to its ability to release intracellular proteases from the lysosomes, it seemed relevant to find out whether a proteolytic enzyme was also responsible for the autolytic breakdown of young rabbit ear cartilage *in vitro*. ϵ -Aminohexanoic acid and other structurally similar compounds and amino acids were used as inhibitors to characterize the enzyme involved.

MATERIALS AND METHODS

Cartilage. Fresh cellular ear cartilage was obtained from young (1-month-old) New Zealand red rabbits (1 kg. weight), stripped of all adhering skin and tissue, cut up into 5 mm.² pieces, weighed (0.750 g.), rinsed quickly twice with

0.9% NaCl and introduced into the incubation buffer. These manipulations were usually completed within 20 min.

In three specific experiments fresh ox articular cartilage, human (adult) tracheal cartilage and human (3-year-old) articular cartilage, stored at -10°, were used.

Chondroitin sulphate. Pure chondroitin sulphate A (sodium salt) prepared from bovine nasal septa (Mathews, Roseman & Dorfman, 1951) was used in electrophoretic and turbidimetric measurements.

Rabbit plasminogen. An acetone-precipitation method has been developed for preparing rabbit plasminogen from rabbit serum. This briefly consists of taking up the final protein precipitate, obtained by the method described by Christenson & MacLeod (1945), in 0.2 M-acetate buffer, pH 4.0, rejecting the precipitate obtained at 35% (v/v) acetone concentration and retaining the precipitate at 50% (v/v) acetone concentration. After dialysing against 1.5 M-NHCl, this rabbit plasminogen preparation was used in an initial experiment. The active enzyme fraction was assayed against casein by the method described by Davidson (1960), each unit being equal to the increase in extinction at 280 m μ /min./ml. of the enzyme fraction.

Buffers. 0.2 M-Acetate-HCl buffers between pH 1 and 2, 0.2 M-acetate buffers between pH 3.6 and 6.0, and 0.2 M-tris buffers between pH 6.4 and 7.4 (Gomori, 1955) were used unless otherwise stated.

Centrifugation. Tissue homogenates were centrifuged in a MSE refrigerated centrifuge (head radius 4.2 in.) at 4°.

Chemical estimations. Hexuronic acid was determined by the modification by Bitter & Ewins (1961) of the carbazole method of Dische (1947), with glucuronolactone as the standard. Hexosamine was estimated by the method of Elson & Morgan (1933), as described by Winzler (1955); D-glucosamine hydrochloride was used as the standard, and hydrolysis was carried out in 4 N-HCl for 4 hr. at 100°. Protein estimations were made by the method of Lowry, Rosebrough, Farr & Randall (1951), with reconstituted freeze-dried serum (Glaxo Laboratories Ltd., Greenford, Middx.) of known protein content as standard. Hydroxyproline was estimated by the method of Neuman & Logan (1950) after hydrolysing in 6 N-HCl for 3 hr. at 100°.

Electrophoresis. Paper electrophoresis in 0.1 M-barbiturate buffer, pH 8.6, was done at a potential difference of 8.3 v/cm. length for 5 hr. Mucopolysaccharide was fixed and stained simultaneously with 5-aminoacridine hydrochloride [0.4% (w/v) in 50% (v/v) ethanol]-toluidine blue [0.1% (w/v) in water] mixture (1:1, v/v) for 30 min. (A. J. Anderson, personal communication). Proteins were stained with aq. 0.1% bromophenol blue.

Turbidimetric studies. To estimate the acid mucopolysaccharide content the turbidimetric method of Di Ferrante (1956) was employed. Testicular hyaluronidase (1500 i.u. of Benger's Hyalase) was used as in the same method to characterize the isolated mucopolysaccharide material.

