Aggregated proteoglycans (70% aggregated), isolated from the Swarm rat chondrosarcoma by extraction with 4 M guanidinium chloride in the presence of protease inhibitors and purified by centrifugation in an associative cesium chloride gradient, were separated into the component parts by centrifugation in a dissociative cesium chloride gradient. The gradient was cut into five equal fractions. The bottom fraction contained 98% of the chondroitin sulfate and 84% of the protein of the aggregate preparation. Sedimentation equilibrium studies on the protein core of this fraction, isolated by column chromatography from chondroitinase ABC digests, suggest that its molecular weight is $2.0 \times 10^6$ to $2.2 \times 10^6$. The intermediate fractions of lower buoyant densities contained hyaluronic acid (0.8% of the total weight of the aggregated preparations) and proteoglycan monomers with fewer chondroitin sulfate chains relative to the protein core than in the bottom fraction. The top fraction, in addition to proteoglycans, contained a link protein. The link protein was separated from the proteoglycans by chromatography on Sephadex G-200 in the presence of 4 M guanidinium chloride. Its molecular weight was estimated to be 40,000. It stabilized complexes of proteoglycan monomers and hyaluronic acid so that they could be seen in the analytical ultracentrifuge at pH 5.8.

The amino acid composition of the link protein differs significantly from that of the protein core of the proteoglycan monomers and from that of the hyaluronic acid binding region of the protein core of the proteoglycans.

In hyaline cartilages, proteoglycans (glycosaminoglycans covalently linked to protein) are the structural components primarily responsible for the characteristic elasticity and reversible resistance to compressive forces (1). These molecules exist as aggregate structures in the extracellular matrix (2). On the basis of work in several laboratories, a model for the structure of proteoglycan aggregates in hyaline cartilages has been proposed (19, 20). The proteoglycan monomer is polydisperse, average molecular weight of $2.5 \times 10^6$ and a range of 1.0 to $4.0 \times 10^6$ (8, 9). It contains about 100 chondroitin sulfate chains, each with an average molecular weight of $2.0 \times 10^4$, and 35 to 50 keratan sulfate chains, each with an average molecular weight of about $5 \times 10^4$ (11–13). The polysaccharide chains are covalently linked to a protein core, which has a weight average molecular weight of $1.8 \times 10^6$ to $2.0 \times 10^6$ (21). One end of the protein core interacted with hyaluronic acid (22).
end is relatively free of polysaccharide chains. The remainder of the protein core provides the attachment points through the hydroxyls of serine and threonine, for the chondroitin sulfate and keratan sulfate chains (22-29). These polysaccharide chains are not uniformly distributed over the protein core. On the protein core of the proteoglycans of bovine nasal septum and trachea, about 60 to 70% of the keratan sulfate chains and less than 10% of the chondroitin sulfate chains are attached to a segment of the protein core immediately adjacent to the segment of the protein core involved in binding to hyaluronic acid (19).

Such proteoglycan monomers can complex with hyaluronic acid (17). The complexes are stabilized by link proteins, which can interact noncovalently with both the protein core of the proteoglycan monomers and with hyaluronic acid (24). Hoffman, et al. (20, 27) have suggested an alternative model for proteoglycan aggregates, in which a specific link protein is not required. They suggest that formation of proteoglycan aggregates is an expression of the polydispersity of the proteoglycans. In their model, proteoglycans of low buoyant density (high protein content) help stabilize aggregate structures of high buoyant density proteoglycans and hyaluronic acid.

In general, the proteoglycan aggregate preparations from the Swarm rat chondrosarcoma have properties very similar to the protein core of the proteoglycan aggregates, in which a specific link protein is not required. They suggest that formation of proteoglycan aggregates is an expression of the polydispersity of the proteoglycans. In their model, proteoglycans of low buoyant density (high protein content) help stabilize aggregate structures of high buoyant density proteoglycans and hyaluronic acid.

Preparation of Dissociative Gradient Fractions of Proteoglycan Aggregates

Proteoglycan aggregates (Al) were isolated from 4 M GdmCl extracts of the Swarm rat chondrosarcoma as previously described (7). The associative gradients were cut into two fractions. The bottom two-fifths or the Al fraction (5) was diluted 1:1 with water and GdmCl was added to increase the concentration of GdmCl to 4 M. CsCl was then added to adjust the density of the solution to 1.50. Dissociative gradients were established by centrifugation in a Beckman Ti-50 rotor at 40,000 rpm for 40 to 48 h at 10°. The gradients were cut into 4 parts, labeled A1-D1 to A1-D4, from bottom to top (5). In most of the experiments, to obtain a finer fractionation, the gradient was cut into 5 equal parts, labeled A1-D1a, A1-D1b to A1-D4.

