Correlated Metabolism of Proteoglycans and Hyaluronic Acid in Bovine Cartilage Organ Cultures*

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Proteoglycans exist in cartilage as complexes in which many proteoglycan molecules are bound to a central filament of hyaluronic acid. Many studies have investigated changes taking place in proteoglycan monomer structure during cartilage catabolism usually under the assumption that hyaluronic acid is a relatively inert metabolic component of the complex. In this paper we present organ culture data supporting a new hypothesis that the catabolism of proteoglycans and hyaluronic acid are coordinate regulated by chondrocytes. The data indicates that: 1) newly synthesized hyaluronate and proteoglycan maintain a nearly constant ratio, almost identical to that existing for the total chemical amounts of these two components in cartilage tissue; 2) these two components are catabolized with virtually identical kinetics; and 3) this catabolic relationship in vitro reflects the loss of hyaluronate and proteoglycans from native, undissociated aggregates as isolated from the tissue. We conclude that hyaluronate catabolism is an integral part of the overall mechanism of proteoglycan resorption in cartilage and that further understanding of this process may be key to the elucidation of the regulatory pathways for proteoglycan resorption in health and disease.

Osteoarthritis is a major crippling disease that causes profound alterations in the integrity and biomechanical function of cartilage and bone. The precise biochemical events involved in the pathogenesis of this disease are not clearly understood, but it is generally agreed that an important early event in the disease is a loss of proteoglycans from the cartilage matrix (1). Maintenance of the appropriate structure and concentration of the proteoglycans is critical for healthy cartilage to resist compressive loads and shield the underlying bone (2). Thus, impairment of mechanisms controlling these processes would be expected to lead to loss of function and disease. A great deal of interest has focused on understanding mechanisms of proteoglycan catabolism. Proteoglycans exist in the cartilage matrix primarily as aggregates in which many proteoglycan monomers are attached to long strands of hyaluronic acid through a specialized binding region near the amino-terminal portion of the proteoglycan core protein, resulting in gigantic complexes with molecular weights on the order of 50 million. The aggregate structure is stabilized by a link protein which effectively "locks" the proteoglycan to the hyaluronic acid backbone (3, 4).

The pioneering studies of Fell and Jubb (5) showed that proteoglycan resorption in cartilage is directly under the control of resident chondrocytes. Sandy et al. (6) subsequently showed that proteoglycans released from cultures of rabbit articular cartilage during the catabolic process had large hydrodynamic sizes but were unable to bind to hyaluronic acid. They proposed that the mechanism of release involved a hydrolytic cleavage above the hyaluronic acid binding region of the proteoglycan core protein. Later studies by other groups have been consistent with this hypothesis (7–9), although the issue has not been definitely settled. An alternate catabolic hypothesis is based on the finding that partially degraded link proteins accumulate in cartilage matrix with increasing age (10) and states that proteolytic cleavage of the link protein inactivates its ability to stabilize the proteoglycan-hyaluronate complex. Catabolism of the third component of the complex, hyaluronate, has not been investigated even though its size and integrity clearly contribute to the overall physical properties of the aggregate complex (11). In this paper we show that hyaluronate is a metabolically active component of the complex and is, as has often been assumed, merely a stable anchor upon which proteoglycans are replaced during the normal course of proteoglycan catabolism.

MATERIALS AND METHODS

Buffers and Experimental Conditions

The following buffers were used: 1) Tris buffer (0.001 M Tris-HCl, 2 mM calcium chloride, pH 7.2); 2) papain digestion buffer (0.05 M sodium acetate, 5 mM EDTA, 0.5 mM dithiothreitol, pH 6.0); 3) 4 M guanidine HCl buffer (4 M guanidine HCl, 0.05 M sodium acetate, pH 5.8); 4) collagenase buffer (0.025 M Tris-HCl, 0.01 M CaCl₂, 0.15 M NaCl, pH 7.4, containing the following protease inhibitor mixture: pepstatin (1 × 10⁻⁴ M), leupeptin (1 × 10⁻⁴ M), N-ethylmaleimide (0.005 M), and phenylmethanesulfonyl fluoride (0.001 M)); 5) 0.5 M guanidine HCl buffer (0.5 M guanidine HCl, 0.05 M sodium acetate, 0.5% CHAPS, pH 5.5, containing protease inhibitors); 6) Tris borate buffer (0.5 M Tris-HCl, 0.1 M boric acid, 0.0036 M sulfuric acid, pH 8.6, in 52% acetonitrile and 12% methanol).

Papain digestions were carried out for 6 h at 60 °C; enzyme concentration was 0.5 mg/ml and the reaction was terminated by addition of icodacetamide to 5 mM. Streptomyces hyaluronidase digestions were done for 5 h at 60 °C and chondroitin ABC lyase digestions for 5 h at 37 °C.

