Mechanisms for Dissociating Proteoglycan Aggregates*

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Preformed CsSO₄ zonal gradients were used to purify aggregates from proteoglycan preparations derived from associative extracts of the Swarm rat chondrosarcoma. Zonal gradients were used in solvents with different concentrations of guanidine HCl and different solvent pH values to study the mechanisms for dissociating the aggregates. Aggregates are stable in concentrations of guanidine HCl up to 1.5 M at pH 6.6. At 2 M guanidine HCl, partial dissociation occurs over 20 h in which a link protein is completely dissociated for every monomer proteoglycan dissociated from the aggregate structure. This suggests that in this solvent disaggregation occurs concurrent with complete separation of link protein from monomer. At solvent pH 2.7 to 3.3 in ionic conditions which normally promote aggregation, dissociation occurs by a mechanism in which the link protein remains associated with monomer. Thus, link protein-monomer complexes dissociate as bimolecular units from hyaluronic acid; such complexes then exhibit physical properties indistinguishable from pure monomers. The link protein-monomer complexes reassociate with hyaluronic acid to form link-stabilized aggregates when the solvent pH is raised to pH 7, i.e., to associative conditions. The study provides additional evidence for the role that link protein-monomer interactions have in proteoglycan aggregate structures.

The previous paper (1) describes a velocity zonal centrifugation procedure utilizing preformed CsSO₄ gradients to separate proteoglycan aggregates from nonaggregated proteoglycans in AA preparations isolated from the Swarm rat chondrosarcoma. Purified aggregates recovered by this procedure represent intact aggregates isolated directly from the tumor since an associative solvent was used in the extraction step (2, 4). Dissociative solvents such as 4 M guanidine HCl effectively separate the aggregate structure into its three components:

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3 The abbreviations used are: AA, a proteoglycan preparation isolated by using an associative (a) extraction solvent (2) followed by an associative CsCl isopycnic gradient isolating proteoglycan from the bottom (A1) fraction (3); AA-D1 and AA-D4, the monomer preparations and link protein-enriched preparations isolated from AA in a subsequent dissociative density gradient either in the bottom (D1) or top (D4) fractions, respectively; HA, an agggregates prepared from partial digests of hyaluronic acid with testicular hyaluronidase and having an average length of 50 monosaccharides, proteoglycan monomers, link proteins, and hyaluronic acid (5–9). Dissociation of aggregates also occurs when the solvent pH is lowered below pH 4 in electrolyte solutions which are associative at higher pH (5, 10). The experiments described in this paper utilized the velocity zonal centrifugation procedure to study the mechanisms for dissociating aggregates in guanidine HCl solutions and at low solvent pH. The results indicate that, while increasing concentrations of guanidine HCl cause separation of all three components of the aggregate, the low solvent pH dissociates a stable complex of proteoglycan monomer-link protein as a unit from hyaluronic acid.

EXPERIMENTAL PROCEDURES

Materials—Hyaluronidase from Streptomyces hyalurolyticus was obtained from Calbiochem. Papain (twice crystallized) and hyaluronic acid (type I) were from Sigma. Oligosaccharides of hyaluronic acid with an average of about 50 monosaccharides, HA₅₀, were isolated from partial digests of hyaluronic acid with testicular hyaluronidase by chromatography on Bio-Gel P-30 as described elsewhere (11). All other materials used in the experiments are described in the preceding paper (1).

Methods: Cesium Sulfate Zonal Gradients—The details for preparing preformed CsSO₄ gradients for zonal centrifugation are given in the preceding paper (1). For some of the experiments described in this paper, the gradients were prepared in the presence of different guanidine HCl concentrations and different solvent pH values. In each of these cases, the two CsSO₄ solutions used to prepare the gradient contained the appropriate solvent, for example, 2 M guanidine HCl, such that the solvent was the same throughout the gradient. Solute samples in each case were dialyzed or diluted into the same solvent in the absence of CsSO₄ and maintained at 4 °C for 16–20 h prior to application onto the gradients. For low pH solvents, the 0.1 M sodium acetate, 0.1 M Tris solvent normally present in the CsSO₄ solutions was replaced with 0.2 M sodium acetate/acetic acid to give the final pH for the experiments. The final solvent conditions are described under "Results."

