The Action of Cathepsin D in Human Articular Cartilage on Proteoglycans

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ABSTRACT In recent years the lysosomal cathepsins have been implicated as important agents in the physiological degradation of various cartilages. In the present study, the nature of cathepsin present in human articular cartilage was investigated by microtechniques and a possible role for cathepsins in the cartilage degradation observed in osteoarthritis was sought. The results of this study indicated that the hemoglobin and proteoglycan-digesting activity in the human cartilage observed is predominantly that of a cathepsin D-type enzyme. This cathepsin D-type enzyme activity was present in two to three times greater amounts in yellowish or ulcerated articular cartilage from patients with primary osteoarthritis than in control "normal" human cartilages. The human cathepsin D-type enzyme, as well as a highly purified cathepsin D from bovine uterus degraded proteoglycan subunit (PGS) maximally at pH 5. Both enzyme preparations were inactive on hemoglobin at pH 6–8, but degraded PGS considerably at neutral pH. The activity of the human cathepsin extract was not affected by reagents which inhibit or activate cathepsins A and B. Neutral proteases which are active on hemoglobin or are inhibited by diisopropylfluorophosphate (DFP) were not detected in these preparations, but contamination by another type of neutral protease cannot be excluded. Chloroquine inhibited the degradation of PGS at neutral pH by the human cartilage enzyme extract.

INTRODUCTION

Observations from several laboratories implicate degradation of matrix components as an important step in the development of osteoarthritis.

In this respect, Bollet advanced the hypothesis that primary osteoarthritis might be perpetuated by abnormal exposure of underlying articular cartilage matrix to a normally occurring synovial fluid hyaluronidase introduced through surface abrasions (1). He and his collaborators found decreased hexosamine and uronate content as well as decreased polysaccharide chain-length in postmortem samples of osteoarthritic cartilage (2, 3). They considered these findings to be evidence for enzymic degradation of the sugar moieties of cartilage matrix proteoglycans. Initiation of this process was postulated to include damage to cartilage cells from abnormal stresses, subsequent lysosomal protease release, and consequent tissue weakening and cartilage surface abrasion (1). An enlarged zone of antigenic sites in osteoarthritic cartilage matrix reactive with fluorescein-labeled antibodies to proteoglycans was shown by Barland, Janis, and Sandson (4); similarly, perichondrocytic halo zones were described by Chrisman (5) in osteoarthritic cartilage. Such data could be explained by chondrocytic enzymic degradation of local matrix proteoglycans. In recent years lysosomal protease rather than hyaluronidase activity became the chief suspected agent of this degradation.

Thomas, McCluskey, Potter, and Weissmann (6) and the Dingle group (7, 8) gave the first clear demonstration that lysosomal protease(s) play a part in the degradation of embryonal chick cartilage matrix. They demonstrated in organ cultures that excess vitamin A led to the release of lysosomal protease(s) and degradation of cartilage matrix. After that report, a growing body of evidence has pointed to lysosomal protease cathepsin D as a ubiquitous agent in causing degradation of cartilage matrices. Acid cathepsin activity resembling that of cathepsin D has been observed in bovine nasal and tracheal cartilage (9) and in bovine costal cartilage (10, 11). Woessner (12) found that cathepsin D is the

major protease in rabbit ear and in chick limb cartilage and that it can degrade cartilage even at neutral pH. Ali and Evans (13) concluded that in rabbit ear cathepsin D "is probably the most important cathepsin involved in the autolysis of cartilage at pH 5.0." Ali also stated (13) that "in monkey articular cartilage cathepsin D may be the only acid protease responsible for cartilage degradation." Most recently Weston (14) prepared a specific antiserum to cathepsin D which inhibited autolytic degradation of cartilage, and Morrison (15), concluded that "the major proteolytic activity in the degradation of cartilage matrix can be attributed to the lysosomal cathepsin D."

In view of these significant developments concerning cathepsin D in animals, the present study was undertaken to investigate cathepsin D activity in human articular cartilage, especially in the early lesions of human osteoarthritis; to compare this activity with that of a highly purified cathepsin D prepared from the bovine uterus (16, 17); and to investigate the degradative action of these enzymes on proteoglycans, especially at neutral pH.

METHODS

13 patients, ages 43–86, all male, with symptomatic primary arthritis, grade II–IV by radiological criteria (18) provided the osteoarthritic cartilage samples for the current study. Five of these patients were undergoing surgery for total hip replacement or for debridement of a knee joint, and eight patients underwent diagnostic arthroscopy of a knee in lieu of a surgical exploration. All cartilage samples were taken from the knee and then only from nonweight-bearing sites subjected only to patellar pressures, viz., that portion of the articular surface exposed in the supracondylar fossa and the under surface of the patella itself. Control tissue was obtained from corresponding "normal" regions of the knee joints of the eight arthroscopy patients and from four additional patients, age 23–62. All of these additional patients were males, and revealed the following diagnoses: meniscal tear, 2 cases; and femoral arterial ischemia, requiring amputation of the involved extremity, 2 cases. Tissue from the knees of two patients with advanced vascular necrosis as judged by X ray and histological criteria were similarly studied. Patients were excluded from the current study if they had overt inflammatory joint disease or had received intra-articular corticosteroid administration within 2 wk before tissue sampling.

To select the biopsy sample, cartilage was viewed either directly or by means of the optical system of a Watanabe arthroscope, and samples 20–50 mg each wet wt, were dissected free, rinsed in 0.9% saline, patted dry on filter paper, and quick-frozen with an alcohol-acetone mixture. The samples were then thawed to 0–5°C and dissected with broken razor blades; samples were trimmed at ×50 magnification to conform to the following criteria based on gross appearance: (a) "normal", glistening white or pale yellow, resilient; (b) brown or dark yellow, nonfibrillated, and within 10 mm of an ulcerated osteoarthritic lesion (Fig. 1); (c) fissured or fibrillated from within an ulceration. Particular care was taken to avoid hypertrophic marginal tissue and the zone of calcified cartilage adjacent to
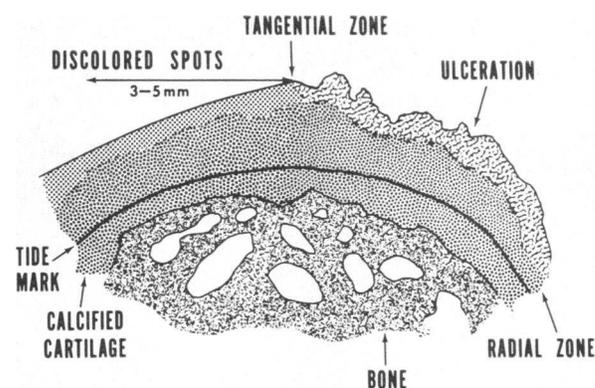

FIGURE 1 Diagram of human osteoarthritic lesion. Sagittal view. Arrow points to the marginal early lesion area.

subchondral bone. Samples (a) comprised the "control" and samples (b) the "discolored" samples used in this study. A small fragment of cartilage was saved for fixation, imbedding, and staining with fast green and safranin O, and the remainder used for biochemical studies.

