Acid Hydrolases in Slices of Articular Cartilage and Synovium from Normal and Abnormal Joints[1] (34633)

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In degenerative joint disease, cartilage digestion occurs in the absence of identifiable chondroclasts. Further, there is no evidence that circulating proteolytic enzymes such as plasmin are involved in cartilage degradation *in vivo* (1). These observations suggest that the enzymes involved in cartilage digestion are derived from chondrocytes and/or synovial cells. Acid hydrolases have been demonstrated in articular cartilage (2, 3) and synovium (4), and if these enzymes do, in fact, play a role in cartilage degeneration, it is not clear whether cartilage or synovium is primarily affected. It has been shown that breakdown products of mucopolysaccharides injected into synovial fluid induce the release of acid hydrolases from synovial cells (5) suggesting that cartilage degradation may occur first.

The present study was designed to determine (a) whether the activity of certain acid hydrolases, known to be lysosomal enzymes, is altered in cartilage degeneration, and (b) whether the earliest changes occurred in cartilage or synovium. The model of continuous compression of joint surfaces (6, 7) was chosen to produce degenerative changes in articular cartilage since this method does not require puncture of the joint cavity or incision of the synovium, and thus changes secondary to hemorrhage and wound healing are eliminated. Joints subjected to continuous compression show pathologic changes in articular cartilage directly in contact with opposing articular surfaces as well as in areas not in direct contact (8, 9). For this reason, these two areas were assayed separately for enzyme activity.

Since efforts to study lysosomal enzymes by the usual methods of homogenization and fractionation of tissue proved to be inadequate for articular cartilage, enzyme activities were determined in thin slices of articular cartilage and synovium.

Enzyme determinations. All enzyme determinations were performed in duplicate and each reaction mixture was gently shaken in a water bath at 37°. After the reaction was stopped and color developed, it was read against a substrate-buffer blank which had been incubated under the same conditions as the sample.

To determine the optimal incubation time for easily detectable substrate hydrolysis by each enzyme fresh-frozen bovine cartilage slices were incubated with an appropriate substrate and at timed intervals 2.0-ml aliquots were removed and the amount of hydrolysis determined. In the rabbit studies only fresh cartilage or synovium was used.

Arylsulfatase was determined by a modified Roy procedure (10). The reaction mixture contained 10.0 ml of 1 m*M* *p*-nitrocatechol sulfate (Sigma Chemical) in 0.1 *M* acetate buffer (pH 5.8) and cartilage slices. To stop the reaction, 0.5 ml of 10% trichloroacetic acid (TCA) was added. Color was developed by adding 0.5 ml of 10% sodium hydroxide in 5% sodium sulfite and read at 510 mμ. The optical densities were compared

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with standards of *p*-nitrocatechol treated in the same manner.

For assay of *β-d*-glucuronidase and *N*-acetyl-*β-d*-glucosaminidase the reaction mixture contained 10.0 ml of 1.0 m*M* solution of *p*-nitrophenyl-B-*d*-glucuronide or *p*-nitrophenyl-*N*-acetyl-B-*d*-glucosaminide (Calbiochem) in 0.1 *M* acetate buffer (pH 5.0) and cartilage slices. 1.0 ml of 10% TCA was added to stop the reaction and 1.0 ml of 3.5 *N* NaOH was added to develop color which was read at 410 mμ. Since substrate hydrolysis occurred about 5 min following the addition of NaOH in the *p*-nitrophenyl-B-*d*-glucuronide assay, the color was determined immediately. The optical densities were compared with standard solutions of *p*-nitrophenol treated in the same manner.

Cathepsin activity was measured by the method of Ali *et al.* (12). The reaction mixture contained cartilage slices suspended in 6.6 ml of 4% denatured hemoglobin brought to 20.0 ml with 0.1 *M* acetate buffer (pH 5.0).