Hydroxyproline determinations were carried by the method of Woessner (12). For all the experiments described below, samples were stored at −20 °C until all the culturing and extraction procedures were carried out. The age of the animals used for the experiments described below ranged between 3 and 8 months (slaughterhouse estimate).

The Abbreviations used are: CHAPS, 3-[(3-cholamidopropyldimethylammonio)-1-propanesulfonic acid; HPLC, high pressure liquid chromatography; DMEM, Dulbecco's modified Eagle's medium.

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**Measurement of the Biosynthesis and Chemical Amounts of Glocosaminoglycans in Total Tissue Fractions**

The Cultures and Extractions—Articular cartilage slices were prepared from the patellar-tibial condyles of two calves as described previously (7). Approximately 1 g of tissue (weight) was placed in each of three batches which were cultured in 15 ml of Dulbecco’s modified Eagle’s medium with high glucose (DMEM) and 20% fetal calf serum (maintenance medium). Medium was changed daily and on days 8, 18, and 23, one batch was radiolabeled by incubation at 37 °C for 16 h in maintenance medium containing 60 μCi/ml [3H]glucosamine and 15 μCi/ml [35S]sulfate. The medium was then removed and the tissues extracted at 4°C in 15 ml of the 4 M guanidine HCl buffer for 24 h. Tissue samples were washed twice at 4 °C with 2 ml of Tris buffer to remove residual guanidine HCl. The washed tissue was incubated at 37 °C in 5 ml of Tris buffer containing 6600 units of type III collagenase (Advance Biofactures, New York). After digestion for 48 h, the tissues were dispersed by homogenization in a glass homogenizer. A remaining residue was incubated in 1 ml of Tris buffer with 850 units of collagenase overnight. This second digest was homogenized and pooled with the first. This procedure essentially dispersed all of the tissue; a small residue contained less than 5% of the total hydroxyproline in the tissue and was not analyzed further.

**Biochemical Analysis of Tissue Extracts**—Chromatography on a Bio-Gel PD-10 column (3 ml) equilibrated in the same buffer, washed with 5 column volumes of buffer, and then eluted with a linear NaCl gradient (0-1.5 M) prepared in the same buffer. The yield of both radiocryptates in the combined breakthrough and gradient fractions was >90%.

Fractions were pooled as indicated by the bars in Fig. 1, A and B, and the peaks analyzed by selective enzyme digestions. Each pool was dried, resuspended in 500 μl of 0.1 M ammonium bicarbonate buffer, pH 8.5, and eluted on a PD-10 column of Sephadex G-25 in the same buffer to remove salts. Fractions eluting in the V_0 were combined, portioned into various tubes for enzyme digestion, and dried. Recoveries were >90%. A set of tubes was analyzed by digestion with Streptomyces hyaluronidase as follows: 1) control sample contained 100 μl of 0.05 M sodium acetate buffer, pH 5; 2) a digestion sample contained 100 μl of this buffer and 10 units of Streptomyces hyaluronidase, and 3) a digestion sample contained the same additions as sample 2 plus 50 μg of a hyaluronic standard. The latter sample determined the elution position of authentic hyaluronic acid digestion products in the analytical Bio-Gel-P-10 column (see below) and ensured that enzyme activity was not rate-limiting. The peaks eluting from DEAE-5 ml of NaCl were also digested with chondroitin ABC lyase as follows: 1) a control sample contained 30 μl of 0.1 M Tris fluoride, pH 8; and 2) a digestion sample contained 30 μl of the same buffer plus 0.4 unit of chondroitin ABC lyase. Samples were chromatographed on a Bio-Gel-P-10 column (0.7 × 100 cm) equilibrated in 0.1 M ammonium bicarbonate, pH 8.5, and eluted at a flow rate of approximately 2 ml/h. Yields were >80%. Fractions containing the oligosaccharide digestion products were pooled as indicated by the bar in Fig. 2B, dried, resuspended in 0.25-0.5 ml of distilled water, and analyzed for hexuronic content by the method of Bitter and Muir (13). The Bio-Gel-P-10 V_0 fractions for the untreated 0.8 M peak (peak 2, 95% of the chondroitin) were pooled, concentrated, and analyzed for hexuronic content (13).