Estimation of proteolytic activity. The following modification of Anson's (1938) method, with bovine haemoglobin as the substrate, was employed to estimate the proteolytic activity. A fraction (1 ml.) of crude enzyme was added to 1 ml. of 0.2 M-acetate buffer, pH 4.0, and mixed with 0.5 ml. of 5% (w/v) haemoglobin solution. After incubation at 37° for 1 hr. 2 ml. of 20% (w/v) trichloroacetic acid was added to stop the reaction and precipitate undigested proteins. After filtration (Whatman no. 3 filter paper) the tyrosine-tryptophan content of the filtrate (1 ml.) was measured with the Folin-Ciocalteu phenol reagent, as given by Anson (1938). The amount of trichloroacetic acid-soluble material released (read against a standard tyrosine calibration curve) by 1 ml. of the crude enzyme fraction in 1 hr. is expressed as $\mu\text{g.}$ of tyrosine equiv. in Fig. 6.

Estimation of acid phosphatase. The method outlined by King & Wootton (1956) with phenyl phosphate as substrate was employed for estimating acid-phosphatase activity in the crude enzyme fraction.

Estimation of chondrolysis. Fresh cartilage (0.750 g.) was incubated in 50 ml. of 0.1 M-buffer at the selected pH in conical flasks at 37° for 24 hr. in a mechanical rotator (45 rev./min.). Penicillin (50 000 units) and streptomycin (50 000 units) were added to prevent bacterial contamination. Samples (4 ml.) were withdrawn at 4 hr. intervals for estimation of mucopolysaccharide released, by determining the hexuronic acid content. After 24 hr. the cartilage was removed for histochemical examination. Chondrolysis (Lack, 1961) refers to the breakdown of cartilage matrix as measured chemically by the release of hexuronic acid-containing acid polysaccharides and histochemically by the loss of metachromatic staining.

Estimation of hexuronic acid and hexosamine in residual cartilage. In an experiment to determine the ratio of acid polysaccharide released into the incubation buffer from cartilage to that remaining in cartilage, the following procedure was adopted. Four samples of heated (at 80° for 40 min.) and four samples of normal unheated cartilage (0.75 g.) were incubated in 50 ml. of 0.1 M-acetate buffer, pH 5.0, in eight 250 ml. conical flasks at 37° and rotated in a mechanical rotator. At fixed intervals the reaction was stopped by carefully removing the residual cartilage from individual flasks with a spatula and introducing it into 4 ml. of water in stoppered boiling tubes. The total hexuronic acid and hexosamine released into the incubation buffer was estimated directly by the methods described under Chemical estimations. To the residual cartilage in 4 ml. of water in stoppered boiling tubes 4 ml. of 10 N-HCl was added and the contents were warmed at 70° in a water bath for 20 min. This produced a complete dissolution of the residual cartilage and, after cooling and appropriate dilu-

tion, representative samples were taken for individual hydrolysis and estimation of hexosamine and hexuronic acid by the methods described under Chemical estimations.

Histology. Cartilage for histochemical examination was fixed in 10% formol saline. In an effort to eliminate possible errors due to differential metachromatic staining, the test and control samples were processed together. They were embedded in the same paraffin block and cut together at approx. 5 μ thickness on a sledge microtome. They were stained together on the same slide simultaneously with a solution of aq. 0.05% Azure A (Hughesdon, 1949). Besides Azure A, Alcian blue, Alcian green and colloidal iron (Mowry, 1958) stains were also used to substantiate and confirm the loss of mucopolysaccharide from the cartilage matrix.

Hypo-osmotic pretreatment of cartilage. Cartilage (0.750 g.) was incubated in 25 ml. of water at 4° for 1 hr. according to the method described by Lucy *et al.* (1961) and incubated subsequently at 37° after adding 25 ml. of 0.2 M-acetate buffer, pH 5.0. Corresponding amounts of cartilage were incubated at 4° for 1 hr. in 25 ml. of 0.2 M-acetate buffer, pH 5.0, alone or after adding NaCl until the concentrations were 0.9% or 1.8% (w/v). To each of these 25 ml. of water was added after incubation at 4° for 1 hr. and these flasks were subsequently incubated at 37° for 24 hr. along with the flasks whose contents received hypo-osmotic treatment, and chondrolysis was estimated as described above.