Three different substrates were used for acid phosphatase activity: 20 m*M* phenyl phosphate (Calbiochem), 50 m*M* B-glycerophosphate (Sigma) and 4.0 m*M* *p*-nitrophenyl phosphate (Calbiochem). The separate reaction mixtures contained 11.0 ml of substrate and cartilage slices. Acid phosphatase activity on the first two substrates was determined by the methods described by Vaes and Jacques (13). When *p*-nitrophenyl phosphate was used as a substrate, the reaction was stopped by the addition of 2.5 ml of 10% TCA. The color developed by addition of 2.5 ml of 3.5 *N* sodium hydroxide was read at 410 mμ. The optical densities of the solutions were compared with those of standard solutions of phenol, inorganic phosphate and *p*-nitrophenol, respectively.

The relationship between wet weight and DNA was evaluated using relatively large samples (up to 75 mg) of fresh-frozen bovine cartilage or synovium. Three extractions with 0.5 *N* perchloric acid at 70° were required to remove 95% of the DNA from cartilage slices or diced synovium. DNA was determined by the method of Burton (11). The tissue wet weight and the total DNA content in bovine articular cartilage or synovium exhibited a linear relationship. A similar relationship was observed between DNA and wet weight of articular cartilage or synovium of normal rabbits using smaller samples (up to 30 mg maximum weight of articular cartilage obtained from a single rabbit knee joint). Thus, all data using slices of tissue were expressed as millimoles of substrate hydrolyzed per milligram of wet weight using constant incubation times.

Studies on rabbit cartilage and synovium. Cartilage degeneration was produced by continuous compression of the joint surfaces in the right knee of 15 anethesized rabbits weighing 4500 to 5000 g. Compression was accomplished by passing a Kirschner wire through the femur above and tibia below the knee joint with the wires joined by rubber bands and the limb immobilized by a groin-to-toe plaster cast. Animals were sacrificed at intervals from 1 to 21 days with an overdose of intravenous pentobarbital. Both left and right knee joints were opened and the synovium immediately removed from the suprapatellar pouch and placed in ice cold 0.25 m*M* sucrose. Cartilage slices were shaved from the femoral condyles and tibial plateau in the areas under direct contact (compressed cartilage). Cartilage shavings were also made from the remainder of the joint in areas which had been immobilized but not in direct contact (uncompressed cartilage). Comparable areas of cartilage were removed from the unimmobilized contralateral knee joint which served as a control (control joint). All specimens were immediately placed in 0.25 *M* sucrose in an ice bath until they could be conveniently blotted with filter paper, transferred to a stoppered weighing bottle and weighed. 5.0 ml of buffered substrate of identical concentration and pH as described for the bovine cartilage enzyme assays were incubated with the cartilage or synovial specimens for appropriate time periods.

In addition, weighed cartilage specimens from compressed (21 days) and control knee joints were assayed for aryl sulfatase activity and protein release at hourly intervals, using

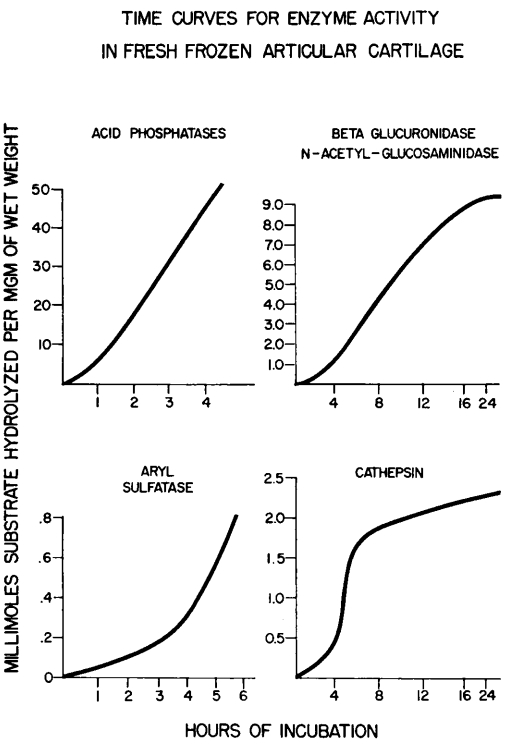

FIG. 1. Acid phosphatase activity using *p*-nitrophenyl phosphate, phenyl phosphate, and β-glycerophosphate; all followed essentially the same time curve pattern. Both β-glucuronidase and *n*-acetyl-B-*d*-glucosaminidase also followed essentially the same time curve pattern.

