Human Cartilage Lysozyme

ROBERT A. GREENWALD, ALAN S. JOSEPHSON, HERBERT S. DIAMOND, and AMBROSE TSANG

From the Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

ABSTRACT The lysozyme content of human cartilage was measured by incubation of lyophilized, powdered cartilage in a variety of buffers and salt solutions, and the factors controlling the binding of lysozyme within cartilage were studied. Lysozyme was extracted from hyaline cartilage by buffers of pH greater than 9.0, by solutions 1 M in monovalent cations, and by solutions 0.12–0.40 M in divalent cations. The ability of cations to extract lysozyme from cartilage agreed with their known affinities for binding to chondroitin sulfate. The total extractable lysozyme content of five samples of human costal cartilage ranged from 1.45 to 3.36 μg lysozyme per mg of cartilage; for five samples of hyaline cartilage from peripheral joints the range was 0.80–3.03 μg lysozyme per mg of cartilage. Cartilage incubated in excess exogenous lysozyme could bind 0.053 equivalents of lysozyme per equivalent of chondroitin sulfate. Fibrocartilage and synovium from knee joints yielded no detectable lysozyme, despite the fact that synovium, a tissue rich in lysosomes, contained measurable quantities of β-glucuronidase. Lysozyme extraction from cartilage was not augmented by incubation with streptolysin S. When incubation was carried out with mild extraction techniques, lysozyme extraction from cartilage tended to parallel uronic acid release, both as a function of time and from one specimen to another. The active material as lysozyme. Lysozyme occurs in human hyaline cartilage as a counterion to polyanionic glycosaminoglycans. Carextracted from cartilage met five criteria for identification tilage lysozyme appears to be extracellular and nonlysosomal. Degradation of cartilage may contribute to the increased serum and synovial fluid lysozyme levels often present in patients with rheumatoid arthritis.

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Dr. Greenwald's present address is Long Island Jewish-Hillside Medical Center, New Hyde Park, N. Y.

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INTRODUCTION

Lysozyme is a highly cationic, low molecular weight (14,300) protein which is widely distributed in the human body. Previous reports (1–3) have documented the common occurrence of elevated levels of serum and/or synovial fluid lysozyme in patients with rheumatoid arthritis. Since lysozyme is present in cellular lysosomes (4), and since lysosomal rupture is associated with inflammation (5), it has been assumed that the source of the increased lysozyme activity is release of the enzyme from the lysosomes of phagocytic cells.

Fleming, who discovered lysozyme 50 years ago, noted that this enzyme was present in joint tissue (6). This finding was confirmed by Meyer and Hahnel (7), who noted the "remarkable" lysozyme content of human and guinea pig rib cartilage. Recently, lysozyme has been identified in canine and bovine cartilages (8), and the effect of lysozyme on the growth of cartilage explants in tissue culture has been studied (9, 10). The present paper reports quantitative data on the lysozyme content of human cartilage and examines the factors which affect the binding of lysozyme within cartilage and which control release of lysozyme when cartilage is incubated in vitro.

METHODS

Tissue specimens. Human costal cartilage was obtained at autopsy from four trauma victims with no gross evidence of connective tissue disease and from one patient with progressive systemic sclerosis. Articular cartilage and synovium were obtained surgically. The tissue samples were frozen without added fluid until processing. The specimens were minced with a vegetable chopper, lyophilized, and then powdered in a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.) with external ice-water cooling. The final product was a coarse powder, usually grey or tan in color, which gave uniform results for 6 months when stored at 4°C.

Reagents. Studies of cartilage lysozyme were performed by incubation of tissue in an extensive series of media, utilizing primarily electrolyte solutions of varying molarities

or buffers of differing pH. Universal buffer (11) was used in experiments involving pH ranges greater than 2 pH U. Values of pH from 2.3 to 11.0 were obtained by addition of 0.2 N NaOH to a stock solution 0.02857 M in each of diethylbarbituric acid, citric acid, potassium dihydrogen phosphate, and boric acid in ratios from 1:10 to 1:1 by volume.