In a final group of experiments, patellar samples were obtained post-mortem from five 52 to 75-yr old males with osteoarthritis. These patellae were kept on ice, washed with cold physiological salt solution; subjected to dissection of cartilage, processed, and graded as described above for biopsy samples.

All the "discolored" cartilage samples in this study corresponded to grade 4–9 (average 6) osteoarthritic cartilage of Mankin, Dorfman, Lipiello, and Zarins (19). The biopsy and postmortem patellar samples were used for enzyme studies and similarly graded surgery samples for the proteoglycan observations.

Extraction of samples for enzyme studies. The cartilage was finely sliced and placed in a microglass homogenizer with 5 vol. 0.005 M phosphate buffer, pH 8.8. After letting stand in the buffer for 24 h at 4°C, the sample was homogenized by hand (in an ice bath) and then centrifuged at 20,000 g and 4°C. The supernate was removed and the pellet was extracted two times more in the same way. The supernates were combined and assayed for cathepsin D activity by the microassay described later. The resuspended pellet was also assayed.

Proteoglycan was extracted from similarly graded samples by the Sajdera-Hascall method (20) scaled down for 50 mg wet wt amounts of cartilage. The weight-average sedimentation coefficients(S)[1] of the proteoglycan was determined by the microtransport method of Pita and Müller (21). The proteoglycan solution was diluted with 0.15 M aqueous KCl to 0.15 mg proteoglycan per ml. All S determinations in this study were carried out at this low concentration to avoid concentration dependence effects on the sedimentation coefficient (22). 10 μl of the diluted solution was placed in a capillary cell (1.0 mm diameter and 1.2 mm long) and centrifuged in a swinging bucket type rotor in the Beckman model L preparative ultracentrifuge (62,000 g, 30 min) (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). After centrifugation the solute boundary traversed a certain distance from the meniscus of the solution, placed

at X_m cm from the rotor center. Then the microcell was sectioned through a previously marked plane on the glass surface, estimated to fall within the upper part of the plateau region. The length H of the column of solution in the upper (centripetal) section was measured. The concentration of proteoglycan in the solution, C_f, as well as the initial loading concentration of the sample, C_o, were determined as uronate. The weight average sedimentation coefficient S was calculated through use of the formula

$$S = \frac{1}{\omega^2 t} \ln \frac{X_m + H}{X_m + H\frac{C_f}{C_o}}$$

which ω is the angular speed used and t the centrifugation time. The error introduced by this method on the value of S is no more than twice the error in the analysis of the initial and final concentrations. When tested on proteins and proteoglycans of known S, the S values were within 5% of experimental error (21). When tested on different concentrations of Rosenberg's proteoglycan fraction PPL3, the S values agreed with those obtained by Rosenberg with the analytical centrifuge (22). Uronate was determined by the Dische method (23) as modified by Bitter and Muir (24).

Batch preparation of cartilage extract. Postmortem patellae were obtained from eight additional 50 to 70-yr old males, without known osteoarthritis. Tissues were kept on ice, cleaned, and washed with ice-cold physiological salt solution and frozen awaiting further preparations. 30 g of the patellar cartilage was diced into cubes and let stand 24 h at 4°C in 50 ml 0.005 M phosphate buffer pH 8.0. Then it was homogenized in a "45" VirTis (VirTis Co., Gardiner, N. Y.) (in an ice bath) for 5 min at 1/2 speed and then for 10–15 min at 3/4 speed. After centrifugation at 130,000 g at 4°C, the supernate was removed and the pellet was extracted two times more. Each supernate was treated with 7.5% aqueous solution of cetylpyridinium chloride (CPC) until a flocculent precipitate formed (25), centrifuged at 4°C, at 130,000 g, and the treatment with CPC repeated two more times. The combined supernates were concentrated to half volume by ultrafiltration on Amicon UM-10 membranes, (Amicon Corp., Lexington, Mass.) 10,000 U of penicillin and streptomycin were added per ml.

The highly purified bovine uterus cathepsin D used in this study was prepared by combining eluates from the first three peaks of the final DEAE—Sephadex gradient chromatography step in the cathepsin D purification procedure
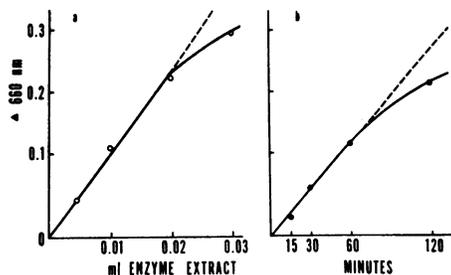

FIGURE 2 Concentration and time curves. Hemoglobin digestion by patellar cathepsin extract at pH 3.1 (average of three experiments). (*a*) 2 h incubation at 37°C. (*b*) 0.02 ml enzyme extract.

previously described (16, 26) and concentrating by ultrafiltration on Amicon UM-10 membranes. It was 1,700-fold purified, had a specific activity of 120 U/mg protein, and contained the cathepsin D multiple forms 4–7 (26) with only a trace of other protein, as shown by disk electrophoresis. It had all the classical properties of cathepsin D (17).

Microassay for cathepsin D. The modified Anson assay, previously described (17), was reduced in volume. The incubation mixture consisted of 0.06 ml acid-denatured hemoglobin (1.5% wt/vol), pH 3.1, 0.02 ml enzyme extract and 0.02 ml 0.05 M citrate buffer, pH 3.1. Incubation was carried out in a 0.4 ml plastic centrifuge tube for 2 h at 37°C. After incubation 0.1 ml 10% trichloroacetic acid (TCA) was added, the mixture was let stand 15 min and then centrifuged in a microfuge (Beckman model 152) at 20,000 g. Tyrosine was determined by mixing 0.1 ml supernate, 0.2 ml 0.5 M NaOH, and 0.06 ml Folin-Ciocalteau reagent (diluted 1 : 3 with water), letting stand 10 min and reading the absorbancy at 660 nm. For blanks, enzyme and substrate were incubated separately and were combined after addition of TCA. In the macroassay (17) 1 U of enzyme is defined as the amount of enzyme producing an absorbancy change of 1.0 at 660 nm. 1 U is equal to the release of 6.6 μeq tyrosine/h incubation, as determined by the absorbancy of a tyrosine standard. In the current study the microassay gave an absorbancy change of 20 for 1 U of enzyme, and therefore, absorbancy measurements were converted to enzyme units by dividing by 20. For the average cartilage extract the assay was linear only up to 1 h incubation and only over the absorbancy range 0–0.25 (Fig. 2). Accordingly, in most cases the amount of enzyme was adjusted to give absorbancy readings within the region of linearity on the concentration curve. Readings above the region of linearity were corrected by extrapolation of the initial linear part of the curve.

