Determination of Ion Activity Coefficients and Fixed Charge Density in Cartilage with ^{23}Na Magnetic Resonance Microscopy

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The sodium concentration and the physicochemical state of sodium ions in biological polyelectrolytes may be an indirect measure of the macromolecular matrix integrity. Nuclear magnetic resonance (NMR) microscopy was used to measure the sodium concentration and to estimate the cartilage matrix fixed charge density (FCD) in control, acid-neutralized, and enzyme-digested bovine nasal cartilage (BNC), a model for human cartilage. The mean ion activity coefficient of sodium in cartilage was calculated from the measured tissue sodium concentration as a function of external NaCl concentration and compared with the predictions of the Debye−Hückel and the modified Manning theories. Our results show that (1) the FCD in bovine nasal cartilage (at physiological NaCl concentrations) is in the range of 0.25−0.35 m, (2) the sodium content measured by NMR microscopy reflects indirectly the changes of FCD induced by changes in pH and enzymatic digestion, and (3) the measured activity coefficient for NaCl in the bovine nasal cartilage matrix is significantly lower than that of an ionic solution of the same salt concentration. An extension of the model developed by Manning for polyelectrolytes is in good qualitative agreement with our experimental results which showed a decrease in the mean activity coefficient with decreased reservoir concentration.

Introduction

Cartilage is a porous biopolymer consisting of a collagenous fiber network supporting a hydrophilic proteoglycan polyelectrolyte gel (Maroudas, 1979; Carney and Muir, 1988). The cartilage extracellular matrix consists of 60%−80% water, 15%−20% collagen, and 5%−12% proteoglycan. The proteoglycan (PG) is a complex macromolecule whose monomer has one core protein and attached approximately 100 chondroitin sulfate and 50 keratan sulfate side chains. Cartilage load-bearing and biomechanical properties are dependent upon the molecular level interactions between the electronegative proteoglycan matrix, liquid phase water, and electrolytes (Carney and Muir, 1988; Mow et al., 1984). The bathing electrolyte solution includes the mobile ions Na^+, K^+, Ca^{2+}, Cl^-, SO_4^{2-}, and HPO_4^{2-} and macromolecules such as hyaluronic acid. Under physiological conditions, the dominant matrix counterion which balances the proteoglycan fixed charge is Na^+. The equilibrium concentration of sodium depends upon the concentration of immobile fixed charged groups on the matrix macromolecules defined as the fixed charge density (FCD). The dominant fixed charge groups on the proteoglycan matrix are sulfate (SO_3^-) and carboxylate (COO^-) (Maroudas, 1979; Carney and Muir, 1988; Mow et al., 1984) which act as weak acids with p*K*'s of 3−3.5 and 1.5−2, respectively (Mathews, 1959; Cleland et al., 1982; Kuettner and Lindenbaum, 1965). At physiological pH, they are fully ionized. For electroneutrality, the counterion Na^+ balances the fixed charges and thus the concentration of sodium can give an indirect measure of the proteoglycan content.

The equilibrium distribution of ions also depends on the ion activity coefficients which give a measure of the change in the chemical potential arising from ionic interactions between ions and matrix fixed charges (Robinson and Stokes, 1955). The mean activity coefficient, $\gamma_\pm$, of a solute in an ideal system or an infinitely dilute solution is defined to be unity. The $\gamma_\pm$ of the solute in a real solution gives a measure of the deviation of the solution properties from those of an ideal system. Measurement of this deviation in cartilage can provide quantitative information about the electrostatic interactions experienced by sodium ions in the nonideal polyelectrolyte matrix of cartilage. Previous studies of biological polyelectrolytes have primarily examined relatively dilute polymer systems (Preston and Snowden, 1973; Magdelenat et al., 1979). In some cartilage systems in which FCD < 0.2 *m*, it has been shown that electrolyte interactions with the matrix are not significant (Preston and Snowden, 1973; Magdelenat et al., 1979; Vanysek, 1990). In this work, we examined cartilage with a relatively high FCD representative of more dense connective tissues in which matrix interactions are thought to be important in understanding ion equilibrium behavior (Parker et al., 1988).

