Investigation of Molecular Motion of Proteoglycans in Cartilage by $^{13}$C Magnetic Resonance

Dennis A. Torchia, Marsha A. Hasson, and Vincent C. Hascall

From the Laboratory of Biochemistry, National Institute of Dental Research, Bethesda, Maryland 20014

$^{13}$C nmr spectral parameters were measured for intact bovine nasal cartilage tissue, the purified proteoglycan aggregate, and chondroitin 4-sulfate. A comparison of integrated intensities obtained for different samples of fresh tissue with an ethylene glycol standard indicated that at least 80% of the total glycosaminoglycan carbons in the tissue contributed to the spectrum. This result was confirmed by intensity measurements obtained at 56° on fresh tissue and at 37° after extensive papain digestion of fresh tissue. Spin lattice relaxation times and nuclear Overhauser enhancements were analyzed in terms of the following models of molecular motion: (a) single correlation time; (b) log $\chi$ distribution of correlation times; and (c) anisotropic motion. The analysis indicates that the segmental motions of glycosaminoglycan chains are characterized by a broad distribution of correlation times centered at about 50 ns.

Slow motion contributions to glycosaminoglycan line widths were reduced by dipolar decoupling ($\gamma H / 2\pi = 65$ kHz). Collagen intensity was observed in dipolar decoupled spectra, but not in scalar decoupled spectra of intact tissue, showing that the type II collagen in cartilage undergoes anisotropic motion like the type I collagen in tendon.

Only glycosaminoglycan resonances were observed in spectra of a solution of proteoglycan aggregate before and after chondroitinase digestion. After subsequent digestion with papain, protein resonances were observed. These results suggest that the protein portions of the proteoglycan aggregate structure, in contrast with the glycosaminoglycan chains, have restricted backbone mobility and consequently a defined backbone structure.

The extracellular matrix of hyaline cartilages consists primarily of two types of structural macromolecules, cartilage type II collagen (1) and proteoglycans. Proteoglycans are large macromolecules with average molecular weights of 2 to $3 \times 10^6$ in which many sulfated glycosaminoglycan chains are covalently bound to a core protein. Typically, about 100 chondroitin sulfate chains, each with an average molecular weight of 20,000, and 50 keratan sulfate chains, each with an average molecular weight of 4 to $8 \times 10^6$, are covalently attached to a single proteoglycan core protein, with an average molecular weight of 200,000 (2). The branched, highly negatively charged proteoglycan molecules occupy large solvent domains per unit mass (2, 3). The proteoglycans are present in the matrix primarily as large aggregates in which 20 to 50 molecules are bound through noncovalent, specific interactions between a portion of the core protein and hyaluronic acid (4-8). Small link proteins stabilize the aggregates (8-10). The organization of the fibrillar collagen with the proteoglycan aggregates in the matrix provides cartilages with their characteristic tissue properties of tensile strength combined with resiliency.

X-ray fiber diffraction studies have shown that a variety of sulfated polysaccharides as well as the proteoglycan aggregate fraction isolated from pig laryngeal cartilage have helical structures when prepared as oriented films (11, 12). These helical structures have been suggested to have a role in determining the mechanical properties of the intact tissue. In contrast, Brewer and Keiser (13) have concluded from estimates of $^{13}$C nmr line widths that chondroitin sulfate chains in the tissue have kinetic freedom like that in solution.

In the present investigation of the molecular structure of cartilage our objectives are the following: (a) measure the fraction of glycosaminoglycan chains which contribute intensity to the scalar decoupled spectrum of intact cartilage obtained under conditions which minimize proteolysis; (b) develop models for the motion of glycosaminoglycan chains which are consistent with measured $T_2$, line width, and NOE values; (c) elucidate the slow motions of the glycosaminoglycan chains and anisotropic motion of collagen in cartilage using high power decoupling; (d) investigate the structure of the proteoglycan protein.

**Experimental Procedures**

Materials—Chondroitinase ABC (Proteus vulgaris) was obtained from Miles Laboratories, dicyclohexylcarbodiimide/trypsin from Worthington Biochemical Corp., and twice crystallized papain from Sigma.

Bovine nasal cartilage was isolated from 1- to 2-year-old steers within 1 h after slaughter. The tissue was freed of extraneous material and chilled on ice until either (a) used directly for nmr experiments (usually initiated within 3 to 4 h) or (b) processed for characterization or for isolation of proteoglycan fractions. For the latter experiments approximately 1-mm slices were prepared with a Stanley Surform as described elsewhere (4). The slices were immediately frozen in liquid $N_2$ and stored at $-30°$ until used. 