Preparation of crude enzyme fraction from cartilage. Fresh rabbit ear cartilage (3.6 g.) was cut into pieces (10 mm.²), washed with 0.9% NaCl and homogenized in 75 ml. of 0.25 M-sucrose solution at 4° for 20 min. in a MSE homogenizer at the top speed of 14 000 rev./min. The homogenate was centrifuged at 2500g for 20 min. The sediment was redistributed in 45 ml. of 0.25 M-sucrose solution. This suspension and the supernatant fraction were then used to determine the proteolytic and acid-phosphatase activities.

Method adopted with synthetic esters. To facilitate the use of small quantities of synthetic esters the following modification was made to the usual method of estimating chondrolysis as given above. Cartilage (0.20 g.) was incubated at 37° in 10 ml. of 0.1 M-acetate buffer, pH 5.0, in the presence of the following esters or amino acids, and the hexuronic acid was estimated in the incubation mixture after 24 hr in a mechanical rotator. The resulting incubated cartilage was then stained for loss of metachromasia. Lysine ethyl ester (0.15 and 0.05 M), lysine methyl ester (0.15 and 0.05 M) and tosylarginine methyl ester (0.15 and 0.05 M) were tested for their inhibitory effects on the release of mucopolysaccharides from cartilage. Arginine (200, 50 and 2 mM) and ornithine (200 and 50 mM) were also tested for their effect on cartilage breakdown.

Extraction of chondromucoprotein. Fresh rabbit ear cartilage (4.732 g.) was incubated at 37° for 24 hr. in 200 ml. of 0.1 M-acetate buffer, pH 5.0. The incubation mixture was centrifuged and the supernatant fluid (50 ml.) added to 2 ml. of aq. 2.5% (w/v) cetyltrimethylammonium bromide. The mixture was diluted with 30 ml. of water and left at 4° overnight to ensure maximum precipitation. The precipitate was centrifuged at 4° and washed three times with 95% (v/v) ethanol solution saturated with NaCl. The precipitate was suspended in 8 ml. of water and dissolved by adding 0.1 ml. of 0.1 N-NaOH. Then 26 ml. of 10% (w/v) perchloric acid was added and the protein precipitate centrifuged down and rejected. The clear supernatant fluid was

dialysed exhaustively against water for 3 days at 4°. Solid NaCl was then added to the dialysed solution to give a concentration of 0.2M, followed by 2 vol. of ethanol. The precipitated mucopolysaccharides were centrifuged down, washed twice with ethanol and dried *in vacuo*. The yield was 20 mg. A solution of this precipitate in water (1%, w/v) was used for characterization studies.

EXPERIMENTAL AND RESULTS

Effect of rabbit plasminogen and ϵ -aminohexanoic acid on the release of hexuronic acid from cartilage. When rabbit ear cartilage (0.75 g.) was incubated at 37° in 0.1M-tris buffer, pH 7.4, alone, in rabbit plasminogen (1000 units) and in rabbit plasminogen plus 0.4M- ϵ -aminohexanoic acid, it was found that there was a maximum release of hexuronic acid in the presence of plasminogen after 24 hr. (Fig. 1). The time lag in the release of hexuronic acid in the presence of plasminogen is probably due to the slow diffusion of the proenzyme into the cartilage matrix and its activation *in situ*. The control, i.e. cartilage in buffer alone, itself showed a substantial rise and this effect was inhibited by ϵ -aminohexanoic acid. This indicated that the autolytic breakdown of cartilage at pH 7.4 was being inhibited by ϵ -aminohexanoic acid and suggested the pos-

sibility that a specific enzyme was involved in this process.

Effect of pH on the release of mucopolysaccharide from cartilage. When cartilage was incubated in buffers of different pH values it was found that the amount of mucopolysaccharide released was pH-dependent, maximum amounts being liberated at pH 5.0 (Fig. 2). Histological examination of the incubated cartilage showed a maximum loss of metachromasia at pH 5 (Plate 1). The inhibition by ϵ -aminohexanoic acid and the pH-dependence suggested that the autolytic breakdown of cartilage was due to an enzyme.