Antiserum to human lysozyme was obtained from Kallestad Laboratories, Inc., Minneapolis, Minn. The antiserum inactivated human lysozyme standards but did not produce precipitin lines on radial immunodiffusion. Egg white lysozyme was obtained from Worthington Biochemical Corp., Freehold, N. J.

Incubation technique. Except as noted otherwise, 20-mg samples of dried powdered tissue were placed in 13 × 100-mm culture tubes to which 1.0 ml of incubation medium was added. The cartilage powder was gently suspended in the medium with a wooden applicator stick to minimize adherence of the powder to the test tube wall. The stoppered tubes were placed in a water bath at 37°C and incubated for 24 hr with constant agitation. After centrifugation at 1500 rpm for 5 min, the supernate was aspirated and the residue washed with an additional milliliter of the incubation reagent. The incubation and wash supernates were pooled. Whenever the incubation medium contained a salt concentration greater than 0.2 M, the pooled supernate was dialyzed overnight in the cold against two changes of normal saline. The dialysis tubing was rated by the manufacturer to retain molecules of molecular weight greater than 12,000; in numerous control experiments there was no evidence of lysozyme leakage during dialysis. In order to test the stability of lysozyme in the media used to extract the enzyme from cartilage, control experiments were performed in all cases by incubation of known amounts of lysozyme in salt solutions or buffers identical with those used for cartilage incubation. The control tubes were also dialyzed against normal saline when the salt concentration exceeded 0.2 M. No precipitation of lysozyme was observed in any control tubes, and with the exception of experiments using LiCl at concentrations greater than 0.75 M, there was no loss of lysozyme activity in any control incubation at the salt concentrations or extremes of pH used to extract lysozyme from cartilage.

Assay techniques. Lysozyme activity was determined by an automated technique to be described in detail elsewhere (12). In brief, an automatic sampler was used to add portions of standard lysozyme solution or supernate to a continuously flowing line of substrate (*Micrococcus lysodeikticus*, 40 mg/100 ml, in 0.066 M phosphate buffer, pH 6.3) which entered a densitometer. The optical density of the substrate was recorded on a strip chart recorder on logarithmic paper. Peaks of decreased optical density were produced on the graph as substrate, acted upon by different amounts of enzyme, entered the flow cell. The decrease in optical density of the substrate was logarithmically related to the lysozyme activity of the sample. Standards for the assay were prepared from purified human lysozyme.[1] The lysozyme concentration in the cartilage incubation supernates was expressed as micrograms lysozyme released per milligram of powdered cartilage.

Hexuronic acid was measured by the carbazole method (13). Assay for β-glucuronidase activity was performed by a standard technique (14).

Measurement of total extractable lysozyme. The total extractable lysozyme content of the hyaline cartilages was determined by exhaustive sequential extraction of a 30 mg portion of each powdered cartilage. The specimens were incubated in 1 ml of 1.0 M NaCl at room temperature for 2 hr, the supernate was aspirated, and fresh reagent was added. This was repeated for a total of five extractions. The five supernates were pooled, dialyzed against normal saline, measured for final volume, and assayed for lysozyme activity. No appreciable amounts of lysozyme could be eluted from cartilage so treated by further washes with the salt solution or by subsequent incubation at 37°C for 24 hr in 1.0 M NaCl.

Characterization of cartilage lysozyme. Cartilage supernates rich in lysozyme activity were acidified or alkalinized by addition of equal volumes of 0.2 M acetate buffer, pH 3.7, or universal buffer, pH 10.5 respectively. The samples were placed in a boiling water bath for 90 sec and then retested for lysozyme activity in comparison to a control diluted with normal saline. Similarly, samples of cartilage were heated to boiling in acid or alkaline buffer, washed twice with normal saline, and then subjected to lysozyme extraction with 1.0 M NaCl (*vide infra*).