Preparation of Samples of Cartilage for nmr Spectra—Unless stated otherwise, cores of cartilage (either 8 mm or 5 mm in diameter

1 The abbreviations used are: $T_2$, $^{13}$C spin lattice relaxation time; $H_n$, external magnetic field; $H_p$, magnetic field in the $^{13}$C rotating frame; $H_p$, magnetic field in the proton rotating frame; $\gamma$, proton magnetogyric ratio; FID, free induction decay; $\Delta$, full linewidth at half maximum; NOE, nuclear Overhauser enhancement (maximum NOE = $3); f, fraction of glycosaminoglycan carbons which contribute to the spectrum of the intact tissue.
Composition of Cartilage—The water content of bovine nasal cartilage was determined by drying weighed slices at 78° over P₂O₅ in vacuo for 24 h. Dried cartilage slices were then hydrolyzed in 6 N HCl or 4 N HCl (about 10 ml/g) under conditions described elsewhere (14) in order to determine amino acid and hexosamine contents, respectively. A Beckman automated amino acid analyzer was used to determine (a) amino acid composition (by the standard buffer programs), (b) hydroxyproline (by eluting with the first buffer at 35° rather than 55°), and (c) hexosamines (5). The relative amounts of chondroitin 4-sulfate and chondroitin 6-sulfate were determined from chondroitinase ABC digests of fresh cartilage slices. The digestion (1 unit of enzyme per 100 mg of tissue suspended in 1 ml of 0.1 M sodium acetate, 0.1 M Tris, pH 7.3) was done for 10 h at 37° with an additional 0.5 unit of enzyme added after 5 h. The supernatant was then analyzed for its relative contents of the unsaturated disaccharide digestion products, 2-acetamido-2-deoxy-3-0-(β-D-glucuronate)-4-sulfoglucuronic acid and 2-acetamido-2-deoxy-3-0-(β-D-glucuronate)-6-sulfoglucuronic acid (see Fig. 1) using the paper chromatographic method developed by Saito et al. (15). Only small amounts of the nonsulfated disaccharide were detected. The summary of these data is presented in Table I.

Preparation of Proteoglycan Fractions—Proteoglycans were extracted from bovine nasal cartilage with 4 M guanidinium chloride using protease inhibitors (14) and conditions (16) described elsewhere. Aggregate samples (referred to previously as A1 preparations) were isolated from the extracts using associative CsCl isopycnic gradients as described elsewhere (8). Aggregate was isolated by similar procedures from the Swarm rat chondrosarcoma (14).

The proteoglycans from the Swarm rat chondrosarcoma contain only chondroitin 4-sulfate. Therefore, peptides with attached chondroitin 4-sulfate chains were prepared from aggregate samples isolated from this tumor. The samples, 5 mg/ml, in 0.1 M sodium acetate, 0.1 M Tris, pH 7.3, were digested 6 h at 37° with trypsin at a final concentration of 50 μg/ml. CsCl was added to the digest (1 g of CsCl/g of solution) to give a density of 1.65 g/ml, and a density gradient was established by centrifugation at 40,000 rpm, 10°, 48 h. The chondroitin 4-sulfate fraction was recovered from the bottom two-thirds of the gradient, dialyzed against 0.5 M sodium acetate, then H₂O, and lyophilized. The preparation contained about 4% peptide, and elution profiles of aliquots on Sepharose 6B indicated that there were an average of about six chondroitin 4-sulfate chains per peptide. (The sample and analyses were kindly provided by Dr. Lawrence L. Faltz, National Institute of Dental Research.)

Enzyme Treatments—Approximately 1 g of fresh cartilage slices (about 1 × 2 × 20 mm) was placed in the 10-mm nmr probe, and a spectrum was determined at 37°. Thin slices of cartilage were used to permit a more rapid diffusion of the enzyme into the tissues. An aliquot of activated papain (125 μg of papain/300 μl of 0.5 M sodium acetate, 0.1 M K₂HPO₄, 0.01 M NaEDTA, 0.01 M mercaptoethanol, pH 6.5) was added directly to the slices. The sample was incubated at 37° for 44 h. After an nmr spectrum was determined, the sample was incubated an additional 12 h at 50°, and another spectrum was measured.

Spectra of solutions (about 350 μl) of bovine nasal A1 in 0.1 M sodium acetate, pH 7.4 (35 mg/ml and 150 mg/ml) were measured. The latter, more concentrated sample was digested with chondroitinase ABC by adding 1.2 units of enzyme dissolved in 60 μl of 0.1 M sodium acetate, pH 7.4, and incubating at 57° for 48 h. The nmr spectrum was determined, and then the sample was dialyzed extensively against solvent to remove digestion products. Another spectrum was measured, and then activated papain (66 μg/30 μl of papain buffer described above) was added. After incubation at 55° for 12 h, a final spectrum was taken.

14C Fourier Transform Spectra—NMR spectra were obtained with a Nicolet Technology 15.09 MHz spectrometer system utilizing a Varian Associates UP-60 magnet. The probe supplied with the spectrometer was extensively modified to maximize performance on the cartilage samples. In the modified configuration a sample of D₂O, containing about 5% of 14C, was placed in the 10-mm nmr probe, and a spectrum was determined at 37°. Thin slices of cartilage were used to permit the rapid diffusion of the enzyme into the tissues. An aliquot of activated papain (125 μg of papain/300 μl of 0.5 M sodium acetate, 0.1 M K₂HPO₄, 0.01 M NaEDTA, 0.01 M mercaptoethanol, pH 6.5) was added directly to the slices. The sample was incubated at 37° for 44 h. After an nmr spectrum was determined, the sample was incubated an additional 12 h at 50°, and another spectrum was measured.

Spectra of solutions (about 350 μl) of bovine nasal A1 in 0.1 M sodium acetate, pH 7.4 (35 mg/ml and 150 mg/ml) were measured. The latter, more concentrated sample was digested with chondroitinase ABC by adding 1.2 units of enzyme dissolved in 60 μl of 0.1 M sodium acetate, pH 7.4, and incubating at 57° for 48 h. The nmr spectrum was determined, and then the sample was dialyzed extensively against solvent to remove digestion products. Another spectrum was measured, and then activated papain (66 μg/30 μl of papain buffer described above) was added. After incubation at 55° for 12 h, a final spectrum was taken.