The validity of the methodology described above for the quantitation of hyaluronate and chondroitin sulfate was tested by an independent method of analysis for samples from the collagenase extract from the day 18 culture sample. The peaks of radioactivity eluting at 0.35 and 0.8 M NaCl from DEAE-Sephacel were separately pooled (see Fig. 1B, bars). Bovine serum albumin (0.5 mg/ml) was added to each as carrier, and the samples were precipitated by adding ethanol (1:10 v/v). After 1 h at room temperature, precipitates were collected by centrifugation at 10,000 g for 30 min at 4°C and dissolved in 40 μl of 0.1 M Tris fluoride, pH 8. Incubation of the samples with papain dispersed insoluble material. After inactivating the papain, 5 μl containing 0.4 unit of chondroitin ABC lyase in the same buffer were added to each sample. After digestion, samples were spotted on cellulose sheets, and thin layer chromatography was done as previously described (14). A mixture of standard chondroitin sulfate and hyaluronic acid disaccharides was spotted on each sheet and visualized by ultraviolet light after chromatography. Sample lanes were divided and cut into 5-mm horizontal strips. The cellulose was scraped off each strip with a razor blade and maintained at 0°C with 1 ml of distilled water followed by gentle swirling for 1 h. Ten ml of Hydro-fluor scintillation solvent were added directly to the vials, and the H and SS radioactivities were determined. Recoveries were between 55-90%.

**Catabolism of Glososaminoglycans in the Total Tissue**

Pooled cartilage slices from two young calves were cultured in maintenance medium in batches for 6 days. One batch (approximately 1 g) was then radiolabeled by incubation for 20 h with 60 μCi/ml [3H]glucosamine and 15 μCi/ml [35S]sulfate. The tissue was washed twice at 37 °C with 15 ml of DMEM to help remove unincorporated isotope and a portion of the tissue (approximately 100-150 mg wet weight) stored for later analysis (the experimental day 0 sample). Duplicate culture vials containing approximately 150-200 mg wet weight of the radiolabeled tissue were set up and maintained in 3 ml of maintenance medium with daily changes. After 30 days of culture, the tissue was extracted with 4°C with 20% glacial acetic acid (0.5 ml) and was then incubated with collagenase as described above. After digestion, the clear collagen extract was removed without homogenizing the tissue. The residual tissue was then digested with papain in 1.5 ml of buffer. The modified extraction protocol was used to determine whether collagenase digestion alone was sufficient to solubilize the hyalurionate and proteoglycan remaining in the tissue after guanidine HCl extraction. All extracts were analyzed by DEAE chromatography essentially as described above. In the case of the papain extracts, aliquots were removed for hydroxyproline analysis, the samples centrifuged, and the supernatants directly applied to DEAE-Sephacel. All the extracts from the day 0 and one of the chase samples were analyzed by Streptomyces hyaluronidase digestion and Bio-Gel P-10 chromatography.

**Catabolism of Glososaminoglycans in the Proteoglycan Aggregate Fraction**

**Tissue Culture and Development of Extraction Procedures**—Tissue slices were cultured in batches as described above. On day 6 (experimental day 0), approximately 1 g (weight) of tissue was radiolabeled by incubation for 20 h with 60 μCi/ml [3H]glucosamine and 15 μCi/ml [35S]sulfate. After washing and further incubation for 2 days to remove unincorporated isotope, two portions of the tissue (approximately 150-200 mg wet weight each) were digested with Streptomyces hyaluronidase. Hank’s balanced salt solution was added to each. Both samples were frozen by immersion in a Dry Ice-ethanol bath and stored (experimental day 2 sample). Another portion of the radiolabeled tissue (150-200 mg wet weight) was placed in a culture vial and maintained for another 18 days in 3 ml of maintenance medium with daily changes. A procedure was developed for the extraction of intact aggregates from the cartilage. A day 2 sample was thawed, portioned into four samples, and each weighed. One was hydrolyzed in 6 N HCl to determine hydroxyproline content so that recoveries could be calculated on the basis of this index. The others were kept moist by adding collagenase buffer. Inhibitors were included in the collagenase buffers to inhibit the action of proteinases of the acidic, cysteine, and serine families. A portion of the tissue was sliced as thinly as possible with a razor blade and maintained at 4°C in 0.5 ml of collagenase buffer (15). The other two portions were rapidly frozen in a small droplet of distilled water and sectioned into 30-μm slices in a cryostat (16). Frozen slices were recovered from the cold cryostat blade rapidly enough to prevent thawing of the tissue and transferred with forceps into a vial containing 0.5 ml of cold collagenase buffer. The manually sliced tissue and one of the cryostat-sectioned tissues were incubated at 37 °C for 5 h after addition of 0.5 ml of collagenase buffer containing 1000 units of the enzyme. The second tissue portion containing 30-μm slices was incubated under the same conditions without addition of the enzyme. Following incubation, guanidine HCl and o-phenanthroline (to block metalloproteinase (collagenase) activity) were added to each sample to a final concentration of 0.5 M and 1 mM, respectively (associative extraction buffer). The tissues were then gently swirled at 4°C. The following day the buffer was removed (associative extract) and the tissues suspended in 1 ml of collagenase
buffer containing 1 mM o-phenanthroline. The tissues were gently homogenized, centrifuged, and the pellets extracted by swiveling in 0.5 M NaOH at 4 °C overnight.

