Studies on the Cathepsins in Elastic Cartilage

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(Received 15 November 1968)

1. The presence of several enzymes in rabbit ear cartilage was examined by a quantitative method that permits the incubation of a fixed weight of cartilage sections ($18\mu\text{m.}$) with an appropriate exogeneous substrate. 2. As the presence of cathepsins B and D in cartilage has already been established, evidence is now provided to show that cathepsins A and C are also present and are maximally active at pH 5. 3. Cathepsin A was recognized by its hydrolysis of benzyloxy-carbonyl-glutamyl-tyrosine and cathepsin C by its hydrolysis of glycyl-tyrosine amide; the cartilage also hydrolysed benzyloxycarbonyl-glutamyl-phenylalanine and benzoyl-DL-phenylalanine 2-naphthyl ester at pH 5. 4. The acid phosphatase activity and the DNA content of cartilage have also been measured to provide a basis for comparison with the cathepsin activity of cartilage obtained from other sites and species.

Degenerative cartilaginous diseases show a characteristic loss of protein-polysaccharide component of the cartilage matrix and this can be observed histochemically by loss of metachromasia (Collins & McElligott, 1960) and chemically by a fall in the chondroitin sulphate concentration (Matthews, 1953; Bollet, Handy & Sturgill, 1963). There is experimental evidence to indicate that the synthesis of chondroitin sulphate is normal in osteoarthritic cartilage, and to account for a fall in the chondroitin sulphate concentration in diseased cartilage a shift in the dynamic equilibrium towards an excessive degradation of the polymeric matrix has been postulated (Collins & McElligott, 1960). This loss of protein-polysaccharide from cartilage matrix in the joint can be brought about by enzymes present in the vicinity of cartilage. Blood proenzyme plasminogen can diffuse into the joint cavity under inflammatory conditions, become activated to the protease plasmin by cytokinase in cartilage, and release the protein-polysaccharide components (Lack, Anderson & Ali, 1961; Lack, 1961; Ali & Evans, 1968). Enzymes in leucocytes (Ziff, Gribetz & Laspalluto, 1960; Lack & Ali, 1967; Weissmann & Spilberg, 1968) and in synovial tissue (Ziff *et al.* 1960; Hamerman, Janis & Smith, 1967; Bollet, Bonner & Nance, 1963) are capable of degrading cartilage polymers under experimental conditions.

Apart from these exogenous enzymes that can attack cartilage, it is evident that there are enzymes in cartilage itself that can bring about the autolysis of this tissue under suitable conditions (Lucy,

Dingle & Fell, 1961; Fell & Dingle, 1963). When cartilage is incubated in buffer at 37° it undergoes maximum breakdown at pH 5 and releases over 50% of its chondromucoprotein content within 24 hr. (Ali, 1964). Extracts of osteoarthritic cartilage have been shown to decrease the relative viscosity of isolated chondromucoprotein (Chrisman & Fessel, 1962; Fessel & Chrisman, 1964). Tourtellotte, Campo & Dziewiatkowski (1963) have reported the isolation and partial purification of an enzyme from cartilage that degrades protein-polysaccharide maximally at pH 4.

As there is no evidence for the presence of any collagenase or hyaluronidase activity in cartilage, the enzymic release of chondromucoprotein at pH 5 has been ascribed to endogenous cathepsins acting on the non-collagenous protein of the matrix (Ali, 1964). 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 presence of a cathepsin B-like enzyme in rabbit ear cartilage has been established by measuring the hydrolysis of benzoyl-arginine amide and benzoyl-DL-arginine 2-naphthylamide (Ali, Evans, Stainthorpe & Lack, 1967). This was facilitated by the use of a quantitative method that permits the incubation of a fixed weight of cartilage, cut into $18\mu\text{m.}$ sections, with the appropriate synthetic or protein substrate (Ali, 1967; Evans, Sayers & Ali, 1967). The present paper provides evidence for the presence of cathepsins A, C and D in rabbit ear cartilage. The acid phosphatase and DNA contents

of this elastic cartilage have also been estimated so as to form a basis for comparison in the future with enzyme activity determinations on other types of normal and pathological cartilage.