Adoption of the method for estimation of chondrolysis. When cartilage was incubated at 37° at pH 5 a time-dependent release of hexuronic acid-containing material into the buffer was noted. When the incubated cartilage was removed, dissolved in 5N-hydrochloric acid and subsequently hydrolysed, the amount of hexuronic acid and hexosamine lost was inversely proportional to the amount liberated into the incubation mixture. Nearly 50% of the total hexuronic acid in cartilage was released into the buffer after incubation at pH 5.0 for 24 hr. (Table 1). Similar results were obtained when hexosamine was estimated instead of hexuronic acid. Further release was observed on extended incubation, but this was much more gradual.

Fig. 1. Release of hexuronic acid-containing material from cartilage at pH 7.4 after incubation with plasminogen and ϵ -aminohexanoic acid. Rabbit ear cartilage (0.75 g.) was incubated at 37° with: Δ , rabbit plasminogen (1000 units); $\bullet$, 0.1M-tris buffer, pH 7.4; $\circ$, rabbit plasminogen in the presence of ϵ -aminohexanoic acid. Samples of incubation mixture were taken at various times and analysed for hexuronic acid. Assays were carried out as described in the text. The results are the average of four experiments.

Fig. 2. Effect of pH on the autolytic degradation of cartilage. Rabbit ear cartilage (0.75 g.) was incubated at 37° for 24 hr. in 50 ml. of buffer at various pH values and the release of hexuronic acid-containing material into the buffer was estimated. Assays were carried out as described in the text.

Table 1. Release at pH 5.0 of material containing hexuronic acid and hexosamine from unheated and heated cartilage

Rabbit ear cartilage (0.75 g.), both unheated and heated (at 80° for 40 min.), was incubated at 37° in 50 ml. of 0.1M-acetate buffer, pH 5.0, for various times. The extent of cartilage breakdown was determined by estimating the hexuronic acid and hexosamine released into the incubation buffer in addition to that remaining in cartilage. Assays were carried out as described in the text.

Time of incubation (hr.)	Hexosamine (mg.)				Hexuronic acid (mg.)			
	0	10	17	24	0	10	17	24
Unheated cartilage	19.0	14.5	12.0	10.2	6.2	4.4	3.4	3.2
Incubation buffer	1.6	4.4	8.4	7.5	0.1	1.5	2.8	2.4
Total recovered	20.6	18.9	20.4	17.7	6.3	5.9	6.2	5.6
Heated cartilage	16.5	18.7	18.7	19.0	5.6	6.0	5.8	5.9
Incubation buffer	1.6	1.4	1.3	1.4	0.1	0.1	0.1	0.1
Total recovered	18.1	20.1	20.0	20.4	5.7	6.1	5.9	6.0

From these results, estimates of hexuronic acid in incubation mixtures were taken to be proportional to the extent of chondrolysis in subsequent experiments. When cartilage was heated at 80° for 40 min. and incubated at 37° at pH 5.0, there was no rise in the concentration of acid polysaccharide in the incubation mixture (Table 1 and Fig. 5). In no experiment could any substantial hydroxyproline release be detected after the incubation of cartilage at 37° at pH 5.0 for 24 hr. This ruled out the action of a collagenase in cartilage breakdown *in vitro*.