The extractability of 35S-labeled material was assessed. The manually sliced material was not effectively dispersed by homogenization following collagenase treatment, and the combined associated extract and homogenate yielded only 45% of the 35S-labeled material. The dissociative extracts from the collagenase-treated and untreated 30-μm slices contained 69 and 31% of the 35S label, respectively. Gentle homogenization of the residues extracted a further 17 and 46%, respectively. The total extraction was 65% for the collagenase-treated sample and 77% for the untreated sample. Separate experiments showed that the collagenase preparations did not degrade proteoglycan aggregate (aAl from the rat Swarm chondrosarcoma (17) generously supplied by Dr. J. Kimura, Rush Presbyterian St. Luke’s Medical Center, Chicago) under experimental conditions comparable to those used here for treatment of the tissues, as determined by velocity sedimentation profiles in cesium sulfate gradients before and after exposure to the enzyme (data not shown).

**Tissue Fractionation and Disaccharide Analysis**—The remaining day 2 and 18 tissue samples were cryostat-sectioned and digested with collagenase. The collagenase-digested tissues were directly homogenized in the associative extraction buffer and the samples centrifuged. The supernatants (2–4 mg of proteoglycan in 1.5 ml) were layered on preformed cesium sulfate density gradients (0.15–0.5 M) and centrifuged at 20,000 rpm for 6 h as described before (18). The buffers used in the detergent contained the inhibitor mix described above including o-phenanthroline. Following centrifugation the gradients were fractionated by carefully introducing fine tubing to the bottom of the tube and pumping cut at a flow rate of approximately 0.6 ml/min. The bottom fractions (Fig. 5, A and C) cosedimenting with a separate aggregate preparation from the rat chondrosarcoma were pooled and dialyzed against 0.5 M guanidine HCl buffer.

Diafiltered samples from the velocity gradients (Fig. 5) were then subjected to equilibrium sedimentation in CsCl gradients (38,000 rpm, 48 h, 10 °C). The bottom fractions of these gradients were pooled as indicated in Fig. 6, B and D, and dialyzed against 40 volumes of papain buffer containing 300 mM NaCl, 0.1% Triton X-100, and 0.5% CHAPS. Recoveries from the velocity and equilibrium centrifugations were each >90%. The samples were digested with papain, precipitated with 4 volumes of ethanol, and centrifuged. The pellets were resuspended in 50 μl of 0.1 M Tris fluoride, pH 8, containing 0.4 unit of chondroitin ABC lyase and digested. The reaction was terminated by addition of 4 volumes of ethanol and overnight storage at −20 °C. Small precipitates were removed by centrifugation in a Microfuge. The supernatants were concentrated by speed vacuum centrifugation and applied to a Partisil 5 F PAC column equilibrated in Tris borate buffer (19). The column was eluted at 1 ml/min, and 0.5-ml fractions were collected. Chromatographic recoveries were between 50–60%. One ml of 35S ethanol containing 0.5% Triton X-100 was added to each fraction and then mixed with 10 ml of Hydrofluor prior to scintillation counting.

**Analysis of Medium Fractions**—The medium collected during the 18 days of culturing was pooled, diluted 1.5-fold with 0.05 M sodium acetate, pH 6, and applied on a 15-ml column of DEAE-Sephacel equilibrated in the same buffer. The loaded column was washed with 30 ml of buffer and then eluted with 45 ml of the same buffer containing 1.5 M NaCl. CHAPS (0.5%) was added to the 1.5 M eluant which was then dialyzed first against 10 volumes of distilled water containing the detergent and then against papain buffer containing 0.5% CHAPS. The sample was digested with papain, concentrated by lyophilization, and dialyzed against 0.1 M ammonium bicarbonate, pH 8.5. A portion was dried, resuspended in 200 μl of 0.1 M Tris fluoride, pH 8, and 1 unit of chondroitin ABC lyase added. After incubation, the sample was treated and analyzed on Partisil 5 F PAC as described above. The fraction that did not bind to the DEAE-Sephacel was dried by lyophilization, resuspended in 5 ml of distilled water, and precipitated with 4 volumes of ethanol. The ethanol supernatants was dried, resuspended in 1 ml of 0.1 M ammonium bicarbonate, pH 8.5, and one-half chromatographed on a Bio-Gel P-10 column equilibrated in the same buffer. All the dialysis steps described above were carried out using Spectra/Por 3500 tubing (nominal cut-off for peptide material = 3500 daltons) in order to prevent loss of intermediate to low molecular weight hyaluronate.