MATERIALS AND METHODS

Cartilage sections. Fresh cellular ear cartilage was obtained from young (1-month-old) New Zealand red rabbits (1 kg. body wt.), dissected free of skin and adhering tissue, and rinsed with 0.9% NaCl buffered at pH 4. The cartilage was transferred into cylindrical blocks and frozen with 0.5 ml. of water and then sectioned (18 μ m.) in a cryostat exactly as described by Ali *et al.* (1967). The sectioned cartilage was collected quantitatively into wide-mouth bottles and could then be used for incubation with an appropriate substrate.

Determination of cathepsin amidase activity. Cartilage sections (0.3 g.) were incubated with 50 mM-*N*- α -benzoyl-arginine amide, or 25 mM-glycyl-tyrosine amide or 25 mM-*N*-acetyl-tyrosine amide, in 5 ml. of universal buffer (Ellis, 1961) at a selected pH. Duplicate samples (0.2 ml.) were removed from the reaction mixture and the ammonia liberated was titrated by the Conway microdiffusion technique as described by Schwert, Neurath, Kaufman & Snoko (1948), with (NH₄)₂SO₄ as the standard.

Determination of benzoyl-DL-arginine 2-naphthylamide hydrolysis. This estimation was performed exactly as described by Ali *et al.* (1967) by a modification of the method described by Nachlas, Plapinger & Seligman (1964).

Determination of benzoyl-DL-phenylalanine 2-naphthyl ester hydrolysis. Cartilage sections (0.3 g.) were incubated at 37° for 24 hr. with 0.2 ml. of 50 mM-benzoyl-DL-phenylalanine 2-naphthyl ester in 10 ml. of universal buffer and sufficient water to give a final volume of 20 ml. Samples (3.5 ml.) were removed at intervals and brought to pH 7 by adding 1 ml. of alkali or concentrated buffer. The β -naphthol released was estimated by coupling it with 1 ml. of 0.4% (w/v) tetra-azotized di-*O*-anisidine [Fast Blue B: Sigma (London) Chemical Co. Ltd., London, S.W.6] as described by Ravin, Bernstein & Seligman (1954). After allowing 10 min. for the chromogenic reaction, 0.5 ml. of 80% (w/v) trichloroacetic acid was added and the colour extracted with 5 ml. of ethyl acetate, and measured at 540 m μ against a water blank. β -Naphthol was used as a standard.

Determination of benzyloxycarbonyl-glutamyl-tyrosine and benzyloxycarbonyl-glutamyl-phenylalanine hydrolysis. Cartilage sections (0.3 g.) were incubated at 37° for 24 hr. with 50 mM-benzyloxycarbonyl-glutamyl-tyrosine or benzyloxycarbonyl-glutamyl-phenylalanine in 5 ml. of universal buffer at a selected pH. Samples (0.6 ml.) were removed at intervals and precipitated by addition of 0.6 ml. of 10% (w/v) trichloroacetic acid and heating at 60° for 15 min. The precipitate was centrifuged down and 0.5 ml. of the supernatant fluid was diluted to 2.5 ml. with water. The free tyrosine or phenylalanine in duplicate 0.2 ml. samples was then determined by the ninhydrin method of Stein & Moore (1948) as described by Rosen (1957). Tyrosine or phenylalanine was used as a standard.

Determination of haemoglobin hydrolysis by cartilage cathepsin. Cartilage sections (0.3 g.) were incubated with 6.6 ml. of a 4% (w/v) denatured bovine haemoglobin solution in 0.2 M-acetate buffer (final volume 20 ml.), pH 4, for 24 hr.

at 37°. Denatured haemoglobin solution was prepared by dissolving dialysed haemoglobin in 6 M-urea. Proteolysis by cathepsins was then measured exactly as described earlier (Ali & Lack, 1965; Ali *et al.* 1967) by a modification of the Folin-phenol method described by Anson (1938).