1.0-ml aliquots for each determination. Protein was determined by the method of Lowry *et al.* (14).

Results. Tissue slice methods. Time curves for the enzymes studied (Fig. 1) showed an initial lag phase that was most pronounced in the cathepsin assay. When the incubation time was continued for 24 hr (cathepsin, glucosaminidase, and glucuronidase), the activities plateaued. The following incubation times were chosen to give optimum color development for those enzymes studied in comparing compressed and normal joints: acid phenyl phosphatase, 2 hr; aryl sulfatase, glucuronidase, and glucosaminidase, 6 hr; and cathepsin, 15–18 hr.

Sites of enzyme action. To determine whether substrate hydrolysis occurred within the slices or as a consequence of enzyme liberation into the buffer, weighed slices of fresh-frozen bovine cartilage were incubated with 2 m*M* 4-nitrocatechol sulfate in 0.1 *M* acetate buffer (pH 5.8) for 4 hr. Comparable weight slices from the same specimen were incubated in the buffer solution with no substrate, and after 4 hr the cartilage slices were removed. Concentrated substrate was then added to make the buffer solution 2 m*M*, and incubated for another hour prior to assay. Only 30% as much substrate was hydrolyzed per milligram of tissue in the specimen incubated initially without substrate.

Effect of membrane disrupting agents on enzyme activity. To determine if enzyme activity in cartilage slices was susceptible to known membrane disrupting agents, comparable weights of cartilage slices from freshly sacrificed rabbits were incubated with 5.0 ml of buffered 1 m*M* *p*-nitrocatechol sulfate in 0.1 *M* acetate buffer (pH 5.8) under the following conditions: (a) in 0.25 *M* sucrose, (b) in buffer alone, (c) in 0.25 *M* sucrose after freezing and thawing the suspended cartilage slices three times in acteone and Dry Ice, and (d) in buffered sucrose made 0.1% with Triton X-100. Enzyme activities were determined on 2.0-ml duplicate aliquots of each reaction mixture after 4 hr incubation.

There was higher aryl sulfatase activity in all cartilage specimens subjected to membrane disrupting agents, compared to a specimen from the same animal suspended in 0.25 *M* sucrose solution. The relative activities expressed as a percentage of those slices incubated in 0.25 *M* sucrose (100%) were buffered substrate without sucrose—191%, cartilage frozen and thawed three times prior to incubation—150%, and buffered substrate made 0.1% with Triton X-100—520%.

Joint compression experiments. In Table I may be seen the results of the effects of joint compression on enzyme activity of cartilage and synovium. Cartilage slices used for assay ranged from 10–20 mg and the synovial specimens from 6–20 mg. The standard deviations for the synovial specimens were greater than those for cartilage, because sampling of synovium as a pure tissue is more difficult than cartilage and the wet weights would thus represent a less homogeneous cellular tissue than cartilage. Even though no abso-

TABLE I. Specific Activity of Enzymes Assayed in Normal and Abnormal Rabbit Joints.[a]