Analysis for Hyaluronic Acid—A modification of the sensitive and specific assay described by Jourdian et al. (12) was used to measure hyaluronic acid contents of fractions. The procedure estimates the amount of the unsaturated hexuronic acid formed during digestion with the hyaluronic acid-specific eliminase from Streptomyces using the thioarbituric acid colorimetric procedure (13). The presence of protein in the samples introduced turbidity in the 1-butanol extracts of the chromophore when the method was used as described (12). Therefore, samples from gradient fractions were treated as follows: (a) fractions were diazylated against 0.1 M sodium acetate, pH 7.0, and then lyophilized. Otherwise, proteoglycans and hyaluronic acid in samples were quantitatively precipitated by the addition of 3 volumes of ice-cold absolute ethanol containing 1.3% (w/v) potassium acetate and the precipitates were collected by centrifugation as described elsewhere (14); (b) the precipitates were dissolved in 0.3 ml of 0.1 M sodium acetate, pH 7.0, 5 mM EDTA, 5 mM cysteine HCl and digested at 60 °C for 5 h with 30 μg of papain; (c) ethanol precipitation was done as described above to remove cysteine. The precipitate was dissolved in 0.3 ml H₂O and then the papain was inactivated by heating at 100 °C for 3 min, and the glycosaminoglycans were then precipitated with ethanol as described above; (d) the final precipitates were dissolved in 50 μl of 0.1 M sodium acetate buffer, pH 5.0, and digested with 5 turbidity-reducing units of Streptomyces hyaluronidase for 5 h at 37 °C; (e) the subsequent development of the
chromophore was done at one-fourth the final volume described by Jourdian et al. (12).

Fig. 1 shows a standard curve for the assay with a sample of hyaluronic acid, indicating the linearity and sensitivity of the method (approximately 0.04 absorbance units/µg of hyaluronic acid). The results given in the inset show that the recovery of a standard amount of hyaluronic acid (8 µg) which was carried through the papain digestion and ethanol precipitation steps either alone (bar A) or with 300 µg of purified monomer proteoglycan (bar C) was quantitative. The presence of excess proteoglycan did not interfere with the assay, and controls with no added hyaluronic acid had absorbance values less than 0.005.

Analysis for Link Protein—Lyophilized fractions or ethanol precipitates of fractions were prepared as described above. The samples were then dissolved in 100 µL of 0.1 M sodium acetate, 0.1 M Tris-Cl, pH 8.0, and digested with chondroitinase ABC (0.2 units) at 37 °C for 90 min. A 35-µL aliquot of 6% sodium dodecyl sulfate and 40 mM dithiothreitol was added to each. After heating at 90 °C for 5 min, the samples were analyzed on 7% polyacrylamide slab gels and stained with Coomassie blue as described elsewhere (15). After destaining, bands corresponding to link protein and proteoglycan core were sliced from the gel in rectangular strips of nearly the same size. Slices from adjacent areas which contained no bands were used as blanks. The dye was extracted from each gel slice by incubation in 0.8 ml of 1% sodium dodecyl sulfate containing 0.02 M NaOH at 37 °C for 24 h with continuous gentle mixing. Each extract was then acidified with 10 µL of concentrated acetic acid to restore the color of the dye, and the absorbance was measured at 590 nm.

Fig. 2 shows a standard curve for the link protein band in analyses of a series of different amounts of a sample of aAl-D4 isolated from the rat chondrosarcoma. The increase in absorbance is nearly linear with concentration at low amounts of link protein but the color response falls off slightly in the 5- to 10-µg range. The assay is sensitive to about 1 µg.