Determination of acid phosphatase activity. Cartilage sections (0.3 g.) were incubated in 10 ml. of 0.2 M-acetate buffer, pH 5, containing 50 mM-sodium β -glycerophosphate, for 24 hr. at 37°. Duplicate samples (0.6 ml.) were withdrawn at intervals and precipitated with 2.5 ml. of 8% (w/v) trichloroacetic acid. After centrifugation, the sediment was discarded and inorganic phosphate content in the supernatant fluid was estimated by the method of Fiske & Subbarow (1925). For determining the pH optimum, the universal buffer described by Ellis (1961) was used but without the phosphate salt. The results are expressed as the amount of phosphorus liberated when compared with KH₂PO₄ as a standard.

Determination of DNA. To a fixed weight of sectioned (18 μ m.) cartilage, 9.5 ml. of water and 0.27 ml. of 60% (v/v) perchloric acid were added and stirred with a glass rod. After standing at 22° for 10 min., the mixture was chilled at 4° for 30 min. and centrifuged. The supernatant fluid was removed carefully and discarded. The sediment was then extracted twice with 5 ml. of 0.5 M-perchloric acid by heating at 70° for 15 min. The DNA content of the extract was then estimated by the diphenylamine method as described by Burton (1956), although the conditions for the colour development were at 100° for 10 min. as described by Dische (1930). Highly polymerized DNA from calf thymus (British Drug Houses Ltd., Poole, Dorset) was used as a standard.

Determination of hexuronic acid release from cartilage slices. Rabbit ear cartilage slices (7.5 mm.², 0.4 g.) were incubated in 10 ml. of 0.2 M-acetate buffer, pH 5, for 24 hr. 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 of the supernatant fluid were withdrawn at intervals and the hexuronic acid content was estimated 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 the amount of hexuronic acid released/ml. of the incubation medium.

EXPERIMENTAL AND RESULTS

Effect of pH on the enzymic activity of cartilage. When rabbit ear cartilage slices (7.5 mm.²) were incubated in buffer of different pH values (containing 10 mM-cysteine) for 24 hr. at 37°, it was found that there was a maximum release of hexuronic acid into the buffer at pH 5 (Fig. 1). This hexuronic acid-containing material has already been characterized as chondromucoprotein, which is released autocatalytically from cartilage at pH 5 (Ali, 1964). When the hydrolysis of benzyloxycarbonyl-glutamyl-tyrosine (cathepsin A), glycyl-tyrosine amide (cathepsin C) and benzoyl-DL-phenylalanine 2-naphthyl ester by cartilage sections was estimated, at various pH values, there

Fig. 1. Effect of pH on the autolytic release of hexuronic acid from cartilage and comparison with other enzymes in cartilage. The autolysis of cartilage was measured by incubating 0.4 g. of slices (7.5 mm.²) of rabbit ear cartilage in 10 ml. of universal buffer, containing 10 mM-cysteine, at 37° for 24 hr. and estimating the release of hexuronic acid into the buffer (○). The hydrolysis of glycyl-tyrosine amide (in 10 mM-cysteine) as ammonia liberated (▲), of benzoyl-phenylalanine 2-naphthyl ester as E_{540} (△), and of sodium β -glycerophosphate as free phosphorus (●), were measured as described in the text after incubating them with 0.3 g. of cartilage sections (18 μ m.) in universal buffer at various pH values at 37° for 24 hr.

