Biochem. J. (1967) **105**, 549
Printed in Great Britain

Characterization of Cathepsins in Cartilage

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(*Received* 8 *March* 1967)

The presence of a cathepsin B-like enzyme in rabbit ear cartilage was established by the use of the synthetic substrates benzoyl-L-arginine amide and benzoyl-DL-arginine 2-naphthylamide. This was facilitated by using a technique that permits the incubation of a fixed weight of thin (18 μ) cartilage sections with an appropriate exogenous substrate. The enzymic properties of cathepsin B in cartilage have been compared with an endogenous enzyme that liberates chondromucopeptide by degrading the cartilage matrix autocatalytically at pH 5. Besides being maximally active at pH 4·7, these cartilage enzymes are enhanced in activity by cysteine and inhibited by arginine analogues, iodoacetamide, chloroquine and mercuric chloride. They are not inhibited by EDTA, di-isopropyl phosphorofluoridate and diethyl *p*-nitrophenyl phosphate. When inhibiting the release of chondromucopeptide from cartilage at pH 5, the arginine-containing synthetic substrates are hydrolysed simultaneously. These enzymes also share the same heat-inactivation characteristics at various pH values, being stable at acid pH and unstable at neutral and alkaline pH. The experimental evidence indicates that a cathepsin B-like enzyme may be partly responsible for the autolytic degradation of cartilage matrix at pH 5.

Early cartilage lesions in osteoarthritis are characterized by a loss of protein–polysaccharide component of the matrix as evidenced histologically by loss of metachromasia (Collins & McElligott, 1960) and chemically by a fall in the chondroitin sulphate concentration (Bollet, Handy & Sturgill, 1963). Studies with [^{35}S]sulphate uptake by cartilage slices *in vitro* have shown an increase in the synthesis of chondroitin sulphate by chondrocytes in osteoarthritic cartilage. To account for a fall in chondroitin sulphate concentration in diseased cartilage a shift in the dynamic equilibrium towards the degradation of the polymeric matrix has been postulated (Collins & McElligott, 1960). Before invoking any exogenous enzyme that may bring about the breakdown of articular cartilage, it has been considered necessary to determine whether an endogenous enzyme in normal cartilage can degrade the matrix under suitable conditions.

The autolytic degradation of embryonic chick cartilage in the presence of excess of vitamin A or under hypo-osmotic conditions was shown by Lucy, Dingle & Fell (1961) to be due to an endogenous intracellular proteolytic enzyme. The release of chondromucoprotein from cartilage was ascribed to the action of a lysosomal protease on the protein–polysaccharide complex of the matrix (Dingle, 1961; Fell & Dingle, 1963). A similar enzyme capable of degrading cartilage maximally at pH 5 was shown by Ali (1964) to be present in rabbit ear cartilage and in ox and human articular cartilage. It released up to 50% of the total cartilage chondromucoprotein within 24 hr. but little or no hydroxyproline. As this enzyme was activated by cysteine, and inhibited by arginine, arginine analogues and iodoacetamide, Ali (1964) postulated the presence of a cathepsin B-like enzyme in rabbit ear cartilage and attributed the autolysis of the matrix at pH 5 to its action on the protein part of chondromucoprotein.

A study was therefore undertaken to characterize the proteolytic enzymes in cartilage by the use of synthetic substrates that are known to be specific for certain cathepsins (Fruton, 1960). The present paper describes the results obtained with benzoyl-L-arginine amide, which is a well-characterized substrate for cathepsin B, and with benzoyl-DL-arginine 2-naphthylamide, which has been shown to be a substrate at pH 5 for cathepsin B (Blackwood & Mandl, 1961) and for trypsin and trypsin-like enzymes at neutral pH (Nachlas, Plapinger & Seligman, 1964; Hopsu & Glenner, 1963).

In early experiments attempts were made to see whether a cartilage extract would hydrolyse the synthetic substrates. These experiments were not always successful because of the difficulty in obtaining either a reproducible extract or a satisfactory homogenate of cartilage. An alternative

procedure was adopted to facilitate the study of the cartilage enzymes. This allows a fixed weight of thin (18 μ) cartilage sections to be incubated with the selected substrate, and a preliminary communication describing part of this work has already been published (Ali, 1967).

MATERIALS AND METHODS

Cartilage sections. Fresh cellular ear cartilage was obtained from young (1-month-old) New Zealand red rabbits (1kg. body wt.), dissected free of skin and adhering tissue and rinsed with 0·9% NaCl buffered at pH4. The cartilage was cut up into 7·5mm.^{2} pieces and weighed into 0·3g. portions. Precautions were taken to prevent dehydration of cartilage and to maintain the temperature at 4°. Cylindrical blocks of frozen cartilage were prepared by freezing 0·3g. of cartilage pieces with 0·5ml. of water in Perspex (acrylic resin) rings (1cm. high, 1·2cm. inside diam.). Blocks of frozen cartilage were removed from the rings, mounted on a microtome chuck and sectioned (18 μ) in a cryostat with knife temperature at −35° and the cabinet temperature at −20°. Frozen cartilage sections were collected quantitatively into wide-mouthed bottles through a funnel made from a sheet of celluloid or aluminium. These manipulations were usually completed within 3–4hr. of obtaining the fresh tissue. Details of the method and equipment are given by Evans, Sayers & Ali (1967).

Cartilage sections were then incubated with the substrate in universal buffer (Ellis, 1961) at 37° for 24hr. in stoppered bottles in which the sections were originally collected. The concentration of the universal buffer was 50mM with respect to each of the four contributory salts (citrate, phosphate, tris and carbonate). A correction was made in the total volume of the incubation system for the 0·5ml. of water introduced with frozen cartilage sections. Streptomycin was included, to prevent bacterial contamination, at a concentration of 1000units/ml. Samples for estimation of the hydrolysed substrate were usually taken from the supernatant fluid after a slight centrifugation (500*g* for 5min.) to sediment the cartilage sections.