Analysis for Proteoglycan—For aliquots from gradients which did not contain guanidine HCl, hexuronic acid concentrations were measured directly with an automated carbazole procedure as described in the preceding paper (1) to estimate proteoglycan contents. In gradients with guanidine HCl, the proteoglycans were precipitated with ethanol as described above and redissolved in 0.2 M sodium acetate, pH 7.2, before analysis for hexuronic acid content.

RESULTS

Centrifugation of aAl-D1 Monomer Proteoglycans in Different Solvents—Preformed Cs2SO4 gradients (0.15 to 0.50 M) were prepared in 2 M guanidine HCl, 4 M guanidine HCl, or 0.2 M acetic acid/sodium acetate, pH 3.3. Aliquots of monomer aAl-D1 (2 mg of proteoglycan in 1 ml) in the same respective solvents were layered on the gradients followed by centrifugation for the indicated times at 25,000 rpm, 11 °C. Fig. 3 shows the hexuronic acid profiles obtained from the gradients. Since each of the solvents has a different density and viscosity (16), different centrifugal times are required for the proteoglycan peak fraction to sediment to the middle fractions; about 12 h for 2 M guanidine HCl, 16 h for 4 M guanidine HCl, and 8 h for the pH 3.3 solvent. Subsequent experiments utilized centrifugal times such that monomers, if present, would migrate to the middle fractions of the gradient. This permits maximum resolution of monomers from intact aggregates, which migrate to the bottom fractions, and from unbound link protein molecules and hyaluronic acid, which remain in the upper fractions of the gradients.

Dissociation of Aggregate—Aggregate was separated from nonaggregated proteoglycans by centrifuging aAl samples on standard preparative preformed Cs2SO4 gradients as described in the preceding paper (1). Aliquots of purified aggregate (about 5 mg in 1 ml) were dialyzed in the cold for 20 h against the correspondingly matched solvent to separate by centrifugation for the indicated times at 25,000 rpm, 11 °C. The results given in the inset show that the recovery of a standard amount of proteoglycan (500 µg) which was carried through the papain digestion and ethanol precipitation steps either alone (bar A) or with 1000 µg of purified monomer proteoglycan (bar C) was quantitative. The presence of excess proteoglycan did not interfere with the assay, and controls with no added hyaluronic acid had absorbance values less than 0.005.

Analysis for Link Protein—Lyophilized fractions or ethanol precipitates of fractions were prepared as described above. The samples were then dissolved in 100 µL of 0.1 M sodium acetate, 0.1 M Tris-Cl, pH 8.0, and digested with chondroitinase ABC (0.2 units) at 37 °C for 90 min. A 35-µL aliquot of 6% sodium dodecyl sulfate and 40 mM dithiothreitol was added to each. After heating at 90 °C for 5 min, the samples were analyzed on 7% polyacrylamide slab gels and stained with Coomassie blue as described elsewhere (15). After destaining, bands corresponding to link protein and proteoglycan core were sliced from the gel in rectangular strips of nearly the same size. Slices from adjacent areas which contained no bands were used as blanks. The dye was extracted from each gel slice by incubation in 0.8 ml of 1% sodium dodecyl sulfate containing 0.02 M NaOH at 37 °C for 24 h with continuous gentle mixing. Each extract was then acidified with 10 µL of concentrated acetic acid to restore the color of the dye, and the absorbance was measured at 590 nm.

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Fig. 1. A standard curve for hyaluronic acid assay and the effect of papain digestion and ethanol precipitation on the reproducibility of the assay (inset). Details for standard assay procedures are described under "Methods." In the inset, bar A shows the optical density at 549 nm obtained for 8 µg of hyaluronic acid without papain digestion and ethanol precipitation; bar B, duplicates of 8 µg hyaluronic acid with the treatments; bar C, duplicates of 8 µg of hyaluronic acid plus 300 µg of purified monomer proteoglycan aAl-D1 with the treatments.

Fig. 2. A standard curve for link protein assay. Details of the assay are described under "Methods." Samples of aAl-D4 isolated from the rat chondrosarcoma were used as a standard.