Portions of other supernates were mixed with antiserum to human lysozyme in ratios ranging from 1:1 to 1:10 and retested for lysozyme activity.

Incubation supernates and human lysozyme standards were subjected to electrophoresis in 1% agarose in barbital buffer at pH 6.8 ($\mu = 0.038$) on 2 × 3 in glass slides for 180 min at a current of 8 ma per slide. After electrophoresis, the slides were overlaid with a suspension of *M. lysodeikticus* in 1% agarose in 0.066 M phosphate buffer, pH 6.3, at 45°C. After cooling, the slides were incubated overnight in a moist chamber at 25°C. The presence of lysozyme on the slide was easily detected as an area of clearing in the previously opaque agarose.

RESULTS

Extraction of lysozyme by variation of pH. When human costal cartilage was incubated in universal buffers, lysozyme was extracted as a function of pH (Fig. 1). Lysozyme release was constant over the pH range from 2.3 to 8.9 and then increased rapidly over the pH range 9.0 to 10.5. All hyaline cartilage specimens yielded ly-

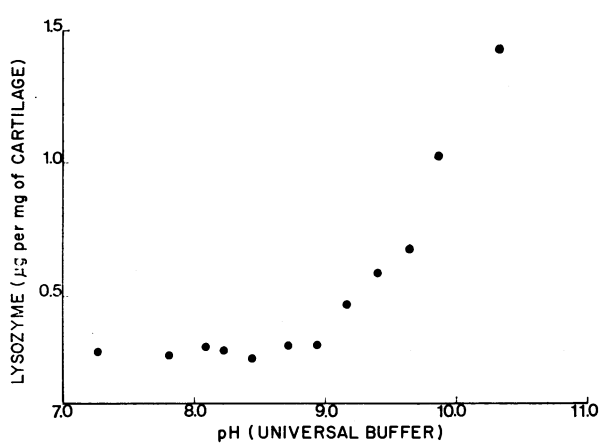

FIGURE 1 Extraction of lysozyme from a representative specimen of costal cartilage as a function of pH (universal buffer).

[1] Kindly provided by Dr. E. Osserman.

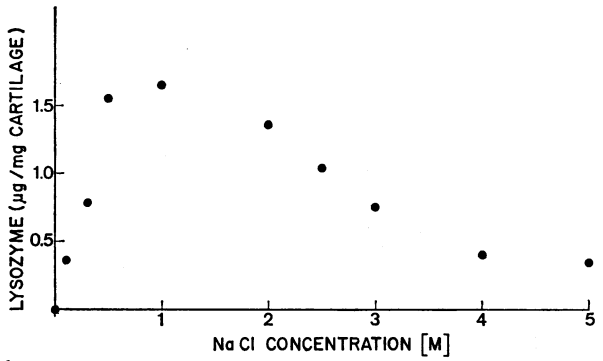

FIGURE 2 Extraction of lysozyme from a representative specimen of costal cartilage by a monovalent cation (Na^+).

sozyme when incubated in a standardized manner at pH 10.5. None of these buffers contained sufficient salts to extract lysozyme by cation exchange. In phosphate buffer at identical pH and cation concentration, lysozyme extraction was the same as that seen with universal buffer. However, an interesting finding was that cartilage incubated in tris buffers over the pH range from 8.0 to 10.3, at molarities from 0.001 M to 0.05 M, released no detectable lysozyme, an effect not attributable to instability of lysozyme in such media.

Lysozyme extraction in salt solutions. Sodium chloride solutions were effective extractors of caritilage lysozyme (Fig. 2). Extraction increased rapidly to a maximum at 1.0 M, and then decreased 50% of this value at 3.0 M. All hyaline cartilage specimens yielded lysozyme when incubated in a standardized manner in 1.0 M NaCl. Similar extraction with a maximum at 1.0 M occurred when KCl solutions were used. LiCl also extracted lysozyme from cartilage with a maximum of 0.75 M, but lysozyme was found to lose activity in LiCl solutions of higher molarities.