**RESULTS**

**Quantitation of Glycosaminoglycans**—In previous publications it was reported that bovine articular cartilage can be cultured under conditions that maintain a steady-state metabolism of constituent proteoglycans (7, 8). The work described in this paper focused on: 1) whether hyaluronate metabolism in cultured bovine articular cartilage also meets steady-state criteria in terms of both biosynthesis and maintenance of tissue concentrations over a period of 3–4 weeks; and 2) whether hyaluronate has a similar extractability as proteoglycans. Previous work showed that these cultures synthesize proteoglycans (7) and hyaluronate3 with linear kinetics over a 24-h period after changing the medium under the culture conditions used in this work.

Bovine articular cartilage was radiolabeled with [3H]glucosamine and [35S]sulfate after 8, 13, and 23 days of culture. Guanidine HCl and collagenase extracts of the tissues which contained >95% of the incorporated 35S label, were treated with papain to release glycosaminoglycans. Fig. 1, A and B, show DEAE elution profiles of these two extracts from the day 8 samples; the chromatographic profiles for days 18 and 23 showed the same peaks (data not shown). The guanidine HCl extract contained two resolvable components eluting at 0.12 M NaCl (unsulfated) and 0.8 M (sulfated). Three labeled peaks were separated in the gradients of collagenase extracts; a sulfated peak eluting with 0.18 M NaCl, an unsulfated peak eluting with a salt concentration of approximately 0.35 M, and a sulfated peak in the region of 0.8 M.

The pooled peaks as shown by the *bars* in Fig. 1 were isolated from extracts of day 8 and 23 samples for digestion with *Streptomyces* hyaluronidase, which has absolute specificity for hyaluronic acid and produces a mixture of hexa- and tetrasaccharides in limit digests (20). Fig. 2 shows the Bio-Gel P-10 elution profiles for the day 8 collagenase, 0.35 M peak eluted before (A) or after (B) enzyme treatment. The

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3 R. M. Mason and C. J. Handley, unpublished observations.
Metabolism of Hyaluronic Acid in Cartilage

Amount of proteoglycan synthesis) determined from the same fractions. The amount of biosynthesis of predominantly chondroitin sulfate by their susceptibility to treatment with the enzyme resulted in a 50% shift of the biosynthesis of sulfated glycosaminoglycans (CS) as measured by "H and "S uptake into these macromolecular components (see "Materials and Methods") over the period of 23 days of culture. B shows the amounts of hyaluronate (HA) in the same cultures as analyzed by "H uptake; the values for days 8 and 23 were directly calculated from Bio-Gel P-10 analysis of Streptomyces hyaluronidase-treated pool I from DEAE chromatography, while the value for day 18 was calculated from the value of pool I by assuming that it contained the same proportion of hyaluronate as days 8 and 23. The analysis of the day 18 pool I by thin layer chromatography (Fig. 2C) showed this calculated value to be correct within a 10% margin of error. C shows the chemical amounts of hyaluronate and sulfated glycosaminoglycans present in the tissues on days 8 and 23 by direct hexuronate analysis of hyaluronate oligosaccharides (Fig. 2B; see bar) and DEAE-isolated sulfated glycosaminoglycans (see "Materials and Methods"). The open bars represent the guanidine HCl extracts, and the cross-hatched bars represent the collagenous extracts.

FIG. 2. Analysis of pools I and II from DEAE-chromatography. A and B represent the analysis of pool I (Fig. 1) by Streptomyces hyaluronidase digestion and Bio-Gel P-10 elution of degradation products from day 8 samples. D shows the analysis of pool II (Fig. 1) by Streptomyces hyaluronidase digestion, and E shows the analysis of the same pool by chondroitin ABC lyase digestion; both panels represent Bio-Gel P-10 analysis of degradation products for day 8 samples. C and F represent analyses of pools I and II, respectively, by chondroitin ABC lyase digestion followed by separation and quantitation of disaccharides by thin layer chromatography for day 18 samples. The arrows represent the migration positions of standard disaccharides.

undigested material eluted in the V5 of the column, while treatment with the enzyme resulted in a 50% shift of the tritiated material to two included peaks. Chromatography of a digest of a separate sample which contained both tritiated material and a hyaluronate standard showed that the two tritiated peaks coeluted with the authentic hyaluronate digestion products. The finding that the same amount of "H-labeled material (50%) was susceptible to Streptomyces hyaluronidase in the presence of a large excess of hyaluronic acid (70% of the total hexuronate) showed that the amount of enzyme used was not rate-limiting for digestion of the radio-labeled materials. No other peak in the DEAE profiles (Fig. 1) contained any material susceptible to Streptomyces hyaluronidase. Analysis of day 23 samples gave the same results (see Fig. 3).