Fig. 3. Zonal centrifugation of proteoglycan monomer aAl-D1 on the Cs2SO4 density gradients in different solvents. Samples of aAl-D1, 2 mg in 1 ml of the same respective solvents as those for the Cs2SO4 density gradients, were centrifuged under the following conditions: (left) in 2 M guanidine HCl (GuCl), 0.2 M sodium acetate, pH 7.2, for the periods indicated; (upper right) in 4 M guanidine HCl, 0.2 M sodium acetate, pH 7.2, for 20 h; (lower right) in 0.2 M sodium acetate, pH 3.3, for the periods indicated. The centrifugations were done at 25,000 rpm at 11 ± 2 °C in a Beckman SW 27.1 rotor.
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the different solvents used in the gradients. After standing at room temperature for 4 h, the samples were layered on the appropriate gradients and centrifuged for the lengths of time as indicated in Fig. 5. Fractions of about 2.4 ml were collected (7/gradient) and each was dialyzed against 1 mM sodium acetate, 1 molar Tris-HCl, pH 7.2, and lyophilized. Each was dissolved in 1 ml of H2O and aliquots were analyzed for contents of proteoglycan, link protein, and hyaluronic acid as described under “Experimental Procedures.”

Fig. 4 shows photographs of sodium dodecyl sulfate-polyacrylamide slab gel analyses for some of the gradients, and Fig. 5 summarizes the overall results for the experiments. The positions of the link protein band and of the two bands which contain chondroitinase-digested proteoglycan core molecules are indicated in Fig. 4. The other bands in the gels are derived from proteins in the chondroitinase enzyme preparation used for the digestions.

In the pH 6.6 standard gradient, the aggregate remained intact and all of the link protein, proteoglycan, and hyaluronic acid sedimented to the bottom two fractions, with the large majority of each in the bottom fraction (Figs. 4 and 5). In the presence of 4 mM guanidine HCl, the three components of aggregate were completely dissociated; the link protein was observed primarily in fraction 1 at the top of the gradient, hyaluronic acid peaked in fraction 2, and the proteoglycan monomer yielded a broad profile with a peak in fraction 4 (Figs. 4 and 5). In gradients with 1 and 1.5 mM guanidine HCl, the aggregate remained intact, indicating that the aggregate is stable to these solvent conditions over an exposure time of at least 24 h (Fig. 5). However, the results for the gradient in 2 mM guanidine HCl indicate that the aggregate is partially dissociated in this solvent. About 79% of the proteoglycan, 60% of the link, and at least 93% of the hyaluronic acid were recovered in the aggregate fraction. The remaining proteoglycan was recovered in the middle, monomer region while most of the remaining link protein was found in the top fractions (Figs. 4 and 5). These results indicate that 2 mM guanidine HCl is sufficiently concentrated to begin to dissociate some of the aggregate during the time of the experiment, about 24 h. Additionally, they suggest that dissociation of a monomer from the aggregate structure in this solvent is accompanied by the release of a link protein as well.

Dissociation of aggregate at lower solvent pH occurs by a different mechanism, however. At pH 2.9 and 3.3, aggregate was not observed; the proteoglycans sedimented to the middle, monomer region of the gradient while hyaluronic acid was recovered primarily in the top two fractions. In contrast with the results in guanidine HCl solutions, however, the link protein was not recovered in the top of the gradient; rather it sedimented with a nearly identical profile as that of the proteoglycans (Figs. 4 and 5). This indicates that link proteins are still associated with the monomers and suggests that dissociation of aggregate at low solvent pH occurs by releasing monomer-link protein complexes from hyaluronic acid without dissociating link protein from monomer.

At pH 3.6, aggregate was not present. However, unlike the results at lower solvent pH, the hyaluronic acid was observed in the middle, monomer region rather than in the top fractions, suggesting that at this pH the hyaluronic acid may interact weakly with the monomer-link protein complex (Fig. 5). When 1 mM guanidine HCl was included in the pH 3.6 solvent, the hyaluronic acid was again observed in the top fractions well resolved from the middle, monomer region. In this solvent, the link protein still remained associated with the monomer.