Characterization of the released product and involvement of a proteolytic enzyme. The hexuronic acid-containing material released from cartilage at pH 5.0 was of high molecular weight and negatively charged, as 90 % of it was recovered by precipitation with the cationic detergent cetyltrimethylammonium bromide. The isolated and purified material was non-diffusible and contained 38 % of hexosamine, 25 % of hexuronic acid and 13 % of protein. The high value for hexosamine is in excess of the theoretical value for pure chondroitin sulphate, as it is not in equal ratio to the accurately determined hexuronic acid by the carbazole method. It is possible that some hexosamine-containing acidic glycoproteins are co-precipitated by the cationic detergent used, and, as they are soluble in perchloric acid and non-diffusible, they would show up as a contaminant in the purified hexuronic acid-containing material. The presence of high percentage of contaminating protein points towards a similar possibility. The presence of glycoproteins other than acid mucopolysaccharides in cartilage has been reported by several workers (Shatton & Schubert, 1954; Anderson, 1962; Anderson, Lack & Ali, 1963). The protein moiety, since it was not precipitated by perchloric acid, was presumably bound to the acid polysaccharide. Moreover, on paper electrophoresis a major component moved with the same mobility as pure chondroitin sulphate but showed streaking characteristic of a viscous mucopolysaccharide. A minor component remained at

the origin and stained for proteins as well as metachromatically.

The turbidimetric studies with 200 μ g. of the isolated sample showed that it contained 71 % of acid polysaccharide when compared with pure chondroitin sulphate. When incubated with hyaluronidase over 90 % of this turbidity disappeared in 16 min. (Fig. 3). These studies indicate that the polysaccharide component is probably released intact from cartilage and is capable of subsequent enzymic hydrolysis by hyaluronidase. This suggests that the cleavage of the polymer from the cartilage took place in the non-collagenous protein component of the mucopolysaccharide by the action of a protease rather than a polysaccharase.

Effect of inhibitors on the release of chondromucoprotein. The inhibitory effect of ϵ -aminohexanoic acid at several concentrations (from 1 mM to 0.4M final concentration) was investigated. There was an exponential rise in the percentage inhibition of chondrolysis at pH 5.0 by increasing concentration of ϵ -aminohexanoic acid, varying from 21 % inhibition at 1 mM to 86 % at 0.4M (Figs. 4 and 7). A straight-line relationship was obtained when percentage inhibition was plotted against the

EXPLANATION OF PLATE 1

Rabbit ear cartilage (0.75 g.) incubated at 37° in buffer (0.1M) in the presence of the following compounds for 24 hr. was removed, fixed and stained with Azure A as described in the Materials and Methods section. Magnification $\times 100$. (A) Acetate buffer, pH 3.6; (B) acetate buffer, pH 5.0; (C) tris buffer, pH 7.4. The following were incubated in acetate buffer, pH 5.0, with the addition of: (D) cysteine (0.05M); (E) lysine (0.4M); (F) arginine (0.2M); (G) ϵ -aminohexanoic acid (0.4M); (H) lysine methyl ester (0.15M); (I) arginine methyl ester (0.15M). Metachromatic staining was retained at pH 3.6 and 7.4 and in the presence of the inhibitors ϵ -aminohexanoic acid, arginine and arginine methyl ester (A, C, F, G and I); metachromatic staining was decreased at pH 5 and in the presence of cysteine, lysine and lysine methyl ester (B, D, E and H).

logarithm of ϵ -aminohexanoic acid concentration, suggesting competitive inhibition. The competitive nature of the inhibitor could not be confirmed further as the substrate concentration could not be varied without affecting the enzyme concentration.

To determine whether the effect of ϵ -amino-hexanoic acid on the rate of chondrolysis was due to a specific inhibition of the enzyme or to a non-specific effect of its high concentration, glycine and lysine in similar concentrations (5 mM to 0.4 M) were tested with cartilage incubated at pH 5.0. Glycine had no effect, and lysine in high concentration (0.4 M) showed a certain enhancement of chondrolysis (Fig. 5). The effects of other amino acids and esters on chondrolysis are summarized in Tables 2 and 3, where the inhibitory effect of arginine and its ester is apparent. All these inhibitory studies with chemical estimations were confirmed by histochemical examination where the metachromatic staining of the matrix with Azure A remained intact in the presence of inhibitors (Plate 1). Again the action of a proteolytic enzyme was suggested, as there was inhibition only in the presence of structurally specific amino acids and esters. The consistent inhibition by arginine, its esters and ϵ -aminohexanoic acid suggests that the enzyme may hydrolyse peptide bonds involving arginine.