The sulfated peaks eluting at 0.8 M NaCl from DEAE chromatography of both extracts (Fig. 1) were identified as predominantly chondroitin sulfate by their susceptibility to chondroitin ABC lyase (>95%) and their resistance to Streptomyces hyaluronidase (Fig. 2, D and E, see also results of disaccharide analyses below). The level of biosynthesis of hyaluronate was determined from the quantitation of the "H radioactivity in the hyaluronate oligosaccharide peaks (Fig. 2B) and the biosynthesis of chondroitin sulfate (used to quantitate proteoglycan synthesis) determined from the amount of "H and "S radioactivity in the 0.8 M peaks (Fig. 2D, Control). The total chemical amounts of these components in the tissue were assessed by hexuronate analyses of the same fractions. The 0.12 M peak from the guanidine HCl extract (Fig. 1A, bar) contained 42 μg of hexuronate/mg of hydroxyproline. However, as mentioned above, the material was insensitive to Streptomyces hyaluronidase, indicating that this material did not contain hyaluronic acid.

The labeled hyaluronate- and chondroitin sulfate-containing peaks from the DEAE chromatography of the day 18 samples were analyzed by direct quantitation of disaccharides produced by chondroitin ABC lyase digestion using thin layer chromatography (Fig. 2, C and F). Sixty percent of the "H-labeled material in the hyaluronate-containing peak comigrated with ΔDi-HA standard, and 36 and 57% of the "H material in the chondroitin sulfate-containing peak comigrated with ΔDi-6S and ΔDi-4S, respectively.

These results agree well with those obtained by analysis of the same peaks from day 8 and 23 samples by the Streptomyces hyaluronidase and the chondroitin ABC lyase procedure as described above.

Biosynthesis and Total Amounts of Glycosaminoglycans in
**Articular Cartilage**—Fig. 3, A and B, show that chondroitin sulfate and hyaluronate were synthesized at essentially constant rates between day 8 and 23 of culture. Hyaluronate comprised 1.1–1.6% of the total labeled glycosaminoglycans (hyaluronate plus chondroitin sulfate) during this time. Further, the chemical amounts of hyaluronate in the tissue were maintained at a ratio of 1.4–1.8% of the total amount of glycosaminoglycan. However, there was a significantly different extractability of hyaluronate from that of proteoglycans. Even though guanidine HCl extracted over 70% of the H-labeled proteoglycan, it failed to extract measurable amounts of [3H]hyaluronate. Fig. 3C also shows that there were no significant amounts of unlabeled hyaluronate in the guanidine HCl extracts.

**Catabolism of Hyaluronate**—The fact that the amount of hyaluronate remained constant while it was actively synthesized suggests that hyaluronate is actively catabolized. The similar ratios of hyaluronate and proteoglycan in the matrix (1.4–1.8%) and in rates of synthesis (1.1–1.6%) suggests that the metabolism of these two components is correlated. Direct evidence for this was sought in an experiment in which articular cartilage was labeled with [3H]glucosamine and [35S]sulfate as precursors and then chased for a period of 26 days. Labeled hyaluronate and proteoglycan were quantitated in the tissues immediately after biosynthesis and at the end of the experiment by DEAE chromatography and Bio-Gel P-10 analysis. Fig. 4A shows that both labeled components were lost with time from one of the cultures, indicating an active catabolism. The estimated time required for 50% of the radiolabeled component to be released from the tissue is shown for [3H]chondroitin sulfate and [3H]hyaluronate (arrows, Fig. 4B). For this individual sample, hyaluronate (t<sub>c</sub> of about 19.5 days) was catabolized with a comparable rate to chondroitin sulfate (t<sub>c</sub> of 25 days). Fig. 4B also shows an independent method for estimating the rate of catabolism of chondroitin sulfate by measuring the amounts of 35S activity released into the culture medium each day. This method bypasses the purification steps since >90% of the [35S]sulfate is incorporated into proteoglycans in these tissues (see Fig. 1). The calculated average t<sub>c</sub> of 29 days for replicate cultures is very close to the average for replicates obtained with H label (27 days).

In this experiment, the guanidine HCl extraction was followed by collagenase digestion without subsequent homogenization of the tissue (see "Materials and Methods"). Fig. 4, C, D, and E, shows that even though the collagenase digest solubilized approximately one-half of the collagen of the tissue, it did not remove significant amounts of the remaining proteoglycans or of the hyaluronate. The association of hyaluronate with the tissue after collagenase treatment persisted in samples chased for 26 days (not shown). Even though more than 50% of the labeled hyaluronate had been lost from the tissue, the extractability of the molecules left behind was not appreciably altered.

**Catabolism of Hyaluronate in Aggregates**—Hyaluronate is found in the pericellular environment in isolated chondrocyte cultures (21). Even though this hyaluronate pool has not been shown directly in intact cartilage, its possible existence or the existence of any other hyaluronate pools apart from the pool involved in proteoglycan aggregation could make a significant contribution to the overall kinetics of hyaluronate metabolism in cartilage cultures. For this reason, an experiment was designed to follow the rate of catabolism of hyaluronate in a native proteoglycan aggregate fraction.