Determination of benzyol-L-arginine amide hydrolysis. Cartilage sections (0·3g.) were incubated with 50mM-*N*-α-benzoyl-L-arginine amide (British Drug Houses Ltd., Poole, Dorset) in 5ml. of universal buffer at a selected pH. Duplicate samples (0·2ml.) were removed from the reaction mixture and the ammonia liberated was titrated by the Conway micro-diffusion technique as described by Schwert, Neurath, Kaufman & Snoke (1948), with ammonium sulphate as the standard. The results are expressed as the amount of ammonia liberated/ml. of the incubation buffer. Unless mentioned otherwise, cysteine (10mM) was included in the buffer–substrate incubation mixture.

Determination of benzoyl-DL-arginine 2-naphthylamide hydrolysis. Cartilage sections (0·3g.) were incubated with 11·5ml. of 2·5mM-*N*-α-benzoyl-DL-arginine 2-naphthylamide (British Drug Houses Ltd.) in sufficient universal buffer to give a final volume of 20ml. at a selected pH. Samples (3·5ml.) were removed at intervals and brought to pH7 by adding 1ml. of sufficient alkali or concentrated buffer. The naphthylamine released was estimated by coupling it with 1ml. of 0·4% (w/v) tetra-azotized di-*o*-anisidine [Fast Blue B; Sigma Chemical Co. (London) Ltd., London, S.W. 6] in 2% (v/v) Tween 40 solution as described by Nachlas *et al.* (1964). After allowing 10min. for the chromogenic reaction, 0·5ml. of 4N-HCl was added and the E_{540} value of the red azo dye solution was measured against a water blank in a Unicam SP.500 spectrophotometer with cells of 1cm. light-path.

Determination of hexuronic acid release from cartilage slices. Rabbit ear cartilage slices (7·5mm.^{2}, 0·4g.) were incubated in 10ml. of universal buffer at a selected pH for 24hr. at 37°. The release of hexuronic acid-containing material (characterized as chondromucoprotein by Ali, 1964) into the supernatant fluid was estimated as an indication of the autolysis of cartilage. Duplicate samples were removed at intervals and their hexuronic acid content was determined by a modification (Bitter & Ewins, 1961) of the carbazole method of Dische (1947). Glucuronolactone was used as the standard and the results are expressed as μg. of hexuronic acid released/ml. of incubation buffer.

Determination of protein hydrolysis by cartilage cathepsins. Cartilage sections (0·3g.) were incubated with 6·6ml. of a 4% (w/v) 'denatured' protein solution in universal buffer (final vol. 20ml.) at a selected pH for 24hr. at 37°. 'Denatured' protein solutions were prepared by making a 4% (w/v) bovine haemoglobin, bovine serum albumin, casein or bovine γ-globulin solution in aqueous 6M-urea. Representative samples (2ml.) were removed at intervals from the incubation system, diluted with 1ml. of water and precipitated with 3ml. of 10% (w/v) trichloroacetic acid. After filtration through Whatman no. 3 filter paper, the E_{280} value of the filtrate was determined in a Unicam SP.500 spectrophotometer with silica cells of 1cm. light-path. This method for the estimation of protein hydrolysis with a similar proportion of protein substrate has been tested and described by Ali & Lack (1965). Occasionally, with haemoglobin as substrate, the trichloroacetic acid filtrates were opalescent and interfered in the estimation procedure. It was found that warming the trichloroacetic acid precipitates at 60° for 15min. and cooling to room temperature produced clear filtrates.

Tyrosine estimations were also made on the trichloroacetic acid filtrates by the Folin–phenol method described by Anson (1938). To 1ml. of the filtrate was added 2ml. of N-NaOH and 0·6ml. of diluted (1:2) Folin–Ciocalteu reagent, and the E_{670} value of the blue solution produced was measured. The amount of aromatic amino acids in the filtrate was estimated against a tyrosine standard and expressed as the amount of tyrosine equiv./ml. of the incubation buffer. It was found that a standard of 30 μg. of tyrosine/ml. usually gave E_{670} 0·390 under the conditions described. The results obtained with the spectrophotometric method (E_{280}) and by the Folin–phenol method (μg. of tyrosine equiv./ml.) are expressed after subtracting the value of the sample removed just before the start of the incubation at 37°.

Protein substrates and chemicals. Light white soluble casein was obtained from British Drug Houses Ltd. Bovine haemoglobin enzyme substrate, bovine serum albumin and bovine γ-globulin were obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. L-Arginine (free base) and di-isopropyl phosphorofluoridate were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Diethyl *p*-nitrophenyl phosphate was obtained from K & K Laboratories Inc., Plainview, N.Y., U.S.A.

EXPERIMENTAL AND RESULTS

Effect of pH *on the enzymic activity of cartilage.* When rabbit ear cartilage slices (7·5mm.^{2}) were incubated in buffer of different pH values for 24hr. at 37°, it was found that there was a maximal release of hexuronic acid into the buffer between pH4·5 and 4·7 (Fig. 1). This hexuronic acid-containing material has already been characterized as chondromucopeptide, which is released autolytically from cartilage under these conditions (Ali, 1964). When the hydrolysis of benzoyl-L-arginine amide (in the presence of 10mM-cysteine) or benzoyl-DL-arginine 2-napthylamide by cartilage sections was estimated under exactly the same conditions, the pH–activity curves overlapped and there was again a peak for maximal activity between pH4·5 and 4·7. This experiment indicated that the autolytic enzyme in cartilage that releases chondromucopeptide and cartilage cathepsin B-like enzyme are maximally active at the same pH.