The micro-Anson assay detected 0.003 U of acid cathepsin activity and, thereby, measured activity in as little as 3 mg wet wt of human articular cartilage. The extraction of the biopsy and postmortem samples removed virtually all the acid cathepsin activity detectable by the microassay. Incubation of remaining pellet yielded negligible traces.

pH activity curves were made using the microassay with both acid-denatured and urea-denatured hemoglobin in 0.2 M citrate buffer (pH 2–5.5) or phosphate buffer (pH 6–8). The denatured hemoglobins were prepared as previously described (17).

Microassay of proteolytic action on proteoglycan subunit (PGS). PGS was prepared from bovine nasal cartilage by the method of Hascall and Sajdera (20). The incubation mixture consisted of 0.15 ml PGS (5 mg/ml in 0.05 M buffer, citrate for pH 3–5.5, and phosphate for pH 6–8) and 0.05 ml enzyme extract brought up to 0.1 ml with buffer of same pH. Incubation was at 37°C for 2 or 20 h. For blanks, substrate and enzyme plus buffer solution were incubated separately and then the enzyme was denatured by boiling for half an hour and combined with the substrate. Immediately after incubation the assay mixture and the blank were chilled and diluted with 0.15 M aqueous KCl to 0.15 mg PGS/ml and S, the weight average sedimentation coefficient of the PGS, was determined by the microtransport method of Pita and Muller (21) as described above, except that a small plastic tube (0.4 ml capacity and 5 mm ID) was used as the sedimentation cell and that, instead of cutting the tube, the solution between the meniscus and any plane in the upper part of the plateau region was removed carefully with a Hamilton syringe. H is the length of the column of solution removed (usually 10 mm).
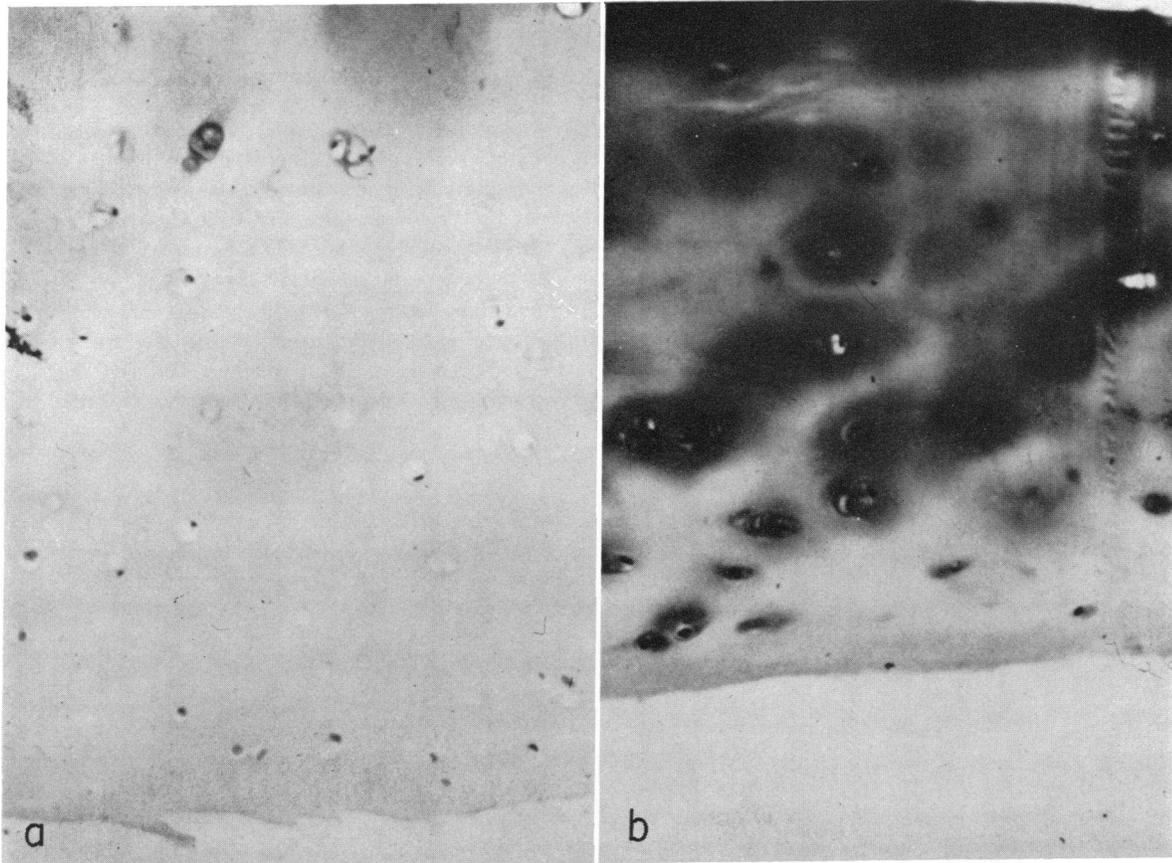
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FIGURE 3 Safranin O and fast green stain of a discolored early lesion sample (*a*) and of a control sample (*b*) from the knee patella revealing altered staining properties for proteoglycans (× 90).

This modification did not enlarge the experimental error described above.

Microviscometry was conducted with an Ostwald-type microviscometer made in this laboratory by F. Muller (vol. 0.15 ml; flow time of H_2O at 36°C: 0.28 min). The studies were done at 36°C. The incubation mixture consisted of 0.1 ml PGS (7.5 mg/ml in 0.23 M buffer, citrate-pH 3–5.5, and phosphate-pH 6–8) and 0.03 ml enzyme mixed with 0.02 ml 0.23 M buffer of the same pH. Heat-denatured enzyme was used in the blanks. The incubation mixture (and blank) was prepared at zero time and introduced immediately into the viscometer. Increase in the molarity of the buffer decreased the viscosity of the PGS solution. This effect was minimal at 0.2 M and therefore 0.23 M buffer was used.