The sodium dodecyl sulfate-polyacrylamide gel analyses in Fig. 4 reveal an additional feature about the proteoglycan monomers. Two bands were observed for the core after chondroitinase digestion, with the more rapidly migrating band accounting for about 20% of the Coomassie blue staining relative to the major, slower migrating band 1. In the pH 3.3 solvent where dissociation was complete, the relative proportion of the band 2 proteoglycan was greater in the upper enzyme preparation. The core and link protein bands were isolated and quantitated as described under “Methods” to provide data for Figs. 5 and 6. Standards (Stnd) are from a chondroitinase ABC-digested aAl preparation and contained: 5 × Stnd = 108 μg of uronic acid and 1 × Stnd = 22 μg of uronic acid. For each slab gel, the top fraction of the gradient is at the left and the bottom fraction is at the right.

FIG. 4. Sodium dodecyl sulfate-polyacrylamide slab gels for fractions from some of the gradients in Fig. 5. A, 0.2 mM sodium acetate, pH 6.6; B, 4 mM guanidine HCl (GuHCl), 0.2 mM sodium acetate, pH 7.2; C, 2 mM guanidine HCl, 0.2 mM sodium acetate, pH 7.2; and D, 0.2 mM sodium acetate/acetic acid, pH 3.3. The electrophoresis was done as described under “Methods.” Bands 1 and 2 indicate the major, large and minor, small core protein bands. L indicates the link protein band. The remaining bands are from the chondroitinase
Reaggregation after Exposure to Low Solvent pH—Aliquots of purified aggregate were exposed to pH 3.3 solvent for 24 h before adjusting the pH to 7.0 for treatment as described in Fig. 7. In one case, aliquots were then kept at room temperature for 2 or 4 h before centrifugation on standard Cs2SO4 zonal gradients (Fig. 7, a and b). Approximately 85 and 91% of the proteoglycans, respectively, were observed in the bottom fractions of the gradients, suggesting that reaggregation was occurring with time at pH 7.

Oligomers, HA-ø.5, of hyaluronic acid were used to test the specificity of the reaggregation process. In previous experiments (11, 17, 18), it was shown that HA-ø.5 oligomers are long enough to accommodate both the hyaluronic acid-binding site of a monomer and the binding site of an adjacent link protein to form link-stabilized ternary complexes with physical properties similar to monomer (11, 18). In one case, HA-ø.5 oligomers were added to an aliquot of aggregate immediately after the pH was adjusted to 7. The amount of oligomer was about 5 to 6 times the amount of macromolecular hyaluronic acid already present in the aggregate sample. After 4 h at room
temperatur for 2 or

Fig. 6. Distributions of aggregating proteoglycans. Larger core proteins (band 1) and smaller core proteins (band 2) on the Cs2SO4 density gradients were estimated by quantitative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Hexuronic acid (hex), core protein band 1 ( ), and core protein band 2 ( ) are indicated for the gradients in 0.2 M sodium acetate/acetic acid, pH 3.3 (a) and in 2 M guanidine HCl, 0.2 M sodium acetate, pH 7.2 (b). Core protein bands before elution are shown on the slab gels in Fig. 4, b and d. Quantification of the two bands was done using known amounts of a chondroitinase digest of aA1-D1 as a standard as described for the link protein assay under "Methods." The bars indicate duplicate values from the same fractions on two different gels.