Effect of pH *on the hydrolysis of proteins by cartilage enzymes.* When cartilage sections were incubated (37° for 24hr.) at various pH values with urea-denatured protein substrates, and the trichloroacetic acid-soluble aromatic amino acids estimated by the spectrophotometric method (E_{280}), it was found that casein, haemoglobin and γ-globulin were hydrolysed maximally at pH4 by the cartilage cathepsin. The results obtained with albumin appeared to have more than one peak between pH3 and 5. With none of these urea-denatured protein substrates was there any evidence of a separate protease activity at neutral or alkaline pH.

To eliminate the possibility of a non-specific absorption at 280mμ distorting the results, the trichloroacetic acid filtrates from the same experiment were analysed for acid-soluble tyrosine by the Folin–phenol method. Urea-denatured casein, haemoglobin and γ-globulin were again found to be hydrolysed maximally at pH4 by the cartilage cathepsin (Fig. 2). Serum albumin appeared to behave differently and gave a peak of maximal hydrolysis at pH3–3·5. Again, with none of these protein substrates was there an indication of neutral or alkaline protease activity.

Urea-denatured casein, γ-globulin and albumin preparations were soluble at all the pH values tested. Haemoglobin (4%, w/v) was soluble in the presence of 6M-urea but appeared to be precipitated in the range pH4·5–7 when incubated at 37°. Representative samples for analysis had to be removed with a wide-mouthed pipette when haemoglobin was used as a substrate. Acid denaturation of casein and haemoglobin at pH2, as described by Press, Porter & Cebra (1960), caused the proteins to become insoluble above pH3 and, as this method proved unsatisfactory for incubation of denatured

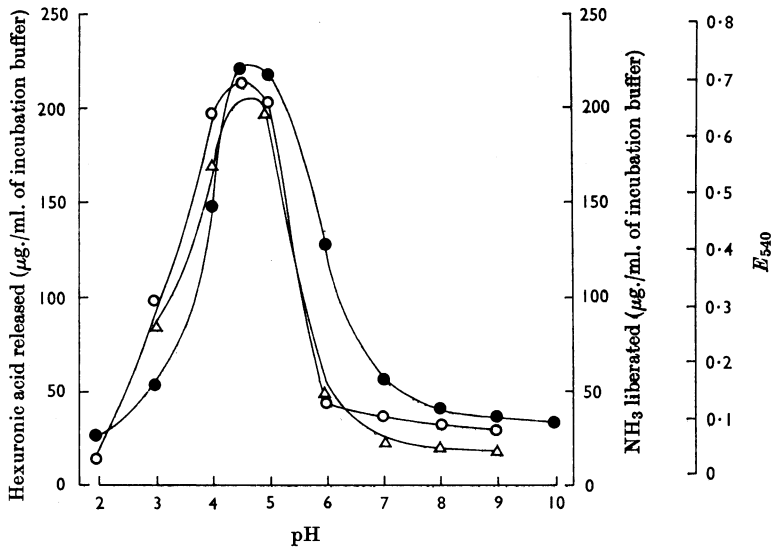

Fig. 1. Effect of pH on cartilage cathepsin activity and on the autolytic release of hexuronic acid from cartilage slices. The autolysis of cartilage was measured by incubating 0·4g. of rabbit ear cartilage slices (7·5mm.^{2}) in 10ml. of universal buffer at 37° for 24hr. and estimating the release of hexuronic acid-containing material into the buffer (○). The hydrolysis of benzoyl-L-arginine amide as ammonia liberated (△) and of benzoyl-DL-arginine 2-naphthylamide as E_{540} (●) were measured as described in the text after incubating them with 0·3g. of cartilage sections (18μ) in universal buffer at various pH values at 37° for 24hr.

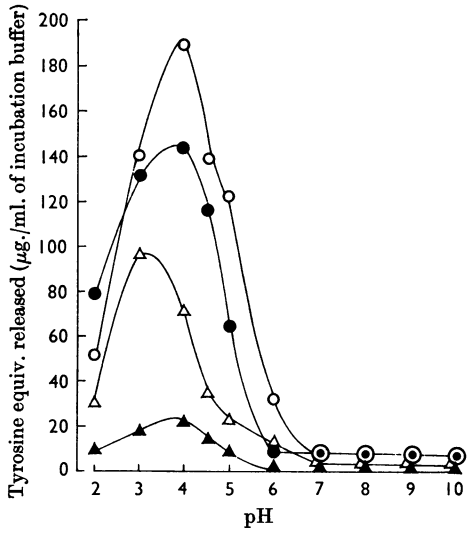

Fig. 2. Effect of pH on the proteolytic activity of cartilage cathepsins. Ear cartilage (0·3g.) sections (18μ) were incubated with urea-denatured casein (○), haemoglobin (●), albumin (△) or γ-globulin (▲) in 20ml. of universal buffer at various pH values for 24hr. at 37°. The trichloroacetic acid-soluble products of protein hydrolysis were measured by the Folin–phenol method and expressed as the amount of tyrosine equiv. liberated as described in the text.