Specific viscosity

$$= \frac{\text{time of flow of incubation mixture (or blank)} - \text{time of flow of water}}{\text{time of flow of water}}.$$

Percent original specific viscosity = 100 times specific viscosity of the incubation mixture divided by that of the blank (with denatured enzyme) measured at the same time interval.

RESULTS

The marginal nonfibrillated early lesion samples showed degenerative changes resulting in loss of normal staining for proteoglycans (Fig. 3, representing five marginal and five control samples). Extraction of the proteoglycans from several similarly graded samples and their sedimentation analysis as described in Methods indicated that a considerable part of their proteoglycan occurred in smaller fragments as compared with the proteoglycans in the "normal" control samples tested (Table I). Although few in number, these technically diffi-

TABLE I

Average Weight Sedimentation Coefficient (*S*) *of Extracted Proteoglycans*

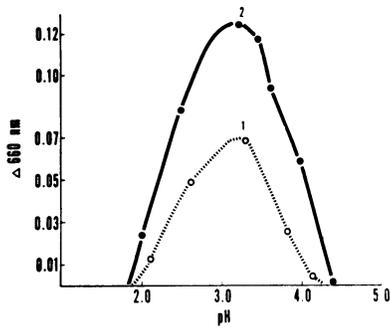| Samples	No. of samples	S
Normal, control	4	48, 30, 37, 28.4
Discolored, marginal zone	3	16, 5.8, 4.1
Aseptic necrosis, ulcerated	1	1.0

FIGURE 4 pH profile of hemoglobin digestion by two biopsy cartilage extracts. No. 1 ∼ 0.004 U in 0.02 ml enzyme extract. No. 2 ∼ 0.006 U in 0.02 ml enzyme extract.

cult to obtain results, taken in conjunction with loss of normal staining for proteoglycan in similar samples indicated that degradation of proteoglycan occurred in these investigated cartilage samples in the marginal zone of the osteoarthritic lesion.

Fig. 4 shows the pH curves of hemoglobin digestion given by two different groups of the biopsy sample extracts. The shape of these curves, the range of pH activity, and the pH maximum of 3.1 were characteristic of cathepsin D (16, 17). These findings indicate that the hemoglobin digesting protease in the marginal human osteoarthritic samples was a cathepsin D-type enzyme.

The "normal" control or "clear" biopsy samples contained an average of 1 to 1.5 U of this cathepsin D activity per g wet wt. In contrast, the samples from the marginal zone of the osteoarthritic lesion contained two

to three times as much activity (Table II). In the larger postmortem human patellar samples only twice as much cathepsin D was observed in the discolored samples as in the controls. This finding may have been due to the larger and less clearly defined pathological areas from which the samples were derived. However, the contrast between the yellow and controls was demonstrated more sharply by the two to three times higher specific activity of the cathepsin in the yellow samples expressed as units per milligram protein in the extract.

To explore further the nature of this cathepsin in human articular cartilage larger batches of enzyme extract were prepared from postmortem patellar-clear cartilage. In general, two-thirds of the enzyme was removed in the first extraction and practically all the rest by the second and third extractions. The treatment with CPC removed all the uronate-containing material, so it would not interfere with the assay, and resulted in a threefold purification of the cathepsin. Practically no enzymic activity was lost by this treatment when care was taken not to use excess CPC above the point of flocculation, but at least three successive treatments were required. The amount of enzymic activity lost by the 2 : 1 concentration by ultrafiltration on Amicon UM-10 membranes was negligible; however, more extensive concentration resulted in some loss. A total of 22.7 U of activity was obtained from a 30 g batch of cartilage.

The pH curves of hemoglobin digestion by this patellar enzyme extract (Fig. 5), using both acid-denatured and urea-denatured hemoglobin, like the pH curves of the biopsy samples, were characteristic of cathepsin D.

TABLE II

Amount of Cathepsin D-type Enzyme in Human Articular Cartilage

	No. of samples	U/g wet wt	mg protein in extract/g wet wt	U/mg protein in extract
In biopsy samples, 30–50 mg				
Normal	5	M* 1.52±0.36		
		R‡ 1.0 − 1.8		
Discolored	4	M 3.68±0.16		
		R 3.4 − 3.8		
P values§		<0.001		
In patellar samples, 200–350 mg				
Normal	6	M 1.0±0.32	5.1±1.52	0.19±0.07
		R 0.6 − 1.5	3.1 − 7.7	0.13 − 0.30
Discolored	7	M 1.90±0.77	4.95±1.34	0.43±0.15
		R 1.4 − 3.4	3.4 − 6.9	0.31 − 0.62
P values§		<0.05	‖NS	<0.01

* M, mean±SD.
‡ R, range.
§ P value, significance of the difference between the mean of the two groups by Student's t test.
‖ NS, No significant difference.

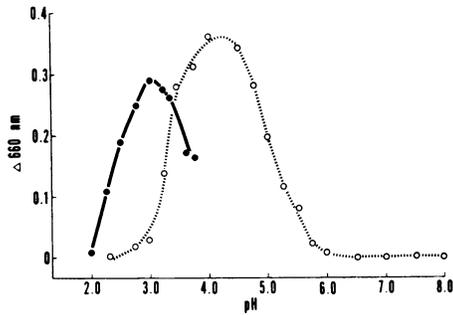628   *A. I. Sapolsky, R. D. Altman, J. F. Woessner, and D. S. Howell*

FIGURE 5 pH profile of hemoglobin digestion by human patellar cartilage extract (average of three experiments). (●), with acid-denatured hemoglobin. (○), with urea-denatured hemoglobin. ~0.015 U in 0.02 ml enzyme extract.
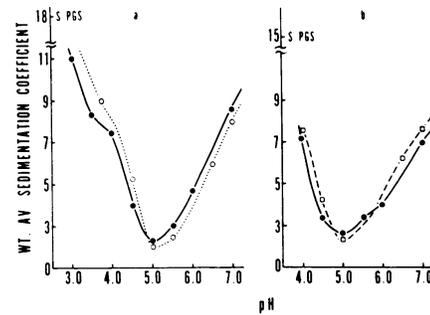

FIGURE 6 Degradation of bovine nasal PGS (average of three experiments). (a) 18S-PGS. (●) by human cartilage cathepsin, 0.07 U, 20 h incubation at 37°C. (○) by purified bovine cathepsin D, 1 U, 2 h incubation. (b) 15S-PGS. (●) by human cartilage cathepsin, 0.07 U, 20 h incubation. (□) by purified bovine cathepsin D, 0.07 U, 20 h incubation.