Collagenase treatment of 30-μm slices, the most effective method found for the extraction of aggregates from the tissue, was used for the analyses described below. Each digest was directly homogenized in the associative extraction solvent (see "Materials and Methods"), and the clarified samples layered on preformed cesium sulfate density gradients. Kimata et al. (17) previously showed that following centrifugation in these gradients for 6 h, aggregates are recovered in the bottom one-fifth of the gradient, while monomers are well separated and recovered in the upper two-thirds of the gradient. Free hyaluronic acid and link protein are recovered in the upper third of the gradient. It was also shown that separation was independent of initial concentration of proteoglycans between 0.4–8 mg/ml.

Fig. 5, A and C, shows the centrifugation profiles for the day 2 and 20 tissue extracts from tissue that was labeled on day 0 and then chased (see "Materials and Methods"). The extraction of 35S-labeled material from the tissue in each case was approximately 60% with 50–60% of this material recovered in the aggregate fraction, tubes 1–6. This is in close agreement with the results of Manicourt et al. (15) using similar procedures to extract native aggregates from rabbit and dog articular cartilage. Thus, the aggregate fraction contains...
tains about 35% of the total tissue proteoglycan.

Fractions 1–6 of the cesium sulfate gradients (Fig. 5, A and C) were pooled and subjected to CsCl density gradient centrifugation. Because the aggregates have high density they will equilibrate in the bottom of the CsCl gradients, whereas any particulate membrane or debris which might have sedimented to the bottom of the Cs2SO4 gradients will float in the CsCl gradients. The aggregate fractions in the bottom of the CsCl gradients (Fig. 5, B and D) were recovered, digested with papain and then chondroitin ABC lyase, and analyzed for disaccharides on an HPLC Partisil 5 PAC column (19). Fig. 6 shows representative elution profiles from this column. There was good separation of all the disaccharides including resolution of the hyaluronate and chondroitin disaccharides. Fig. 7A shows that both radiolabeled chondroitin sulfate and hyaluronate are lost from the aggregate fraction during the chase. Fig. 7B shows the calculated \( t_1/2 \) for \([\text{H}]\)chondroitin sulfate and \([\text{H}]\)hyaluronate: 17 and 20 days, respectively. The former value corresponds closely with that obtained for the total release of \( ^{35}S \)-labeled material from the tissue (\( t_1/2 = 18 \) days, B). The percent of \([\text{H}]\)hyaluronate of the total \([\text{H}]\)labeled glycosaminoglycans in aggregate was 2.5–3%. Analysis of the rest of the fractions from the velocity gradients showed that they contained chondroitin sulfate, but not hyaluronate. Fig. 6B shows a representative profile, and Table I shows the disaccharide analyses for the various samples.

Analysis for Hyaluronate in the Medium—Attempts were made to detect hyaluronate degradation products in the medium fractions. The media collected from the 18 days of chase were pooled and fractionated on a DEAE-Sephacel column. The bound fraction which would be expected to contain any large to relatively small molecular weight hyaluronate contained only chondroitin sulfate (Table I). Experiments with standard oligosaccharides produced by Streptomyces hyaluronidase digestion of hyaluronate showed that the tetrasaccharide is not bound by the column, while the hexasaccharide is weakly bound (eluted by 0.1 M NaCl). Thus, the unbound fraction from the DEAE chromatography was concentrated and precipitated with 3 volumes of ethanol. The hexa- and tetrasaccharides would be soluble in this procedure. The ethanol supernatant was analyzed by Bio-Gel P-10 chromatography. All of the ethanol-soluble material was recovered in the \( V_t \) of this column. Thus, there was no evidence for the presence of hyaluronate oligosaccharides in the medium.

**FIG. 5.** Separation of native proteoglycan aggregates from cultured tissues. A and C represent velocity sedimentation profiles of associative tissue extracts from days 2 and 18 of the experimental protocol for analysis of native aggregates (see “Materials and Methods”). Fractions from these gradients were pooled as indicated by the bars in the figure. Fractions 1–6 were subjected to an equilibrium run on cesium chloride gradients; the profiles from these gradients are shown in B and D. The bars under these panels indicate the fractions pooled for analysis by chondroitin ABC lyase and HPLC.

**FIG. 6.** HPLC analysis of disaccharides from the native aggregate pool. A represents the chromatography of disaccharides produced by chondroitin ABC lyase digestion of the papain-treated aggregate from the experimental day 2 (Fig. 5B, bar). B represents the chromatography of the disaccharides from the material in the velocity gradients, fractions 7–16 for the day 2 sample (Fig. 5A, middle bar). HA, hyaluronate; OS, chondroitin disaccharides.