**TABLE I**

<table>
<thead>
<tr>
<th>Composition of bovine nasal septum cartilage from 1- to 2-year-old steers</th>
<th>Dry Weight Tissuea (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenb</td>
<td>40</td>
</tr>
<tr>
<td>Noncollagenous proteinc</td>
<td>8</td>
</tr>
<tr>
<td>Proteoglycan proteind</td>
<td>4</td>
</tr>
<tr>
<td>Chondroitin 4-sulfate</td>
<td>39</td>
</tr>
<tr>
<td>Chondroitin 6-sulfate</td>
<td>5</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>7</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a H₂O constitutes 75% of the tissue.

b From hydroxyproline content assuming that the collagen contains 10% hydroxyproline.

c From amino acid composition of cartilage slices after subtracting the contribution from collagen content.

d Hascall and Sajdera (2).

Total chondroitin sulfate was estimated from the galactosamine content of the tissue and the relative proportion of the 4- and 6-epimers from chondroitinase digests as described under "Experimental Procedures."

From glucosamine content.

Hardingham and Muir (6).
watts of root mean square power to the probe, which was sufficient to rotate the $^{13}$C magnetization by 90° in 6.8 μs ($\gamma_1 H_1$ about 35 G). The power was provided by an ENI 3100-L power amplifier. Normally, a

A second probe, accepting a 6-mm tube, was used to obtain the dipolar decoupled spectra (17-20). This probe was "homebuilt" according to design specifications generously provided to us by Dr. D. L. VanderHart of the National Bureau of Standards. Decoupling power was provided by an ENI 3100-L power amplifier. Normally, a field of 0.8 G ($\gamma_2/2\pi = 3.5$ kHz) was used for scalar decoupling and a 15 G field ($\gamma_3/2\pi = 65$ kHz) was used for dipolar decoupling. The $^{13}$C coil had dimensions of 6 mm x 18 mm and produced an $H_1$ field of about 60 G which rotated the magnetization through 90° in 3.9 μs. Since the 6-mm probe required only about 20% as much sample as the 10-mm probe, the former probe was used to obtain solution spectra when sample quantities were limited, as was the case with the solutions of bovine nasal aggregate. The sensitivity of the 6-mm probe was 40 to 45% that of the 10-mm probe. Sample temperatures in the Dewarded probes were regulated with a Variarn temperature controller and were measured with a copper-constantan thermocouple. The temperature of intact cartilage was measured (in the nmr probe) on a sample having a concentric hole into which the thermocouple was inserted.

**Data Acquisition**—Free induction decay signals were accumulated in a NIC-80 data system (12 K total memory) equipped with a NIC-293 controller and a Diablo disc drive. The disc interactive Nicolet program version 10.06 was used to perform the usual mathematical operations on the FID. Data were obtained in single channel and in the quadrature modes. The latter provided $\sqrt{2}$ times better sensitivity than single channel operation. The images in the quadrature mode were seldom more than a few per cent, hence, the software correction to normalize the two FID signals was seldom employed. Software-controlled pulse widths and delays were generated by timers in the NIC-293.

**Absolute Intensity Measurements**—Throughout the course of these experiments, the gain of the spectrometer was monitored by periodic measurement of the signal intensity of a neat ethylene glycol sample having a height equal to that of the $^{13}$CC coil. The integrated signal intensity mean varied less than 10% over the time period of the experiments. Before each measurement, the input impedence of the probe plus sample was tuned to 50 Ω. The integrated intensity of the power spectrum of an H2O sample was also periodically measured and compared with the glycol intensity to monitor sensitivity of the spectrometer.

Cartilage samples were prepared for absolute intensity measurements by packing the tissue to a height equal to that of the liquid in the standard glycol sample and then tuning the input impedance of the probe plus sample to 50 Ω. Hence, the integrated intensity of the liquid in the standard glycol sample was used to determine the uncertainty in determining the signal intensity of the samples was the curvature in the base-line which resulted from transients in the FID. Although the base-line curvature could be approximately determined from a spectrum of H2O, the net uncertainties in the integrated intensities of static samples were determined from static sample data in the 80- to 140-ppm region. The uncertainties in determining the signal intensity of static samples were used in calculating the absolute intensities of the spectra.

**Determination of nmr Relaxation Parameters**—Each NOE value was determined (using a 90° pulse sequence with $t_1$ at least 0 times greater than the largest $T_1$) from a comparison of a spectrum obtained using continuous broadband decoupling with that obtained using gated broadband decoupling with the 90 MHz transmitter gated on only during acquisition of the FID. The gated spectrum was multiplied by a scale factor and then subtracted in the NIC-80 computer from the continuously decoupled spectrum. The scale factor which yielded a null difference signal in the 80 to 140 ppm region of the experiment as the null curve. Line widths of the glycosaminoglycan backbone carbons were determined using a computer simulation procedure described previously (21).