They revealed a pH maximum of 3.1 with the acid-denatured and of 4.0 with the urea-denatured hemoglobin. These curves were very similar to the corresponding pH curves of the crude and the 1000-fold purified bovine uterus cathepsin D (17), and of our most highly purified (single band on disk electrophoresis) bovine (16), as well as other cathepsin D reported in the literature (27–29). Moverover, like cathepsin D (17), this enzyme extract showed no degradative activity on hemoglobin in the neutral pH range. Also, iodoacetamide (24 mM in the incubation mixture) which completely inhibited cathepsin B in absence of cysteine (30) had no effect in the current study at pH 3.1, 4.0, and 4.5, with citrate buffer. The absorbancy reading at these pHs given in Fig. 5 were the same with or without iodoacetamide. The same amount of iodoacetamide likewise had no effect at pH 4.0, 4.5 and 5.0 with acetate buffer and acid-denatured hemoglobin prepared as above but with acetate instead of citrate buffer; nor at pH 5.0, 6.0, and 7.0 with citrate buffer and casein as substate. The pH optimum of cathepsin B1 on hemoglobin and acetate buffer is 4.0–4.5 (31). All of these data indicated that the hemoglobin-digesting activity in these human articular cartilage extracts was predominantly that of a cathepsin D-type enzyme. It also virtually excludes (except for possible traces) cathepsin B1 as well as those neutral proteases which digest hemoglobin (32–41). The apparent increase in activity with urea-denatured hemoglobin as substrate was not due to any activation of the enzyme but is possibly due to urea increasing the solubility of the reaction products in TCA (42).

Two bovine nasal PGS preparations were made, one with a weight average sedimentation coefficient of 18 and the other of 15. Fig. 6 depicts the degradation of these PGS preparations by both the highly purified bovine uterus cathepsin D and by the human patellar enzyme extract at various pH. Incubation of the PGS blanks at

pH 3.5–7 (with denatured enzyme) affected their sedimentation coefficient value only slightly, in a variable manner (0–12% decrease in S value). Both the human and the bovine enzymes digested the PGS maximally at pH 5.0 all the way down to a weight average S of about 2. Although both enzyme preparations had no action on hemoglobin at neutral pH, both degraded the PGS considerably at pH 7, breaking down the 18S PGS to a weight average S of 8–9 and the 15S PGS to a weight average S of about 7, under the given conditions (Fig. 6). On 20 h incubation at pH 7, 1 U of the bovine cathepsin D degraded the 18S PGS further to uronate-containing products with a weight average S of 6; and 1.5 U, to a weight average S of about 4.

The pH profiles of the 15S PGS obtained by the microviscometry (Fig. 7) for both the patellar and the purified bovine cathepsins paralleled that given by the sedimentation analysis. Again they revealed a pH maximum around 5 and also substantial degradation of the
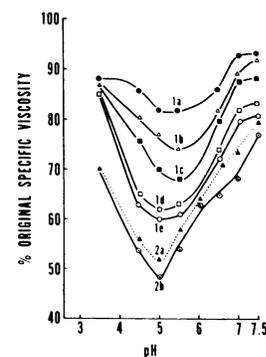

FIGURE 7 pH profile of PGS degradation obtained by microviscometry. Incubation mixture as given in Methods. The 15S PGS was used. (1) human cartilage enzyme extract, 0.07 U: (a) 15 min, (b) 30 min, (c) 60 min, (d) 180 min, (e) 20 h. (2) purified bovine cathepsin D, 1 U: (a) 180 min, (b) 20 h.
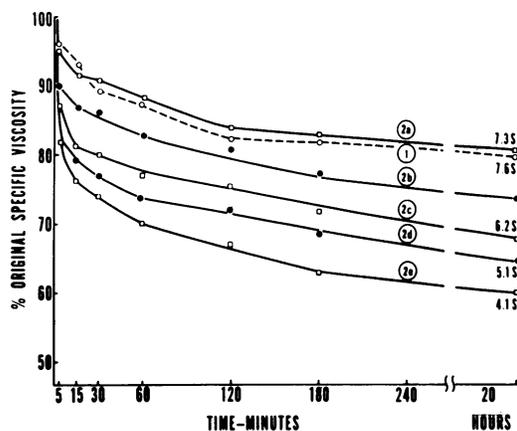
FIGURE 8 Viscometry of PGS degradation at pH 7.0. 15S PGS, 5 mg/ml in pH 7.0 phosphate buffer, 0.23 M in incubation mixture. (1) by human cartilage enzyme extract, 0.07 U. (2) by purified bovine cathepsin D: (2a) 0.07 U, (2b) 0.25 U, (2c) 0.5 U, (2d) 1.0 U, (2e) 1.5 U. The S values were the sedimentation coefficients determined after 20 h incubation with the enzyme.

PGS in the neutral pH range (pH 6.5–7.5). Considerable degradation at neutral pH was demonstrated also by the viscometery at pH 7 with varying amounts of enzyme (Fig. 8).

The viscometry (Fig. 8) and the sedimentation (Fig. 9) experiments showed that the degree of degradation of the PGS depended both on the amount of enzyme and the time of incubation. The PGS digestion by both the bovine and the human cartilage enzyme at neutral pH was exponential (Fig. 9). Even at neutral pH the degradation was comparatively fast; 1 U of the bovine cathepsin D was sufficient to degrade the PGS in 30 min to 70% of its original specific viscosity and to 60% of its initial S value.

The human cartilage enzyme extract also degraded a sample of protein-polysaccharide complex (PPC, kindly supplied by Dr. Lawrence Rosenberg of New York University) from an S value of 33.7 to pieces of 9.5S after 20 h incubation at pH 7.

The PGS digestion at pH 7 by the human cartilage enzyme extract (Table III) was not affected by iodo-
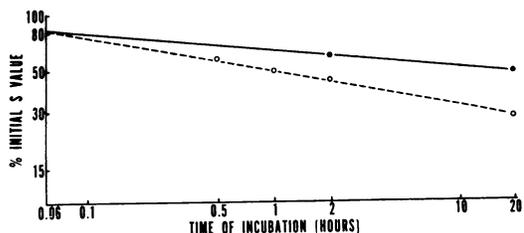

FIGURE 9 Degradation of bovine nasal PGS at pH 7.0. Double-log plot. (●) by human cartilage cathepsin, 0.07 U, (○) by purified bovine cathepsin D, 1 U.

acetamide which inhibits cathepsin A and B, nor by cysteine which activates these cathepsins. Nor was it inhibited by diisopropylfluorophosphate (DFP) which inhibits several neutral proteases (32, 39, 40). These reagents had no effect on the PGS degradation also at pH 4.5. On the other hand, chloroquine inhibited the PGS digestion quite considerably at neutral pH (Table III).

