Characteristics of the Protein-Keratan Sulfate Core and of Keratan Sulfate Prepared from Bovine Nasal Cartilage Proteoglycan

VINCENT C. HASCALL AND RICK L. RIOLO
From the School of Dentistry, The University of Michigan, Ann Arbor, Michigan 48104

SUMMARY

Protein-keratan sulfate core was isolated from bovine nasal cartilage proteoglycan after enzymatically removing chondroitin sulfate with chondroitinase AC from Flavobacterium heparinum. The core preparation exhibited a broad, approximately Gaussian distribution of buoyant densities in CsCl density gradients (between 1.43 and 1.58 g per ml with an average of 1.51 g per ml). Chemical and physical analyses of subfractions from the density gradient indicate that the core macromolecules have a wide polydispersity in molecular weights (350,000 to 550,000, with an average of 450,000). Molecules with larger molecular weights have greater buoyant densities and higher ratios of keratan sulfate to protein. The data suggest that the core molecules contain a protein (or combination of proteins) with a molecular weight of about 200,000 to which different amounts of keratan sulfate are attached. Differences in average keratan sulfate chain lengths and chain numbers appear to contribute to the polydispersity of the protein-keratan sulfate core.

Keratan sulfate was isolated from the core preparation after proteolysis with papain and purification with DEAE-cellulose chromatography. Chemical and physical analyses of the keratan sulfate indicate that the polysaccharide exhibits a large polydispersity of molecular weights with a weight average molecular weight of about 8,500 and a z-average molecular weight of about 11,000, and that polysaccharide molecules with larger molecular weights have higher ratios of glucosamine to galactosamine. Alkali treatment of the keratan sulfate selectively destroys threonine and serine as it does for other skeletal keratan sulfates. After alkaline treatment the keratan sulfate chromatographs on Sephadex G-100 with a higher retention volume, $K_d = 0.6$, than that for the untreated preparation, $K_d = 0.3$, which indicates that the sizes of the molecules have been significantly reduced. The data are consistent with the hypothesis that the papain-prepared keratan sulfate consists of molecules which contain two polysaccharide chains, each with an average of about six repeat disaccharides, connected through a peptide bridge. Each chain probably contains a terminal sialic acid residue and is attached to the protein with a glycosidic bond between galactosamine and a threonine or serine residue. The high concentration of glutamic acid in the fraction suggests that the polysaccharide chains are also attached in some way to this amino acid. Such a model suggests that the intact proteoglycan macromolecules each contain about 60 keratan sulfate chains of variable lengths built onto the protein core structure.

EXPERIMENTAL PROCEDURE

Materials and Methods—CsCl (optical grade) was purchased from Schuchardt Biochemicals. Fluorocarbon FC-43 was purchased from Beckman Instruments. N-(Morpholino)ethane sulfonic acid was purchased from Calbiochem. All other chemicals were reagent grade.

Procedures for the analysis of hexosamines, amino acids, uronic acid, 4,5-unsaturated uronic acid, sialic acid, neutral sugars, protein, and potassium ion were described previously (1).

Chondroitinase AC from F. heparinum, prepared by Seikagaku

1 The proteoglycan subunit preparation is referred to as subunit in the text. The protein-keratan sulfate core preparation isolated after treatment of subunit with chondroitinase AC from F. heparinum is referred to as core (1).

* This work was supported by National Institute of Dental Research Grant DE-02731.
Fine Biochemicals, Tokyo, Japan according to the procedure of Yamagata et al. (2), was purchased from Miles Research Laboratory. Papain (twice crystallized) was purchased from Worthington Biochemicals.

Proteoglycan subunit was prepared from bovine nasal cartilage as described previously (3). Subunit was treated with chondroitinase AC and the potassium salt of protein-keratan sulfate core isolated as described previously (1).

**Analytical Centrifugation of Core in CsCl Density Gradient**—The potassium salt of core was dissolved overnight directly into a CsCl solution with an initial density of 1.50 g per ml. An aliquot, with 2 mg per ml, was placed in one sector of an analytical ultracentrifuge cell equipped with a 12-mm Epon double- sectored centerpiece and a negative 1° wedge upper window; an aliquot of the CsCl solvent was placed in the other sector to provide a reference blank. The cell was centrifuged at 36,000 rpm in a model E ultracentrifuge at 20° for 48 hours to establish an equilibrium density gradient. Schlieren patterns of the equilibrium gradient were photographed with the analyzer set at an angle of 45°. The vertical distance between the solution and solvent patterns in the photographs were measured at different radial positions in order to estimate the gradient of refractive index, dn/dr, for the solute. The area under the schlieren solute curve was determined by a procedure similar to that described by Silpananta et al. (4). The areas of successive radial trapezoids at close intervals were summed across the entire pattern. The area of each trapezoid was weighted by a factor (ri2/rc2) to correct for the radial dilution which occurs in the sectors of the cell, where ri is the radius of the midpoint of any given trapezoid and r, was chosen as the radius in the pattern where the solute-solvent curves cross. The solvent densities at different radial positions were determined from the solvent pattern as described by Chervenka (Reference 5, Equations 21 to 23). The integral curve, which shows the relative solute concentration at points within the gradient, was then plotted as a function of solvent density.

The average partial specific volume for the potassium salt of core was estimated from the average buoyant density of the sample in the CsCl gradient as described by Silpananta et al. (4). Two assumptions were made: (a) the equivalence of core is 1.1 meq per g as estimated from its potassium content (1), and (b) essentially none of the charges are neutralized by excess counter-ion, α = 0 (4).

**Preparative Centrifugation of Core in CsCl Density Gradient**—An equilibrium gradient was established in a preparative centrifuge with conditions designed to give a range of densities similar to the analytical gradient described above. A core solution, 2 mg per ml in CsCl with an initial density of 1.50 g per ml, was centrifuged for 70 hours in an SW 50.1 rotor at 36,000 rpm, 20°. A Beckman tube slicer was used to separate the gradient into 10 approximately equal portions, referred to as Fractions 1 to 10 from the top to the bottom of the gradient. The densities of the fractions were determined with a 500-μl pipette as a pycnometer.