The simulation program required the intensity, chemical shift, and line width of each resonance as input. The relative intensities of the chondroitin 4-sulfate and chondroitin 6-sulfate signals were taken as 10:1 (Table I). Keratan sulfate was not included in the simulation since the keratan sulfate available was insufficient to obtain the needed chemical shift data. Chemical shifts of chondroitin 4-sulfate were measured from a 88.7 MHz spectrum of the chondroitin sulfate fraction isolated from trypsin-digested rat chondrosarcoma proteoglycan aggregate as described above. (This spectrum was kindly obtained by Dr. William Egan, National Institute of Child Health and Human Development.) The chondroitin 6-sulfate chemical shifts were determined by a computer simulation of the published high temperature spectrum of this material (22). Initial values of the protonated carbon line widths of the intact cartilage spectrum, Fig. 2b, were estimated by inspection of the 80- to 140-ppm region of the spectrum and were then varied until a satisfactory simulation, Fig. 3a, was obtained. The agreement between the experimental and calculated spectra was significantly better when two or more line widths, rather than a single line width, were assumed in the simulation, indicating that the observed line shape was determined by a distribution of line widths. Uncertainties in line shape due to both sensitivity limitations and the overlap of individual carbon resonances precluded the determination of a unique line width distribution. However, the line shape was sufficiently well defined to show that at least two components, broad ($\Delta < 120$ Hz) and narrow ($\Delta < 40$ Hz), were present with the broad line component predominant. The simulation in Fig. 3a was obtained assuming that 80% of the signals had widths of 120 Hz while the remaining signals had widths of 40 Hz. This simulation was as good as was achieved using three components in the distribution and faithfully reproduces the experimental spectrum except for the shoulder at about 125 ppm. The shoulder may represent the contribution of keratan sulfate which was not included in the calculated spectrum. On the basis of the 25 to 30 Hz carbonyl, carboxyl, and methyl line widths, we ascribe about 50 Hz of the observed width to instrumental sources and variations in magnetic susceptibility of the sample. Carbonyl, carboxyl, and methyl lines broadened to about 60 Hz, Fig. 3a, when thin rectangular pieces of cartilage, rather than tightly fitting circular discs were packed into the nmr tubes, Fig. 2b. Displacement of the air between the rectangular pieces with water reduced the measured carbon, carboxyl, and methyl widths to about 10 Hz (with no digital filtering of the FID). However, the presence of added water greatly accelerated endogenous proteolysis and, hence, the intact cartilage samples studied contained no added water. The $T_2$ values (Table I) were determined from simulations of measured conversion-recovery spectra (21).

**Correlation Time Calculations**—The solid curves, Fig. 6, relating the nmr parameters to the single isotropic correlation time were calculated using standard formalism (23-26). Previously described procedures (21, 27, 28) were used to calculate the curves corresponding to the distribution of correlation times model and the anisotropic reorientation model.

**Dipolar Decoupling Experiments**—$^{13}$C spectra are normally obtained with a field of sufficient power ($\gamma_2/2\pi \approx 2$kHz) applied at the proton resonance frequency to remove the proton-carbon scalar coupling. Since dipolar interactions are about 100 times larger than scalar interactions, the latter are not affected by scalar decoupling. In contrast with scalar interactions, dipolar interactions are averaged out by fast isotropic molecular reorientations which result in spectra having line widths of a few hertz or less. Macromolecules in the tissues of cartilage are in a state of restricted motion which are slow or spatially restricted and incomplete over aging of the dipolar interaction occurs, leading to very broad line widths. Application of a strong field, $\gamma_2/2\pi \approx 60$ kHz, at the proton resonance frequency virtually eliminates line width contributions arising from static dipolar interactions as well as from line motions $T_1 < T_2$ for which $r \approx (\gamma_2 H_1)^{1/2}$. Hence, application of dipolar decoupling to systems having slow or restricted motion will produce spectra with sharper lines or greater intensity, or both, than spectra obtained using scalar decoupling (17-20). Such differences between dipolar decoupled and scalar decoupled cartilage spectra provide evidence for restricted motion in the tissues as discussed in the text.

**RESULTS AND DISCUSSION**

The resonances in the spectrum of the proteoglycan aggregate sample of bovine nasal cartilage2 (Fig. 2a) have been assigned (13) to the carbons in the sulfated glycosaminoglycan chains, primarily chondroitin sulfate, in the proteoglycan molecules. With the exception of small resonances in the aromatic (65 ppm) and aliphatic (155 to 180 ppm) regions, the signals seen in the spectrum of the intact cartilage have the same chemical shifts as those of the aggregate in solution and therefore have been assigned (13) in the glycosaminoglycan spectrum.
chains in the tissue. The tissue spectrum, Fig. 2b, exhibits broader lines than the spectrum of the aggregate in solution, Fig. 2a, indicating that motion of the glycosaminoglycan chains is more restricted in the tissue, since $^{13}$C line widths decrease with increasing chain mobility. However, the fact that the line widths observed in the tissue are $\leq 100$ Hz implies that the vast majority of correlation times characterizing reorientation of the C–H vectors in the glycosaminoglycan are less than 1 ps (13). This statement applies only to that fraction ($f$,) of the glycosaminoglycan chains which contribute intensity to the spectrum. In order to obtain a quantitative measure of $f$, one must obtain a spectrum under conditions in which the integrated intensity of the nmr signal is proportional to the number of carbons which contribute to the signal. The spectrum observed for bovine nasal cartilage in Fig. 2c was obtained under such conditions, i.e. the time between 90° pulses was 8 times larger than the $T_1$ values of the protonated carbons, and the NOE was suppressed using gated decoupling. Furthermore, the spectrum was obtained in less than 1.2 h on tissue freshly isolated as described under "Experimental Procedures." The $T_1$ values were obtained using 4096 transients accumulated in (a), 8192 transients accumulated in (b) and (c). The glucuronate and galactosamine moieties are respectively designated GA and GalNAc. Chemical shift scale is in parts per million from external CS.$\gamma$.