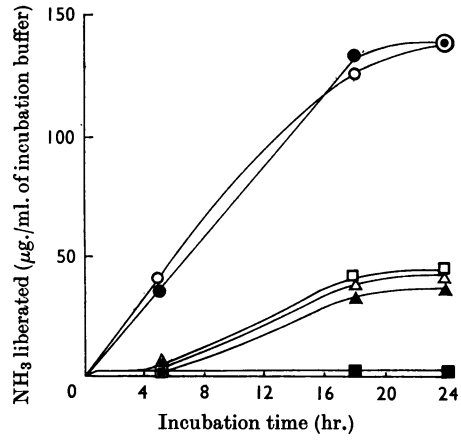

Fig. 3. Effect of various compounds on the hydrolysis of benzoyl-L-arginine amide by cartilage cathepsin. Rabbit ear cartilage (0·3g.) sections (18μ) were incubated with 50mM-benzoyl-L-arginine amide, in the presence of 10mM-cysteine, in 5ml. of universal buffer, pH5, at 37° for 24hr. Samples were withdrawn at intervals for estimation of the amount of ammonia liberated after incubation in the presence of buffer alone (●), 0·1% (v/v) Triton X-100 (○), 0·1M-chloroquine (△), benzoyl-DL-arginine 2-naphthylamide (▲), 2mM-mercuric chloride (□) and 0·1M-arginine (■).

protein with cartilage sections, it was not employed in this study. Any denaturation effect of urea on cartilage cathepsin was not apparent, as the cathepsin activity was higher against urea-denatured substrate than against untreated protein substrate.

Hydolysis of benzoyl-L-arginine amide by cartilage cathepsin. When benzoyl-L-arginine amide was incubated with cartilage sections at various pH values, it was hydrolysed maximally at pH4·7 (Fig. 1). The enzyme hydrolysing benzoyl-L-arginine amide at pH5 appears to be in a partially active state in cartilage and requires 10mM-cysteine for further activation (Ali, 1967). Preheating the cartilage sections at 80° for 15min. inactivated the enzyme completely. Iodoacetamide (25mM) inhibited 66% of the cysteine-activated enzyme and inactivated the cartilage cathepsin completely in the absence of cysteine. These experiments indicated the dependence of cartilage cathepsin activity on thiol reagents and showed it to be similar to cathepsin B found in other tissues.

The hydrolysis of 50mM-benzoyl-L-arginine amide at pH5 in the presence of 10mM-cysteine was proportional to the amount of cartilage in the range 0·1–0·4g. With 0·4g. of cartilage sections approx. 30% of the substrate was hydrolysed within 24hr. at pH5. Increasing the amount of cartilage to 1g. increased the total volume of the incubation system and the result obtained with such a high amount of cartilage is unsatisfactory for this reason.

The hydrolysis of benzoyl-L-arginine amide by a cysteine-activated cathepsin in 0·3g. of cartilage sections was inhibited by 70% by 0·1M-chloroquine and by about 50% by 50mM-chloroquine (Fig. 3). Triton X-100 (0·1%, v/v) or 0·2M-lysine did not have any effect on the activity of the cartilage enzyme. EDTA (1mM) enhanced the catheptic activity by 18%. Di-isopropyl phosphorofluoridate (1mM) and diethyl *p*-nitrophenyl phosphate (1mM) did not inhibit cartilage cathepsin B. Arginine (0·1M) inhibited the cysteine-activated cartilage cathepsin completely and mercuric chloride (2mM) inhibited the activity by nearly 70%. Benzoyl-DL-arginine 2-naphthylamide (2mM) inhibited the reaction by 77% (Fig. 3). It was found that benzoyl-DL-arginine naphthylamide, while inhibiting the hydrolysis of benzoyl-L-arginine amide, was itself hydrolysed (Figs. 3 and 5). This may indicate that the arginine-containing synthetic substrates act as competitive inhibitors when present together and may imply that they are hydrolysed by the same cathepsin.

Hydrolysis of benzoyl-DL-arginine 2-naphthylamide by cartilage cathepsin. When benzoyl-DL-arginine 2-naphthylamide (1·5mM) was incubated for 24hr. at 37° with 0·3g. of cartilage sections (18μ) at various pH values, it was hydrolysed maximally

at pH 4·7 (Fig. 1). Preheating the cartilage sections at 80° for 15min. in water and subsequently incubating them at pH 5 with synthetic substrate appeared to inactivate the cartilage cathepsin completely (Fig. 5). When benzoyl-DL-arginine 2-naphthylamide was incubated at pH 5 with increasing amounts of sectioned cartilage and the extent of hydrolysis estimated, the results shown in Fig. 4 were obtained. It is clear that the hydrolysis of the synthetic substrate is dependent on the cartilage (enzyme) concentration and is proportional to it in the range 0·1–0·5g. under the experimental conditions. This experiment also indicates the validity of using a fixed quantity of sectioned cartilage as a method of detecting the presence of specific enzymes in cartilage with synthetic or exogenous substrates.

The hydrolysis of benzoyl-DL-arginine 2-napthylamide by cartilage cathepsin at pH 5 was inhibited almost completely by the presence of 25mM-iodoacetamide (Fig. 5). Cysteine (20mM) interfered with the 2-naphthylamide colour reaction with Fast Blue B and produced a yellow opalescence. The effect of cysteine on the hydrolysis of benzoyl-DL-arginine 2-naphthylamide could not therefore be evaluated. Dannenberg & Bennet (1964) have reported similar interference by cysteine in their experiments on the hydrolysis of *N*-benzoyl-DL-phenylalanine β-naphthyl ester by rabbit phagocytes when they used a similar estimation procedure for 2-naphthol. Mercuric chloride (2mM) inhibited the hydrolysis of benzoyl-DL-arginine 2-naphthylamide by cartilage cathepsin completely at pH 5, and chloroquine (50mM) also inhibited the hydrolysis by nearly 80%. Di-isopropyl phosphorofluoridate (1mM) and diethyl *p*-nitrophenyl phosphate (1mM) did not inhibit the cartilage cathepsin. The presence of EDTA (1mM) appeared to enhance the catheptic hydrolysis of benzoyl-DL-arginine 2-naphthylamide by 80%.