Aliquots of the two center fractions, 5 and 6, from the gradient were diluted to a solute concentration of about 2 mg per ml and the CsCl concentration adjusted to give an initial density of 1.50 g per ml for each. The samples were then centrifuged to equilibrium in a model E ultracentrifuge at 56,000 rpm, 20°, 48 hours. Data from photographs of the equilibrium schlieren patterns were integrated to give curves of the relative solute concentration as a function of density within the gradient as described above.

Portions of each of the ten fractions from the preparative gradient were dialyzed at 4° against several changes of 0.25 M KCl. The protein, hexosamine, amino acid, and unsaturated hexuronic acid contents of each fraction were determined. The results of these analyses were used to estimate the amounts of protein, keratan sulfate, and residual chondroitin sulfate attachment oligosaccharide in each fraction.

**Sedimentation Equilibrium Centrifugation of Core Fractions**—Aliquots of Fractions 4, 6, and 8 which had been dialed against 0.25 M KCl were investigated with sedimentation equilibrium centrifugation. Three solute concentrations were prepared in 0.25 M KCl for each of the three fractions; approximately 0.24, 0.16, and 0.08 mg per ml. Each channel on the solvent side of a three-sample, 12-mm, Kel F centerpiece in a cell assembly equipped with sapphire windows received 0.12 ml of 0.25 M KCl diluted. Each channel on the solute side received 0.02 ml of fluorocarbon FC-43 and 0.09 ml of one of the three solute concentrations for one of the fractions. The assembled cell was centrifuged at 11,000 rpm and 20° in an AN-J rotor. Interference patterns of the equilibrium distributions were photographed after 36 and 48 hours of centrifugation. The fringe displacements and the radial positions in the patterns were determined. The radii of the bottoms of the solute columns, r, were determined from the positions of the solute-fluorocarbon interfaces in patterns from early times in the centrifugal runs. Data from solvent-solvent blanks indicated that baseline corrections to the solute-solvent patterns were unnecessary. The interference patterns observed at 36 and 48 hours were the same within experimental error.

For the three core fractions studied, the centrifugal conditions described above achieve the criteria described by Yphantis (6) for the meniscus depletion method of determining apparent molecular weights for the solute. Therefore, the data obtained for each equilibrium distribution were analyzed with a computer program developed by Roark and Yphantis (7) in order to calculate the reduced number average, Mn(r), and reduced weight average, MW(r), molecular weights at the different radial positions, r, within the patterns. These were converted to apparent number average, M, and apparent weight average, MW, molecular weights from the relation

\[ M(r) = \frac{\sigma_0(r) RT}{\omega^2(1 - \bar{e})} \]  

The value of the density of the solvent, ρ, for 0.25 M KCl at 20° was calculated from International Critical Tables to be 1.0101 g per ml (8). Values of the partial specific volumes of the solutes, \( \bar{e} \), were determined from the average composition of each fraction with values of 0.72 ml per g for the protein and of 0.62 ml per g for the polysaccharide portion of the molecules. The value for the protein was calculated from the amino acid composition of subunit as described previously (9). The value for the polysaccharide was estimated from the value of \( \bar{e} \) for the potassium salt of the core preparation as is discussed below.

The values of \( M_n(r) \) for each solute concentration obtained...
for each fraction were plotted against values of the concentrations, $c(r)$, at different radial positions in the patterns, and also against the parameter $(r^2 - r^2)/2$ as suggested by Yphantis (6). Weight average and z-average molecular weights for the fractions were estimated from the data as discussed below.

Preparation of Keratan Sulfate from Core—Core, 25 mg, was dissolved in 5 ml of 1 m potassium acetate, 0.005 m sodium ethylenediaminetetraacetate, 0.05 m $N$-(morpholino)ethane sulfonic acid, pH 6.4. A 50-μl aliquot of mercaptoethanol was added and the solution was incubated in a capped culture tube at 37°. Approximately 0.3 mg of papain in 0.25 ml of the same buffer was activated at 37° for 30 min with 5 μl of mercaptoethanol. The activated enzyme was added to the core solution and incubation continued at 37° for 8 hours. Another aliquot with 0.3 mg of activated enzyme was added and incubation was continued for an additional 12 hours.

An aliquot of the digest was chromatographed on a Sephadex G-100 column (55 x 0.9 cm); 0.1 m KCl was used as an eluent. The hexosamine and unsaturated uronic acid contents of some of the fractions from the column were determined. The excluded volume, $V_e$, and the included volume, $V_i$, of the column were determined with aliquots of proteoglycan subunit and of glucuronolactone, respectively, by analyzing eluent fractions for uronic acid.

The remainder of the digest was dialyzed twice against 25 volumes of 0.05 m sodium acetate, pH 4.5. A small amount of insoluble material was removed by centrifugation and discarded. Control experiments indicated that 6 to 10% of the glucosamine, representing smaller keratan sulfate molecules, was lost in the dialysis step.

Purification of Keratan Sulfate with DEAE-Chromatography—Mammex DEAE (0.93 meq per g) was washed with 0.5 m NaOH, H2O, 0.1 m HCl, H2O, 0.1 m NaOH, H2O, and then equilibrated with 0.05 m sodium acetate, pH 4.5. An aliquot with about 5 mg of keratan sulfate in 0.05 m sodium acetate, pH 4.5, was applied to a DEAE-column with a resin volume of 10 ml. The column was eluted with 15 ml of the buffer and then with successive 5-ml aliquots of buffer which contained 0.1 m KCl, 0.2 m KCl, ..., 1.0 m KCl. The eluent was collected in 5-ml aliquots which were analyzed for potassium ion concentration and for hexosamine contents.