For experimental monitoring of ion equilibrium there are several well-established methods of determining activity coefficients; however, no routine noninvasive methods exist for biological systems. Nuclear magnetic resonance (NMR) imaging and spectroscopy of ^{23}Na nuclei has been suggested as a possible probe of ions in tissue and it has been used to obtain the sodium concentration in a variety of tissues (Kohler and Kolodny, 1992; Ra et al., 1986; Boada et al., 1994; Lesperance, 1992). The NMR intensity can be related to the sodium content, and in previous preliminary work it was shown that sodium NMR can be used to measure the FCD of cartilage (Lesperance, 1992). In this work Lesperance et al. measured the FCD of articular cartilage explants by measuring the ^{23}Na concentration with NMR spectroscopy and then applying the Donnan equilibrium theory to determine the FCD. They showed that ^{23}Na spectroscopy detected the total tissue sodium by comparing the NMR determined concentrations to measurements using both desorption and inductively coupled plasma emission spectroscopy. Furthermore, they showed with ^{23}Na NMR imaging that spatial variations in sodium concentration across heterogeneous articular cartilage correlated with variations in tissue glycosaminoglycan. Additional work by Broada et al. (1994) further established

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the quantitative value of ^{23}Na NMR imaging to measure sodium content although their work pointed to the necessity of accounting for the fraction of sodium having an extremely short T_2 with sequences having very short echo times.

Unfortunately, previous work used oversimplified theoretical models for data interpretation. For example, the ideal Donnan equilibrium was assumed in the work by Lesperance et al., thus variations of ion activity coefficients with the FCD, reservoir pH, and concentrations were ignored. The ideal Donnan equilibrium might be applicable for dilute polyelectrolyte solutions, but for equilibrium involving a dense polyelectrolyte such as cartilage, the general Donnan equilibrium is more appropriate.

In this study, we used the general Donnan equilibrium except for the enzyme-digested samples, which was reasonable since in enzyme-digested samples, the polyion concentration is relatively very low (0.03 *m*) and the ideal Donnan equilibrium may be applied. Such an approach leads us to important information about the polyion−counterion interactions in cartilage by comparing the experimentally determined mean ion activity coefficients with predictions of polyelectrolyte models. NMR microscopy was used to allow simultaneous measurement of both the cartilage matrix sodium and the reservoir sodium concentration as an absolute reference. Specifically, we address the following questions: (1) Can ^{23}Na NMR imaging be used to monitor changes in the fixed charge density (FCD) of cartilage brought about by changes in pH and by enzymatic digestion? (2) What are the equilibrium sodium ion concentrations and activity coefficients for bovine nasal cartilage (BNC) at physiological salt concentrations? (3) How does the ion activity coefficient vary with changes in ionic conditions and can the behavior be understood in terms of existing theories for polyelectrolytes?

Background and Theory

The equilibrium condition for simple electrolytes in two compartments, e.g. a simple electrolyte bath and a polyelectrolyte solution, is that the chemical potential should be equal. For 1−1 valence salts, the Donnan equilibrium condition is given by

$$\left(\frac{\hat{\gamma}_\pm}{\gamma_\pm}\right)^2 = \left(\frac{m_+ m_-}{\hat{m}_+ \hat{m}_-}\right) \tag{1}$$

where $\gamma_\pm$, m_+, and m_- are the mean ionic activity coefficient and concentrations of cation and anion, respectively, in the bath and $\hat{\gamma}_\pm$, $\hat{m}_+$, $\hat{m}_-$ are their counterparts in the polyelectrolyte.

Using the electroneutrality condition, $z_+m_+ + z_-m_- = 0$ for the bath and $z_+\hat{m}_+ + \mathrm{FCD} + z_-\hat{m}_- = 0$ for the polyelectrolyte solution, for monovalent ions eq 1 yields the equilibrium equation:

$$\hat{\gamma}_\pm = \frac{\gamma_\pm m_+}{\sqrt{\hat{m}_+(\hat{m}_+ - \mathrm{FCD})}} \tag{2}$$

where FCD is the fixed charge density of the polyelectrolyte.