Comparison of Size of Proteins in A1 and A1-D Fractions

Chondroitin sulfate chains were removed from the core proteins by incubation with chondroitinase ABC for 60 min at 37°, as previously described (7). The glycosaminoglycans in these fractions were also separated and quantified by the use of the microradioloue column procedure of Antonopoulos et al. (28). The amount of glucosamine and galactosamine in each fraction was determined. After digestion of the A1-D1 fraction through A1-D3 fractions with papain or treatment with 0.5 M sodium hydroxide for 24 h at 4°, the relative sizes of the chondroitin sulfate chains in the fractions were estimated by gel permeation, using analytical columns of Sepharose 6B.

In the electrophoretic analyses, samples were applied to 3 or 7% polyacrylamide gels in the presence of sodium dodecyl sulfate and mercaptoethanol and run as described by Weber and Osborn (29). The gels were stained with Coomassie blue (29) or periodic acid-Schiff reagent for carbohydrate (30). The molecular weights of the separated components were estimated by comparing their positions in the gels with those of proteins of known molecular weights (29, 31).

Column Chromatography

Analytical columns (0.9 x 165 cm) were packed with either Sepharose 2B, Sepharose 4B, or Sepharose 6B. Elution and analyses of the effluent by automated procedures were as previously described (7).

For preparative purposes, columns (0.9 x 100 cm) were packed with Sepharose G-200 (medium) or Sephadex G-150 (medium) in 4 M GdmCl, 0.05 M sodium acetate, pH 5.8. The samples were applied in then eluted with the above solvent. The flow rate was 1.4 to 1.5 ml/h at a hydrostatic pressure of about 10 cm. Fractions of 0.7 ml were collected.

Preparation of Protein Core of Al-D1a

The protein core of the proteoglycan monomer (Al-D1a) was prepared by chondroitinase ABC digestion and it was purified as previously described (7). The material eluted from the Sepharose 4B column at Kav = 0.60 was examined in the analytical ultracentrifuge. To this end, this fraction was dialyzed three times, for 12 h each time, against 100 volumes of 0.5 M sodium acetate, pH 7.0, at 4°. The final dialysate was used for the preparation of reagent blanks and for determinations of the retardation for analysis in the analytical ultracentrifuge.

The systematic nomenclature of Heinegdrd is used throughout this report (5). Sequential steps are indicated by letters; A indicates associative (low salt) and D, dissociative (4 M GdmCl) gradient steps, with a number to indicate the gradient fraction. A1 indicates the bottom two-fifths of an associative gradient, with A2, A3, and A4 each being one-fifth portions of gradient going from bottom to top. Sequential treatment of a sample is indicated by a hyphen and the next steps. The term A1-D1 indicates the bottom fraction from a dissociative gradient when the A1 fraction is used.

1 Beckman Instruments has recently cautioned against the use of high concentrations of salts that may form crystals in lower parts of gradients. For alternate procedure see Ref. 13 or contact Beckman Instruments, Palo Alto, Ca.
Table I

Composition of fractions of dissociative gradient when AI preparation of proteoglycans extracted from Swarm rat chondrosarcoma was used

| Fraction  | Density (g/ml) | Total protein (mg) | Total GalN (µmol) | Total uronic acid (µmol) | GalN/µmol protein | GluN/GalN |
|-----------|----------------|-------------------|-------------------|-------------------------|-----------------|-----------|
| A1-D1a    | 1.609          | 13.80             | 198               | 229                     | 14.30           | 0.144     |
| A1-D1b    | 1.583          | 0.53              | 2.42              | 3.24                    | 4.55            | 0.149     |
| A1-D2     | 1.485          | 0.40              | 1.10              | 2.24                    | 2.75            | 0.178     |
| A1-D3     | 1.428          | 0.27              | 0.57              | 1.32                    | 2.42            | 0.107     |
| A1-D4     | 1.405          | 1.52              | 0.40              | 0.65                    | 0.26            | 0.200     |
| A1-D4-G200| 0.86           | 0.13              | 0.13              | 1.00                    | 1.00            |           |
| A1-D4-G200b| 0.70        | 0.08              | 0.08              | 0.01                    | 1.00            |           |

These ratios of GluN to GalN found in hyaluronan of the materials without prior separation by the procedure of Antonopoulos et al. (28).