The keratan sulfate fraction which eluted between 0.1 and 0.4 m KCl was lyophilized. It was then dissolved in 10 ml of H2O and dialyzed against 0.25 m KCl. The final solution, about 0.1 mg per ml, was representative of about 80% of the original keratan sulfate in the core preparation.

Analysis of DEAE-purified Keratan Sulfate—The amino acid, hexosamine, neutral sugar, and sialic acid contents of the keratan sulfate isolated from the DEAE-column were determined.

Two solute concentrations, approximately 0.40 and 0.27 mg per ml, were investigated with sedimentation equilibrium centrifugation in the model E ultracentrifuge. The samples were centrifuged at 20° at a series of different rotor velocities, 40,000, 36,000, 32,000, and 26,000 rpm. The centrifugal time at each velocity was sufficient to achieve equilibrium distributions.

Because the molecular weights of the keratan sulfate are small, the centrifugal conditions were insufficient to attain negligible solute concentrations at the air-solution meniscus, and the meniscus depletion procedures described above could not be used. In order to estimate the total solute concentrations in suitable units, the fringe concentrations, $c(r)$, were plotted against $r$ values for each solute concentration at each rotor velocity. The area under each curve between the radial positions of the air-solution meniscus, $r_m$, and the solution-fluorocarbon meniscus, $r_s$, was determined by integrating the best second order least squares fits through successive segments of the graph. The calculated areas were then plotted against values of the reciprocal of the square of the angular velocity, $1/ω^2$. The intercepts at the ordinate for the straight lines through these data provide estimates of the total areas, and hence the total solute concentrations, which would be observed if the rotor velocity were sufficient to achieve negligible solute concentrations at the air-solution meniscus.

The parameter, $\ln(c(r))/ω^2$, was plotted against $r^2/2$ for each set of data in order to evaluate the relative effects of solute polydispersity and nonideal behavior on the equilibrium patterns. Weight average and z-average molecular weights were estimated from the analyses with Equations 12 and 13 of Reference 5 with a value of 0.06 ml per g assumed for the partial specific volume of the solute.

RESULTS AND DISCUSSION

Isopycnic Centrifugation of Core in CsCl Gradients—Because proteins have lower buoyant densities than polysaccharides, the high proportion of protein to polysaccharide in the core preparation indicates that these molecules will have lower buoyant densities than the proteoglycans from which they were prepared. Further, any polydispersity in the ratio of polysaccharide to protein in the molecules would be reflected by differences in their buoyant densities. This suggests that isopycnic CsCl density gradients can be used to investigate the characteristics of the core preparation.

Fig. 1 shows a picture of the schlieren pattern observed in the analytical ultracentrifuge after an aliquot of core in CsCl was centrifuged to equilibrium. The solute molecules are spread throughout the gradient, with perhaps a small amount of solute extending to the bottom of the cell. The integral curve of the measured differences between the solute and solvent patterns calculated from Fig. 1 is plotted as a function of density within the gradient in Fig. 2. The area under the right lobe of the schlieren pattern in the density gradient was greater than the area under the left lobe. This is indicated by the fact that the

![Fig. 1 (left). Photograph of the schlieren pattern observed for core after centrifugation at 56,000 rpm, 20° for 72 hours in a CsCl gradient with an initial density of 1.50 g per ml. The initial solute concentration was 2 mg per ml.

Fig. 2 (right). The relative solute concentration of core in the CsCl gradient shown in Fig. 1 is plotted against the density at points within the gradient. The solid circles are values on a Gaussian curve with a mean of 1.507 g per ml and a standard deviation of 0.023 g per ml.](http://www.jbc.org/content/4531/2/4531/F1.large.jpg)
between 1.45 and 1.57 g per ml. The center of the distribution in Fig. 2. About 90% of the total material was recovered in Fig. 3 (solid circles). The curve is very similar to that shown were recentrifuged in the analytical centrifuge after the initial fractions 4 through 8, which correspond to gradient densities ties, subfractions of core were prepared from a preparative den-

sity gradient which extended over approximately the same range of densities. The total amounts of solute recovered in the gradient. Solute molecules are spread from densities below 1.45 g per ml to densities above 1.55 g per ml in an approximately Gaussian distribution. The solid circles in Fig. 3 are points on a Gaussian curve with a mean of 1.507 g per ml and a standard deviation of 0.023 g per ml. Such a broad range of densities suggests that the solute buoyant densities are poly-
disperse although solute diffusion also undoubtedly contributes in part to the width of the observed band. A value of 0.66 ml per g was calculated for the average partial specific volume of the potassium salt of the core preparation from the mean buoyant density of 1.507 g per ml for the sample in the CsCl gradient (4). Protein, with a partial specific volume of 0.72 ml per g, constitutes 44% of the core (1). If the partial specific volumes are additive, the data suggest that the partial specific volume of the remaining polysaccharide portions of the preparation should be around 0.62 ml per g. These values were used to estimate the partial specific volumes for the sedimentation equilibrium experiments discussed below.

In order to correlate chemical and physical properties of the molecules with the observed differences in solute buoyant dens-
ties, subfractions of core were prepared from a preparative den-
sity gradient which extended over approximately the same range of densities. The total amounts of solute recovered in each fraction are plotted against the density of each fraction. The curves of Figs. 4 and 10 show that the relative solute concentrations of each fraction at different densities in the gradient. Although the two distributions overlap considerably, the centers of each are well separated, 1.50 g per ml for fraction 5 and 1.52 g per ml for fraction 6. This verifies the fact that the fractions contain populations of molecules with different average buoyant densities.

Because the sulfated keratan sulfate portion of the core macro-
molecule has a higher buoyant density than the protein, the results suggest that the molecules vary in their relative contents of this polysaccharide and of protein. The chemical analyses of the fractions are consistent with this interpretation. Fig. 3 shows that the relative percentages of protein in the fractions (open circles) decrease while those of keratan sulfate (open boxes) increase as the densities of the fractions increase. This indicates that core molecules which have higher ratios of polysaccharide to protein have higher buoyant densities.