Integrations were used with 4096 transients accumulated in (a), 8192 transients accumulated in (b) and (c). The glucuronate and galactosamine moieties are respectively designated GA and GalNAc. Chemical shift scale is in parts per million from external CS.$\gamma$.

The integrated intensities were converted into weight of $^1$C by calibrating the instrument with a sample of neat ethylene glycol to determine the integrated intensity/scan/mg of $^1$C as described under "Experimental Procedures." The weight of mobile glycosaminoglycan was then readily calculated from the chemical structure and the weight of $^{13}$C in the backbone of the glycosaminoglycan chains. The integrated intensity accounts for all the glycosaminoglycan, measured by weight, in the tissue (Table II). Although this result suggests that all the glycosaminoglycan chains in the tissue are mobile ($f, = 1$), we emphasize that the uncertainty in $f,$ is large, $\pm 20\%$, primarily because of uncertainties in the shape of the base-line (see under "Experimental Procedures").

Because of the large uncertainty in $f,$ two other methods were used to estimate the fraction of mobile glycosaminoglycan chains in the tissue. First, intensities from a spectrum of a fresh tissue sample were compared with intensities obtained after the tissue had been treated with papain; and, second, the intensities of a fresh tissue were measured at several temperatures. The line widths were significantly smaller in the papain-treated spectrum, Fig. 3b, resulting in greater peak heights. The integrated intensities in the 80 to 120 ppm range, however, were the same as found in Fig. 3a, within experimental error, 20%. The papain-treated sample exhibited new signals in the 120 to 190 ppm range arising from protonated carbons in flexible peptides released by the papain digestion. When the digest was continued for 12 h at 56°, substantial increases in peptide resonance intensities, Fig. 3c, were observed. These resonances appeared because most of the noncollagenous protein and some collagen in the tissue were digested under these conditions. The 80 to 120 ppm region of the spectrum, which contains resonances due to the glycosaminoglycan chains, however, was unchanged by the higher temperature digestion. Fig. 4 contains spectra at 4° and 56° of the fresh tissue sample whose spectrum at 37° is shown in Fig. 2b. Comparison of Fig. 2b and 4b shows the expected sharpening of spectral lines at the higher temperature as well as peptide resonances in the 120 to 190 ppm range. The latter result is a consequence of temperature-accelerated endogenous proteolysis or thermal denaturation, or both. Significantly, the integrated intensity of the glycosaminoglycan resonances in the 80 to 120 ppm range is the same at 56° as at 37°. At 4° the line widths are so large that the uncertainty in the intensity measurement is about a factor of 2; hence, quantitative comparison with intensity measured at 37° is not possible. Taken together, the above results provide strong evidence that at least 80% of the glycosaminoglycan chains contribute intensity to the nmr spectrum in native bovine nasal cartilage and hence are mobile.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dry weight</th>
<th>Weight of glycosaminoglycan</th>
<th>Weight of glycosaminoglycan from nmr$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>227</td>
<td>119</td>
<td>118</td>
</tr>
<tr>
<td>2</td>
<td>246</td>
<td>119</td>
<td>140</td>
</tr>
<tr>
<td>3</td>
<td>238</td>
<td>114</td>
<td>115</td>
</tr>
<tr>
<td>4</td>
<td>246</td>
<td>119</td>
<td>119</td>
</tr>
</tbody>
</table>

* Integrations extended over the eleven protonated carbons in the 80 to 140 ppm region of the spectra.

* Experimental uncertainty ± 15%.