Preparation of High Molecular Weight Hyaluronic Acid

The preparation of the hyaluronic acid from umbilical cords (2 mg in 1 ml of 0.5 M sodium acetate buffer, pH 7.0) was fractionated by the use of a Sepharose 2B column. Elution was with 0.5 M sodium acetate buffer, pH 5.8. The fraction with Kav of 0.30 was designated as the high molecular weight fraction (HA-H). The other hyaluronic acid sample (HA-L), estimated average molecular weight of 120,000, was more polydisperse than the preparation from umbilical cords. On the same column its Kav was 0.61.

Interaction of A1-D1a with Hyaluronic Acid and Link Protein

Solutions of the materials were prepared as follows: A1-D1a was dissolved in 4 M GdmCl 0.05 M sodium acetate buffer, pH 5.8, at a concentration of 5.8 mg/ml; the hyaluronic acid (HA), or HA-H, was dissolved in 0.5 M sodium acetate buffer, pH 7.0, at a concentration of 360 µg/ml; and the purified link protein (A1-D4-G200b) in 4 M GdmCl, 0.05 M sodium acetate buffer, pH 5.8, at 221 µg/ml. The solutions were mixed in varying proportions and diluted to 1.1 ml with 4 M GdmCl. Incubation was performed in 0.5 M sodium chloride, pH 5.8. To promote aggregation, the mixtures were dialysed at 4°C against three changes of 0.5 M GdmCl, 0.05 M sodium acetate buffer, pH 5.8, each for 12 h. The final dialysate was used as the reference blank in the ultracentrifugal studies. Aliquots of the retentates were also chromatographed on Sepharose 2B columns.

Analytical Ultracentrifugation

Sedimentation Velocity – To determine the effect of the purified link protein on the stabilization of complexes of proteoglycan monomer (A1-D1a) and hyaluronic acid, samples were centrifuged at 40,000 rpm in a 12-mm double-sector cell with sapphire windows. The temperature was 20°C. Schlieren patterns were photographed with the camera lens focused on the midplane of the cell. The photographic plates were scanned with a Nikon comparator.

Sedimentation Equilibrium – Sedimentation equilibrium experiments, using the meniscus depletion method of Yphantis (32), were performed on the proteoglycan core (A1-D1a) of rat chondrosarcoma, as described by Hascall and Riolo for chondroitinase-treated proteoglycan of bovine nasal septa (21). Protein concentrations were 164, 112, and 88 µg/ml. An ANI rotor was used at speeds of 9,000, 10,000, and 11,000 rpm and at a temperature of 20°C. A partial specific volume of 0.67 g/ml was calculated from a dry weight protein content of 51.2%, assuming a partial specific volume of 0.72 g/ml for protein and 0.52 g/ml for the carbohydrate portion. The data were collected and calculated as described by Hascall and Riolo (21). Apparent weight average molecular weights were obtained by extrapolation to zero protein concentration.

Characteristics of A1-D Fractions – A typical distribution of protein, hexuronic acid, and galactosamine in a dissociative cesium chloride gradient of A1 preparations from the Swarm rat chondrosarcoma is shown in Table I. About 84% of the protein and 98% of the galactosamine and hexuronic acid are present in the bottom fifth of the gradient. In this fraction (A1-D1a) the ratio of galactosamine to protein is higher than in any of the other fractions; the ratio of galactosamine to protein decreases as the buoyant density of the fractions decreases. On the other hand, the ratio of gluconosamine to galactosamine in the proteoglycans increases as the buoyant density of these decreases. Realizing that hyaluronic acid was present in fractions of intermediate buoyancy, the gluconosamine of the hyaluronic acid was eliminated from the estimate of the ratios of gluconosamine to galactosamine by the use of the procedure of Antonopoulos et al. (28) for the estimation of hyaluronic acid in the fractions.