The presence of 40mM-benzoyl-L-arginine amide inhibited by 50% the hydrolysis of benzoyl-DL-arginine 2-naphthylamide by cartilage cathepsin at pH 5 (Fig. 5). While inhibiting this reaction, benzoyl-L-arginine amide was found to be hydrolysed simultaneously. The concentration of benzoyl-DL-arginine 2-naphthylamide could not be increased beyond 1·5mM in the incubation system because of its relative insolubility in aqueous medium. A stock solution containing 2·5mM-benzoyl-DL-arginine 2-naphthylamide appeared to be almost a saturated aqueous solution.

Effect of heat and various compounds on the hydrolysis of proteins by cartilage cathepsin. Preheating 0·3g. cartilage sections at 80° for 15min. appeared to inactivate the cartilage cathepsin involved in the hydrolysis of urea-denatured casein and haemoglobin at pH 5. Mercuric chloride (1mM) and iodoacetamide (25mM) inhibited the hydrolysis

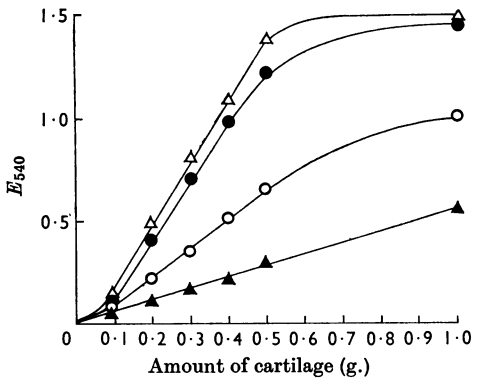

Fig. 4. Effect of cartilage concentration on the hydrolysis of benzoyl-DL-arginine 2-naphthylamide. Portions of sectioned cartilage (0·1–1·0g.) were incubated with 1·5mM-benzoyl-DL-arginine 2-naphthylamide in 20ml. of universal buffer, pH 5, at 37° for 24hr. Samples were withdrawn and analysed for the naphthylamine liberated, by determining E_{540} as described in the text, after incubation for 2hr. 30min. (▲), 7hr. (○), 17hr. (●) and 24hr. (△).

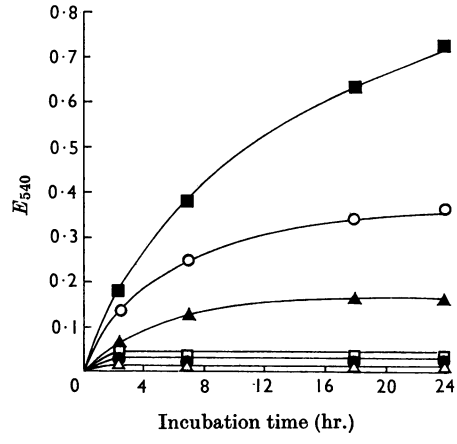

Fig. 5. Effect of heat and various compounds on the hydrolysis of benzoyl-DL-arginine 2-naphthylamide by cartilage cathepsin. Cartilage (0·3g.) sections were incubated with 1·5mM-benzoyl-DL-arginine 2-naphthylamide in 20ml. of universal buffer, pH 5, at 37° for 24hr. Samples were withdrawn at intervals for estimation of liberated naphthylamine, by determining E_{540} as described in the text, after incubation in the presence of buffer alone (■), 40mM-benzoyl-L-arginine amide (○), 50mM-chloroquine (▲), 25mM-iodoacetamide (□) and 2mM-mercuric chloride (●). Preheated (80° for 15min.) cartilage sections were also incubated with buffer and substrate under exactly the same conditions (△).

of haemoglobin by cartilage cathepsin at pH 5 by 40% and 65% respectively. When the acid-soluble tyrosine was estimated by the Folin–phenol method, cysteine (25mM) reduced the Folin–phenol reagent directly and produced a blue colour, and hence its effect on haemoglobin hydrolysis could not be evaluated. When the hydrolysis of haemoglobin by cartilage cathepsin at pH 5 was measured spectrophotometrically at 280mμ, there appeared to be a substantial increase in activity in the presence of cysteine.

Chloroquine, benzoyl-L-arginine amide and benzoyl-DL-arginine 2-naphthylamide interfered in both the estimation methods because of their aromatic ring structure, and their possible inhibitory effects on haemoglobin hydrolysis could not be evaluated. EDTA (1mM), di-isopropyl phosphorofluoridate (1mM) and diethyl *p*-nitrophenyl phosphate (1mM) did not inhibit the cartilage cathepsin.

Inhibition of hexuronic acid release from cartilage slices. Rabbit ear cartilage slices (7·5mm.², 0·4g.) were incubated at pH 5 in 10ml. of 0·1M-acetate buffer at 37° for 24hr. The release of hexuronic acid (chondromucopeptide) was estimated in the incubation buffer as an indication of the autolytic breakdown of cartilage *in vitro*, as has been substantiated by Ali (1964). Benzoyl-L-arginine amide (50mM) and benzoyl-DL-arginine 2-naphthylamide (2mM) inhibited the release of hexuronic acid from cartilage slices at pH 5 (Fig. 6). When the hydrolysis of the synthetic substrates was estimated from the final 24hr. incubation sample, in the same experiment, it was found that 48μg. of ammonia/ml. was liberated from benzoyl-L-arginine amide and the naphthylamine colour estimation gave E_{540} 0·386 (compare with Figs. 3 and 5). This experiment indicated the competitive nature of the inhibition of hexuronic acid release from cartilage by arginine-containing synthetic substrates as they are hydrolysed simultaneously.