Table I presents data for the fractions from the gradient. The

| Fraction | Protein | Glucuronate | Galactosamine | Uronic acid | Uronate to protein | GlcN to protein | GalN to protein | GlcN to protein |
|----------|---------|------------|---------------|-------------|------------------|----------------|----------------|----------------|
|          | mg/ml   | um/ml      | um/ml         | um/ml       | um/ml            | um/ml          | um/ml          | um/ml          |
| 1        | 0.18    | 0.04       | 0.015         | c           | c                | 2.7            | 0.08           | 0.22           |
| 2        | 0.18    | 0.06       | 0.021         | 0.06        | 0.36             | 2.9            | 0.12           | 0.33           |
| 3        | 0.25    | 0.17       | 0.055         | 0.08        | 0.32             | 3.1            | 0.22           | 0.68           |
| 4        | 0.36    | 0.33       | 0.088         | 0.13        | 0.36             | 3.8            | 0.24           | 0.91           |
| 5        | 0.49    | 0.47       | 0.115         | 0.16        | 0.38             | 4.1            | 0.97           | 1.12           |
| 6        | 0.43    | 0.58       | 0.128         | 0.16        | 0.37             | 4.5            | 0.30           | 1.35           |
| 7        | 0.29    | 0.47       | 0.094         | 0.11        | 0.39             | 5.0            | 0.32           | 1.62           |
| 8        | 0.17    | 0.34       | 0.063         | 0.05        | 0.28             | 5.3            | 0.37           | 2.01           |
| 9        | 0.09    | 0.21       | 0.032         | c           | c                | 6.5            | 0.55           | 2.32           |
| 10       | 0.06    | 0.18       | 0.025         | c           | c                | 7.0            | 0.41           | 3.00           |

* Estimated from the Lowry procedure (1).
* Estimated from the thiobarbituric acid procedure (1).
* Too little material to estimate.
ratio of unsaturated uronic acid to protein in the fractions from the middle portion of the gradient are relatively constant which suggests that the amount of the chondroitin sulfate attachment oligosaccharide in each fraction is in a constant proportion to its protein content. The ratio of glucosamine to galactosamine increases for molecules which have higher keratan sulfate to protein ratios. Data discussed below suggest that the ratio of glucosamine to galactosamine is an indication of the molecular size and possibly of the lengths of the keratan sulfate chains. The result suggests that the average polysaccharide chain lengths are greater in fractions which have a higher percentage of keratan sulfate. The ratio of galactosamine to protein also increases gradually from Fractions 4 to 9. If the galactosamine content is an indication of the number of keratan sulfate chains in the molecules, the results suggest that the number of chains per protein may also increase somewhat for fractions with a higher percentage of keratan sulfate.

The amino acid contents of Fractions 3, 5, 7, and 9 are given in Table II. The profiles are all very similar and do not show significant trends across the gradient.

**Table II**

Amino acid profiles for fractions of core from CsCl density gradient

| Amino acid    | Fraction | 3 | 5 | 7 | 9 |
|---------------|----------|---|---|---|---|
| Lysine        |          | 27| 23| 21| 25|
| Histidine     |          | 19| 12| 11| 14|
| Arginine      |          | 41| 36| 35| 34|
| Aspartic acid |          | 82| 68| 66| 64|
| Threonine     |          | 52| 61| 59| 50|
| Serine        |          | 136|138|125|137|
| Glutamic acid |          | 143|146|151|141|
| Proline       |          | 104|119|120|120|
| Glycine       |          | 132|118|121|118|
| Alanine       |          | 77 |71 |72 |73 |
| Half-cystine  |          | 13 |9  |11 |11 |
| Valine        |          | 42 |50 |47 |55 |
| Methionine    |          | 6  |4  |4  |4  |
| Isoleucine    |          | 27 |33 |30 |30 |
| Leucine       |          | 75 |77 |72 |63 |
| Tyrosine      |          | 15 |17 |17 |18 |
| Phenylalanine |          | 33 |35 |35 |35 |

Fig. 5. The apparent number average molecular weights observed at different radial positions in sedimentation equilibrium patterns for the indicated fractions are plotted against the concentrations observed at the radial positions within the patterns (measured in millimeters on the photographic plates, pmm). Three initial solute concentrations, approximately 0.24 mg per ml (●), 0.16 mg per ml (■), and 0.08 mg per ml (▲), were observed for each fraction.

**Sedimentation Equilibrium Centrifugation of Core Fractions—**

One model which is consistent with the physical and analytical data discussed above is that the molecules in the core preparation contain a protein structure of a certain molecular weight to which different amounts of keratan sulfate are attached. Such a model predicts that molecules with higher ratios of keratan sulfate to protein would also have higher molecular weights. To test this hypothesis, Fractions 4, 6, and 8 from the preparative CsCl gradient were investigated in the analytical ultracentrifuge in order to estimate molecular weights by procedures developed by Yphantis (6).

For each fraction, three solute concentrations in the proportions of 1:2:3 (▲:■:●) were investigated. The results from the experiments are indicated in Figs. 5 to 7. Consider the results for Fraction 6 which consists of macromolecules from near the center of the core distribution. Fig. 5b shows the apparent number average molecular weights, \( M_n(r) \), observed at any point \( r \) within the equilibrium distributions plotted against the total concentration, \( c(r) \), at that point. Each different initial solute concentration gives a different curve, with the most dilute concentration yielding the highest values for \( M_n(r) \). If the solute were monodisperse with a single molecular weight, the curves for the different dilutions would superimpose because \( M_n(r) \) at any point within the distribution would depend only upon the total solute concentration at that point (6). The fact that three distinct curves were observed shows that the preparation contains solute molecules with a large polydispersity in molecular weights. Similarly, the results for Fractions 4 and 8 indicate that they also are polydisperse in solute molecular weights. This is consistent with the data given in Fig. 4 above where it was shown that the buoyant densities of molecules in adjacent fractions from the CsCl gradient overlapped considerably.