As the buoyant densities of the proteoglycans in a dissociative gradient decrease, their molecular weights apparently decrease; they are retarded to a progressively greater extent on a column of Sepharose 2B (Table II). This, however, is not a reflection of shorter chondroitin sulfate chains. It appears, Table II, that the average length of the chondroitin sulfate chains prepared by either digestion with papain or treatment with 0.5 M sodium hydroxide, is the same in the proteoglycans at all levels of the dissociative gradient. These data in conjunction with the data in Table I, which clearly indicate a decreas-
Table II

Size of proteoglycans and chondroitin sulfate chains at decreasing buoyant densities of dissociative gradient when A1 preparation from Swarm rat chondrosarcoma was used

The fractions were reduced and alkylated for the analysis on the Sepharose 2B columns. For analysis on the Sepharose 6B columns, the fractions were either treated with papain (a) or with 0.5 M sodium hydroxide (b) as described under "Experimental Procedures." Skewness was calculated as $K_m$, of the mode/$K_m$, of 50% of the material from $V_m$. Polydispersity was calculated as the interval of 50% of the material from the mode/$V_m - V_m$.

| Fraction | $K_m$, 2B | $K_m$, 6B | Skewness on 2B | Polydispersity on 2B |
|----------|------------|------------|----------------|---------------------|
| A1-D1a   | 0.14       | 0.48*      | 1.0            | 0.2                 |
| A1-D1b   | 0.16       | 0.56, 0.50*| 1.1            | 0.4                 |
| A1-D2    | 0.24       | 0.46, 0.50*| 1.1            | 0.4                 |
| A1-D3    | 0.42       | 0.50, 0.50*| 1.7, 1.4*      | 0.8, 0.6*           |

The protein core of the proteoglycans decreases in size as the buoyant density of the proteoglycan decreases. The proteoglycan fractions were treated with chondroitinase ABC to remove the chondroitin sulfate chains (95% digestion). Such a treatment produced protein cores which still carried the linkage region of neutral sugars and an average of 1.5 repeat disaccharides of the chondroitin sulfate chains. When these samples, after reduction with $\beta$-mercaptoethanol, were electrophoresed in 3% polyacrylamide gels in the presence of sodium dodecyl sulfate, it was found (Fig. 1) that the mobility of the protein cores increased as the buoyant density of the proteoglycan fractions decreased. This decrease in the size of the protein cores occurred in discrete steps, since as seen in the electrophoretograms, the protein cores in any given fraction contained one or more clearly separated protein entities. In A1-D1a only a single protein component was seen. Two components were readily discerned in A1-D1b; a slower migrating component was in higher concentration than a faster migrating component (Fig. 1B). The reverse was seen in the case of A1-D2 (Fig. 1C). A third protein component was additionally present in the A1-D3 fraction; its mobility (Fig. 1D) was even greater than that of the two protein components seen in A1-D1b and A1-D2. The protein core of the proteoglycans in the A1-D4 is primarily of the size of the fastest migrating protein component in the A1-D3 fraction. The A1-D4 fraction, however, also contains two other protein components, whose mobility is less than that of the major protein component, but decidedly faster than that of the two protein components seen in A1-D1b and A1-D2. Each of the fractions was recentrifuged in a dissociative isopycnic cesium chloride gradient at the same starting density. The buoyant density of the fractions was not altered. Nor were the protein/uronic acid ratios changed thereby. Moreover, the electrophoretograms of the recentrifuged fractions were indistinguishable from the electrophoretograms of the fractions before recentrifugation.

For comparison, the protein components in A1 preparations are shown in Fig. 1F. It is apparent from such electrophoretograms that in A1 preparations there is a mixture of proteoglycans, since their protein cores vary in size. It is further apparent that in such preparations a protein core of lowest mobility is present in highest concentration.

To obtain an estimate of the molecular weight of the protein core in the A1-D1a fraction (Fig. 1A), the A1-D1a fraction was treated with chondroitinase ABC, as above. The resultant protein core was examined in the analytical ultracentrifuge, using the techniques of sedimentation to equilibrium. On extrapolation of the apparent molecular weights to zero protein concentration, it was found that the molecular weight of the protein core was in the range of $2.0 \times 10^6$ to $2.2 \times 10^6$.