Because of the large uncertainty in $f,$ two other methods were used to estimate the fraction of mobile glycosaminoglycan chains in the tissue. First, intensities from a spectrum of a fresh tissue sample were compared with intensities obtained after the tissue had been treated with papain; and, second, the intensities of a fresh tissue were measured at several temperatures. The line widths were significantly smaller in the papain-treated spectrum, Fig. 3b, resulting in greater peak heights. The integrated intensities in the 80 to 120 ppm range, however, were the same as found in Fig. 3a, within experimental error, 20%. The papain-treated sample exhibited new signals in the 120 to 190 ppm range arising from protonated carbons in flexible peptides released by the papain digestion. When the digest was continued for 12 h at 56°, substantial increases in peptide resonance intensities, Fig. 3c, were observed. These resonances appeared because most of the noncollagenous protein and some collagen in the tissue were digested under these conditions. The 80 to 120 ppm region of the spectrum, which contains resonances due to the glycosaminoglycan chains, however, was unchanged by the higher temperature digestion. Fig. 4 contains spectra at 4° and 56° of the fresh tissue sample whose spectrum at 37° is shown in Fig. 2b. Comparison of Fig. 2b and 4b shows the expected sharpening of spectral lines at the higher temperature as well as peptide resonances in the 120 to 190 ppm range. The latter result is a consequence of temperature-accelerated endogenous proteolysis or thermal denaturation, or both. Significantly, the integrated intensity of the glycosaminoglycan resonances in the 80 to 120 ppm range is the same at 56° as at 37°. At 4° the line widths are so large that the uncertainty in the intensity measurement is about a factor of 2; hence, quantitative comparison with intensity measured at 37° is not possible. Taken together, the above results provide strong evidence that at least 80% of the glycosaminoglycan chains contribute intensity to the nmr spectrum in native bovine nasal cartilage and hence are mobile.
assumed that reorientation of the internuclear C—H bond axis is isotropic and is characterized by a single correlation time. If reorientation proceeds by rotational diffusion, then \( r = 1/(6R) \), where \( R \) is the rotational diffusion coefficient. A more complicated situation occurs when two or more diffusion constants are required to describe the motion. The reorientation of an axially symmetric rigid body, such as a cylinder or an ellipsoid of revolution, is described by two diffusion constants, \( R_1 \) and \( R_2 \), which characterize, respectively, the rates of reorientation parallel and perpendicular to the symmetry axis. Woessner (30) derived expressions relating the nmr parameters to the diffusion constants and \( \theta \), the angle between the C—H axis vector and the rotational symmetry axis. The dashed curves in Fig. 6 illustrate the results of this calculation for \( \theta = 60^\circ \) and \( R_1/R_2 = 10 \). When \( \theta = 0^\circ \), the C—H vector is parallel to the symmetry axis and the solid curves, Fig. 6, apply, since reorientation is completely characterized by the single diffusion constant \( R = 1/2R_1 \).

A third motional model, which has often been applied to the analysis of dielectric and nmr relaxation data of polymers, assumes that isotropic reorientation is characterized by a distribution of correlation times rather than a single correlation time \( 27, 28, 31, 32 \). A distribution of correlation times describes more realistically the reorientation of a polymer backbone, since a polymer can have many conformations, each characterized by a different correlation time. To describe the distribution of chain motions, we will use a normalized log \( x^2 \) distribution introduced by Schaefer (27) and defined as

\[
G_x(s) = (p\mu)^p - e^{-p\mu} / \Gamma(p)
\]

with

\[
s = \log \left[ 1 + \left( b - 1 \right) \theta / \pi \right]
\]

\( G_x(s) \) is the probability of finding a correlation time \( \tau \) corresponding to \( s = s(\tau) \). The width of the distribution is governed by the variables \( p \) and \( b \), and the use of a log \( (s/\tau) \) argument in defining \( G \) allows very broad distributions to be employed. For \( p > 1 \), \( \tau \) approximately equals the most probable value of \( \tau \).

The nmr parameters plotted as a function of \( \tau \) in Fig. 6 (dashed-dot curves) were calculated by means of a distribution, having \( p = 14 \) and \( b = 1000 \), which had been used to
analyze nmr data obtained for synthetic polymers (27) and elastin (29). These parameters yield a very broad asymmetric distribution of correlation times as seen in Fig. 7. The correlation times in the neighborhood of 10 ns determine $T_1$ and NOE values, while the large correlation times, $>100$ ns, determine the linewidth.

**Analysis of $T_1$ and NOE Values**—Because the isotropic single correlation time model involves no unknown parameters, it will be used first to discuss the relaxation data. The correlation times, $\tau$, were calculated from measured relaxation times, Table III, and the solid curves, Fig. 6, for the cartilage and chondroitin 4-sulfate samples. As seen in column 1, Table IV, for each sample the difference between the correlation time calculated from the $T_1$ value and that calculated from the NOE value is larger than can be accounted for by experimental errors in $T_1$ and NOE values.

The internal consistency of the analyses improved significantly when $\tau$ values were calculated using the log $\chi^2$ distribution of correlation times model, dashed-dot curves in Fig. 6. As seen in column 2, Table IV, the $\tau$ value calculated from the $T_1$ value agrees with that calculated from the NOE value for each sample.

Although the log $\chi^2$ distribution of isotropic correlation times provides a self consistent analysis of the data, this does not exclude the possibility of anisotropic motion. The dashed curves in Fig. 6 apply for anisotropic reorientation described by two diffusion constants, $R_1$ and $R_2$, where $R_1 = 10 R_2$ and the angle between the C$-\text{H}$ bond axis and the symmetry axis equals 60°. As is seen from column 3, Table IV, the correlation times $\tau_A = (1/R_2)$ obtained from the nmr parameters for this model show internal consistency as good as that obtained with the single correlation time isotropic model, column 1, Table IV. The consistency of the analysis could be improved by assuming a distribution of asymmetric motions, but the number of unknown parameters involved precludes the possibility of obtaining any new information. Although $\theta = 60°$ was chosen arbitrarily, the correlation times calculated from the nmr parameters change by less than a factor of two as $\theta$ varies from 40° to 140°. Furthermore, if a value of $\tau_1$ is chosen, the nmr parameters vary by no more than ±35% (from their mean values) as $\theta$ changes from 30° to 150°. Hence the approximate equality of measured $T_1$ and NOE values for the various glycosaminoglycan backbone carbons does not imply that motion is isotropic.