	Acid phosphatase [12]	Glucos-aminidase [11]	Cathepsin [14]	Glucuronidase [14]	Sulfatase [14]
Compressed cartilage	5.80 (1.85)	6.01 (3.51)	1.40 (0.75)	2.42 (2.24)	3.77 (1.88)
Control joint	5.47 (1.35)	5.51 (2.04)	1.52 (0.76)	1.93 (1.24)	2.95 (1.88)
Uncompressed cartilage	5.75 (1.08)	6.19 (2.34)	1.83 (1.25)	2.61 (2.33)	3.68 (1.39)
Control joint	6.37 (1.83)	5.71 (2.29)	2.23 (1.44)	2.05 (1.74)	2.80 (0.84)
Synovium compressed joint	9.75 (8.14)	10.98 (3.90)[b]	4.26 (3.71)	8.81 (5.85)[c]	9.52 (6.13)[b]
Synovium control joint	7.63 (5.81)	6.84 (1.83)	3.12 (2.21)	4.24 (2.44)	5.14 (1.92)

[a] Mean values of all enzyme determinations from day 1–21 postcompression of joint. Areas of cartilage in direct contact (compressed) and areas immobilized but not in direct contact with opposing articular surface (uncompressed) are compared against comparable areas from unimmobilized control joint. Values represent millimoles of substrate hydrolyzed per milligram of tissue. Figures in parentheses indicate standard deviations and the figures in brackets indicate the number of experiments for each assay. Probability values at bottom of table refer to differences in enzyme concentrations between control joints and compressed joints.

[b] $p < .01$.

[c] $p < .05$.

lute differences in enzyme activity could be determined in articular cartilage slices when all values were pooled from days 1 through 21, there were certain significant elevations in enzyme activity when the results were analyzed on the basis of time postcompression (Fig. 2). For example, the ratios of enzyme activity in cartilage between compressed and control specimens for aryl sulfatase in nine experiments carried out during the first 6 days ranged from 0.86 to 1.41, while the ratios in five experiments carried out between days 7 and 21 ranged from 1.18 to 1.75. The times of significant change in ratio of enzyme activity varied from day 3 to 6 following initiation of joint compression for each enzyme. Using this method of expression (Fig. 2), there were two enzymes that showed significant differences in activity between cartilage slices from the compressed joints when compared to comparable areas from control joints. Glucosaminidase showed an increase in ratio in areas not under compression during the last 16 days of treatment, while aryl sulfatase showed an increase in ratio in both compressed and uncompressed areas after 6 days of treatment.

Changes in enzyme activity in the synovium were more dramatic, and absolute changes were noted in the mean values in the enzyme activities of glucosaminidase, glucuronidase, and sulfatase in the synovium from immobilized joints when compared to control joints using all data from days 1 through 21 (Table I). In addition, when comparing the ratios of enzyme activity of synovium from the compressed joints with the control joints on the basis of the time

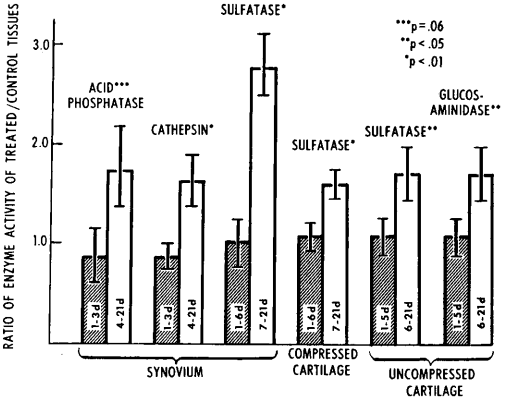

FIG. 2. Relative enzyme activity during 21 days of joint compression. Ratios of enzyme activity in immobilized vs. control joints based on time of increase in ratio. Only those enzymes showing significant differences are listed. Designations of tissue source and standard deviations are identical to Table I. Days included for division of data are shown in each column. Probability values refer to increase in activity after X-days of treatment.