Fig. 2. Hydrolysis of benzyloxycarbonyl-glutamyl-tyrosine and benzyloxycarbonyl-glutamyl-phenylalanine by cartilage cathepsins. Sections of rabbit ear cartilage (0.3 g.) were incubated with 50 mM-benzyloxycarbonyl-glutamyl-tyrosine in 5 ml. of universal buffer, pH 5, at 37° for 24 hr. The release of tyrosine in 0.2 ml. samples was determined, as described in the text, after incubation in the presence of buffer alone (○), 10 mM-cysteine (●), 20 mM-iodoacetamide (▲) or after heat-inactivation (80° for 15 min.) of cartilage enzyme in buffer alone (△). The hydrolysis of benzyloxycarbonyl-glutamyl-phenylalanine by cartilage cathepsin under exactly the same conditions both with (□) and without (■) cysteine is also shown.

was a peak of maximum activity at pH 5 (Fig. 1). This experiment and its results should be compared with a similar pH curve for cathepsin B activity (benzoyl-arginine amide and benzoyl-DL-arginine 2-naphthylamide as substrates) with a maximum at pH 5 (Ali *et al.* 1967). Ear cartilage showed acid phosphatase activity with a peak at pH 5 but appeared to be devoid of any alkaline phosphatase activity at pH 9 or 10.

Hydrolysis of cathepsin A substrate by cartilage enzyme. When the cathepsin A substrate benzyloxycarbonyl-glutamyl-tyrosine was incubated with 0.3 g. of cartilage sections at 37° for 24 hr. it was hydrolysed maximally at pH 5. There was only a slight enhancement of the catheptic activity in the presence of 10 mM-cysteine but substantial inhibition in the presence of iodoacetamide (Fig. 2). Preheating the cartilage sections at 80° for 15 min., before incubation with substrate, abolished the cathepsin activity. Cartilage sections, at pH 5 and in the presence of cysteine, also hydrolysed benzyloxycarbonyl-glutamyl-phenylalanine, which Iodice, Leong & Weinstock (1966a) have reported as a cathepsin A substrate. There was no activity in the absence of cysteine (Fig. 2 and Table 1).

Hydrolysis of cathepsin C substrate by cartilage enzyme. When the cathepsin C substrate glycyl-tyrosine amide was incubated with 0.3 g. of cartilage sections at 37° for 24 hr., it was hydrolysed maximally at pH 5 in the presence of cysteine (Fig. 1). The absence of cysteine led to a loss in total activity

Fig. 3. Hydrolysis of cathepsin and acid phosphatase substrates by cartilage enzymes. Sections of rabbit ear cartilage (0.3 g.) were incubated with appropriate substrate in universal buffer, pH 5, at 37° for 24 hr. Samples were removed at intervals and the hydrolysis of glycyl-tyrosine amide, as ammonia liberated, with 10 mM-cysteine (▲) and without cysteine (×), of benzoyl-DL-phenylalanine 2-naphthyl ester as E_{540} (●) and of sodium β -glycerophosphate as phosphorus released (■), were measured as described in the text. The activities against these substrates after preheating the cartilage sections at 80° for 15 min. are shown by △, ○ and □ respectively.

Table 1. *Determination of cartilage cathepsin activity against various substrates*

Sections (18 μ m.) of rabbit ear cartilage (0.3 g.) were incubated with each substrate at pH 5 for 24 hr. at 37° and the amount of substrate hydrolysed was then determined as described in the text. Haemoglobin hydrolysis was measured at pH 4.

Substrate	Cathepsin	Hydrolysis (%)			
		With 10 mm-cysteine	Without cysteine	With 20 mm-iodoacetamide	After heating at 80° for 15 min.
Benzoyloxycarbonyl-Glu-Tyr (50 mm)	A	50	32	10	7
Benzoyloxycarbonyl-Glu-Phe (50 mm)	A	20	0	—	—
Benzoyl-Arg-NH ₂ (50 mm)	B	18	8	0	0
Benzoyl-DL-Arg-2-naphthylamide (1.5 mm)	B	—	4	0	0
Gly-Tyr-NH ₂ (25 mm)	C	34	10	—	0
Acetyl-Tyr-NH ₂ (25 mm)	—	1	1	—	0
Benzoyl-DL-Phe-2-naphthyl ester (0.5 mm)	?	—	18	—	0
Haemoglobin (264 mg.), pH 4	D	—	17	3	0