The amino acid profiles of the proteoglycan fractions, expressed in residues per 1000 amino acid residues are given in Table III. Certain progressions are apparent. As the buoyant density of the fractions decreases, the protein core contains progressively more lysine and arginine and less threonine and serine. There is also an increase in the ratio of glucosamine of oligosaccharides (1% cetylpyridinium chloride fraction) to galactosamine of chondroitin sulfate as the buoyant density of the proteoglycan fractions decreases (Table I). Since the proteoglycan of the Swarm rat chondrosarcoma lacks keratan sulfate, this decrease in the number of chondroitin sulfate chains attached to the protein core provides an explanation for the reduced intensity of this linkage site in the A1-D fractions.
of the link protein was different than that of the hyaluronic
fraction of a dissociative gradient on a column of Sephadex G-
resin in polyacrylamide gels, one protein component was found
hyaluronic acid-binding peptide derived from the protein core
When such pools of the fractions were examined by electropho-
resis indicated by the bar above Peak II were pooled.

From columns of Sepharose 2B.

ported (7) that when hyaluronic acid is at about this concen-
ature in a mix of hyaluronic acid and proteoglycan monomer
present in Fractions Al-D2 and Al-D3 and accounted for 0.8%
(Fig. 3, A and B). Its mobility was greater than that of the
complex from the proteoglycan monomers in the Al-D4
fraction. Additionally, it has more aspartic acid, alanine, tyrosine, and phenylalanine and less threonine, serine, and
glycine than the protein core in the Al-Dla fraction.

Examination of the papain digests of the Al-D fractions for
the glycosaminoglycans in them by the method of Antonopou-
lou et al. (29) indicated that most of the hyaluronic acid was
in terms of residues per 1000 amino acid residues, also has more lysine and argin-
ine and less threonine and serine than the protein core of the
proteoglycans in the Al-Dla fraction. Additionally, it has more
aspartic acid, alanine, tyrosine, and phenylalanine and less proline and glycine than the protein core in the Al-Dla fraction.

Nature of Link Protein – The separation of the link protein
(A1-D4-G200,) from the proteoglycan monomers in the Al-D4
fraction of a dissociative gradient on a column of Sephadex G-
200 with 4 M GdmCl as the eluent was as shown in Fig. 2. The
fractions indicated by the bar above Peak II were pooled.

When such pools of the fractions were examined by electropho-
resis in polyacrylamide gels, one protein component was found
(Fig. 3, A and B). Its mobility was higher than that of the
hyaluronic acid-binding peptide derived from the protein core
of the Al-Dla fraction (Fig. 3C). The amino acid composition
of the link protein was different than that of the hyaluronic
acid-binding peptide and that of the protein cores of the proteo-
glycans in the A1-D fractions (Table III). In terms of residues
per 1000 amino acid residues, there was significantly more
lysine, arginine, aspartic acid, glycine, half-cystine, tyrosine,
and phenylalanine and less threonine, serine, glutamic acid,
alanine, valine, and methionine in the link protein than in the
hyaluronic acid-binding peptide. Significantly more aspartic
acid, alanine, half-cystine, tyrosine, and phenylalanine and
less threonine, serine, and methionine were found in the link
protein than in the protein cores of the proteoglycans in all of
the A1-D fractions.

Small amounts of glucosamine and galactosamine were
found in preparations of the link protein. In view of the
identity of the ratio of these hexosamines in the preparations
of the link protein and the ratio of these hexosamines in the
proteoglycans (A1-D4-G200,) in the A1-D4 fraction, it is possi-
able that the link protein preparations (A1-D4-G200,) may still
contain a small amount of proteoglycan. Caterson and Baker
(36) also found these amino sugars to be present in link pro-
tein(s) isolated from bovine nasal septa. Attempts to deter-
mine whether other sugars were present in the link protein
preparations have been unsuccessful, possibly because of the
small amounts of material used. If there are other sugars, the
sum of these is probably less than 2% of the weight of the link
protein, as suggested by the use of phenolsulfuric acid assay
(7) and by the use of the electrophoretic procedure of Segrest
and Jackson (31). That the carbohydrate content may be very
low is also suggested by the virtual absence of periodic acid-
Schiff-positive staining of the link protein in acrylamide gels
after electrophoresis. In line with this is the virtual absence of
neutral sugars in the effluents in which the link protein appeared when columns of Sephadex G-200 were used for
separating it from proteoglycans in the A1-D4 fraction (Fig. 2).