For dilute simple electrolyte solutions, Debye−Hückel theory assumes that long range interionic forces dominate over short-range electrostatic and ion−solvent interactions such as Van der Waals forces and ion−dipole interactions. Therefore, it corrects for the reduction in the free energy of a single isolated ion due to the presence of neighboring ions and the mean electrolyte activity coefficient is given by

$$\log \gamma_\pm = -A|z_+z_-|I^{1/2} \tag{3}$$

The constant A is defined by the expression $A = 1.8246 \times 10^6/(\epsilon T)^{3/2}$ which depends on the dielectric constant of the solvent (ϵ) and its absolute temperature (T) (Robinson and Stokes, 1955; Hamer and Wu, 1972). The parameter I is the ionic strength of the solution expressed as $I = {}^1/_2[m_+z_+^2 + m_-z_-^2]$. When the size of the ion is taken into account, the Debye−Hückel equation becomes:

$$\log \gamma_\pm = \frac{-A|z_+z_-|I^{1/2}}{1 + BI^{1/2}} \tag{4}$$

where $B = 1.4495$. Long-range interactions vary as the square root of concentration, while the short-range forces depend linearly on concentration. In dilute solutions up to 1 *m*, the short-range forces can be accounted for empirically by adding a linear term to the expression above, while for more concentrated solutions higher order terms in I are required. The full expression is given by Hamer and Wu (1972).

Analogous to Debye−Hückel's limiting law for dilute simple electrolyte solutions, Manning developed a limiting law for polyelectrolyte solutions (Manning, 1969). The basic assumptions of Manning's formulation are (1) the polymer chain is modeled as an infinitely long, rodlike line with a uniform linear charge density (and thus, the end effect and free energy change accompanying chain conformational change are ignored), and (2) the interactions between fixed ion groups on the same chain and between those on different chains are screened by interactions between mobile ions and polyions. In spite of these assumptions, many experimental results are in reasonable agreement with this model.

A characteristic parameter, ξ, representing the ratio of electrostatic to kinetic energy is defined as

$$\xi = e^2/(\epsilon kTb) \tag{5}$$

where ϵ is the dielectric constant of the system. The mean intercharge spacing, b, is calculated from the contour length of the polyelectrolyte (end-to-end distance in the state of maximum extension) and the number of charge groups on the chain. The central result of the theory is that systems characterized by $\xi > 1$ are unstable and will "condense" a sufficient number of counterions to reduce ξ to a value just less than 1. The mean ion activity coefficient for polyelectrolyte systems with $\xi < 1$ is

$$\ln \hat{\gamma}_\pm = \frac{-\xi X}{2(X + 2)} \tag{6}$$

and for $\xi > 1$

$$\ln \hat{\gamma}_\pm = \frac{-\xi^{-1}X}{4(\xi^{-1}X + 2)} \ln\left[\frac{\xi^{-1}X + 1}{X + 1}\right] \tag{7}$$

where $X = C_e/C_s$, the concentration ratio of polyelectrolyte to simple electrolyte or co-ions. Manning's ion condensation theory is applicable to systems with low concentrations of all ionic species. At finite polyion concentrations, this theory consistently overestimates the mean activity coefficient. Manning's treatment was modified for application to solutions with a low polyelectrolyte concentration and a finite salt concentration (Wells, 1973a,b; Kwak, 1973). Assuming the additivity of excess free energies, the mean activity coefficient of the mobile ions was calculated from the superposition of the excess free energies arising from (1) interactions between mobile ions and polyions and (2)

mutual interaction between mobile ions:

$$\hat{\gamma}_{\pm} = \gamma^{PM}\gamma^{MM} \tag{8}$$

γ^{PM} is the mean ion activity coefficient given in Manning's original theory (eqs 6 and 7) and represents the contribution from polyion−mobile ion interactions. The factor γ^{MM} describes the interaction between mobile ions.

Experimental Methods

Sample Preparation. Bovine nasal cartilage (BNC) was chosen as a model system due to its homogeneous structure and widespread availability and use in cartilage research. Fresh BNC was obtained from Shamrock Meats, Inc., Los Angeles, and transported in an ice bath to the experimental site in Santa Barbara within 3 h of sacrifice. The BNC samples were approximately 80 mm × 30 mm × 5 mm. BNC plugs were cut with a tissue punch (i.d. 4 mm), treated as described below and then equilibrated in the requisite solution at 4 °C for approximately 12 h. Prior to NMR imaging, all samples were equilibrated to room temperature for at least 90 min.