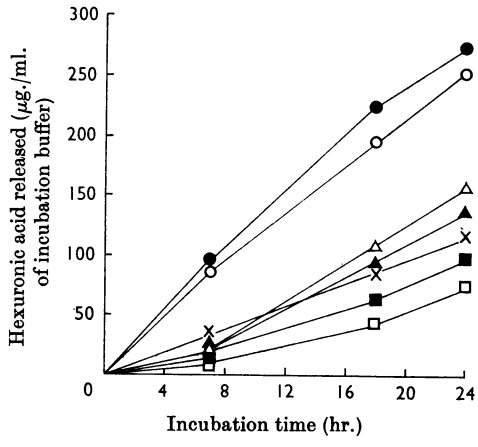

Fig. 6. Effect of various compounds on the release of hexuronic acid-containing material from cartilage slices. Rabbit ear cartilage (0·4g.) slices (7·5mm.²) were incubated in 10ml. of 0·1M-acetate buffer, pH 5, at 37° for 24hr. Samples were withdrawn at various times for estimation of the hexuronic acid released after incubation in the presence of buffer alone (○), 25mM-cysteine (●), 25mM-mercuric chloride (△), 50mM-benzoyl-L-arginine amide (▲), 25mM-iodoacetamide (×), 2mM-benzoyl-DL-arginine 2-naphthylamide (■) and 20mM-chloroquine (□).

Iodoacetamide (25mM), mercuric chloride (2mM) and chloroquine (20mM) inhibited the release of hexuronic acid-containing material from cartilage slices by 52%, 36% and 70% respectively (Fig. 6). Triton X-100 (0·1%, v/v) interfered with the carbazole reaction for hexuronic acids. But, when the released chondromucopeptide was estimated after precipitation with cetyltrimethylammonium bromide, it was found that Triton X-100 had no effect on the release of acid mucopolysaccharide at pH 5 or at pH 7. EDTA (1mM), di-isopropyl phosphorofluoridate (1mM) and diethyl *p*-nitrophenyl phosphate (1mM) did not inhibit the release of hexuronic acid from cartilage slices at pH 5.

Thermal stability of cartilage cathepsin at various pH values. As the enzyme involved in the autolysis of cartilage appeared to be very similar to that hydrolysing the arginine-containing synthetic substrates at pH 5, it was decided to compare their thermal stabilities at different pH values. Rabbit ear cartilage (0·3g.) sections (18μ) were incubated at various pH values for 1hr. at 56° in 2ml. of universal buffer. After this treatment the pH was brought to near 5 by the addition of N-sodium hydroxide or N-hydrochloric acid and incubated with universal buffer, pH 5, at 37° for 24hr. with the appropriate substrate as described in the Materials and Methods section. A similar experiment was carried out in which 0·4g. samples of cartilage slices were incubated at various pH values for 1hr. at 56° and subsequently brought to pH 5, and the release of hexuronic acid into the buffer was measured. The results obtained are shown in Fig. 7.

The enzyme hydrolysing benzoyl-L-arginine amide and benzoyl-DL-arginine 2-naphthylamide and the enzyme responsible for the release of chondromucopeptide from cartilage behaved identically in their stabilities at different pH values. They were all inactivated in the neutral and alkaline pH region, and were more stable at acid pH values, showing maximum stability in the range pH 3–5·5. The cartilage enzyme hydrolysing urea-denatured haemoglobin at pH 5 showed exactly the same instability in the neutral and alkaline pH region. Although it showed maximal

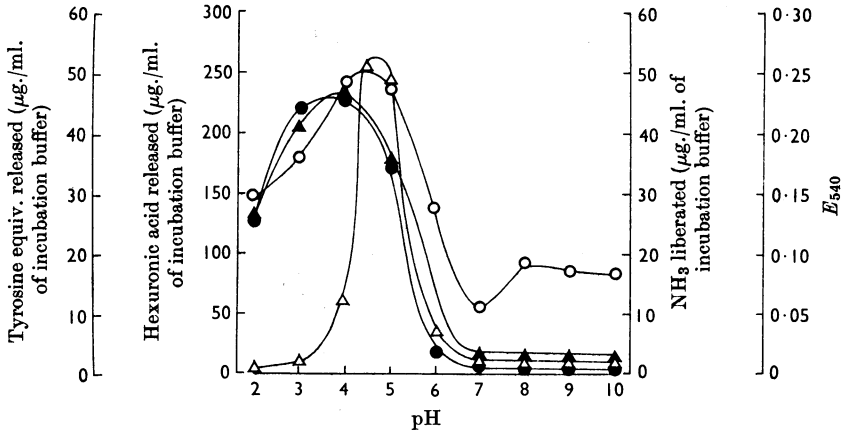

Fig. 7. Stability of cartilage cathepsins at 56° at different pH values. Rabbit ear cartilage (0·3g.) sections (18μ) or cartilage (0·4g.) slices (7·5mm.^{2}) were incubated in universal buffer at various pH values at 56° for 1hr. The reaction mixtures were then brought to pH5 with acid or alkali and incubated in universal buffer, pH5, with appropriate substrate at 37° for 24hr. The autolysis of cartilage slices was measured by the release of hexuronic acid-containing material into the buffer (▲). The hydrolysis of benzoyl-L-arginine amide was measured by the ammonia liberated (●), the hydrolysis of benzoyl-DL-arginine 2-naphthylamide by E_{540} (○) and the hydrolysis of haemoglobin by the acid-soluble tyrosine liberated (△).

stability in the range pH4–5·5, as did the enzyme responsible for the hydrolysis of the other substrates, it was nevertheless completely inactivated at pH2 and 3, possibly owing to the inactivation process going to completion at extremely acid pH.

To compare the stability of the cartilage autolytic enzyme towards heating at different pH values with that reported for cathepsin D (Press *et al.* 1960), cartilage slices were incubated at various pH values at 37° for 2hr. and then brought to pH5, and the release of hexuronic acid into the buffer was measured as described. There was maximal release of hexuronic acid after treatment of cartilage slices at pH2. The enzyme lost some of its activity with the increase in pH and the release at pH10 was only 65% of that at pH2. This indicated that the autolytic enzyme was more stable in the acid pH range than at alkaline pH and is in contrast with cathepsin D, which is completely stable at pH10, becomes unstable in the acid pH range and loses all its activity at pH2.