and the preheating of cartilage sections at 80° for 15 min. abolished the activity altogether (Fig. 3). The pH-heat stability of this cathepsin was exactly the same as that reported earlier for cathepsin B and the autolytic enzyme in cartilage (Ali *et al.* 1967): that is, it was maximally stable only at pH 4 and 5 after heating the cartilage sections at 56° for 1 hr.

Effect of cartilage enzyme on chymotrypsin substrates. Two other synthetic substrates, which are known to be hydrolysed by chymotrypsin at neutral pH, were incubated with 0.3 g. of cartilage sections at 37° for 24 hr. Acetyltyrosine amide was not hydrolysed by the cartilage cathepsin at pH 5, even in the presence of 20 mm-cysteine (Table 1). Benzoyl-phenylalanine 2-naphthyl ester was hydrolysed maximally at pH 5 (Fig. 1) and could be measured very conveniently with the azo-dye-reaction method. There appeared to be a substantial enzymic hydrolysis of this substrate even at neutral pH. When examining the activity at various pH values it was important to have appropriate controls at alkaline pH as the substrate appeared to undergo spontaneous breakdown when incubated at pH 8, 9 and 10 at 37° for 24 hr. Preheating the cartilage sections at 80° for 15 min. abolished the cathepsin activity at pH 5 (Fig. 3). Cysteine interfered with the azo-dye reaction and produced a yellow opalescence. Attempts at removing cysteine, after incubation with cartilage and before the colour reaction, by precipitating with 20 mm-*p*-chloromercuribenzoate failed to give a satisfactory method.

Hydrolysis of acid phosphatase substrate. When 0.3 g. of cartilage sections was incubated with 50 mm-sodium β -glycerophosphate in buffer at different pH values it was hydrolysed maximally at pH 5 (Fig. 1). There was no evidence of any alkaline phosphatase activity in cartilage against this substrate. The cartilage acid phosphatase

activity was completely abolished by preheating the cartilage sections at 80° for 15 min. (Fig. 3). The pH-heat stability (56° for 1 hr.) of acid phosphatase in cartilage was very similar to that of other cartilage cathepsins and autolytic enzymes reported by Ali *et al.* (1967): that is, it was maximally stable at pH 4 and 5 and was inactivated at acid (pH 2 and 3) and alkaline (pH 7–10) values.

Estimation of total DNA content and enzymic activity of cartilage. DNA was extracted and estimated from different weights of sections of rabbit ear cartilage and it was found to be proportional to the amount of cartilage, thus establishing the usefulness of the method employed (Fig. 4). The total DNA content of 0.3 g. of rabbit ear cartilage was 460 μ g. and this value will serve as a unit of comparison when the catheptic activities of other types of cartilage are measured.

The percentage hydrolysis of various substrates by 0.3 g. of cartilage sections is shown in Table 1. It is clear that all four cathepsins, that is A, B, C and D, are present in rabbit ear cartilage. Cathepsin A activity, as measured by benzoyloxycarbonyl-glutamyl-tyrosine hydrolysis, was not all abolished by preheating the cartilage at 80° for 15 min., and apart from indicating that the enzyme is relatively heat-stable, it is possible that there was some spontaneous breakdown of the substrate in this assay. The hydrolysis of benzoyl-L-arginine amide and benzoyl-DL-arginine 2-naphthylamide by cartilage cathepsin B has been described elsewhere (Ali *et al.* 1967), but the total activity is summarized here. Both cathepsin B and cathepsin C activities were very dependent on cysteine activation and were inhibited by iodoacetamide.