There are two reasons for considering the possibility of anisotropic motion. First, each disaccharide unit bears two negative charges; hence, it is reasonable to suppose that the polyanionic chains adopt extended conformations to minimize
Molecular Motion of Cartilage Proteoglycans

3623
coulombic repulsions. Second, glycosaminoglycan chains in oriented films have extended helical conformations (11, 33). Perrin's theory of reorientation of rigid ellipsoids of revolution predicts that a rod-like particle having \( R_1 = 10 R_2 \) has a long axis, \( a_1 \), equal to 6.5\( a_2 \), where \( a_2 \) is the diameter of the ellipsoid (30, 34). Thus the nmr data is compatible with the presence of local rod-like glycosaminoglycan conformations having axial dimensions whose ratio is 6.5.

A substantially larger axial ratio is compatible with the measured parameters provided that backbone carbon \( \theta \) values assume a small range. However, the precise axial ratio cannot be obtained since the range of \( \theta \) is unknown.

Analysis of Glycosaminoglycan Linewidths—Although the \( T_1 \) and NOE values calculated using the log \( x^2 \) distribution (\( p = 14, b = 1000, \tau = 65 \text{ ns} \)) agree with the measured parameters, the calculated line width, 500 Hz, is 5 to 25 times larger than the glycosaminoglycan line widths obtained from the cartilage spectrum, Table III. This result indicates that the long tail of the log \( x^2 \) function overemphasizes the large correlation times. Accordingly, a 100 Hz line width is calculated (\( \tau = 65 \text{ ns} \)) when correlation times >500 ns are deleted from the distribution by truncating the distribution at \( b \) in Fig. 7. Thus, deleting these larger correlation times which comprise only 13% of the distribution yields a calculated line width which equals that found for the broad component of the glycosaminoglycan spectrum. Correlation times >250 ns comprise 20% of the distribution and their deletion (truncation at \( a \) in Fig. 7) yields a 35 Hz calculated line width, in approximate accord with the 20 Hz line width estimated for the narrow component of the glycosaminoglycan spectrum. In each case truncating the log \( x^2 \) function had virtually no effect (<2%) on calculated \( T_1 \) and NOE values, since these parameters are determined by correlation times in the neighborhood of 10 ns.

Although the truncated log \( x^2 \) distribution accounts for the line widths, this function is deficient in two respects. First, slow motions (\( \tau >500 \text{ ns} \)) are completely neglected whereas dipolar decoupling experiments (discussed below) show that such motions are present. Second, all motions are assumed to be isotropic. In spite of these deficiencies we have used the truncated log \( x^2 \) function since it illustrates two important features of the chain motion: (a) a broad distribution of \( \tau \) values in the range of the Larmor frequency is needed to account for \( T_1 \) and NOE values, (b) the large correlation times, although comprising a minor fraction of the distribution, determine the line widths.

This latter point and the result that at least two line widths must be used to simulate the glycosaminoglycan spectrum, Fig. 9a, is strong evidence that the distribution of slow motions is not the same for all backbone carbons in a glycosaminoglycan chain. Dynamic heterogeneity is also suggested by the chemical structure of the proteoglycan molecule, since one end of each glycosaminoglycan chain is free and presumably more mobile than the other end of the chain which is attached to the core protein. In particular, one would expect increasing resistance to the cooperative slower motions involving longer chain segments as one moves inward from the free end of the chain.

Dipolar decoupling greatly reduces the line width heterogeneity, Fig. 8a and b. Corrected line widths of 40 Hz (80% of the signal) and 20 Hz (20% of the signal) are obtained from the computer simulation, Fig. 8b, of the dipolar decoupled spectrum. This result strongly supports the idea, discussed above, that the slow motions of glycosaminoglycan chains in intact cartilage are heterogeneous. The sharpened glycosaminoglycan lines, Fig. 8b, show that the dipolar broadening of the scalar decoupled spectrum, Fig. 8a, due to slow motion is reduced when the dipolar interaction is suppressed by high power decoupling.

In addition to sharpening the lines of the glycosaminoglycan carbons, dipolar decoupling dramatically increases the intensity in the 120 to 190 ppm range. The large background signal seen in this region, Fig. 8a, is due to collagen carbons, which have lines that are too broad to detect (>3 kHz) using low power decoupling. The fact that a large collagen signal is obtained without cross-polarization with a 2-s interval between 90° pulses implies that at least the side chains of the collagen in the tissue undergo rapid internal motion. Hence, the type II collagen in the bovine nasal cartilage (35) exhibits mobility which is like that observed (20) for type I collagen in tendon.

Mobility of Proteoglycan Protein—The nmr data provide strong evidence that the glycosaminoglycan chains in the intact tissue are segmentally mobile. However, we were unable to obtain unambiguous information about the molecular dynamics of the proteoglycan protein since we could not distinguish resonances of the proteoglycan protein from those due to the other, more abundant proteins (Table I) in the tissue. For...
this reason, we studied spectra of samples, consisting primarily of proteoglycan aggregates, which did not contain extraneous protein material. The $37^\circ$ spectrum of a highly viscous solution of the aggregate, Fig. 10a, in which the concentration, 150 mg/ml, approximates the aggregate concentration in the tissue, exhibits line widths which are about twice those in the aggregate spectrum, Fig. 2a obtained at a lower concentration, 35 mg/ml, and more closely resemble line widths of the tissue, Fig. 2b. This observation is consistent with the expectation that motion should be more restricted in the more viscous solution.