Proteolytic and acid-phosphatase activities of cartilage extract. The homogenized cartilage-cell

fractions sedimenting at 2500 g did not show any proteolytic activity at pH 4.0, and only 30 % of the acid-phosphatase activity. The supernatant fraction, which according to de Duve's (1959) method of fractionation would include the lysosomes, showed proteolytic activity and 70 % of the total acid-phosphatase activity. The pH-activity curve of the proteolytic activity in the dialysed crude enzyme fraction showed that it had a broad peak with an acid pH optimum (Fig. 6). 0.4 M- ϵ -Amino-hexanoic acid inhibited 40 % of the proteolytic activity against haemoglobin at pH 4.0. Attempts to homogenize the cartilage and fractionate the sub-cellular components were unsuccessful, as there is no gentle way of homogenizing cartilage that would leave the particulate elements intact.

Other experiments with cartilage. There was no significant increase of chondrolysis at pH 5.0 in rabbit ear cartilage previously subjected to hypo-osmotic conditions. Lucy *et al.* (1961) reported increased chondrolysis of chick embryonic cartilage under similar conditions. In fact the presence of sodium chloride [0.9 % and 1.8 % (w/v)] in the

Fig. 3. Depolymerization of isolated acid mucopolysaccharide by hyaluronidase. Acid mucopolysaccharide (2 mg.), released from cartilage after autolysis at pH 5.0 (see the Materials and Methods section), was incubated at 37° with hyaluronidase (1500 i.u.) in 10 ml. of 0.15 M-acetate buffer, pH 5.6, and the decrease in turbidity was estimated at various times as described in the text.

Fig. 4. Effect of ϵ -aminohexanoic acid concentration on the release of hexuronic acid-containing material from cartilage. Rabbit ear cartilage (0.75 g.) was incubated at 37° in 50 ml. of 0.1 M-acetate buffer, pH 5.0, for 24 hr. Samples were withdrawn at various times for estimation of hexuronic acid after incubation in buffer alone (■) or in the presence of following concentrations of ϵ -aminohexanoic acid; 1 mM (□); 0.05 M (▲); 0.1 M (△); 0.2 M (●); 0.4 M (○). Assays were carried out as described in the text.

Fig. 5. Effect of amino acids and of heat on the release of hexuronic acid-containing material from cartilage. Rabbit ear cartilage (0.75 g.) was incubated at 37° in 50 ml. of 0.1M-acetate buffer, pH 5.0, for 24 hr. Samples were withdrawn at various times for estimation of hexuronic acid after incubation in the presence of: buffer alone (●); 0.4M-lysine (○); 0.4M-glycine (△); 0.4M-ε-aminohexanoic acid (▲). Results with heated cartilage (at 80° for 40 min.) instead of normal cartilage in buffer alone are also shown (□). Assays were carried out as described in the text.

Table 2. Effect of amino acids and esters on the release of hexuronic acid from cartilage

Rabbit ear cartilage (0.2 g.) was incubated at 37° in the presence of various amino acids and synthetic esters in 10 ml. of 0.1M-acetate buffer, pH 5.0, for 24 hr. and the incubation mixture analysed for hexuronic acid in two different experiments. Assays were carried out as described in the text.

Amino acid or ester added (final concn.)	Hexuronic acid released (µg./ml. of incubation buffer)	Percentage inhibition
Expt. I		
None	155.0	0
Lysine ethyl ester (0.15M)	174.0	0
Lysine ethyl ester (0.05M)	153.0	1.2
Lysine methyl ester (0.15M)	170.8	0
Lysine methyl ester (0.05M)	181.2	0
Tosylarginine methyl ester (0.15M)	8.4	94.6
Tosylarginine methyl ester (0.05M)	44.2	71.5
Expt. II		
None	142.0	0
Arginine (0.2M)	18.8	86.8
Arginine (0.1M)	10.0	93.0
Arginine (0.02M)	24.0	84.0
Ornithine (0.2M)	138.0	3.0
Ornithine (0.05M)	148.0	0

incubation mixture resulted in a more rapid and increased chondrolysis at pH 5.0 (Fig. 7).