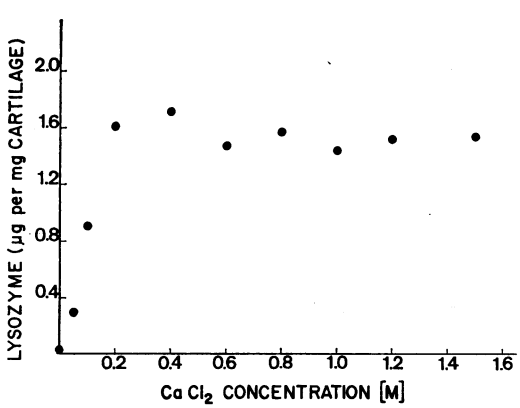

FIGURE 3 Extraction of lysozyme from a representative specimen of costal cartilage by a divalent cation (Ca^{++}).

TABLE I

Effectiveness of Different Cations in Extraction of Lysozyme from Cartilage

Cation	Lowest peak molarity	Lysozyme yield
		μg/mg
K^+	1.0	2.13
Na^+	1.0	1.43
Mg^{++}	0.40	1.57
Ca^{++}	0.20	1.72
Ba^{++}	0.12	2.07

Divalent cations were also potent in the extraction of lysozyme from cartilage. In $CaCl_2$ solutions (Fig. 3), extraction was maximal at a salt concentration of 0.2 M and no decrease in lysozyme release was noted at concentrations up to 1.5 M. Whereas maximum lysozyme extraction with monovalent cations was attained at strengths of 1 M, divalent cations were effective at molarities from 0.12 to 0.40, and yielded more lysozyme than did monovalent cations (Table I). When the cations K^+, Na^+, Mg^{++}, Ca^{++}, and Ba^{++} were arranged in order by the lowest molarity at which maximum lysozyme ex-

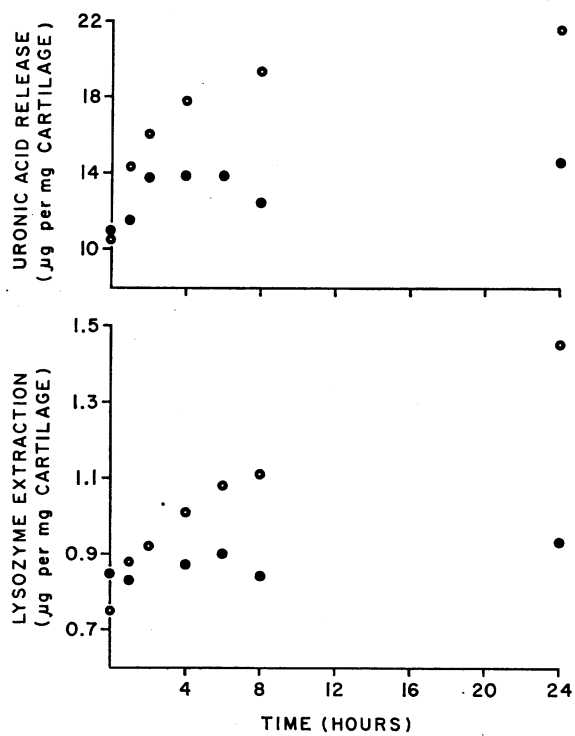

FIGURE 4 Lysozyme extraction and uronic acid release from a representative specimen of human costal cartilage in 1.0 M NaCl as a function of time: Open circles, 37°C; closed circles, 4°C. Curves of identical shape were observed in universal buffer, pH 10.5.