Slices of rabbit ear cartilage (0.4 g. wet wt.), when incubated in 0.2 M-acetate buffer, pH 5, at 37° for 24 hr., released a total of 2.9 mg. of hexuronic acid into the incubation buffer. This was found to be 72% of the total hexuronic acid content of the

Fig. 4. Estimation of the DNA content of rabbit ear cartilage. Increasing wet weights of samples of rabbit ear cartilage were sectioned ($18\mu\text{m.}$) and extracted with perchloric acid as described in the text. The DNA content of the extracts was then determined with highly polymerized calf thymus DNA as standard.

original cartilage slices when examined after hydrolysis in 6ml. of 6M-hydrochloric acid at 80° for 15min. Under these conditions of hydrolysis 400mg. wet wt. of cartilage slices also had a protein content of 50.4mg., as determined by the method described by Lowry, Rosebrough, Farr & Randall (1951).

Acid phosphatase in 0.3g. of cartilage sections, when allowed to hydrolyse 50mM-sodium β -glycerophosphate at pH5 for 24hr. at 37° , liberated 3.65mg. of phosphorus, which amounts to about 23.5% of the original substrate.

The percentage of haemoglobin hydrolysed was measured by estimating the total amount of trichloroacetic acid-soluble aromatic amino acids released into the incubation buffer by cartilage cathepsin, as compared with the total obtained when 6.6ml. of 4% (w/v) haemoglobin was hydrolysed in 5M-hydrochloric acid for 15min. at 100° . Cartilage sections (0.3g.) liberated a total of 2.85mg. of tyrosine equivalents from 264mg. of haemoglobin, at pH4 in 24hr. at 37° , and this appeared to be 17% of the total aromatic amino acid content in the substrate.

DISCUSSION

The DNA content of rabbit ear cartilage, which was found to be 1.5mg./g. wet wt. of ear cartilage, is in the range of values published for other types of cartilage obtained from different species. Mankin & Baron (1965) reported a value of about 1mg. of DNA/g. wet wt. of adult rabbit articular cartilage obtained from the distal surface of the

femur. They used the indole method described by Bonting & Jones (1957). Simmons & Kunin (1967), using the diphenylamine method described by Burton (1956), have reported a value of about 0.652mg. of DNA/g. wet wt. of epiphyseal cartilage obtained from adult rats. The higher value obtained by our method of extraction of DNA from cartilage sections is understandable in view of the fact that young rabbit ear cartilage is more cellular than articular or epiphyseal cartilage.

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 before (Ali, 1964). The ability of enzymes in cartilage sections to hydrolyse benzoyl-arginine amide and benzoyl-DL-arginine 2-naphthylamide maximally at pH5 indicated the presence of cathepsin B in cartilage (Ali, 1967; Ali *et al.* 1967). By using the same quantitative techniques the presence in rabbit ear cartilage of cathepsins A and C has now been shown by the hydrolysis at pH5 of their specific synthetic substrates benzyloxycarbonyl-glutamyl-tyrosine and glycyl-tyrosine amide. The cartilage cathepsins B and C even respond to the same activator (cysteine) and inhibitor (iodoacetamide) as the characterized cathepsins from spleen and liver (Fruton, 1960), thus indicating that they are similar in specificities to the respective cathepsins from other tissues.

The hydrolysis of benzyloxycarbonyl-glutamyl-phenylalanine by cartilage enzyme appeared to be dependent on activation by cysteine and this is in contrast to the known properties of cathepsin A from ox spleen, which does not require cysteine for the hydrolysis of benzyloxycarbonyl-glutamyl-tyrosine (Fruton, 1960). Iodice *et al.* (1966a), working with purified enzyme from chicken and rabbit muscle, have described benzyloxycarbonyl-glutamyl-phenylalanine as a substrate for cathepsin A. The activity of purified cathepsin A, when obtained from different tissues and species, has been shown to vary in its specificity towards synthetic substrates and in its response to activators and inhibitors (Fruton, 1960; Iodice *et al.* 1966a). It is therefore only tentatively that the cartilage enzyme hydrolysing benzyloxycarbonyl-glutamyl-phenylalanine has been classified as cathepsin A in the text and Table 1. It is also not clear which cartilage cathepsin is responsible for the hydrolysis at pH5 of the chymotrypsin substrate benzoyl-DL-phenylalanine 2-naphthyl ester. It does not seem to have the right configuration to be a substrate for cathepsin B or C (Fruton, 1960).