Fig. 6c shows the \( M_n(r) \) calculated for Fraction 6 plotted against the parameter \( (\gamma_2^2 - \gamma_2^0)/2 \). These curves illustrate the nonideality, or concentration dependence, of the \( M_n(r) \) values independent of the polydispersity in solute molecular weights (6). Three distinct curves were observed for the different solute dilutions, which indicates that the macromolecules exhibit marked concentration effects in the equilibrium distributions.

In the analyses of the meniscus depletion method, Yphantis showed that the apparent weight average molecular weight, \( M_w \), for the entire sample at any given initial solute concentration is the value of \( M_n(r) \) at the bottom of the equilibrium distribution, i.e. \( M_n(r_b) \). These values can be obtained for each
Fig. 6. The apparent number average molecular weights are plotted against the parameter \((r^2 - r'^2)/2\) for each set of data given in Fig. 5. The extrapolated values (open symbols) were determined from first order least squares lines through the data and represent the apparent weight average molecular weights for each initial solute concentration.

Fig. 7. The apparent weight average molecular weights determined from the intercepts in Fig. 6 are plotted against initial solute concentrations for each fraction. The intercepts of these curves at zero solute concentration provide estimates of the actual weight average molecular weights for the three fractions (Table III).

fraction from the data in Fig. 6 by extrapolating the curves to values of \((r^2 - r'^2)/2\) of zero as is indicated by the open symbols (O, D, A). These values, in turn, can be extrapolated to zero initial solute concentration to yield the actual weight average molecular weights, \(M_w\), for the samples, as is shown in Fig. 7.

### Table III

| Fraction | \(\dot{\gamma}^a\) | \(M_w\) \(\times 10^5\) | Percentage protein | Mol wt of protein | \(W_\mathrm{av}\) |
|----------|-----------------|-----------------|-------------------|-----------------|-----------------|
| 4        | 0.672           | 4.12            | 52                | 2.14            | 4.65            |
| 6        | 0.665           | 4.60            | 45                | 2.06            | 5.25            |
| 8        | 0.660           | 4.73            | 40                | 1.90            | 5.55            |

\(^a\) Values of \(\dot{\gamma}\) were estimated from the composition of the fractions with \(v = 0.72\) ml per g for the protein and \(v = 0.62\) ml per g for the polysaccharide.

First order least squares lines were used to determine all extrapolated values.

Table III summarizes the results. The \(M_w\) values for Fractions 4, 6, and 8 increase for those preparations which have higher buoyant densities and hence higher ratios of keratan sulfate to protein. Fractions 5 and 6 contain molecules from the middle of the distribution. Thus the value of \(M_w\) for Fraction 6 is probably close to that for the entire distribution. The values for \(M_w\) were calculated in the same manner as the values for \(M_w\), starting with values for \(M_w\) (9). The ratios of \(M_w/\overline{M}_w\) for each fraction are 1.13 to 1.15 which is another indication of the molecular weight polydispersity exhibited by each.

An estimate of the molecular weight of the protein portion of each of the fractions can be made from the values of \(\overline{M}_w\) and the percentage of protein in each. The values are between 190,000 and 215,000; they agree well with a previous estimate of 180,000 suggested on the basis of physical and chemical analyses of proteoglycan subunit (9). The results are consistent with a model for the subunit preparation in which the macromolecules have a core protein structure of a definite molecular weight, around 200,000, to which different amounts of both chondroitin sulfate (9) and keratan sulfate are attached.

In the preparative gradient, the protein content varies from about 56 to 37% between Fractions 3 and 9. This suggests that the molecular weights for the core macromolecules range from 350,000 to 550,000 if the protein has a molecular weight of 200,000.

### Papain Digestion of Core—Core was digested with papain, and an aliquot was chromatographed on Sephadex G-100 as described under "Experimental Procedure." Keratan sulfate eluted from the column in a single, very broad peak as is indicated by the curve for glucosamine (open circles) in Fig. 8b. The keratan sulfate was retarded by the gel \((K_d = 0.35)\) in contrast to a sample of chondroitin sulfate prepared from subunit, which was essentially excluded from the column \((K_d = 0.05)\). This suggests that the molecular weights for the core macromolecules range from 350,000 to 550,000 if the protein has a molecular weight of 200,000.

### Experimental Procedure—The keratan sulfate molecules are highly polydisperse in molecular weight and considerably smaller than the chondroitin sulfate chains. Most of the galactosamine (solid circles) elutes as a broad peak within the column and before the unsaturated uronic acid peak (open triangles). This suggests that the galactosamine in the core is associated with fractions from the data in Fig. 6 by extrapolating the curves to values of \((r^2 - r'^2)/2\) of zero as is indicated by the open symbols (O, D, A). These values, in turn, can be extrapolated to zero initial solute concentration to yield the actual weight average molecular weights, \(M_w\), for the samples, as is shown in Fig. 7.

Values of \(\dot{\gamma}\) were estimated from the composition of the fractions with \(v = 0.72\) ml per g for the protein and \(v = 0.62\) ml per g for the polysaccharide.

First order least squares lines were used to determine all extrapolated values.

### Table III

| Fraction | \(\dot{\gamma}\) | \(M_w\) \(\times 10^5\) | Percentage protein | Mol wt of protein | \(W_\mathrm{av}\) |
|----------|-----------------|-----------------|-------------------|-----------------|-----------------|
| 4        | 0.672           | 4.12            | 52                | 2.14            | 4.65            |
| 6        | 0.665           | 4.60            | 45                | 2.06            | 5.25            |
| 8        | 0.660           | 4.73            | 40                | 1.90            | 5.55            |

\(^a\) Values of \(\dot{\gamma}\) were estimated from the composition of the fractions with \(v = 0.72\) ml per g for the protein and \(v = 0.62\) ml per g for the polysaccharide.