Fig. 7. Reaggregation at pH 7.0 of aggregate which was first dissociated at pH 3.3. Aggregate was dialyzed against a 0.2 M sodium acetate/acetic acid, pH 3.3, at 4 °C for 24 h. Aliquots (1.25 mg) were centrifuged after the following: a, 2 h after the pH was raised to 7.0; b, 4 h after the pH was raised to 7.0; c, 4 h after the pH was raised; d, 4 h after the pH was raised to 7.0 followed by the addition of HA-ø.5 oligomers just prior to centrifugation; e, 4 h after the pH was raised to 7 with HA-ø.5 (100 µg) added just after the pH was raised; f, an aliquot of the original aggregate sample maintained at pH 7.0; g, an aliquot of the original aggregate in pH 7.0 to which HA-ø.5 (100 µg) was added followed by incubation for 2 h. The per cent above each peak indicates the proportion of hexuronic acid in fractions from tube 1 to tube 16 to total hexuronic acid. The per cent in parentheses indicates the proportion in these fractions excluding hexuronic acid due to HA-ø.5 oligomers.
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The interaction of monomer with hyaluronic acid is reversible (11, 19-22) whereas link protein-stabilized aggregates are much more stable (11, 18, 19, 23, 24). Thus, while the experiments shown in Fig. 7, a, b, and e, show that monomer is able to reassociate with hyaluronic acid after raising the solvent pH to 7, they do not test for stabilization of the aggregate by functional link protein. The results of the experiments shown in Fig. 7, c and d, however, provide evidence that the link protein also retains its functionality after exposure to low solvents. HA-oligomers were added to aliquots which had been adjusted to pH 7 either 2 or 4 h previously. After an additional 2 h in the first case or immediately in the second, the mixtures were centrifuged. About 22 and 7% of the proteoglycan-related hexuronic acid, respectively, was observed in the middle, monomer regions of the gradients. These values were close to those obtained in Figs. 7, a and b. The results indicate that aggregates formed prior to the addition of the oligomers remained intact whereas the presence of the oligomers prevented additional aggregation. Since monomers do not remain associated with macromolecular hyaluronic acid in the absence of link protein when HA-oligomers are present (see Fig. 8e below), the experiments in Fig. 7, c and d, indicate the presence of functional link protein in the reassociated aggregates.

Fig. 7f indicates that the original sample which was not exposed to low solvent pH contained only proteoglycan aggregates. The addition of HA-oligomers to another aliquot of the original sample, Fig. 7g, did not lead to any appreciable disaggregation since the hexuronic acid was observed essentially only in the top fractions (the oligomers) and the bottom fractions (aggregate).

Interaction of Aggregating Monomer with Hyaluronic Acid—A series of experiments was designed to determine whether the interaction of monomers with hyaluronic acid under the conditions described in Fig. 8 is stable in standard CsSO₄ zonal gradients. Monomer was isolated from purified aggregate using a 4 M guanidine HCl dissociative CsCl isopycnic centrifugation. A sample of the monomer was dialyzed into 0.2 M sodium acetate, pH 7. An aliquot was run directly on a standard zonal gradient (Fig. 8a). Essentially all of the proteoglycan was recovered in the middle, monomer region with less than 8% of the hexuronic acid in the bottom fraction (fractions 21-26). When 0.5% (w/v) of macromolecular hyaluronic acid was added, followed by incubation at room temperature, about 65% of the proteoglycans sedimented to the bottom fractions with 35% (shaded area) sedimenting in the monomer region.

The proportion in the bottom fraction increased to about 80% for a 1% mixture of hyaluronic acid with monomer, which has been shown previously (7) to be nearly optimal for proteoglycan-hyaluronic acid mixtures. At 2% hyaluronic acid, the amount of proteoglycan sedimenting further into the gradient than the monomer region was still about 60% but more was found in the fractions immediately above the bottom, indicative of smaller complexes, i.e. fewer monomers per hyaluronic acid molecule. These results indicate that the proteoglycan-hyaluronic acid interaction is stable to a large extent to the conditions of the zonal gradients. In the experiment shown in Fig. 8e, hyaluronic acid (2%) was added to aggregating monomer. After incubation at room temperature for 2 h, HA-oligomers (10%) were added and incubation continued for 2 additional h before centrifugation. The proteoglycans in this case were recovered almost solely in the monomer region of the gradient. This demonstrates the reversibility of the proteoglycan-hyaluronic acid interaction since equilibrium in the mixture would favor proteoglycan-oligomer interactions with each macromolecular hyaluronic acid molecule having only a few monomers associated with it. This result is in sharp contrast with the experiment in Fig. 7g where the oligomers were added to link protein-stabilized aggregates.

