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Digestion of Articular Cartilage by Papin *in vitro*.* (31905)

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Crude papain and its purified, crystalline protease have been shown to have marked chondrolytic properties following systemic(1, 2), intraosseous(3) and intra-articular injection(4) of various species of laboratory animals. Reported studies of the effect of papain on cartilage *in vitro* have been essentially qualitative and somewhat contradictory, due possibly to variations in the preparations of papain that were used. Allison *et al*(5) stated that the ear cartilage of an adult rabbit became soft and lost its metochromatic staining properties following its immersion in a solution of crude papain at 37°C for 22 hours. Slack(6) found that the digestion of rat costal cartilage by purified papain for 48 hours resulted in the release of most of the polysaccharides into the supernatant solution. Curtiss and Klein observed that 75% of the hexosamine of bovine articular cartilage was lost when cartilage slices were incubated in a solution of an unspecified type of papain at 37° for 10 days, but only minimal dissolution of cartilage was observed(7).

It has been recently found that multiple intra-articular injections of papain protease can produce bony ankylosis of a joint(4). This led to the present quantitative investigation of the digestion of articular cartilage by papain *in vitro* by measuring the rate of appearance of various degradation products of cartilage during its disintegration in a solution of papain.

Materials and methods. Articular cartilage was excised with a scalpel from the upper tibia and lower femur of a 2-year-old steer about 5 hours after it had been slaughtered. The cartilage slices were immediately diced into fragments about 2 mm on edge, weighed out in 200 mg portions and stored at —10°C in tightly capped vials.

Papain protease, twice crystallized, was

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purchased from Worthington Biochemical Corp. Enzyme assay was performed using I^{131}-labeled albumin(8) to establish that the enzyme was at maximum activity prior to use. For kinetic study, 1.6 g of the diced articular cartilage were immersed in 40 ml of a solution of papain, 1 mg/ml, in M/15 phosphate buffer, pH 7.2, containing .001 M ethylene-diaminetetraacetate (versene), and activated with .03 M cysteine hydrochloride. The solutions were warmed at 37°C before mixing the enzyme with buffer and activators. The flask containing the cartilage and enzyme was shaken mechanically in a water-bath at 37°C until completion of the experiment. Five ml of solution were removed from the reaction mixture immediately after mixing and at 30, 60, 90, and 120 minutes when the digestion was terminated by filtering the solution through a porcelain crucible. The residual cartilage was dried overnight at 95°C and weighed. Nitrogen determination by the micro-Kjeldahl technic was performed on a portion of this cartilage and on the various samples of solution. The remainder of the cartilage and each sample of solution were hydrolyzed in 4 N HCl at 100°C for 8 hours. Determination of hydroxyproline content by the method of Miyada and Tappel(9) and of hexosamine by Schloss' technic(10) was performed on the hydrolyzed samples of cartilage and filtrate.

A modification of the experiment to permit serial gravimetric and chemical analysis of the residual cartilage at each half-hour interval was also performed using 200 mg cartilage samples in 5 ml of the enzyme solution, the same ratio employed in the primary experiment. A batch of tubes containing cartilage and enzyme was placed in the water bath at the start of the run and the reaction halted in 2 of the tubes at 30, 60, 90, and 120 minutes. Analysis of the supernatant solution and cartilage was done as in the initial part of the study.