Papain Digestion of Core—Core was digested with papain, and an aliquot was chromatographed on Sephadex G-100 as described under "Experimental Procedure." Keratan sulfate eluted from the column in a single, very broad peak as is indicated by the curve for glucosamine (open circles) in Fig. 8b. The keratan sulfate was retarded by the gel \((K_d = 0.35)\) in contrast to a sample of chondroitin sulfate prepared from subunit, which was essentially excluded from the column \((K_d = 0.05)\), where the elution volume, \(V\), of a component is given by

\[
V = V_r + K_d(V_i - V_r) \tag{2}
\]

The results suggest that the keratan sulfate molecules are highly polydisperse in molecular weight and considerably smaller than the chondroitin sulfate chains. Most of the galactosamine (solid circles) elutes as a broad peak within the column and before the unsaturated uronic acid peak (open triangles). This suggests that the galactosamine in the core is associated with

Fractions 1, 2, and 10 contained too little material for reliable estimates; they represent, at most, 4% of the molecules on either edge of the distribution. For these reasons they were not included in the calculation.
suggest that the ratio of glucosamine to galactosamine is involved in the attachment region between keratan sulfate and protein in proteoglycans (10-13). The results indicate that galactosamine, and unsaturated uronic acid in fractions obtained when dialyzed papain digest of core was chromatographed on DEAE-cellulose as described under "Experimental Procedure." Fig. 9a shows the recovery of hexosamines in fractions eluted from the column with a stepwise KCl gradient; the dashed line indicates the recovery of glucosamine from the column. The results show that the ratio of glucosamine to galactosamine steadily increased across the gradient from 1.5 at 0.03 M KCl to 8 at 0.6 M KCl. The results are complementary to those observed with Sephadex G-100 chromatography. Keratan sulfate molecules with larger molecular weights as indicated by the greater ratio of glucosamine to galactosamine are bound more tightly to the DEAE column and require stronger ionic strengths to elute them. If there is one sulfate in each repeat disaccharide in keratan sulfate, molecules with higher molecular weights, and hence more sulfate residues per molecule, would have a greater affinity for the cationic resin (13). The results may also reflect varying degrees of sulfation for the molecules, as was reported for a similar experiment with keratan sulfate isolated from whale nasal cartilage (10). In this case, however, molecules with larger molecular weights would also have a proportionately higher degree of sulfation.

**Chemical analyses of DEAE-purified keratan sulfate before and after treatment with NaOH**

| Component | Untreated | Treated |
|-----------|-----------|---------|
| Hexosamines | | |
| Glucosamine | 1.000 | 1.000 |
| Galactosamine | 0.152 | 0.056 |
| Amino acids* | | |
| Aspartic acid | 0.063 | 0.066 |
| Threonine | 0.092 | 0.027 |
| Serine | 0.130 | 0.073 |
| Glutamic acid | 0.236 | 0.245 |
| Proline | 0.251 | 0.233 |
| Glycine | 0.075 | 0.072 |
| Alanine | 0.081 | 0.077 |
| Valine | 0.044 | 0.038 |
| Isoleucine | 0.031 | 0.025 |
| Leucine | 0.033 | 0.033 |
| Tyrosine | 0.042 | 0.011 |
| Phenylalanine | 0.072 | 0.070 |
| Neutral sugars | | |
| Galactose | 0.080 | 0.039 |
| Mannose | 0.096 | 0.096 |
| Fucose | Trace | |
| Sialic acid* | 0.247 | |

* A sample was treated with 0.5 M NaOH, 48 hours, 25°C before analysis for amino acids and hexosamines.
* Amino acids account for 15% of the untreated sample.
* Sialic acid accounts for 12% of the untreated sample.
the structure of skeletal keratan sulfate (10-12). The content of galactose and glucosamine in the untreated preparation are the same. Mannose, with a concentration of 1 residue per 11 of glucosamine, and a small amount of fucose were also present. Xylose was absent which indicates that the preparation contained no residual chondroitin sulfate oligosaccharides. Amino acids and sialic acid accounted for about 15 and 12% of the preparation, respectively.

The treatment with alkali destroyed 0.057 μm of serine, 0.065 μm of threonine, and 0.096 μm of galactosamine per μm of glucosamine. The results are in agreement with those reported for keratan sulfate isolated from whale nasal cartilage and are compatible with the suggestion that O-glycosidic bonds between galactosamine on the one hand and serine and threonine on the other, provide one kind of attachment between keratan sulfate and protein (10-12). The sum of the alkali-labile serine and threonine residues accounts for about 1 residue for every 8 residues of glucosamine.

Fig. 10b shows the elution patterns of the untreated (open circles) and NaOH-treated (open triangles) keratan sulfate on Sephadex G-100. The treated sample is more retarded by the gel (Kd = 0.60) than the untreated (Kd = 0.30). This indicates that the alkali reduced the molecular weights of the molecules. The treated molecules are still relatively large since they elute ahead of the completely included volume, Vd, which suggests that the alkali does not hydrolyze the polysaccharide randomly. The results, as discussed below, are consistent with the hypothesis that the NaOH splits the keratan sulfate into halves. The ratio of glucosamine to galactosamine for the untreated keratan sulfate, Fig. 10a, decreases for molecules with lower molecular size. A comparison of the patterns in Fig. 10 (open circles) with results in Fig. 9 indicate that some of the smaller molecular weight species have been removed in the purification steps. This is also reflected in the higher glucosamine to galactosamine ratio, 6.5, for the DEAE-isolated keratan sulfate, as compared with 4.0 for the core preparation.