The effect of bath concentration on intratissue sodium concentration was studied by measuring intratissue sodium concentration at nine bath NaCl concentrations (0.05, 0.15, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2, and 2.0 *m*) at pH 7. To minimize sample-to-sample variability, nine BNC plugs were obtained from the same piece of BNC sample. The homogeneity of the tissue is such that sample-to-sample variability from a given piece of BNC is less than 5%. Each of the plugs was then equilibrated in one of the nine baths for 12 h before the measurements of intratissue sodium concentration were performed.

The effect of fixed charge density on the equilibrium behavior was studied by measuring intratissue sodium concentration at five bath concentrations (0.05. 0.15, 0.3, 0.5, 0.8 *m*) for low FCD samples. Five BNC plugs were obtained from the same piece of BNC sample. Their FCD was decreased by protonation with acidic saline. Since p*K* values of the fixed charge groups on proteoglycan matrix, sulfate (SO_3^-) and carboxylate (COO^-), are 3.0−3.5 and 1.5−2.0, respectively (Manning, 1969; Wells, 1973a,b; Kwak, 1973), FCD starts to drop significantly only when pH drops below the average p*K* of the fixed charge groups. Therefore, in this study, we used 0.15 M pH 3 NaCl solution to equilibrate the BNC plugs for 12 h. Each of the five acid-treated BNC plugs was then equilibrated in one of the five NaCl baths for 12 h before the measurements of intratissue sodium concentration were performed.

Reduced FCD from degenerative disease was simulated by treating BNC plugs with trypsin (Sigma, , 10 mg/mL) for 24 h at 37 °C. This digest primarily degrades the noncollagenous matrix proteoglycans and effectively reduces FCD. After the trypsin treatment, the BNC plugs were equilibrated in a 0.05 *m* NaCl bath for 12 h prior to NMR studies.

At the end of representative experiments, the proteoglycan/collagen concentration ratios of the samples were measured biochemically. The cartilage was first digested and the proteoglycan content was measured as sulfated glycoaminoglycans (GAG) (Goldberg and Kolibas, 1990; Farndale et al., 1982) and the collagen content was measured as hydroxyproline (HYP) (Woessner, 1961). The ratio of GAG:HYP gives a measure of the proteoglycan content of the matrix.

Nuclear Magnetic Resonance. Sodium NMR concentration measurements were performed on cartilage plugs loaded into glass tubes (o.d. 5 mm) and filled with incubation solution which acted as an internal reference for the determination of tissue sodium concentration. The measurements were performed on a Chemagnetics CMX spectrometer and an 11.6 T Magnex magnet. The ^{23}Na resonance frequency was 131.9 MHz. Actively shielded gradients (Doty Scientific, Columbia, SC) were used to produce linear magnetic field gradients of 80 G/cm. A prototype rf assembly consisting of a resonant saddle coil (length 10 mm, i.d. 5 mm) was used to provide an approximately homogeneous B_1 field over the cylindrical volume. The rf field, mapped by imaging a saline-filled glass tube, was uniform to within 5% over the total sample volume. The 90° pulse width for the rf probe was 15−20 ms. The sample temperature was maintained at 25 °C throughout each experiment.

The spin−spin relaxation behavior of ^{23}Na in cartilage has been shown to be multiexponential (Jelicks et al., 1993). At 131.9 MHz, the short component of T_2 is in the range of 2−5 ms and the long component is 16−32 ms (Dai and McFarland, 1996). To obtain an image reflecting the total sodium signal, a free induction decay (FID) projection image was obtained by acquiring the ^{23}Na NMR signal in the presence of a gradient applied immediately after a 90° spin excitation (Ra et al., 1986; Callaghan, 1991). Acquisition of an FID projection minimizes signal loss due to transverse relaxation and produces image profiles proportional to the total sodium content (Boada et al., 1994).

FID projections were acquired with both receiver and acquisition delays set to 5 μs, a repetition delay (TR) of 500 ms and a frequency encoding gradient applied along the axis of the NMR tube. The T_1 of sodium in normal BNC at room temperature is approximately 20 ms (Dai and McFarland, 1996). Data were Fourier transformed with phase and baseline correction to give the sodium distribution along the axis of the tube.