Interaction of Link Protein with Hyaluronic Acid and with
Proteoglycan Monomers – In the absence of hyaluronic acid or
of proteoglycans, the link protein was retarded on a column of
Sepharose 2B; it appeared in the effluent near the Vp of the

\begin{table}[h]
\centering
\caption{Amino acid compositions of materials separated by centrifugation of A1 in dissociative gradient compared with that of link protein and of hyaluronic acid (HA) binding region of proteoglycan monomers from Swarm rat chondrosarcoma.}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Amino acid & A1-Dla & A1-Dlb & A1-D2 & A1-D3 & A1-D4-G200 & A1-D4-G200b & HA binding region
\hline
residues/1000 residues
\hline
Lysine & 18 & 27 & 37 & 48 & 52 & 49 & 36
Histidine & 24 & 17 & 17 & 22 & 22 & 18 & 24
Arginine & 29 & 35 & 35 & 48 & 48 & 58 & 49
Aspartic acid & 73 & 82 & 84 & 99 & 99 & 131 & 106
Threonine & 91 & 84 & 76 & 67 & 65 & 51 & 68
Serine & 135 & 123 & 116 & 99 & 84 & 61 & 83
Glutamic acid & 133 & 142 & 139 & 128 & 131 & 106 & 135
Proline & 83 & 79 & 77 & 64 & 64 & 52 & 59
Glycine & 138 & 126 & 127 & 115 & 102 & 109 & 91
Alanine & 64 & 71 & 73 & 77 & 76 & 83 & 92
\(1,3\)-Cystine & 7 & 11 & 14 & 11 & 13 & 24 & 10
Valine & 54 & 51 & 51 & 51 & 59 & 47 & 60
Methionine & 5 & 8 & 7 & 11 & 12 & 3 & 7
Isoleucine & 28 & 25 & 25 & 24 & 24 & 25 & 34
Leucine & 74 & 63 & 69 & 70 & 79 & 82 & 83
Tyrosine & 16 & 25 & 18 & 12 & 25 & 54 & 28
Phenylalanine & 27 & 29 & 28 & 34 & 35 & 46 & 37
\hline
\end{tabular}
\end{table}
FIG. 2. Gel filtration of the A1-D4 fraction on Sephadex G-200 in the presence of 4 M GdmCl. The materials in the peaks were pooled as indicated by the bars above the peaks. The material in Peak I and Peak II were designated A1-D4-G200I and A1-D4-G200II (link protein), respectively, on the basis of absorbance at 280 nm and content of hexuronic acid. The column was prepared and eluted as described in the text. Absorbance at 280 nm, -; protein, - - - -; hexuronic acid, A - - - -; total sugar as glucose by phenolsulfuric acid, A - - - -; samples were dialyzed against 0.5 M sodium acetate, pH 7.0, before protein and total sugar were determined.

FIG. 3. Disc electrophoresis of link protein (A1-D4-G200I) and of the hyaluronic acid-binding peptide of the protein core of an A1 preparation. A, 10 μg of A1-D4-G200, subjected to discontinuous electrophoresis. The arrow indicates the tracking dye. B, 10 μg of A1-D4-G200I in a 7% polyacrylamide gel in the presence of sodium dodecyl sulfate. C, densitometric tracing of a 7% polyacrylamide gel when 10 μg of the hyaluronic acid-binding peptide was electrophoresed under the same conditions used in B.

FIG. 4. Gel filtration of link protein and of hyaluronic acid. An analytical column of Sepharose 2B was used as described in the text. Link protein alone (-----); hyaluronic acid alone (-----); link protein and hyaluronic acid in combination (protein, - - - -; and hexuronic acid, - - - -).

FIG. 5A. Only a single component was seen when such preparations were examined in the analytical ultracentrifuge (Fig. 5F). On the addition of link protein to the A1-D1a, neither the schlieren pattern nor the elution profile (Fig. 5C) from the Sepharose 2B column were altered. However, most, if not all of the link protein was in association with the proteoglycan in that none of the link protein was detected at Vf of the Sepharose 2B column. When a mixture of hyaluronic acid and proteoglycan was used the schlieren pattern was again as that seen with the proteoglycan alone (Fig. 5F'), but the elution profile was markedly altered (Fig. 5B). The elution profiles of a mixture of link protein, hyaluronic acid, and proteoglycan monomers were comparable to the elution patterns of a mixture of hyaluronic acid and proteoglycan (Fig. 5, D and E), but now the schlieren patterns indicated that some of the proteoglycan was in the form of aggregates (Fig. 5, G and H).