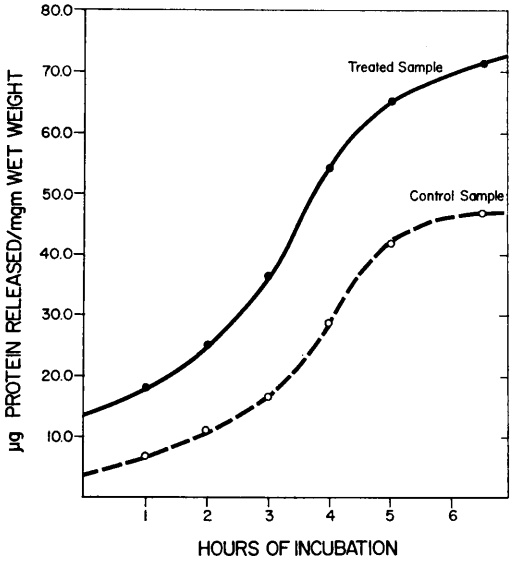

FIG. 3. Protein release from cartilage slices from immobilized and control joints. Note similarity of curves to cathepsin time curve in Fig. 1.

postcompression, cathepsin showed a significant elevation after day 3 and sulfatase showed a significant elevation after day 6 (Fig. 2). There was a trend ($p = .06$) for elevation of acid phosphatase after day 3.

Release of protein into the incubation medium was significantly greater by cartilage slices from compressed joints than from controls (Fig. 3). The differences in amounts released was greater with increasing time. Sulfatase activity was also found to be increased in the treated specimens when compared to the control (Fig. 4).

Discussion. When articular cartilage was subjected to homogenization and centrifugal fractionation according to deDuve (15), the variation in enzyme activity from animal to animal was remarkably large, even in normal tissue. Also, the distribution of enzyme activity in the subcellular fractions revealed a higher concentration in the nuclear and supernatant fractions than is normally found in soft tissues. These wide variations in enzyme activity and distribution in normal articular cartilage are felt to be the result of several factors: (a) Homogenization of articular cartilage requires a vigorous abrasive force to release chondrocytes from their protein-polysaccharide matrix and although uniform times for homogenization were used, it is unlikely that a constant number of cells were liberated each time; (b) The vigorous homogenization required may either solubilize or put into a colloidal state varying amounts of collagen which react in the Lowry method for protein determination; (c) The homogenization would also cause variable disruptions of cellular and subcellular membranes releasing different amounts of enzyme into solution, and Sucrose interferes with the Lowry procedure for protein determination (16) and in the initial work, the assays for protein were carried out in 0.25 *M* sucrose. Thus, it is apparent that when lysosomal enzyme activity of cartilage homogenates is expressed in the classical manner as activity per milligram of protein of each subcellular fraction there is considerable possibility for large variations and error. For the above reasons, the tissue slice method of enzyme assay was adopted and results were expressed in millimoles of substrate hydrolyzed per milligram of wet weight which was shown to be linearly related to DNA or cell number. Even though a linear relation between DNA and

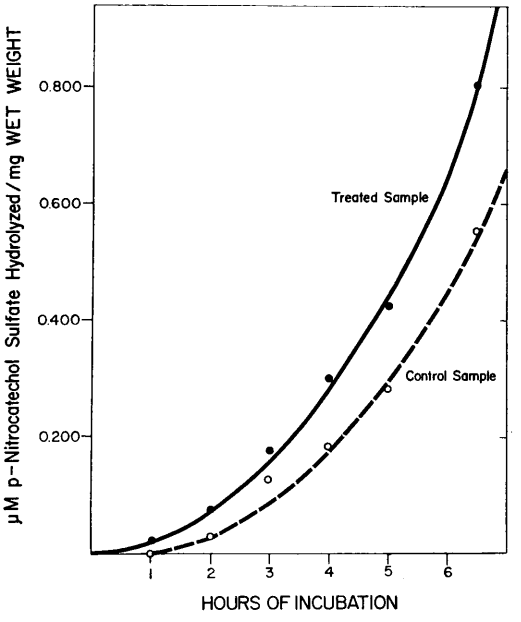

FIG. 4. Sulfatase activity from cartilage slices from immobilized control joints.

wet weight exists in normal cartilage and synovium, the possibility remains that comparisons of abnormal with normal cartilage slices may represent different degrees of cellularity.