To convert sodium intensities to molal concentrations, it was assumed that all sodium ions in BNC are NMR visible (Lesperance et al., 1992). Each signal intensity was calibrated with respect to the bath sodium concentration and then normalized to the tissue water to yield the intratissue sodium ion concentration. For this purpose, it was necessary to measure the water content of the BNC samples. By using gravimetric analysis of BNC samples after treatment with ethanol and then drying to constant weight, we found both normal and acid-neutralized (pH 3) BNC contained 78 ± 5% water by weight. Thus, the concentration of sodium in BNC is given by

$$[Na^+]_t = \frac{1}{0.78}\frac{I_t}{I_b}[Na^+]_b \tag{9}$$

where subscripts b and t represent the bath solution and the tissue, respectively. $[Na^+]_b$ is the concentration of sodium in the incubation solution, and I_t and I_b are the measured image signal intensities. The water content of enzyme-digested BNC was ∼5% higher than for normal BNC which is within the experimental error.

Determination of Fixed Charge Density. In normal cartilage, the high density of matrix fixed charges results in Donnan exclusion of the co-ions from the bath electrolyte (Helfferich, 1962). The FCD can then be determined directly by measuring the concentration of sodium in cartilage equilibrated in a dilute bath solution, i.e. FCD ≈ $[Na^+]_t$ when the bath is dilute (Maroudas and Thomas, 1970). In our study, the dilute bathing solution used was 0.05 *m* NaCl aqueous solution. Since $[Na^+]_t = [Cl^-]_t$ + FCD, the error of the FCD measurement is equal to $[Cl^-]_t$ which is given by eq 1:

$$[Cl^-]_t = \left(\frac{\gamma_\pm}{\hat{\gamma}_\pm}\right)^2 \frac{[Na^+]_b}{[Na^+]_t}[Cl^-]_b \quad (10)$$

It follows that if a dilute bath of 0.05 *m* is used, $[Cl^-]_t$ or equivalently the error on the FCD measurement is less than 0.05 *m*.

For samples equilibrated in solutions at low pH (pH < 4), the concentration of hydrogen ions in the tissue cannot be ignored and must be included in the FCD calculation. The electroneutrality condition gives

$$FCD = [cation]_t = ([Na^+]_t + [H^+]_t) \quad (11)$$

If we assume that the concentration ratio $[Na^+]_t/[Na^+]_b$ is equal to $[H^+]_t/[H^+]_b$ (cf. Helfferich, 1962), then the FCD in acid-neutralized BNC (pH < 4) can be calculated from

$$FCD = \left([Na^+]_t + \frac{[Na^+]_t}{[Na^+]_b}[H^+]_b\right) \quad (12)$$

Therefore, in systems with low pH, the FCD was calculated using the equation above to account for the excess protons present in the system.

In enzyme-digested BNC, the tissue FCD is very low (<0.1 *m*) and the concentration of co-ions in the tissue is no longer negligible compared to matrix fixed charges. Thus, to accurately measure tissue FCD, a measurement of $[Cl^-]_t$ is required. In this study, instead of measuring $[Cl^-]_t$ directly which is extremely difficult due to very low NMR sensitivity of chlorine, we assumed that the activity of NaCl in enzyme-digested BNC is approximately equal to that in the external solution. This assumption is justified in very low FCD polyelectrolytes where chemically the ionic species behaves the same as in free solution. If the activity coefficients in both phases are equal, eq 2 simplifies to the ideal Donnan equilibrium equation and, in the case of NaCl, it is given by

$$\frac{[Na^+]_b^2}{[Na^+]_t([Na^+]_t - FCD)} = 1 \quad (13)$$

Equation 13 was used to calculate the FCD of trypsin-treated samples.

Calculation of Activity Coefficients. The mean activity coefficient of sodium chloride in BNC was calculated using eq 2 with the known bath sodium concentration, the experimentally measured tissue sodium concentration, and the experimentally determined FCD values. The mean activity coefficient of the NaCl bath was calculated from the empirical relation given by Hamer and Wu (1972).

Results and Discussion

Figure 1 shows intratissue sodium concentrations as a function of bath concentrations for normal BNC at pH 7. The sodium concentration was obtained from the ^{23}Na signal intensity measured by NMR microscopy of the BNC relative to the surrounding reservoir signal which had a known ^{23}Na concentration. Possible sources of error include variations in the NMR signal intensity across the sample, error in the water content determination, and physiological sample-to-sample variability as described above. At all reservoir concentrations, the Donnan potential maintains a concentration gradient which decreases with increasing reservoir concentration. The experimental data were compared to values calculated assuming ideal Donnan equilibrium (solid line). For high reservoir salt concentrations, the results were in good qualitative agreement with the predictions of ideal Donnan equilibrium. Deviations at low reservoir concentrations were attributed to interionic interactions which should be accounted for by the mean ion activity coefficient.