DISCUSSION

Early attempts at characterization of the cathepsins in cartilage were frustrated by the inability to obtain a satisfactory homogenate or an enzymically active extract of cartilage. Moreover, at pH5, which is the pH for maximum activity of cathepsins, the high acid mucopolysaccharide content of the extract interfered in the protease assay by its capacity to form electrostatic complexes with proteins (Meyer, Palmer & Smyth, 1937; Badin & Schubert, 1955; Anderson, 1963). The specific inhibitory effect of polyanions on enzymes has been reviewed by Bernfeld (1966). Incubation of a fixed quantity of sectioned (18μ) cartilage eliminated these difficulties and permitted the endogenous cathepsins to hydrolyse extraneous substrates, so that information could be obtained on the most suitable conditions for enzyme action. Incubation of sectioned cartilage with synthetic substrates also held the advantage that any cofactors essential for the activity of the autolytic enzyme were not eliminated by extraction or purification.

The reasons for the assumption that a cathepsin is involved in the autolysis of cartilage at pH5 rather than a collagenase or a polysaccharase have been outlined by Ali (1964). The ability of cartilage sections to hydrolyse benzoyl-L-arginine amide maximally at pH5 and the dependence of this activity on thiol agents indicated the presence of a cathepsin B-like enzyme in cartilage. The specificity of the cartilage cathepsin was further confirmed by using another arginine-containing synthetic substrate, benzoyl-DL-arginine 2-naphthylamide. This trypsin substrate has been shown by Blackwood & Mandl (1961) to be hydrolysed at pH5 by cathepsin B present in rat liver, kidney and spleen.

The behaviour of the cartilage cathepsin towards benzoyl-L-arginine amide and benzoyl-DL-arginine 2-naphthylamide was almost identical when the pH optimum, thermal stability at different pH values and inhibition by various compounds were studied.

When present together each appeared to inhibit the catheptic hydrolysis of the other, while being hydrolysed simultaneously, presumably by the same enzyme. Although more benzoyl-L-arginine amide (20%) was hydrolysed by cartilage cathepsin than benzoyl-DL-arginine 2-naphthylamide (5%), the latter provided an easier and more sensitive method for the estimation of cartilage cathepsin because of the chromogenic reaction of the free naphthylamine. It should even be possible to use benzoyl-DL-arginine 2-naphthylamide as a histochemical substrate for the study of cartilage enzymes at pH 5 by using a method described for study of the trypsin-like activity of human mast cells at neutral pH (Hopsu & Glenner, 1963). The difference in the amount of the two substrates hydrolysed may depend on the different concentrations used in the method and also because a racemic mixture of benzoyl-DL-arginine 2-naphthylamide was used.

The finding by Whitehouse & Cowey (1966) that the antimalarial drug chloroquine inhibits the release of chondromucopeptide from ox nasal or tracheal cartilage slices at pH 5 has been confirmed in our experiment with rabbit ear cartilage slices. Moreover, it has been shown in our study that a possible mechanism of the inhibitory action of chloroquine may be through its ability to inhibit cathepsin B, which may be involved in the autolysis of cartilage. It is possible that the mechanism of chloroquine inhibition of cathepsin B action involves reaction with thiol groups. Quinones are known to interact with protein thiol groups (Schubert, 1947), and Gerber (1964) has found that chloroquine will bind to certain thiol groups of human serum albumin and will inhibit the reduction of ferricyanide by cysteine.

The hydrolysis of benzoyl-L-arginine amide by cartilage cathepsin B was much more dependent on activation by cysteine, whereas the autolytic enzyme in cartilage was not, as the addition of cysteine only supplemented the partially active enzyme. This implied the presence in cartilage of a reducing substance that could keep the enzymes in active (reduced) state while hydrolysing chondromucoprotein inside the cartilage, but requiring additional reducing agent when diluted in an aqueous medium for hydrolysis of benzoyl-L-arginine amide. This is consistent with the fact that inactive (oxidized) papain when injected intravenously into rabbits becomes active (reduced) *in situ* in cartilage, and Thomas (1964) has accordingly postulated the presence in cartilage of a reducing factor or system.

The evidence in favour of the presence of cathepsin B and other cathepsins in the lysosome-rich fraction of several tissues has been summarized by de Duve, Wattiaux & Baudhuin (1962) and confirmed by Bouma & Gruber (1966). Triton X-100, which is known to release lysosomal hydrolases by disrupting the lysosomal membrane, had no effect either on the release of hexuronic acid from cartilage slices or on the hydrolysis of synthetic substrates by cartilage cathepsin. This may be due to the prolonged incubation period (24hr.) employed and in the experiment with cartilage sections to the fact that the chondrocyte diameter is reckoned to be 40 μ and the sections were cut at 18 μ, thus liberating subcellular particles into the hypo-osmotic medium at pH 5. The lysosomal membrane may have also been disrupted by a cycle of freezing and thawing of the cartilage sections. Triton X-100 had no activating effect on any inactive protease in cartilage as is the case for a rabbit kidney protease active at neutral pH (Ali & Lack, 1965).

By none of the four techniques employed in our experiment for the detection of cartilage enzyme activity was there any evidence of a neutral protease activity in normal rabbit ear cartilage. Fessel & Chrisman (1964) have, however, ascribed the loss in viscosity of chondromucoprotein by cell-free extracts of pathological human cartilage to the action of an enzyme active at neutral pH. The cartilage cathepsin active at pH 5 does not appear to be dependent on activation by heavy-metal ions, as it was not inhibited by EDTA but was slightly enhanced in its presence. The cartilage cathepsin was also insensitive to organophosphorus compounds, distinguishing it from the di-isopropyl phosphorofluoridate-sensitive esterases and peptidases.