TABLE II

Lysozyme Extraction (micrograms per milligram) from Joint Tissues

Tissue	Age	Sex	Diagnosis	NaCl*	Buffer**	Total lysozyme
Costal cartilage	25	M	Accident victim	1.43	0.68	2.06
Costal cartilage	48	F	Accident victim	0.89	0.40	1.45
Costal cartilage	21	M	Accident victim	1.67	1.28	2.52
Costal cartilage	22	M	Accident victim	2.53	1.64	3.36
Costal cartilage	45	F	Scleroderma	1.52	0.44	2.62
Mean values for five costal cartilages:				1.61	0.89	2.40
Articular hyaline cartilage (hip)	65	F	Fractured femur	0.69	0.33	1.38
Articular hyaline cartilage (hip)	85	M	Fractured femur	1.45	0.65	3.03
Articular hyaline cartilage (knee)	65	M	Fractured patella	0.81	0.30	1.31
Articular hyaline cartilage (knee)	43	M	Villondular synovitis	0.21	0.17	0.80
Articular hyaline cartilage (knee)	25	F	Trauma to knee	0.99	0.43	1.83
Mean values for five hyaline articular cartilages:				0.83	0.38	1.67
Articular fibrocartilage (knee)	34	M	Torn meniscus	Trace	Trace	—
Articular fibrocartilage (knee)	30	F	Torn meniscus	Trace	Trace	—
Articular fibrocartilage (knee)	25	F	Torn meniscus	Trace	Trace	—
Synovium (knee)	43	F	Rheumatoid arthritis	0	0	—
Synovium (knee)	44	F	Rheumatoid arthritis	0	0	—

* Results of single incubation in 1.0 M NaCl.

‡ Results of single incubation in universal buffer, pH 10.5.

traction occurred, this order agreed with their known affinities for binding to chondroitin sulfate (15).

Time course of lysozyme extraction. Lysozyme extraction from costal cartilage as a function of incubation time was found to be dependent upon temperature (Fig. 4). At 37°C, an initial wash of the tissue (equivalent to incubation for zero time) yielded 52% of the total lysozyme extracted from a parallel sample incubated for 24 hr. The remaining lysozyme was obtained in small increments as the incubation time was increased. In another sample of the same cartilage incubated at 4°C, the initial recovery of lysozyme was similar but extraction did not increase with incubation up to 24 hr. As a function of time, uronic acid release, measured on the same specimens, paralleled lysozyme extraction.

Lysozyme content of different tissues. Five costal cartilage specimens, eight samples of articular cartilage, and two pieces of synovium were analyzed for lysozyme release and for total lysozyme content (Table II). Standardized 24 hr incubation in either 1.0 M NaCl or universal buffer of pH 10.5 yielded reproducible amounts of lysozyme for a given specimen. Incubation in the salt solution always yielded more lysozyme than did the buffer. Articular hyaline cartilage yielded less extractable lysozyme than did hyaline costal cartilage. Three samples of articular fibrocartilage yielded only trace amounts of lysozyme. Neither reagent extracted detectable amounts of lysozyme from the two samples of synovial tissue.

By exhaustive sequential extraction with 1.0 M NaCl, the total extractable lysozyme content of the hyaline cartilages was determined (Table II). For five specimens of costal cartilage extractable lysozyme ranged from 1.45 to 3.36 μg lysozyme per mg cartilage. For five samples of articular hyaline cartilage from hip and knee joints, total extractable lysozyme ranged from 0.80 to 3.03 μg lysozyme per mg cartilage.

Lysozyme binding by cartilage. The lysozyme content of normal costal cartilage represented only 2% of the total lysozyme which this cartilage could bind. A cartilage specimen weighing 44 mg, from which all endogenous lysozyme was extracted, was incubated in 3.0 ml of a solution containing 2.03 mg egg white lysozyme per ml. Lysozyme binding by the cartilage, measured by determination of residual, unbound lysozyme, and by subsequent re-extraction with 1.0 M NaCl, was 5,136 μg of lysozyme. Assuming that dry cartilage powder is 30% chondroitin sulfate, and utilizing the values of 1670 and 300 for the equivalent weights of lysozyme and chondroitin sulfate, respectively (16), the uptake of lysozyme by the cartilage corresponded to a binding affinity of 0.053 equivalents of lysozyme per equivalent of chondroitin sulfate.