In this series of experiments, Fractions A1-D1a through A1-D3 were recombined in the original proportions and then examined in the ultracentrifuge. The aggregated form of the proteoglycans was not seen. The aggregated form of the proteoglycans also was not seen when to such mixtures of the A1-D1a through A1-D3 fractions the proteoglycans (A1-D4-G200) of the A1-D4 fraction were added in amounts proportional to that originally present in the A1-D4 fraction. These data strongly suggest that the proteoglycans of low molecular weight do not stabilize hyaluronic acid-proteoglycan complexes.

Link protein in sufficient amounts was not available to extend these experiments so that a stoichiometric relation of link protein to proteoglycan in aggregate preparations could be precisely established. However, as shown in Table I, all of the protein in an A1-D4 fraction was subfractionated into proteoglycans of low buoyant density (A1-D4-G200I) and link protein (A1-D4-G200II). The weight of the latter, 0.76 mg, is equivalent to 0.020 μg, assuming a molecular weight of 40,000 for the link protein. The amount of protein core in the A1 preparation from which this amount of the link protein was isolated can be estimated by summing the amounts of protein in the A1-D1a through A1-D4 fractions minus the protein in the A1-D4-G200I subfraction. Supposedly, only 70% of this protein in the form of proteoglycan monomers was in the aggregate form, as suggested by the areas of the peaks seen in schlieren patterns of this A1 preparation. With this factor in
Proteoglycan monomers in the aggregated form is 0.055 × 10^6, molecular weight of 200,000, the calculated concentration of link protein/proteoglycan monomer is 0.36. The dissociation of proteoglycan aggregates from adult hyaline cartilage is in the middle of the gradient.

In the upper portions (Al-D4 fraction) of dissociative gradients of proteoglycan aggregates from cultures of chick limb-bud chondrocytes contain only a link protein of 45,000 molecular weight (13). Interestingly, proteoglycan aggregates isolated from chick limb buds at stage 25 also contain a protein of Mr = 45,000, but by stage 35 a link protein of Mr = 40,000 is additionally present (39). Pita et al., using ultramicro methods, found only a link protein of Mr = 45,000 in the intercellular fluid from epiphyseal growth cartilage of young rats. In the proteoglycan aggregates from the Swarm rat chondrosarcoma only one link protein, molecular weight of 40,000, was found (7). Moreover, it was shown that the Al-D4 fractions in which it was present could substitute for Al-D4 fractions from bovine nasal septa, which contain two link proteins, in the stabilization of proteoglycan aggregates of the bovine nasal septa. In turn, the Al-D4 fraction of the bovine nasal septa could substitute for the Al-D4 fraction of the Swarm rat chondrosarcoma in the stabilization of aggregates of the proteoglycans from the rat chondrosarcoma.

The link protein of the Swarm rat chondrosarcoma has been isolated in highly purified form and has been partially characterized. It has been shown to be capable of stabilizing complexes of well characterized proteoglycan monomers with hyaluronic acid. Recently, Caterson and Baker (36) using more complicated procedures, isolated a mixture of two link proteins from bovine nasal septa. From their brief report, it would seem that the material isolated by them is comparable in many respects to the link protein of the rat chondrosarcoma, but due to the reagents employed during isolation may be inactive.

The molecular weight of the link protein is 40,000, as previously reported (7). Its amino acid composition differs significantly from that of the hyaluronic acid binding region of the protein core of the proteoglycan monomers. Its amino acid composition is also significantly different from that of the intact protein cores of even the most buoyant proteoglycan fractions, i.e., Al-D3 and Al-D4–G200. The link protein differs in yet another way from the proteoglycan monomers: it is insoluble in water.

In vitro, the differences between complexes of proteoglycans and hyaluronic acid and proteoglycan aggregates are 2-fold. The latter is more stable and it is less sensitive to treatment with trypsin (22). The nonequivalent interactions of the link protein with both the hyaluronic acid and the protein core probably bring these three components into a spatial relationship such that the link protein and a portion of the protein core of the proteoglycans are less accessible to trypsin. The hyaluronic acid of the proteoglycan aggregates is also protected. Faltz et al. (30) have reported that even after limited digestion of proteoglycan aggregates with trypsin and chondroitinase ABC the hyaluronic acid of the complex yielded fragments the molecular weights of which were 7,000 to 10,000 (18 to 25 repeat disaccharides).