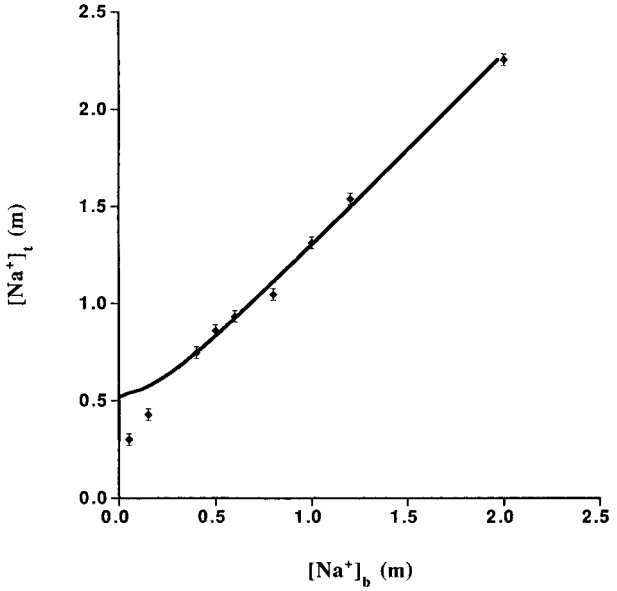

Figure 1. Sorption of sodium ions by normal bovine nasal cartilage at 25 °C for bath concentrations, $[Na^+]_b$, of 0.05 *m* to 2.0 *m* at pH = 7. Errors in the tissue sodium concentrations, $[Na^+]_t$, were set to 5% of the lowest measured sodium concentration. The solid line was calculated assuming ideal Donnan equilibrium.

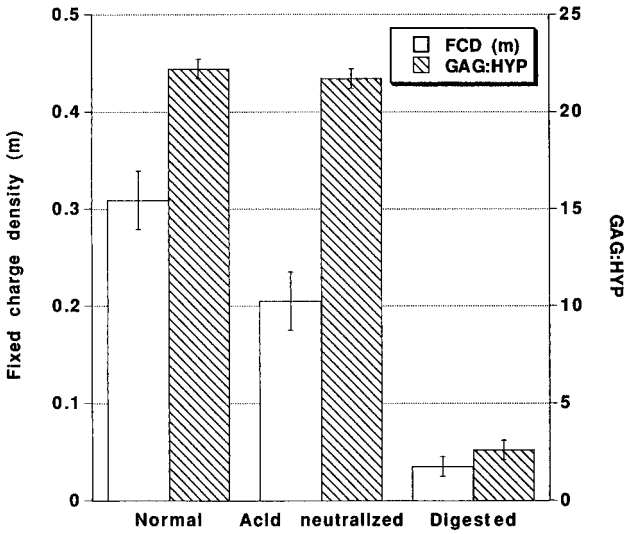

Figure 2. Fixed charge density of normal, acid-neutralized (pH 3), and trypsin-treated BNC measured at 25 °C. The FCD for normal, acid-neutralized, and trypsin-treated BNC were measured when the bath NaCl solution concentration was 0.05 *m*. Also shown are the biochemical results plotted as a ratio of glycosaminoglycan (GAG) to hydroxyproline (HYP). These results give a measure of the proteoglycans relative to the collagen content of the BNC.

To calculate the mean activity coefficient using eq 2, a measure of the matrix FCD is first required. We determined the FCD of normal, acid-neutralized, and trypsin-treated BNC (Figure 2) as described above. The errors are the sum of the systematic error described previously plus the experimental error of 5% in the intensity measurement. The FCD of normal BNC was, therefore, 0.31 ± 0.03 *m*. For acid-neutralized BNC, the concentration of ionized fixed charge groups was significantly reduced compared to pH 7 BNC. The reduction in FCD at pH 3 may be related to the protonization of carboxylate groups in the BNC since