DISCUSSION

The term cathepsin D refers to a class of very closely related acid cathepsins which digest acid-denatured hemoglobin maximally at pH 3.0–3.5 and urea-denatured hemoglobin around pH 4.0 and are not affected by sulfhydryl and serine reagents (17). Cathepsin D does not hydrolyze the usual small synthetic peptides but digests the B chain of insulin with a specificity closely related to that of pepsin. Quite a number of enzyme preparations, including some highly purified ones, have been identified as cathepsin D-type enzymes chiefly by their pH activity curves, without testing their specificity on B chain of insulin (27, 43, 44). In fact, de Duve, Wattiaux, and Bandhuin (45) and Woessner (46) have suggested that any lysosomal acid cathepsin degrading hemoglobin around pH 3.5 is probably cathepsin D.

The acid cathepsin reported here in the human articular cartilage is undoubtedly a cathepsin D-type enzyme. The size and shape of the pH activity profile, the narrow pH range, and lack of effect by iodoacetamide on the hemoglobin digestion as well as lack of effect of cysteine and DFP on its action on proteoglycan are suggestive that this cathepsin D-type enzyme may be the major hemoglobin and proteoglycan-digesting protease in the human cartilage studied here. Woessner (46) found that cathepsin D accounts for almost all the hemoglobin-

TABLE III
Digestion of Bovine Nasal PGS by the Human Cartilage Cathepsin at pH 7.0

*Incubation mixture	‡S
Control (blank)	18.0
No addition	8.8
+ IAA, 25 mM	8.0
+ Cysteine, 10 mM	9.2
+ DFP, 10 mM	7.3
+ Chloroquine, 20 mM	15.3

Average of two experiments; incubation, 20 h at 37°C.
* 0.05 ml enzyme extract (0.07 U) plus 0.05 ml phosphate buffer (0.05 M, pH 7.0) mixed with 0.15 ml PGS (5 mg/ml in 0.05 M phosphate buffer, pH 7.0). In control heat-denatured enzyme extract used. The additions were preincubated with the 0.1 ml enzyme + buffer solution for 15 min at room temperature.
‡ Weight average sedimentation coefficient.

digesting activity in rabbit ear and chick cartilage. Ali (13) likewise found cathepsin D to be the major protease in rabbit ear cartilage but found present also cathepsins A, B, and C. However, he found only cathepsin D in monkey and human articular cartilage (47).

Lysosomal enzymes, including cathepsin D, have been implicated in many biological processes of degradation and repair (16, 48), and more specifically, a pronounced rise in the content of cathepsin D has been found in a variety of species at tissues sites wherein connective tissue is in the transitional state of remodelling (49). Therefore, it was not surprising to find in the early lesions of human osteoarthritis and in the discolored patellar samples tested a two- to threefold increase in cathepsin D-type enzyme. Likewise, Ali[2] reported recently that he found a significant rise in level of cathepsin D activity in diseased human articular cartilage as compared with normal. A similar increase of cathepsin level has been noted in menisci of rheumatoid knee joints (50) and of cathepsin D in synovial membrane of patients with rheumatoid arthritis (51, 52).

Hamerman, Janis, and Smith (53) observed that cultured synovial membrane cells from rheumatoid arthritic patients released protease which infiltrated and depleted the matrix of human articular cartilage. However, Granda, Ranawat, and Posner (51) found that, although there occurred a marked increase of cathepsin D-type enzyme in the rheumatoid synovial membrane, the level of cathepsin D activity was the same in the osteoarthritic synovial membrane as in the normal. Also, a recent check made in this laboratory of the cathepsin D level in the synovial fluid of three osteoarthritic patients showed it to be 0.05 U/g or less, i.e., 1/70 or less the level in the early lesion osteoarthritic cartilage.[3] Accordingly, in the present study, the increase in cathepsin D in the osteoarthritic cartilage was considered to be derived chiefly from the chondrocytes in the cartilage.

The average of 1–1.5 U of cathepsin D activity per g wet wt in the normal mature human cartilage samples and of 4 U in the early osteoarthritic lesions may be compared with 1.5 of the same units found by Woessner (12) in rabbit ear cartilage and 5.2 U in the limb bud cartilage of the 12 day-old chick embryo. This amount of enzyme activity is more than sufficient to account for both the physiological and pathological degradation of cartilage proteoglycans if the enzyme is available at acid pH.

The pH profiles of PGS digestion by the human cartilage enzyme extract and by the highly purified bovine

[2] Ali, S. Y., Enzymic degradation of cartilage in osteoarthritis. Unpublished report presented at the Workshop on Osteoarthritis sponsored by the University of Miami School of Medicine, 14–15 December 1971.

[3] Unpublished observations of the authors and Dr. Leon Cuervo.

cathepsin D, whether obtained by sedimentation or viscometry (Figs. 7 and 8), resembled each other, both showing a pH maximum of 5 and both extending into the neutral pH range. A similar pH optimum of 5 was found by the Dingle-Barrett group (15, 54) for proteoglycan and cartilage digestion by cathepsin D from human rabbit and chicken liver, as well as for the autolytic degradation of cartilage. As in the present case, they likewise found that this activity extended into the neutral pH range. Dingle (54) noted that the pH maximum shift of 2 pH U from the pH optimum for hemoglobin digestion might be due to the large number of negatively charged groups present in proteoglycans. Also, this shift of the pH maximum might be the reason for the present pH activity curve of cathepsin D proteoglycan digestion (but not hemoglobin digestion) extending into the neutral pH range.

Extracellular cathepsin D could not account for the degradation of proteoglycan in cartilage unless it could digest proteoglycans at near-neutral pH, the pH shown previously in this laboratory by micropuncture to exist within the matrix of mammalian articular cartilage.[3] In the present investigation both the human cartilage enzyme and the highly purified cathepsin D degraded PGS quite substantially at neutral pH.

Still, in the present investigation, the finding of substantial degradation of PGS at neutral pH leads one to suspect possible contamination by a neutral protease. Some neutral proteases occur in synovial fluid (33). Fessel and Chrisman (55) observed viscosity reducing activity at neutral pH on chondromucoprotein of 7.6% by normal and of 12.2% by fibrillated human osteoarthritic cartilage extracts. However, lack of activity on hemoglobin at neutral and slightly alkaline pH by both the highly purified bovine cathepsin D and the human cartilage cathepsin D-type enzyme extract eliminates any known neutral proteases which are active on hemoglobin. This finding excludes many of the neutral proteases reported in the literature (32–41), most of which originate from leukocytes.