Adult human tracheal cartilage, adult bovine articular cartilage and young (3-year-old) human articular cartilage also showed maximum mucopolysaccharide release when incubated at pH 5.0 (Table 4). This release was again inhibited by 0.4M-ε-aminohexanoic acid. This points to the fact that a similar enzyme is involved in the breakdown of different types of cartilage even from different species.

Table 3. Effect of cysteine and iodoacetamide on the release of hexuronic acid from cartilage

Rabbit ear cartilage (0.75 g.) was incubated at 37° in 50 ml. of 0.1M-acetate buffer, pH 5.0, for 24 hr. in the presence of cysteine or iodoacetamide, and the amount of hexuronic acid released into the incubation buffer was estimated in samples removed at intervals. Assays were carried out as described in the text.

Time of incubation (hr.)...	Hexuronic acid released (µg./ml. of incubation buffer)		
	4	12	24
Compound added (final concn.)			
None	8	50	87
Cysteine (0.05M)	15	62	100
Iodoacetamide (5 mM)	7	35	65
ε-Amino-hexanoic acid (0.4M)	2	6	24

Fig. 6. Effect of pH on the proteolytic activity of the crude enzyme fraction from cartilage. Rabbit ear cartilage was homogenized in 0.25M-sucrose and the homogenate centrifuged at 2500g. The supernatant obtained was dialysed against water and the proteolytic activity determined at different pH values, with haemoglobin as the substrate. For the buffers used and other details see the Materials and Methods section.

Fig. 7. Effect of hypo-osmotic pretreatment on the degradation of cartilage. Rabbit ear cartilage (1.0 g.) was pre-treated in water at 4° for 1 hr. (as described in the Materials and Methods section) and adjusted to pH 5.0, by the addition of sufficient 0.2M-acetate buffer to give a final volume of 50 ml. Incubation was then carried out at 37° for 24 hr. with and without the addition of NaCl. ○, Hypo-osmotic pretreatment plus ε-aminohexanoic acid (0.4M); ●, hypo-osmotic pretreatment only; △, direct incubation at 37°; ▲, hypo-osmotic pretreatment and incubation in 0.9% NaCl; □, direct incubation in 0.9% NaCl; ■, direct incubation in 1.8% (w/v) NaCl. Assays were carried out as described in the text.

Table 4. Effect of ε-aminohexanoic acid on the release of hexuronic acid from different types of cartilage

Cartilage (which was retained at -10°) was thawed, weighed and incubated at 37° in 0.1M-acetate buffer, pH 5.0, for 24 hr., and the amount of hexuronic acid released in the presence and absence of 0.4M-ε-aminohexanoic acid was measured. Assays were carried out as described in the text.

	Hexuronic acid released (µg./ml. of incubation buffer)	
	ε-Amino- hexanoic acid absent	ε-Amino- hexanoic acid present
Human articular cartilage (0.75 g. in 50 ml. of buffer)	83.0	36.5
Human tracheal cartilage (0.2 g. in 10 ml. of buffer)	48.0	29.6
Ox articular cartilage (0.5 g. in 50 ml. of buffer)	37.0	19.0

DISCUSSION

It is becoming increasingly evident that in many tissues the neutral proteinases participate in the physiological protein turnover, whereas cathepsins are active mainly under pathological conditions or in metabolic disorders (Fruton, 1960; Marks & Lajtha, 1963). It was thus interesting to detect in cellular cartilage what seemed to be a proteolytic degradation of the matrix under optimum conditions for an endogenous enzyme at pH 5.0. Further studies indicated that this enzyme attacks peptide bonds involving arginine in the non-collagenous protein of the mucopolysaccharide in the matrix, because it was consistently inhibited by arginine and its analogues. Its activation by cysteine and slight inhibition by iodoacetamide further showed that it is probably cathepsin B (Fruton, 1960), which is present in other tissues in the lysosome-rich fraction (de Duve, Wattiaux & Baudhuin, 1962), and is known to split the arginine amide esters besides hydrolysing proteins and peptides at the arginine residue.