Results. Base values of cartilage. Approximately 7 g of articular cartilage were obtained from the knee joint of each of 2 steers. The results of the measurements of nitrogen, hydroxyproline, hexosamine, ash, and water of untreated cartilage are shown in Table I. From these direct determinations, the values of collagen, chondroitin sulfate and non-collagenous protein were calculated using these factors: collagen was derived by multiplying the hydroxyproline value by 7.15(11). For chondroitin sulfate, the value of hexosamine was multiplied by 3.1, a factor based upon the structural formula of chondroitin sulfate. Non-collagen protein (NCP) was derived from the formula: NCP = 6.25 [total nitrogen — (collagen nitrogen + hexosamine nitrogen)], with collagen nitrogen assumed to be 18.68% of collagen(11). No previously published analysis of these constituents in bovine articular cartilage was found, but comparison with the results of Anderson *et al* for human articular cartilage (12) and Eichelberger *et al*(13) who studied articular cartilage of puppies, shows markedly similar findings in the 3 species except for the considerably higher water content of bovine articular cartilage. The organic components comprised 87.9% of the dry cartilage. Ash was found to measure 4.2%. Retained water may account for the remaining 7.9%.

TABLE I. Composition of Bovine Articular Cartilage.

Constituent	% of dry weight
Nitrogen	13.3 ± .8 (31)
Hydroxyproline	7.6 ± .7 (30)
Hexosamine	5.3 ± 1.0 (23)
Ash	4.2 ± .2 (6)
Collagen	54.0
Chondroitin sulfate	16.4
Non-collagen protein	17.5
% water in fresh cartilage	85.3 ± 3.6 (30)

The value of ± 1 standard deviation is shown where a direct determination was made of the constituent. Numbers in parentheses indicate number of determinations performed. Values of collagen, chondroitin sulfate and non-collagen protein were derived by formulas cited in text.

Papain digestion of cartilage. The amount of each measured component of cartilage found in the filtrate at each 30-minute interval is shown in Fig. 1 as a percentage of the measured total amount of that constituent in the residual cartilage and filtrate. The most striking finding is the speed and completeness of chondroitin sulfate release as shown by the hexosamine curve. The breakdown of non-collagen protein follows closely behind the

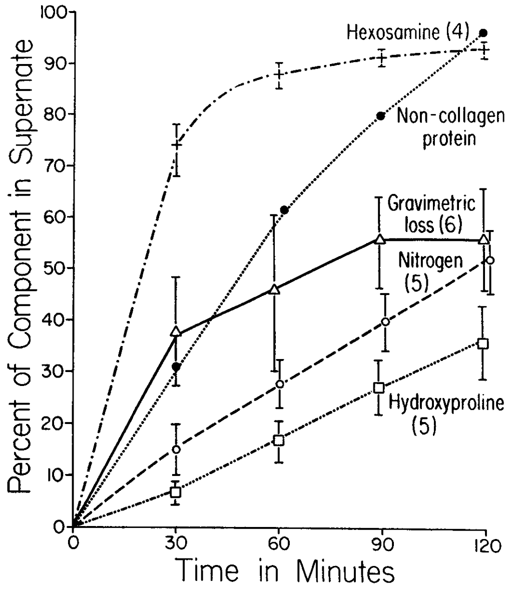

Fig. 1. Amount of hexosamine, nitrogen and hydroxyproline present in the supernatant solution at each time interval is shown as a percentage of the total of that constituent measured in filtrate and cartilage. Gravimetric loss at each time interval as percent of dry cartilage is also shown. Vertical lines represent range of 1 standard deviation from the mean. Figures in parentheses indicate number of determinations performed. Percent of non-collagen protein present in the supernate was derived from the experimental data using formulas cited in text.

chondroitin sulfate appearance. The hydroxyproline curve indicates an initial lag in collagen breakdown followed by an accelerated rate after 30 minutes. Collagenolysis is still proceeding relatively briskly at the end of 2 hours although the overall rate of cartilage breakdown has slowed markedly. Dissolution of cartilage in the control tubes was slight, but measurable. Mean gravimetric loss at 2 hours was 8% in control tubes containing buffer, cysteine and versene but only 4% in phosphate buffer alone.

In Table II the expected cartilage weight loss on the basis of the total amount of the organic constituents found in the supernatant is compared with the average measured weight loss at each half-hour interval. The difference between the calculated and actual weight loss may be accounted for by the presence in cartilage of inorganic salts and retained water. These substances appear to go into solution during the first 30 minutes. We have no explanation for the progressively smaller difference between expected and measured gravimetric loss of the later time intervals.