In addition to being active on hemoglobin at neutral pH, the neutral proteases of Janoff and Zeligs (32), LoSpalluto, Fehr, and Ziff (39) and Mounter and Atiyeh (40) were ruled out by the fact that DFP inhibited them but had no effect on the PGS degradation by the present human cartilage enzyme extract.

In contrast to the above neutral proteases, Weissmann and Davies (56) and Weissmann and Spilberg (57) recently reported a neutral protease from rabbit leukocytes which was inactive on neutral hemoglobin but degraded proteoglycan maximally at pH 7.3. However, its pH activity peak was sharply distinct and well separated from that of the acid protease present in the same leukocyte extract, whereas here the neutral pH activity was

not distinct but occurred as the tail of the pH profile. If such a neutral protease, which was inactive on neutral hemoglobin but degraded proteoglycan were present as a contaminant in the highly purified cathepsin D from bovine uterus, this protease would have had to be carried with cathepsin D intimately through the many various purification steps and thus would have to resemble cathespin very closely. Nevertheless, contamination by such a protease cannot be ruled out.

Chloroquine stabilizes the lysosomal membrane and, at least in part, thereby inhibits the autolysis of cartilage (58). However, here it inhibited degradation of PGS in a particulate-free system (Table III). Chloroquine at pH 7 is a doubly protonated cation and may inhibit by binding to the negatively charged PGS in a way similar to its binding to DNA (59). In any event, and whether contamination by a neutral protease is involved or not, chloroquine inhibited the degradation of PGS at neutral pH.

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REFERENCES

1. Bollet, A. J. 1967. Connective tissue polysaccharide metabolism and the pathogenesis of osteoarthritis. *Adv. Intern. Med.* **13**: 33.
2. Bollet, A. J., J. R. Handy, and B. C. Sturgill. 1963. Chondroitin sulfate concentration and protein-polysaccharide composition of articular cartilage in osteoarthritis. *J. Clin. Invest.* **42**: 853.
3. Bollet, A. J., and J. L. Nance. 1966. Biochemical findings in normal and osteoarthritic articular cartilage. II. Chondroitin sulfate concentration and chain length, water, and ash content. *J. Clin. Invest.* **45**: 1170.
4. Barland, P., R. Janis, and J. Sandson. 1966. Immunofluorescent studies of human articular cartilage. *Ann. Rheum. Dis.* **25**: 156.
5. Chrisman, O. D. 1969. Biochemical aspects of degenerative joint disease. *Clin. Orthop. Related Res.* **64**: 77.
6. Thomas, L., R. T. McCluskey, J. L. Potter, and G. Weissmann. 1960. Comparison of the effects of papain and vitamin A on cartilage. I. The effects in rabbits. *J. Exp. Med.* **111**: 705.
7. Lucy, J. A., J. T. Dingle, and H. B. Fell. 1961. Studies on the mode of action of excess of vitamin A. II. A possible role of intracellular proteases in the degradation of cartilage matrix. *Biochem. J.* **79**: 500.
8. Fell, H. B., and J. T. Dingle. 1963. Studies on the mode of action of excess of vitamin A. VI. Lysosomal protease and the degradation of cartilage matrix. *Biochem. J.* **87**: 403.
9. Cowey, F. K., and M. W. Whitehouse. 1966. Biochemical properties of anti-inflammatory drugs. VII. Inhibition of proteolytic enzymes in connective tissue by chloroquine

10. (resochin) and related antimalarial/antirheumatic drugs. *Biochem. Pharmacol.* **15**: 1071.
10. Dziewiatkowski, D. D., C. D. Tourtellotte, and R. D. Campo. 1968. Degradation of protein-polysaccharide (chondromucoprotein) by an enzyme extracted from cartilage. *In* Chemical Physiology of Mucopolysaccharides. G. Quintarelli, editor. Little, Brown and Co., Boston.
11. Dziewiatkowski, D. D., V. C. Hascall, and S. W. Sajdera. 1970. The effect of cathepsins from varied sources on protein-polysaccharide (PP-L) of bovine costal cartilage and on proteoglycan subunit (PGS) of bovine nasal cartilage. *Calcif. Tissue Res.* **4**(Suppl.): 64.
12. Woessner, J. F., Jr. 1969. Lysosomal enzymes and connective tissue breakdown. *In* Inflammation Biochemistry and Drug Interaction. J. D. Houck, and A. Bertelli, editors. Excerpta Medica Foundation, Publishers, Amsterdam.
13. Ali, S. Y., and L. Evans. 1969. Studies on the cathepsins in elastic cartilage. *Biochem. J.* **112**: 427.
14. Weston, P. D. 1969. A specific antiserum to lysosomal cathepsin D. *Immunology.* **17**: 421.
15. Morrison, R. I. G. 1970. The breakdown of proteoglycans by lysosomal enzymes and its specific inhibition by an antiserum to cathepsin D. *In* Chemistry and Molecular Biology of the Intercellular Matrix. E. A. Balazs, editor. Academic Press, Inc. Ltd., London. **3**: 1683.
16. Sapolsky, A. I. 1969. Multiple forms of the cow uterus cathepsin D. Doctoral Dissertation. University of Miami, Miami, Fla.
17. Woessner, J. F., Jr., and R. J. Shamberger, Jr. 1971. Purification and properties of cathepsin D from bovine uterus. *J. Biol. Chem.* **246**: 1951.
18. Kellgren, J. H., and J. S. Lawrence. 1957. Radiological assessment of osteo-arthrosis. *Ann. Rheum. Dis.* **16**: 494.
19. Mankin, H. J., H. Dorfman, L. Lipiello, and A. Zarins. 1970. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. *J. Bone Jt. Surg. A. Am. Vol.* **53A**: 523.
20. Hascall, V. C., and S. W. Sajdera. 1969. Proteinpolysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates. *J. Biol. Chem.* **244**: 2384.
21. Pita, J. C., and F. J. Müller. 1972. Ultracentrifugal studies in capillary cells. I. Determination of sedimentation coefficients. *Anal. Biochem.* **47**: 395.
22. Rosenberg, L., S. Pal, R. Beale, and M. Schubert. 1970. A comparison of proteinpolysaccharides of bovine nasal cartilage isolated and fractionated by different methods. *J. Biol. Chem.* **245**: 4112.
23. Dische, Z. 1947. A new specific color reaction of hexuronic acids. *J. Biol. Chem.* **167**: 189.
24. Bitter, T., and H. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* **4**: 330.
25. Scott, J. E. 1960. Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. *Methods Biochem. Anal.* **8**: 145.
26. Sapolsky, A. I., and J. F. Woessner, Jr. 1972. Multiple forms of cathepsin D from bovine uterus. *J. Biol. Chem.* **247**: 2069.
27. Balasubramaniam, K., and W. P. Deiss, Jr. 1965. Characteristics of thyroid lysosomal cathepsin. *Biochim. Biophys. Acta.* **110**: 564.
28. Barrett, A. J. 1967. Lysosomal acid proteinase of rabbit liver. *Biochem. J.* **104**: 601.