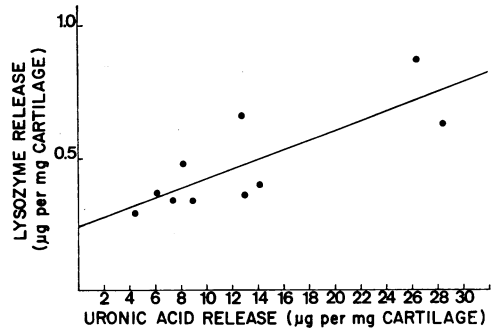

FIGURE 5 Lysozyme extraction and uronic acid release from hyaline cartilage incubated in phosphate buffer. The correlation is statistically significant ($P = 0.003$).

Relationship of lysozyme extraction to release of β-glucuronidase and uronic acid. When the powdered tissues were incubated in 0.1 M phosphate buffer, $\mu =$ 0.21, at pH 7.4, all of the hyaline cartilage specimens, but not the synovium, yielded lysozyme. However, the synovial extracts did contain β-glucuronidase activity (approximately 0.2 Fishman U/mg tissue), whereas this lysosomal enzyme was not found in any of the cartilage specimens. Furthermore, when cartilage specimens were incubated with streptolysin S, an agent which ruptures lysosomes (17), β-glucuronidase activity remained undetectable and lysozyme extraction was not increased. Simultaneous measurement of release of uronic acid and lysozyme extraction for 10 cartilage specimens incubated in phosphate buffer (Fig. 5) revealed that lysozyme release increased as uronate release increased ($r = +0.81$).

Characterization of cartilage lysozyme. The active principle extracted from cartilage in these experiments met five criteria for identification as lysozyme. Lysis of the test organism, *M. lysodeikticus,* is a necessary property of the enzyme. The activity of the supernatants was inhibited at salt concentrations greater than 0.2 M (18). Boiling at alkaline pH resulted in an 80% loss of activity, whereas boiling in acid had no such effect. Similar treatment of powdered cartilage produced the same effect upon the amount of lysozyme which was subsequently extracted from the cartilage. This property of heat stability in acid but not alkaline media is a characteristic of all animal lysozymes (19). Lysozyme activity in the supernates was sequentially abolished by the incremental addition of goat antiserum to human lysozyme. Finally, both the human lysozyme standards and the active principle in the cartilage extracts had cathodal mobility on electrophoresis at pH 8.6.

DISCUSSION

The major constituents of cartilage are collagen and acid mucopolysaccharides (predominantly chondroitin sulfate) which together make up at least 80% of the dry weight of cartilage. Most techniques for the extraction of cartilage components yield macromolecular protein-polysaccharide complexes, and most research on the noncollagenous proteins of cartilage has been directed at study of the "core" protein which is covalently linked to carbohydrate. Less attention has been paid to other protein components of cartilage. The present study demonstrates that lysozyme is a protein found in human hyaline cartilage, both costal and articular, in amounts ranging from 0.15 to 0.35% dry weight.

Alexander Fleming (6) noted that lysozyme was ubiquitous in the human body and that a specimen of patella had the greatest lysozyme activity of any mammalian tissue tested. With the exception of a similar observation by Meyer and Hahnel, (7), little attention was paid to cartilage lysozyme until Kuettner, Guenther, Ray, and Schumacher demonstrated that the lysozyme content of calf scapula cartilage varied in different zones of the tissue as delineated histologically, with the greatest amounts found in ossifying cartilage (8). It is difficult to compare their results with ours quantitatively, since they used egg white lysozyme as a standard for their assay, which has a different specific activity than the human enzyme (20), and because they used a diffusion technique for lysozyme measurement, a method which gives different results than that used in the current paper (12). These authors have also shown that lysozyme can exert profound effects upon the growth of cartilage explants in tissue culture (9, 10) and that some of these effects are shared by protamine, another highly cationic substance. They concluded that lysozyme in these tissues was nonlysosomal and primarily extracellular (21, 22).