**FIG. 7.** Catabolism of glycosaminoglycans in the native aggregate fraction. A shows the total amounts of \( ^{3}H \)-labeled sulfated glycosaminoglycans (CS) and hyaluronate (HA) on days 2 and 20 of the experimental protocol; analysis was as shown in Figs. 5 and 6. B shows the total loss of \( ^{35}S \) activity from these cultures. The arrows indicate the \( t_1/2 \) calculated for the \( ^{35}S \)- and \( ^{3}H \)-labeled components; the latter were calculated from analysis of A.

**DISCUSSION**

The results of this study support the novel hypothesis that the metabolism of both hyaluronate and proteoglycans in cartilage is highly coordinated. The data presented support this conclusion in that: 1) newly synthesized hyaluronate and proteoglycans maintain a nearly constant ratio that is very similar to that existing between the total chemical amounts of these two components in the steady-state culture system;
2) the catabolism of these two components occurs with virtually identical kinetics; and 3) this catabolic relationship holds for the loss of both hyaluronde and proteoglycans from native, undissociated aggregates isolated from intact tissue. This latter finding argues that the kinetic interrelationship seen in whole tissue is not the fortuitous result of the coexistence of various hyaluronde pools turning over with different kinetics. Furthermore, we have investigated general protein catabolism by chondrocytes in this system and found that the t<sub>1/2</sub> of this pool is much faster than for hyaluronde, chondroitin sulfate, or the core protein of proteoglycan. This indicates that the rate of release of the aggregate components is not simply a reflection of the general rate of protein catabolism in the tissue.

These new data suggest that it is important to introduce the problem of hyaluronde processing into any hypothesis dealing with the mechanism of proteoglycan catabolism. In previous work we found that the release of proteoglycans from the matrix is a highly targeted process in which proteoglycan monomers destined for removal from the matrix are excised without leaving partially truncated proteoglycans behind (7, 8). The majority (>90%) of the molecules released into the culture medium are of large hydrodynamic size and are comparable to, or slightly smaller than, newly synthesized molecules (8); <10% of the proteoglycans released into the medium are completely degraded, presumably by lysosomes, as monitored by the generation of free [35S]sulfate from catabolized proteoglycan.

It cannot be ascertained at this point which is the decisive or primary event that releases a proteoglycan monomer from its complex association in the tissue, but it is clear that the major portion of the protein core of the proteoglycan escapes extensive processing. Hyaluronde is lost from the tissue with similar kinetics as for proteoglycans, but no detectable large hyaluronic acid molecules or hyaluronic acid oligosaccharide fragments were detected by the methodology used. The fate of the catabolized hyaluronde will be the focus of a separate investigation.

A number of cell types including liver endothelial cells (22), 3T3 fibroblasts (23), and embryonic myocardial cells (24) have been shown to metabolize hyaluronde by receptor-mediated endocytosis followed by extensive intracellular degradation. The possibility that cartilage proteoglycan aggregate catabolism involves a similar process is considered in the following hypothesis. Proteinase(s) activated close to or on the surface of the cell may cleave proteoglycan monomers preferentially in the hyaluronic acid binding region and in the link protein, releasing large fragments from the aggregate complex while the hyaluronde backbone is internalized for intracellular degradation. Dingle and Dingle (25) originally suggested that proteoglycan catabolism occurs in close vicinity to the cell and provided indirect evidence for this by showing that proteoglycans introduced into small cavities in cultured cartilage remain unhydrolyzed while adjacent cartilage undergoes extensive resorption. In addition, the observation of Saklatvala and Bird (26) that cytochalasin B inhibits the rapid proteoglycan losses induced by interleukin 1 suggests that an intact cytoskeleton is vital to the catabolic process and thus implies a cell surface involvement. However, the possible existence of extracellular neutral hyaluronidases, yet unidentified, which contribute to the overall process of hyaluronde resorption cannot be ruled out.

Whatever the exact mechanisms involved in the catabolism of hyaluronde acid, this study clearly shows that this process is an integral part of the mechanism of proteoglycan aggregate metabolism. This might reflect a highly regulated process by which whole aggregates become catabolized, thereby insuring that aggregate structures containing molecules damaged by proteolysis or by wear and tear of the tissue do not accumulate in the matrix.

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* T. I. Morales and V. C. Hascall, unpublished observations.

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**Table I**

| Sample                  | % disintegrations/min H<sup>3</sup> in HPLC 
|-------------------------|------------------------------------------|
| Cesium sulfate fractions (7-16), day 3 | (1)* 10 20 69 
| Cesium sulfate fractions (7-16), day 18 | (1) 15 22 61 
| Cesium sulfate fractions (17-19), day 2 | (1) 9 23 67 
| Cesium sulfate fractions (17-19), day 18 | (1) 9 30 60 
| Media, pooled, day 18 | (0.001) 11.5 29 59 

* The values of percent of hyaluronde disaccharides are in brackets to indicate that there were no distinct peaks in this area of the elution profile (refer to Fig. 6).

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