The involvement of cathepsins in the disruption of the limiting lysosomal membrane at pH 5 and the subsequent autolysis of tissues by lysosomal hydrolases has been shown by Beaufay & de Duve (1959). Mego & McQueen (1965) have attributed the degradation of denatured ^{131}I-labelled albumin in mouse liver mitochondrial–lysosomal fraction to cathepsin B, as it was maximal at pH 5, activated by 40mM-cysteine and completely inhibited by 10mM-iodoacetamide. It is therefore possible that cathepsin B is also involved in the autolysis of cartilage, as it responds similarly to the same conditions as measured by the release of hexuronic acid from cartilage or by the hydrolysis of arginine-containing synthetic substrates.

Although the evidence in this study indicates the similarity if not the identical nature of the cartilage cathepsin B and the autolytic enzyme in cartilage, it should not be assumed that cathepsin B is the only enzyme responsible for this autolysis. It is possible that cathepsin B acts in conjunction with other cathepsins or enzymes in the degradation of cartilage matrix, for Iodice, Leong & Weinstock (1966) have shown that purified cathepsins A and D from skeletal muscle when incubated together hydrolysed haemoglobin twice as much as the total

of their individual activities. Barrett (1966*a*,*b*) has shown that a purified acid protease from rabbit liver resembling cathepsin D can degrade isolated chondromucoprotein at pH 4. The thermal stability of the cartilage autolytic enzyme at various pH values appears to be different from that of cathepsin D reported by Press *et al.* (1960). A true comparison of this property may have to await the isolation and purification of the cartilage enzyme, as the values for cathepsin D stability refer to a purified enzyme.

We are grateful to the Nuffield Foundation for a grant which has made this work possible, to Dr J. D. Fitzgerald of Imperial Chemical Industries Ltd. (Pharmaceutical Division) for a gift sample of chloroquine diphosphate, and to Mr D. C. J. Sayers for assistance in sectioning the cartilage.

REFERENCES

Ali, S. Y. (1964). *Biochem. J.* **93**, 611.
Ali, S. Y. (1967). *Biochem. J.* **102**, 10C.
Ali, S. Y. & Lack, C. H. (1965). *Biochem. J.* **96**, 63.
Anderson, A. J. (1963). *Biochem. J.* **88**, 460.
Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Badin, J. & Schubert, M. (1955). *J. clin. Invest.* **34**, 1312.
Barrett, A. J. (1966*a*). *Nature, Lond.*, **211**, 1188.
Barrett, A. J. (1966*b*). *Biochem. J.* **101**, 30P.
Beaufay, H. & de Duve, C. (1959). *Biochem. J.* **73**, 604.
Bernfeld, P. (1966). In *The Amino Sugars*, vol. 2B, p. 213. Ed. by Balazs, E. A. & Jeanloz, R. W. New York: Academic Press Inc.
Bitter, T. & Ewins, R. (1961). *Biochem. J.* **81**, 43P.
Blackwood, C. & Mandl, I. (1961). *Analyt. Biochem.* **2**, 370.
Bollet, A. J., Handy, J. R. & Sturgill, B. C. (1963). *J. clin. Invest.* **42**, 853.
Bouma, J. M. W. & Gruber, M. (1966). *Biochim. biophys. Acta*, **113**, 350.
Collins, D. H. & McElligott, T. F. (1960). *Ann. rheum. Dis.* **19**, 318.
Dannenberg, A. M. & Bennet, W. E. (1964). *J. Cell Biol.* **21**, 1.
de Duve, C., Wattiaux, R. & Baudhuin, P. (1962). *Advanc. Enzymol.* **24**, 291.
Dingle, J. T. (1961). *Biochem. J.* **79**, 509.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Ellis, D. A. (1961). *Nature, Lond.*, **191**, 1098.
Evans, L., Sayers, D. C. J. & Ali, S. Y. (1967). *J. med. Lab. Tech.* **40**, no. 4.
Fell, H. B. & Dingle, J. T. (1963). *Biochem. J.* **87**, 403.
Fessel, J. M. & Chrisman, O. D. (1964). *Arth. Rheum.* **7**, 398.
Fruton, J. S. (1960). In *The Enzymes*, vol. 4, part A, p. 233. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Gerber, D. A. (1964). *Arth. Rheum.* **7**, 193.
Hopsu, V. K. & Glenner, G. G. (1963). *J. Histochem. Cytochem.* **11**, 520.
Iodice, A. A., Leong, V. & Weinstock, I. M. (1966). *Arch. Biochem. Biophys.* **117**, 477.
Lucy, J. A., Dingle, J. T. & Fell, H. B. (1961). *Biochem. J.* **79**, 500.
Mego, J. L. & McQueen, J. D. (1965). *Biochim. biophys. Acta*, **100**, 136.
Meyer, K., Palmer, J. W. & Smyth, E. M. (1937). *J. biol. Chem.* **119**, 501.
Nachlas, M. M., Plapinger, R. E. & Seligman, A. M. (1964). *Arch. Biochem. Biophys.* **108**, 266.
Press, E. M., Porter, R. R. & Cebra, J. (1960). *Biochem. J.* **74**, 501.
Schubert, M. (1947). *J. Amer. chem. Soc.* **69**, 712.
Schwert, G. W., Neurath, H., Kaufman, S. & Snoke, J. E. (1948). *J. biol. Chem.* **172**, 221.
Thomas, L. (1964). *Biophys. J.* **4**, 207.
Whitehouse, M. W. & Cowey, F. K. (1966). *Biochem. J.* **98**, 11P.