The markedly cationic nature of the lysozyme molecule (iso-electric point = 10.4) suggests that it is bound in cartilage as a counterion to the polyanionic glycosaminoglycans of that tissue. Such binding is known to occur in aqueous solution (16), where salts can bring about dissociation of the two molecules by interference with ionic binding. Lysozyme extracted from animal cartilage appears to be similarly bound (10). Titration of the cationic amino groups of the lysozyme molecule by incubation in basic buffers would be expected to dissociate the lysozyme-mucopolysaccharide complex and lead to lysozyme release, as occurred in the present experiments. The shape of the pH elution curve, with a rapid increase above pH 9.0, suggests that lysine, of which there are five residues in human lysozyme (20), may be a cationic site at which binding occurs. However, the finding of decreased lysozyme extraction from cartilage in NaCl concentrations above 2 M suggests that lysozyme may be capable of other types of binding to cartilage components when its configuration is altered by changes in the ionic environment.

It appears unlikely that diffusion of lysozyme from the bloodstream or from interstitial fluids can account for the lysozyme content of cartilage. The lysozyme content of animal cartilage is greatest in that portion of the tissue farthest from the synovial fluid interface (8). There is evidence that lysozyme is synthesized in animal cartilage grown in tissue culture (21). The large, fixed molecules of which cartilage is constructed tend to prevent the entry into the tissue of other large molecules, a phenomenon known as the excluded volume effect (15). Iron dextran, of molecular weight 7000 (half that of lysozyme) is a molecule that is thus excluded (23).

Cartilage lysozyme appears to be of nonlysosomal origin. Synovial tissue from patients with rheumatoid arthritis, which is known to be rich in lysosomes (24) contained no lysozyme extractable by our techniques. Furthermore, β-glucuronidase, a known lysosomal enzyme (5) was detected only in the synovial extracts and not in cartilage. Although it was not anticipated that intact lysosomes would survive the techniques of tissue preparation utilized here, an agent was added to the cartilage which ruptures lysosomes, and there was no demonstrable increase in the amount of lysozyme extracted.

The increased concentration of lysozyme in hyaline cartilage (up to 1000 times greater than that of serum) suggests that cartilage is a potential source of the increased serum and synovial fluid lysozyme levels present in patients with rheumatoid arthritis. Release of glycosaminoglycans during incubation of cartilage paralleled the extraction of lysozyme for 10 different specimens incubated in a buffer at physiologic pH. A similar parallel was noted when cartilage supernates were studied as a function of the time and temperature of incubation. These experiments were performed under mild extraction conditions which resulted in release of only a small fraction of the total cartilage mucopolysaccharide. It should not be inferred that under vigorous extraction conditions with reagents which release much greater quantities of proteoglycan, e.g., 3 M $MgCl_2$ or 4 M guanidinium chloride, that lysozyme extraction would parallel the release of carbazole-reactive material. Cartilage from patients with rheumatoid arthritis has a decreased glycosaminoglycan content (25). Lysozyme release from cartilage may accompany the loss of cartilage glycosaminoglycans in diseases such as rheumatoid arthritis.

In addition, we have reported elsewhere (26) studies in which cartilage dissolution was induced in vivo by the injection of papain into rabbits, resulting in the release of chondroitin sulfate into the serum and a twofold rise in serum lysozyme activity. Patients with the arthritis of systemic lupus erythematosus have normal serum lysozyme levels (12) and do not usually manifest destruction of cartilage. The increased serum and synovial fluid lysozyme levels which occur in patients with rheumatoid arthritis are likely to be the result of cartilage degradation as well as lysosomal rupture during inflammation.

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