The extensive and detailed work on cultures of chick embryonic cartilage in the presence of an excess of vitamin A has led to the conclusion that the vitamin acts on the lipoprotein membrane of lysosomes, releasing the hydrolytic enzymes and particularly a cathepsin that facilitates a rapid degradation of the cartilage matrix (Dingle, Lucy & Fell, 1961; Dingle, 1962; Fell & Dingle, 1963). It was also reported that the chick embryonic rudiments were capable of maximum autolytic degradation at pH 5.0 even in the absence of vitamin A owing to the presence of a cathepsin, as has been observed in the present experiments. This is relevant to the lysosome concept, as de Duve (1959) has pointed out that the limiting lysosome membrane is capable of autolytic breakdown at pH 5.0, probably owing to its content of cathepsin active at that pH (de Duve & Beaufay, 1959).

The presence of cathepsins in cartilagenous tissues is of special significance, as Thomas (1956) and Potter *et al.* (1960) have demonstrated *in vivo* and *in vitro* the gross depletion of cartilage matrix when papain is allowed to be activated *in situ*. Similar work with a more physiological proteolytic enzyme plasmin, injected into rabbits in conjunction with experimentally enhanced permeability conditions, leads to the breakdown of cartilage matrix and to the release of acid mucopolysaccharide in urine (Lack *et al.* 1961; Lack, 1961). An acid cathepsin has been reported to be responsible for the resorption of connective tissue of the human uterus on post-partum involution (Woessner & Brewer, 1963). It has been further confirmed that the cleavage of the peptide bonds in connective tissue by proteolytic enzymes is a prerequisite for any

subsequent attack by any collagenase or polysaccharase leading to the complete degradation and dissolution *in vitro* (Bazin & Delaunay, 1961; Partington & Wood, 1963). This evidence implicates the much more important pathological role of proteolytic enzymes in connective-tissue disorders.

It has been established that proteolytic enzymes attack the non-collagenous protein moiety that holds the chondroitin sulphate polymers in the cartilage matrix (Shatton & Schubert, 1954; Muir, 1958; Partridge, Davis & Adair, 1961; Anderson, 1962). It is thus possible that the malfunctioning of an intracellular protease in cartilage may have pathological repercussions leading to chondrolysis followed by connective-tissue disorders. Dingle (1962) has suggested that the destruction of articular cartilage is related to the activation of lysosomal enzymes in cells that are closely apposed to the cartilage. Our experiments with rabbit ear cartilage and the involvement of a cathepsin in its degradation point towards very similar conclusions. If these assumptions are correct then the inhibitory effect of ϵ -aminohexanoic acid and other arginine analogues may assume an obvious clinical significance. The inhibition of catheptic activity by arginine and ϵ -aminohexanoic acid clearly allows the cartilage matrix, as observed in our experiments *in vitro*, to be preserved biochemically, histologically and macroscopically.

SUMMARY

1. Rabbit ear cartilage has been shown to contain a proteolytic enzyme capable of autolytic degradation of cartilage with an optimum at pH 5.0 *in vitro*. Chondrolysis has been followed chemically by the release of chondromucoprotein into the incubation buffer and histochemically by loss of metachromasia from cartilage matrix.

2. A similar enzymic breakdown has also been shown to take place in human and ox articular cartilage and human tracheal cartilage.

3. Besides inhibiting chondrolysis at neutral pH ϵ -aminohexanoic acid has been shown to be a potent inhibitor of this enzyme at pH 5.0.

4. In addition to the inhibition by ϵ -aminohexanoic acid the inhibition by arginine and its esters points to the possible involvement of cathepsin B. Its possible origin in the lysosomes and its role in connective-tissue disorders is discussed.

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