Although the aggregate spectrum obtained at high concentration, Fig. 10a, does not show evidence of protein resonances, it is possible that the attached glycosaminoglycan chains restrict segmental motion of the peptide backbone to the extent that protein line widths are over twice those of the glycosaminoglycan chains. Due to the low protein content of the aggregate (Table I) such lines could not be distinguished from the noise. Thus, the absence of protein lines from Fig. 10a does not imply that the protein backbone is rigid.

The possible hindrance of protein segmental motion by the glycosaminoglycan chains was reduced by digesting the aggregate with chondroitinase ABC which removed the chondroitin sulfate chains from the core protein. The digestion produced striking changes in the spectrum as can be seen by comparing Fig. 10a with Fig. 10b. The spectrum of the digested aggregate shows new resonances (e.g. the unsaturated C$_1$H resonance in Fig. 10b) as a result of the new covalent structures, Fig. 1b, which result from chondroitinase digestion. Furthermore, the oligosaccharide tumbles far more rapidly than the glycosaminoglycan chains because their molecular weight is much lower and the solution viscosity is markedly reduced after the digestion. Significantly, protein resonances are still absent, in spite of the much reduced solution viscosity and the absence of chondroitin sulfate chains to hinder peptide segmental motion.

The oligosaccharide digestion products were removed from the sample by dialysis in order to permit the low intensity protein peaks to be more readily detected. Fig. 10c shows that only 10 to 15% of the glycosaminoglycan intensity remains after dialysis, primarily from undigested keratan sulfate and chondroitin sulfate linkage oligosaccharides still covalently attached to the protein. Since protein resonances were still not observed, the solution was then digested with papain. A comparison of Fig. 10c with Fig. 10d shows that papain digestion did not affect the oligosaccharide resonances, but new resonances (shaded in Fig. 10d) are seen in the positions expected for carbons in amino acid residues. The relative distribution of the shaded signal intensity is in qualitative agreement with that predicted for amino acids which are known to be present in the proteoglycan protein (2). This result indicates that the intact polypeptide chains of the proteoglycan protein do not exhibit segmental motion, since amino acid resonances are absent from Figs. 10b and 10c but present in Fig. 10d. After digestion with chondroitinase, residual oligosaccharide chains attached to the protein impose some restrictions on segmental motion of the polypeptide backbone. However, it seems unlikely that the short, highly mobile oligosaccharides (note the sharp lines in Fig. 10c: as compared with Fig. 10a) could so restrict the protein motion that protein signal intensities could not be observed. Hence, we conclude that the proteoglycan protein, in contrast with the polysaccharide, has highly restricted backbone motion arising from a well defined backbone structure.

**CONCLUSIONS**

The nmr results provide strong evidence that the protein constituents of bovine nasal cartilage have well defined secondary structures. In the case of collagen this is, of course, expected since a triple-stranded helical structure is assumed by this protein in the native state. It is interesting that collagen spectral intensity is observed upon dipolar decoupling since this shows that anisotropic motion is present even in the highly organized collagen fibrils. The nmr spectra do not provide information about the geometry of the secondary structure of the proteoglycan protein. That a secondary structure exists and does not depend upon the presence of the glycosaminoglycan chains is indicated by the absence of protein resonances in the spectrum of the chondroitinase-treated aggregate.

The highly restricted protein motion contrasts with the segmental mobility of the glycosaminoglycan chains in solution and in intact tissue. Interchain interactions occur with greater probability in the tissue, due primarily to the higher concentration of glycosaminoglycan chains, resulting in more restricted motion than found in solution. The chemical structure of the proteoglycan suggests that glycosaminoglycan chains are dynamically heterogeneous, behavior which is manifest in the multiple line widths which contribute to the scalar decoupled tissue spectrum. Using the log $x^2$ distribution to represent the broad range of motions in the segmentally mobile chains, one obtains a self-consistent analysis of the $T_1$ and NOE data. The observed line widths can also be accounted for by suitable truncation of the large correlation time contributions to the distribution. The success of the truncated log $x^2$ function in accounting for the nmr parameters does not imply...
that this distribution represents the true distribution of motions of the glycosaminoglycan chains. The experimental data are not sufficiently accurate or extensive enough to determine a distribution function uniquely, and many details of the motion (e.g. possible anisotropy) remain to be determined. However, whatever the precise nature of the glycosaminoglycan chain motion, it is clear that at least 80% of the glycosaminoglycan chains are characterized primarily by segmental motions having correlation times \( \leq 500 \text{ ns} \). Hence, the proteoglycan molecules hold the anionic polysaccharide chains within the tissue matrix but do not fix their spatial positions. The highly ordered conformations observed in oriented films are apparently not common in the native tissue.

Acknowledgments – We are indebted to Dr. D. L. VanderHart for advice and assistance in building the probe used to obtain dipolar decoupled spectra. Mr. Rolf Tschudin collaborated in making the spectrometer modifications that were needed for dipolar decoupling. Ms. C. E. Sullivan assisted with sample preparation and obtained several of the spectra.

REFERENCES

32. Healy, F., and Beguin, A. (1976) Polymer 17, 399-408
Investigation of molecular motion of proteoglycans in cartilage by 13C magnetic resonance.

D A Torchia, M A Hasson and V C Hascall