29. Press, E. M., R. R. Porter, and J. Cebra. 1960. The isolation and properties of a proteolytic enzyme, cathepsin D, from bovine spleen. *Biochem. J.* **74**: 501.

30. Ali, S. Y., L. Evans, E. Stainthorpe, and C. H. Lack. 1967. Characterization of cathepsins in cartilage. *Biochem. J.* **105**: 549.

31. Otto, K. 1971. Cathepsin B1 and B2. Purification from bovine spleen and properties. *In* Tissue Proteinases. A. J. Barrett, and J. T. Dingle, editors. American Elsevier Publishing Co., Inc., New York.

32. Janoff, A., and J. D. Zeligs. 1968. Vascular injury and lysis of basement membrane *in vitro* by neutral protease of human leukocytes. *Science* (*Wash. D. C.*). **161**: 702.

33. Wood, G. C., R. H. Pryce-Jones, and D. D. White. 1971. Chondromucoprotein-degrading neutral protease activity in rheumatoid synovial fluid. *Ann. Rheum. Dis.* **30**: 73.

34. Marks, S., and A. Lajtha. 1965. Separation of acid and neutral proteinases of brain. *Biochem. J.* **97**: 74.

35. Dannenberg, A. M., Jr., and E. L. Smith. 1955. Proteolytic enzymes of lung. *J. Biol. Chem.* **215**: 45.

36. Haschen, R. J., and K. Krug. 1966. Distribution patterns of proteolytic enzymes in normal and leukaemic human leucocytes. *Nature* (*Lond.*). **209**: 511.

37. Kazakova, O. V., and V. N. Orekhovich. 1963. Isolation and properties of cathepsins from transplanted rat sarcomeM-1. *Vopr. Med. Khim.* **9**: 63.

38. Lebez, D., M. Kopitar, V. Turk, and I. Kregar. 1971. Comparison of properties of cathepsin D and E with some new cathepsins. *In* Tissue Proteinases. A. J. Barrett and J. T. Dingle, editors. American Elsevier Publishing Co., Inc., New York.

39. LoSpalluto, J., K. Fehr, and M. Ziff. 1971. Intracellular proteases and digestion of immunoglobulins. *In* Tissue Proteinases, A. J. Barrett and J. T. Dingle, editors. American Elsevier Publishing Co., Inc., New York.

40. Mounter, L. A., and W. Atiyeh. 1960. Proteases of human leukocytes. *Blood.* **15**: 52.

41. Wasi, S., R. K. Murray, D. R. L. MacMorine, and H. Z. Movat. 1966. The role of PMN-leucocyte lysosomes in tissue injury, inflammation and hypersensitivity. II. Studies on the proteolytic activity of PMN-leucocyte lysosomes of the rabbit. *Br. J. Exp. Pathol.* **47**: 411.

42. Wojtowicz, M. B., and P. Odense. 1970. The effect of urea upon the activity measurement of cod muscle cathepsin with hemoglobin substrate. *Can. J. Biochem.* **48**: 1050.

43. Barrett, A. J. 1970. Cathepsin D. Purification of iso-enzymes from human and chicken liver. *Biochem. J.* **117**: 601.

44. Iodice, A. A., V. Leong, and I. M. Weinstock. 1966. Separation of cathepsins A and D of skeletal muscle. *Arch. Biochem. Biophys.* **117**: 477.

45. de Duve, C., R. Wattiaux, and P. Bandhuin. 1962. Distribution of enzymes between subcellular fractions in animal tissue. *Adv. Enzymol.* **24**: 291.

46. Woessner, J. F., Jr. 1965. Acid hydrolases of connective tissue. *Int. Rev. Connect. Tissue Res.* **3**: 201.

47. Ali, S. Y. 1970. Biochemical evidence for lysosomes in chondrocytes and variation of cathepsin activity in different types of cartilage. *Fed. Proc.* **29**: 553 (Abstr.)

48. de Duve, C., and R. Wattiaux. 1966. Functions of lysosomes. *Annu. Rev. Physiol.* **28**: 435.

49. Woessner, J. F., Jr. 1971. Cathepsin D. Enzymic properties and role in connective tissue breakdown. *In* Tissue Proteinases. A. J. Barrett and J. T. Dingle, editors. American Elsevier Publishing Co., Inc., New York.

50. Wegelius, O., M. Klockars, and K. Vainio. 1970. Content of fibrocartilagenolytic enzymes and viscosity of homogenates of joint menisci in rheumatoid arthritis. *Scand. J. Clin. Lab. Invest.* **25**: 41.

51. Granda, J. L., C. S. Ranawat, and A. S. Posner. 1971. Levels of three hydrolases in rheumatoid and regenerated synovium. *Arthritis Rheum.* **14**: 223.

52. Luscombe, M. 1963. Acid phosphatase and catheptic activity in rheumatoid synovial tissue. *Nature* (*Lond.*). **197**: 1010.

53. Hamerman, D., R. Janis, and C. Smith. 1967. Cartilage matrix depletion by rheumatoid synovial cells in tissue culture. *J. Exp. Med.* **126**: 1005.

54. Dingle, J. T. 1971. The immunoinhibition of cathepsin D-mediated cartilage degradation. *In* Tissue Proteinases. A. J. Barrett and J. T. Dingle, editors. American Elsevier Publishing Co., Inc., New York.

55. Fessel, J. M., and O. D. Chrisman. 1964. Enzymatic degradation of chondromucoprotein by cell-free extracts of human cartilage. *Arthritis Rheum.* **7**: 398.

56. Weissmann, G., and D. T. P. Davies. 1969. A neutral, lysosomal protease active on protein polysaccharides and histones. *Arthritis Rheum.* **12**: 703. (Abstr.)

57. Weissmann, G., and I. Spilberg. 1968. Breakdown of cartilage proteinpolysaccharide by lysosomes. *Arthritis Rheum.* **11**: 162.

58. Weissmann, G. 1965. Lysosomes. *N. Engl. J. Med.* **273**: 1143.

59. Cohen, S. N., and K. L. Yielding. 1965. Spectrophotometric studies of the interaction of chloroquine with deoxyribonucleic acid. *J. Biol. Chem.* **240**: 3123.