Molecular Weight Determination for Keratan Sulfate by Sedimentation Equilibrium Centrifugation—Molecular weight parameters for DEAE-purified keratan sulfate were calculated from sedimentation equilibrium data as described under “Experimental Procedure.” Fig. 11 shows graphs of the parameter, ln(c(r))/ω², against r²/2 for three different angular velocities and two different initial solute concentrations. The factor 1/ω² is included to normalize the data observed for the different centrifugal speeds. Otherwise the graphs are simply a plot of ln c against r²/2, and the slopes of the curves provide information about the solute molecular weights. The curves show an upward curvature which indicates that the preparation has a marked polydispersity in molecular weights. This is verified by the molecular weights calculated from the curves, Table V; the value of Mw, for the preparation, around 11,000, is significantly higher than that for Mw, around 8,500. The curves do...
not exhibit pronounced concentration dependence; in the absence of nonideality solute behavior the set of curves for each initial solute concentration would be parallel but displaced vertically. This is supported by the fact that the molecular weight values given in Table V do not vary appreciably with initial solute concentration nor with different centrifugal speeds. As the angular velocity is increased, the solute molecules which remain at the air-solution meniscus are proportionately enriched in smaller molecular weight species. The values of \(M_{\text{min}}\) listed in Table V illustrate this phenomenon. They indicate that the preparation contains molecules with molecular weights below 4000.

The molecular weight parameters of the preparations discussed above depend upon the actual values for the partial specific volumes of the molecules. For the core fractions, values for \(\bar{\varepsilon}\) were estimated from the average compositions of the fractions and from values of \(\bar{\varepsilon}\) for the protein and polysaccharide (keratan sulfate plus chondroitin sulfate attachment oligosaccharide) portions of the molecules that were determined in part from the average buoyant density of core in CsCl gradients. For the DEAE-purified keratan sulfate a value of 0.60 ml per g was assumed. This provides a reasonable estimate for the preparation if keratan sulfate has a partial specific volume comparable to chondroitin sulfate, reported to be 0.54 ml per g (14) or 0.59 ml per g (15) for the sodium salt. The presence of 15\% peptide in the DEAE-purified keratan sulfate, then, would increase \(\bar{\varepsilon}\) to a value near that used in the calculations above. For either the core frictions or the keratan sulfate preparation, a change in \(\bar{\varepsilon}\) by \(\pm 0.02\) ml per g from the values used will change the calculated molecular weights by about \(\pm 5\%\).

General Discussion—The chemical analyses for the DEAE-purified keratan sulfate fraction are similar to those reported by Toda and Seno (10, 11) for keratan sulfate isolated from whale nasal cartilage. These authors showed that N-acetylmuraminic acid is released from the keratan sulfate by neuraminidase. Since the sialate residues in core are also released by a sialidase (1) and are part of the keratan sulfate portion of the molecules (Table IV), it is likely that they are also N-acetylmuraminic acid residues at nonreducing ends of the polysaccharide chains. Toda and Seno presented evidence that most of the galactosamine in their keratan sulfate preparations is linked to hydroxyl groups of threonine and serine through O-glycosidic bonds. The galactosamine was converted to a precursor of Kuhn's chromagen (16, 17) in 0.5 M NaOH, 25\°, 48 hours, concomitant with a loss of galactosamine and of threonine and serine. These results are consistent with the observed alkaline lability of galactosamine, serine, and threonine in the DEAE-purified keratan sulfate (Table IV). The milder alkaline treatment described for subunit and core (1) apparently is less effective in \(\beta\)-eliminating the O-glycosidic bonds to keratan sulfate in contrast to those which provide the attachment for chondroitin sulfate chains since threonine is less labile. Similarly, Heinegard\(^4\) has found that galactosamine, threonine, and, to a large extent, serine are stable when keratan sulfate from bovine tracheal proteoglycan is treated with 0.1 M LiOH, 3\°, 103 hours. Toda and Seno also provide evidence that the precursor of Kuhn's chromagen as well as significant amounts of amino acids, primarily glutamic acid, remained attached to keratan sulfate after the alkaline treatment. Similarly, Heinegard\(^4\) observed that after mild alkaline treatment and extensive proteolysis with a number of enzymes, the tracheal keratan sulfate contains glutamic acid and proline as the predominant amino acids, and that they are in equimolar ratios with galactosamine. These results suggest that glutamic acid (or glutamine) which is present at a concentration of 1 residue for every 4.6 residues of glucosamine, Table IV above, also provides an attachment point for keratan sulfate to the protein as was also suggested by Mathews and Cifonelli (18). Thus, there may be two different types of attachment for the keratan sulfate chains to protein in the cartilage proteoglycans.

The data given in Figs. 8 and 10 above suggest that the molecular sizes, and hence molecular weights, of the papain-prepared keratan sulfate correlate with an increase in the ratio of glucosamine to galactosamine. This result is similar to that reported by Antonopoulos et al. (13) who analyzed keratan sulfate from human nucleus pulposus with gel and ion exchange chromatography. They suggested that the average chain lengths of the molecules could be estimated from the ratio of glucosamine to galactosamine. The DEAE-purified keratan sulfate molecules have a weight average molecular weight of about 8500. With the composition indicated in Table IV, the molecules would have about 13 repeat disaccharides, assuming the normal structure for keratan sulfate. They would each have about 1 mannose, 2 galactosamine, 2 to 3 glutamic acid, 2 to 3 sialic acid, and almost 2 alkaline-labile (serine plus threonine) residues. The chromatographic behavior of alkaline-treated keratan sulfate indicates that the molecular weights of the molecules are reduced, probably to about one-half their original value. These results agree with those of Bruy et al. (19) who showed that NaOH treatment of skeletal keratan sulfate caused a significant decrease of molecular weights. It is possible, then, that the papain digest of core contains doublet structures, two keratan sulfate chains separated by a short peptide, in an analogous manner to the doublet structure for chondroitin sulfate chains isolated from proteoglycans after trypsin digestion (20). The alkaline treatment would split the doublet structure by \(\beta\)-elimination of the polysaccharide chains from O-glycosidic bonds, but would still leave some peptidic material, presumably attached through glutamic acid (or glutamine), covalently bound to the single polysaccharide chains.