The enzymes studied have been previously shown to be lysosomal enzymes (13); however, some of the activity measured using the tissue slice technique may represent acid hydrolases not "bound" in lysosomes, since it is necessary to isolate a specific sedimentable fraction in order to fulfill the definition of a lysosmal enzyme (15). The striking increase in sulfatase activity following treatment with membrane disrupting agents suggests that this enzyme probably is of lysosomal origin. However, it is possible that the increase in activity was derived from other subcellular particles or from cytoplasmic enzyme since these membrane disrupting agents would very likely break the cell membrane as well as lysosomal membranes.

The relative lack of enzyme activity when cartilage was preincubated in buffer solution without substrate suggests that a significant amount of substrate hydrolysis occurs within the tissue substance rather than as a result of enzyme being released from the tissue into the incubation medium. The initial lag phase, seen in all assays, is interpreted as the time required for the substrate to penetrate the tissue. If this assumption is correct, it is not surprising that the cathepsin assay showed the lowest activity and the longest lag phase, when one considers the size of the substrate molecule (hemoglobin). It is very likely that hydrolysis of hemoglobin occurred after enzyme was released from the tissue into the buffer since hemoglobin is probably too large to penetrate into the cells, and if the figures for sulfatase activity are transposed to cathepsin, only 30% of the enzyme present is released into the solution from cartilage slices.

No detectable differences in enzyme activity were present in the compressed cartilage compared to a control specimen early in the experiment (1–3 days). Sulfatase was the only enzyme which increased significantly in areas of direct compression as well as in areas not under compression. Glucosaminidase was the only other enzyme which showed an increase in activity but only after the second week of treatment and only in areas of cartilage not under compression. The presence of increased activity in areas of cartilage not under direct compression has certain possible explanations. The amount of tissue available for assay in areas not under compression is large compared to those areas under direct compression, and the assay techniques may not have been sensitive enough to pick up differences in activity in the smaller samples. In addition, it is well known that these areas eventually degenerate (8, 9).

The rise in all enzyme activities in the synovium was prominent between 3 and 6 days and is consistent with the proliferative synovitis, leukocyte, and round cell infiltration seen on histologic examination.

Although there was an elevation of certain enzyme activities in cartilage from joints subjected to compression, it is unlikely that this represents an increase in the amount of enzyme in the sample, since as much as a 5-fold increase in activity was demonstrated in cartilage subjected to membrane disrupting agents, such as Triton X-100. The increase in enzyme activity might reflect an increase in the rate of penetration of substrate into or in the rate of diffusion of the reaction products from cells of degenerating tissue. Regardless of the mechanism, certain enzyme activities in damaged cartilage are greater than those in normal cartilage.

It is difficult to establish from this study whether synovitis or cartilage degeneration occurred first. Although increased enzyme activity did occur earlier in the synovium, *i.e.* around day 3, the possibility cannot be ruled out that some breakdown of cartilage might have occurred at the same time or even earlier with the liberation of chondrotin sulfate which has been shown to cause synovitis (5).

Summary. Five acid hydrolases, *i.e.*, glucosaminidase, sulfatase, phosphatase, cathepsin, and glucuronidase were identified in normal articular cartilage and synovium of rabbit knee joints by incubating tissue slices with appropriate substrates. These enzymes were also assayed in cartilage and synovium of rabbit knee joints which had been subject-

ed to continuous compression from 1 to 21 days. Significant elevation of enzyme activity occurred in the first two enzymes in the cartilage and in the first four enzymes from the synovium of the treated joints. Studies with tissue slices preincubated with and without substrate suggest that most of the hydrolysis of substrate occurs within the tissue, rather than as a result of enzyme liberation into the incubation medium.

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ERRATUM

Vol. **132**, No. 3 (1969): note that the article, "Phosphoprotein Synthesis in Epidermis During Acantholysis," by Richard H. Decker and Nancy J. McMahon appears on pages 1178-1182. This was incorrectly printed in the Table of Contents and Author Index as page 1128.