Discussion. For most of the measurements made during the kinetic study the size of one standard deviation is quite large, but this is not surprising in a 2-phase system where the volume and surface area of the cartilage fragments varied considerably. Nevertheless, these results show clearly that purified papain rapidly disintegrates bovine articular cartilage *in vitro* at a physiological temperature and pH. It appears that the initial effect of papain is to split off chondroitin sulfate from its complex with the non-collagenous matrix protein. This finding is consistent with that of Muir who studied the effect of papain on chondromucoprotein extracted from pig laryngeal cartilage(14). She found that solutions of this complex underwent an 80% fall in viscosity 10 minutes after the addition of minute quantities of crystalline papain (1 μg/ml) at a pH of 5.5. A comparable effect could be achieved only by using 1 N NaOH or pepsin at pH 1.9. The fall in viscosity was associated with separation of the chondroitin sulfate from the protein moiety.

The sequence in the appearance of organic constituents of cartilage in our study is consistent with the structural model of the chondromucoprotein complex postulated by Partridge *et al*(15) who described this substance as a central core of non-collagen protein with approximately 20 to 30 chains of chondroitin sulfate attached to the periphery of the pro-

TABLE II. Amount of Major Organic Constituents Separated from Cartilage as Determined from Degradation Products in Supernatant Solution at Various Intervals During Papain Digestion of Articular Cartilage. The figures represent each constituent as the percentage of total cartilage. The sum of these percentages is compared with the percentage of weight lost as determined by direct weighing of the dried residual cartilage at each interval.

	Reaction time (min)			
Constituent	30	60	90	120
Collagen	3.8	9.2	14.6	19.4
Chondroitin sulfate	12.1	14.4	14.9	15.1
Non-collagen protein	6.3	10.0	13.9	16.9
% of total cartilage in supernatant	22.2	33.6	43.4	51.4
% of gravimetric loss of residual cartilage	38.0	46.0	56.0	56.0

tein. The relationship of this complex to the collagen fiber is still to be elucidated.

Collagen breakdown occurs during the degradation of articular cartilage by papain although native collagen is not susceptible to digestion by papain at the temperature and pH employed in this experiment(16). Denatured collagen, however, is readily digested by papain. The fact that hydroxyproline appears in the filtrate only after chondromucoprotein breakdown is much advanced, suggests that denaturation of collagen occurs as chondromucoprotein is lost from the cartilage. It is also possible that collagen breakdown is catalyzed in some way by the products of cartilage breakdown.

Summary. The digestion *in vitro* of diced fragments of bovine cartilage by a 0.1% solution of papain was investigated over a 2-hour period. At half-hour intervals, measurements were made of the gravimetric loss of cartilage, and of the amount of cartilage nitrogen, hexosamine, and hydroxyproline present in the supernatant solution. From these determinations, the quantities of chondroitin sulfate, collagen and non-collagen protein released from the cartilage were calculated and their total compared with the weight loss of cartilage. During the first hour, the cartilage lost 88% of the chondroitin sulfate and 63% of the non-collagen protein. After 2 hours, more than 90% of each of these components were found in the supernatant solution. Collagen breakdown proceeded at the rate of about 9% per half-hour. Gravimetric loss measured 56% of the dry weight of the cartilage at the end of 2 hours.

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Iron Reversal of Serum Inhibited Respiration by *Bacillus subtilis*.* (31906)

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Bactericidal effects of serum against *Bacillus subtilis* have been studied manometrically during growth of the microorganism in a nutrient broth(1). In this growth system it was found that serum antirespiratory activity was proportional to serum bactericidal activity, that the antirespiratory agent was a serum globulin, and that ferric iron in excess of the amount needed to saturate transferrin re-

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