DISCUSSION

The experiments described in this report utilized preformed CsSO₄ zonal gradients in different solvents to study dissociation of purified proteoglycan aggregates. At pH 6.6 in guanidine HCl solutions up to 1.5 M, the aggregate remained associated whereas at 2.0 M, partial dissociation over 20 h was observed. In this latter solvent, for each monomer dissociated from the aggregate, a link protein was also dissociated from both monomer and hyaluronic acid. In this case, the rate-limiting step may be destabilization of link protein function and its release from the aggregate, allowing concurrent dissociation of monomer from hyaluronic acid. In solvents with pH 2.9-3.3, the link protein remains tightly bound to monomer, and both dissociate from hyaluronic acid. In this case, the solvent appears to reverse the interaction of hyaluronic acid with both the binding sites in the hyaluronic acid-binding region of monomer and in the link protein, perhaps by protonation of carboxyl groups on the glucuronic acid.
residues in the hyaluronic acid, since these groups are major determinants in the interaction with monomer (26).

The link protein-monomer association appears to remain stable at the low solvent pH, clearly separating from the hyaluronic acid in the zonal gradients. Additionally, the link protein-monomer complex is able to reassociate with hyaluronic acid to form link protein-stabilized aggregates. This was demonstrated by the stability of reformed aggregates in the presence of HA-50 oligomers in contrast to the instability of complexes of monomer with hyaluronic acid in the same conditions. The demonstration that monomer-link protein complexes can exist independent of hyaluronic acid provides additional support for the recently reported evidence (18) that such a complex is probably an intermediate in the formation of proteoglycan aggregates from newly synthesized and secreted proteoglycans in chondrocyte cultures. The C5s04 zonal gradients, then, can be used at low solvent pH to isolate the mono-mer-link protein complexes for further study.

The monomer fraction isolated from purified aggregate contained two populations of proteoglycans, both able to aggregate. The minor population (~20% of the total) was slightly smaller in hydrodynamic size, sedimented slightly slower in dissociative zonal gradients, and yielded a core preparation after chondroitinase digestion which migrated further into 7% sodium dodecyl sulfate-polyacrylamide gels. It is likely that this minor population represents a selective breakdown product of the larger proteoglycans in the major population which either accumulates in the tumor matrix with time or is generated during the extraction and isolation steps. It is unlikely to be a separate biosynthetic product since in studies reported elsewhere (25), it is shown that the aggregating proteoglycan population synthesized in culture by chondrocytes derived from the chondrosarcoma appears to have a uniform core protein.

Acknowledgments—We wish to thank Dr. S. DeLuca for the heparonic acid analyses and Dr. C. B. Caputo for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We are also grateful to Thelma Prather for excellent technical assistance. K. K. is very indebted to Dr. George R. Martin, National Institute of Dental Research, for continuing support.

Note Added in Proof—Since this paper was submitted, Franzen et al. (28) described an experiment in which purified link protein was mixed with purified monomer under associative conditions and the mixtures centrifuged in sucrose-stabilized velocity gradients. Link protein, as estimated by immunological procedures, sedimented with the monomer.

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2 Oike et al. (27) have shown that commercial preparations of chondroitinase ABC contain some proteolytic activity. However, it is unlikely in this case that enzyme digestion yielded the smaller and minor band on the gels because the relative content of this band to the larger major band in each fraction is reflective of the distribution of minor proteoglycans on the gradient (see Fig. 6a). It is likely that proteolytic activity in the chondroitinase ABC would produce similar proportions of the minor component throughout.
Mechanisms for dissociating proteoglycan aggregates.
K Kimata, V C Hascall and J H Kimura

J. Biol. Chem. 1982, 257:3827-3832.

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