Proteoglycan aggregates have been shown to have a greater thermal stability than complexes of hyaluronic acid and proteoglycan monomers (40). Additionally, both Hardingham and Muir and Swann et al. (41) have recently shown that complexes of proteoglycans and hyaluronic acid can be detected by

DISCUSSION

Link protein, which stabilizes the complex of proteoglycan monomers and hyaluronic acid, is separable from the latter two components of aggregate preparations by centrifugation in a dissociative cesium chloride gradient. It is found, as is a small amount of proteoglycans of relatively low molecular weight, at the top of the gradient. The bulk of the proteoglycan monomers are at the bottom of the gradient and the hyaluronic acid is in the middle of the gradient.

In the upper portions (Al-D4 fraction) of dissociate gradients of proteoglycan aggregates from adult hyaline cartilages (38) and from a human chondrosarcoma, two proteins have been found, with molecular weights estimated by polyacrylamide gel electrophoresis as 40,000 and 45,000. Proteoglycan aggregates from cultures of chick limb-bud chondrocytes contain only a link protein of 45,000 molecular weight (13). Interestingly, proteoglycan aggregates isolated from chick limb buds at stage 25 also contain a protein of Mr = 45,000, but by stage 35 a link protein of Mr = 40,000 is additionally present (39). Pita et al., using ultramicro methods, found only a link protein of Mr = 45,000 in the intercellular fluid from epiphyseal growth cartilage of young rats. In the proteoglycan aggregates from the Swarm rat chondrosarcoma only one link protein, molecular weight of 40,000, was found (7). Moreover, it was shown that the Al-D4 fractions in which it was present could substitute for Al-D4 fractions from bovine nasal septa, which contain two link proteins, in the stabilization of proteoglycan aggregates of the bovine nasal septa. In turn, the Al-D4 fraction of the bovine nasal septa could substitute for the Al-D4 fraction of the Swarm rat chondrosarcoma in the stabilization of aggregates of the proteoglycans from the rat chondrosarcoma.

The link protein of the Swarm rat chondrosarcoma has been isolated in highly purified form and has been partially characterized. It has been shown to be capable of stabilizing complexes of well characterized proteoglycan monomers with hyaluronic acid. Recently, Caterson and Baker (36) using more complicated procedures, isolated a mixture of two link proteins from bovine nasal septa. From their brief report, it would seem that the material isolated by them is comparable in many respects to the link protein of the rat chondrosarcoma, but due to the reagents employed during isolation may be inactive.

The molecular weight of the link protein is 40,000, as previously reported (7). Its amino acid composition differs significantly from that of the hyaluronic acid binding region of the protein core of the proteoglycan monomers. Its amino acid composition is also significantly different from that of the intact protein cores of even the most buoyant proteoglycan fractions, i.e., Al-D3 and Al-D4–G200. The link protein differs in yet another way from the proteoglycan monomers: it is insoluble in water.

In vitro, the differences between complexes of proteoglycans and hyaluronic acid and proteoglycan aggregates are 2-fold. The latter is more stable and it is less sensitive to treatment with trypsin (22). The nonequivalent interactions of the link protein with both the hyaluronic acid and the protein core probably bring these three components into a spatial relationship such that the link protein and a portion of the protein core of the proteoglycans are less accessible to trypsin. The hyaluronic acid of the proteoglycan aggregates is also protected. Faltz et al. (30) have reported that even after limited digestion of proteoglycan aggregates with trypsin and chondroitinase ABC the hyaluronic acid of the complex yielded fragments the molecular weights of which were 7,000 to 10,000 (18 to 25 repeat disaccharides).

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1. T. Oegema, unpublished data.
2. J. C. Pita, F. J. Noller, and J. E. Madruga, personal communication.
3. T. E. Hardingham and H. Muir, personal communication.
the use of an ultracentrifuge, but only if the pH of the solution is above 7 and the concentration of the hyaluronic acid is greater than 1%. Such complexes were nearly completely dissociated at pH 5.8, the value used in the present study to demonstrate the stabilization of proteoglycan aggregates by the link protein.

The results of the present studies reaffirm the link protein as an entity different from proteoglycans of low buoyant density in which the protein to carbohydrate ratio is high. It is probable that in aggregates of hyaluronic acid, proteoglycan monomers and link protein the latter two components are in a 1:1 relationship. It may be that it promotes and then stabilizes complexes of proteoglycan monomers with hyaluronic acid in cartilages so that they function as they do.

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