The protein portion of the core molecules appears to have a uniform molecular weight of about 200,000 and essentially the

\[ M_a \] and \[ M_i \] were calculated according to Equations 12 and 13 of Reference 5. A value for \(\bar{\varepsilon}\) of 0.60 ml per g was used.

\[ M_{\text{min}} \] values are calculated from the initial slopes at \(r_a \) for the curves in Fig. 11.

| Speed | M\(_{\text{max}}\) | M\(_a\) | M\(_i\) | M\(_{\text{min}}\) | M\(_a\) | M\(_i\) |
|-------|----------------|------|------|----------------|------|------|
| 40,000 | 4,940 | 8,370 | 10,500 | 3,590 | 8,340 | 11,100 |
| 32,000 | 6,280 | 8,580 | 10,900 | 3,260 | 8,350 | 11,000 |
| 26,000 | 7,410 | 8,830 | 10,900 | 6,410 | 8,600 | 11,300 |

\(^a\) M\(_a\) and \(M_i\) were calculated according to Equations 12 and 13 of Reference 5. A value for \(\bar{\varepsilon}\) of 0.60 ml per g was used.

\(^b\) \(M_{\text{min}}\) values are calculated from the initial slopes at \(r_a\) for the curves in Fig. 11.
same amino acid composition independent of the amount of keratan sulfate attached. These data are consistent with the hypothesis that proteoglycan subunit isolated from bovine nasal cartilage contains a single core protein (or proteins). They do not rule out other possibilities such as similar populations of molecules with similar, overlapping physical characteristics. The electron microscopic observations of Rosenberg, Hellmann, and Kleinschmidt (21) indicate that the backbone of the subunit molecule extends almost 4000 Å. More than 1000 amino acid residues would be required for an extended polypeptide of such a length. This would necessitate a protein of molecular weight between 1 to 2 \( \times 10^9 \). The data presented in this paper are consistent with such a model.

If there is 1 galactosamine residue per keratan sulfate chain as the data suggest, the average number of chains for a protein core of 200,000 would be about 60, each with an average of four to five repeat disaccharides and 1 or more sialic acid residues. This indicates that there would be about half as many keratan sulfate chains as chondroitin sulfate chains built onto the protein core of the subunit macromolecules. Variable chain lengths and, to a lesser extent, variable chain numbers result in the observed molecular weight polydispersity of the core molecules.

Acknowledgments—We express our gratitude to Dr. D. Dzie-wiatkowski and Dr. John Gregory for helpful suggestions in writing the manuscript, and to Mrs. Jan Everett for preparing the manuscript.

REFERENCES

1. HASCALL, V. C., RIIOLO, R. L., HAYWARD, J., AND REYNOLDS, C. C. (1972) J. Biol. Chem. 247, 4521-4528
2. YAMAGATA, T., SAITO, H., HABUCHI, O., AND SUZUKI, S. (1968) J. Biol. Chem. 243, 1523-1535
3. HASCALL, V. C., AND SAIDER, S. W. (1969) J. Biol. Chem. 244, 2384-2396
4. SELPANATA, P., DUNSTON, J. R., AND OGSTON, A. G. (1968) Biochem. J. 109, 43
5. CHEVENA, C. H. (1969) in A Manual of Methods for the Analytical Ultracentrifuge Spino Division of Beckman Instruments, Inc., Palo Alto, Calif.
6. YPHANTIS, D. A. (1964) Biochemistry 3, 297-317
7. ROARK, D., AND YPHANTIS, D. A. (1969) Ann. N.Y. Acad. Sci. 164, 245
8. International Critical Tables, 1928
9. HASCALL, V. C., AND SAIDER, S. W. (1970) J. Biol. Chem. 245, 4020-4030
10. Toda, N., AND SENO, N. (1970) Biochem. Biophys. Acta 205, 227
11. SENO, N., AND Toda, N. (1970) Biochem. Biophys. Acta 215, 544-546
12. SENO, N., MEYER, K., ANDERSON, B., AND HOFFMAN, P. (1965) J. Biol. Chem. 240, 1005-1010
13. ANTONOPOULOS, C. A., FRANSON, L. A., CARRILLO, S., AND HEINEGARD, D. (1969) Acta Chem. Scand. 23, 2616
14. LUSCOMBE, M., AND OPHIS, C. F. (1967) Biochem. J. 103, 103
15. ETREEING, E. J., AND YANG, J. I. (1968) J. Biol. Chem. 243, 1306
16. KUEN, R., AND KREUGER, G. (1956) Chem. Ber. 89, 1473
17. AMINOFF, D., BIXLEY, W. W., SCHAFFER, R., AND MOWRY, R. W. (1970) in The Carbohydrates (Pigman, W., AND HORTON, D., eds), Vol. II B, 2nd Ed., pp. 745-748, Academic Press, New York.
18. MAZIES, M. B., AND CIFONELLI, J. A. (1965) J. Biol. Chem. 240, 4140
19. BRAY, B. A., LIEBERMAN, R., AND MEYER, K. (1967) J. Biol. Chem. 242, 3373
20. MAZIES, M. B. (1971) Biochem. J. 125, 37
21. ROSENBERG, L., HEILMANN, W., AND KLEINSMITP, A. K. (1970) J. Biol. Chem. 245, 4123-4130
Characteristics of the Protein-Keratan Sulfate Core and of Keratan Sulfate Prepared from Bovine Nasal Cartilage Proteoglycan

Vincent C. Hascall and Rick L. Riolo

J. Biol. Chem. 1972, 247:4529-4538.

Access the most updated version of this article at http://www.jbc.org/content/247/14/4529

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/14/4529.full.html#ref-list-1