The presence of cathepsin D has been evident from the ability of cartilage extracts or sections to hydrolyse haemoglobin (Lucy *et al.* 1961; Ali, 1964) and other protein substrates (Ali *et al.* 1967) maximally at about pH4. In fact, Woessner

(1967*a,b*) has succeeded in isolating and purifying a cathepsin D-like enzyme from rabbit ear cartilage. As cathepsin D has been consistently shown to have substantial proteolytic activity (Press, Porter & Cebra, 1960; Barrett, 1967) it is probably the most important cathepsin involved in the autolysis of cartilage at pH 5.

The proteolytic activity of cathepsin B against a variety of protein substrates has been established in numerous reports (Greenbaum, Hirschowitz, Shoichet, 1959; Barnhart, 1960; Otto, 1968). It is therefore possible that it shares the autolytic effect of cathepsin D in rabbit ear cartilage, especially in view of the inhibitory effect of arginine analogues and arginine-containing synthetic substrates on the release of chondromucoprotein at pH 5 (Ali, 1964; Lack & Ali, 1967; Ali *et al.* 1967). It is not clear whether cathepsins A and C have a significant protease role in cartilage autolysis at pH 5 as their specificity is restricted to a certain special configuration in their substrates that is usually not encountered physiologically (Planta & Gruber, 1961; Planta, Gorter & Gruber, 1964; Mettrione, Neves & Fruton, 1966). It is possible that their action is supplementary to that of cathepsins B and D. Iodice *et al.* (1966*a*) have shown that the hydrolytic activity of purified cathepsins A and D from skeletal muscle when incubated together with haemoglobin was twice the total of their individual proteolytic activities.

This quantitative method used in the assay of cartilage cathepsins was adopted as an alternative after failing to obtain a satisfactory homogenate or a reproducible extract of cartilage. The need for freezing the cartilage slices for sectioning in a cryostat in this method may affect the true or total activity of some enzymes in cartilage that are sensitive to one cycle of short freezing-and-thawing. Since frozen-and-thawed tissues have been shown to retain their subcellular morphology (Trump, Young, Arnold & Stowell, 1965), their enzymes are less sensitive to physical maltreatment than are purified enzymes in an artificial environment. As we have demonstrated the presence in cartilage of enzymes that have not been shown previously by any other quantitative technique, we may rely on this method as reflecting, as closely as possible, the enzymic activities of viable cartilage and chondrocytes.

Various tissues have been shown to undergo autolysis at acid pH values from the action of endogenous cathepsins (Baird, 1964; Umana & Dounce, 1964; Mego & McQueen, 1965; Iodice, Leong & Weinstock, 1966*b*; Woessner & Brewer, 1963). The autolysis of rabbit ear cartilage can be followed quantitatively by measuring the release of chondromucoprotein from the matrix. A study of the breakdown of articular cartilage by endo-

genous cathepsins is now in progress and the estimation of the acid phosphatase activity and the DNA content of rabbit ear cartilage will provide a basis for comparison. There are indications (S. Y. Ali, unpublished work) that in monkey articular cartilage cathepsin D may be the only acid protease responsible for cartilage degradation as the other cathepsin activities are not evident by the methods used successfully with rabbit ear cartilage.

We are grateful to Professor C. H. Lack for his interest and encouragement and to Miss Elizabeth Stainthorpe for skilled technical assistance. This work was supported by a grant from the Arthritis and Rheumatism Council.

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