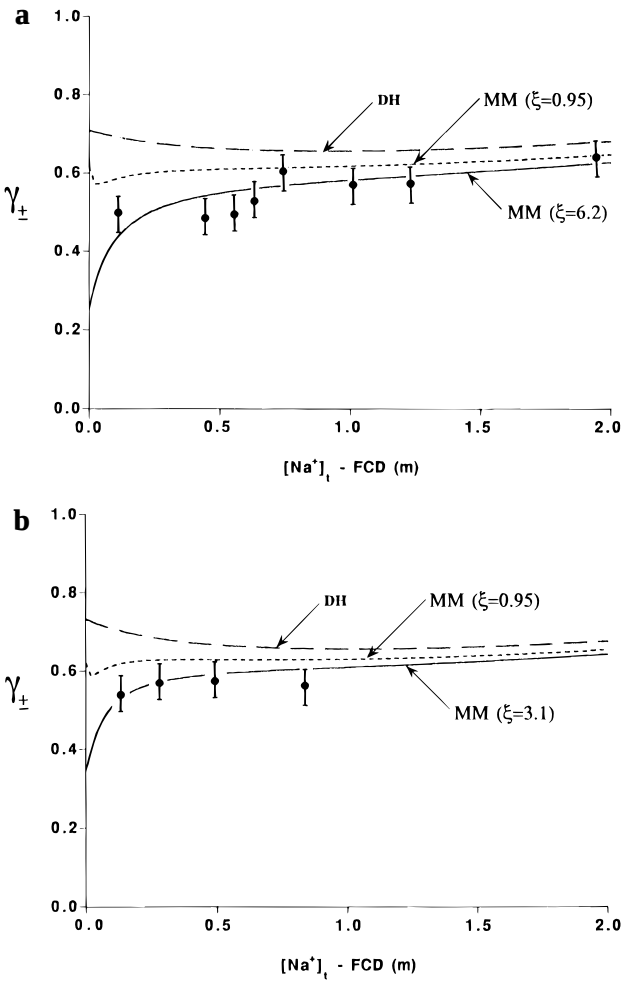

Figure 3. Mean ion activity coefficients, $\gamma_{\pm}$, of NaCl in (a) normal BNC and (b) acid-neutralized BNC shown as a function of the co-ion concentration ($[Na]_t$ − FCD). The data point values were calculated using the general Donnan equilibrium equation, eq 2, and the measured FCD and $[Na^+]_t$ values. DH line: theoretical predictions from extended Debye−Hückel theory. Two predictions based on the Manning theory (MM lines) but with different ξ values are also shown.

the carboxylate p*K* is 3.0−3.5. Trypsin digestion of the BNC resulted in a significant reduction in matrix fixed charges, Figure 2. Partial digestion of the proteoglycan elements by the trypsin and subsequent diffusion of the GAG elements out of the matrix are responsible for the observed change. A similar process is thought to occur in degenerative human diseases and aging where a decrease in GAG content occurs over time. The FCD dropped by a factor of 10 from 0.3 *m* to 0.03 *m* for a GAG:HYP change from 22 in normal BNC to 2.5 in the digested samples. Though this change in GAG:HYP is large compared to what might be expected in human diseases, it suggests that correlations might be established between NMR-determined tissue sodium content and the extent of degeneration. These findings are in contrast to human joint fluid measurements of GAG:HYP which remain approximately constant throughout life.

Figure 3a shows the mean ion activity coefficients for sodium chloride in normal BNC tissue calculated using eq 2, the general Donnan equilibrium equation, for the experimental sorption data shown in Figure 1, and the normal BNC FCD data shown in Figure 2. The experimentally derived values are shown as a function of the co-ion, Cl^-, concentration which we take as the difference between the measured tissue sodium concentration and the FCD. At physiological concentrations (0.15 *m*) and 25 °C, NaCl solutions have an activity coefficient $\hat{\gamma}_{\pm} = 0.755$ (Vanysek, 1990). We found $\hat{\gamma}_{\pm}$ in BNC for a reservoir NaCl concentration of 0.15 *m* to be significantly less, $\hat{\gamma}_{\pm} = 0.48 \pm 0.05$. Thus, the matrix fixed charges are more effective at lowering the system's internal energy by attractive interactions between sodium ions and the ionic matrix than an equivalent number of mobile co-ions. Effectively, the counterion/polyion pairs are closer together on average and have a greater attractive energy than counterion/co-ion pairs. Overall, $\hat{\gamma}_{\pm}$ is seen to increase with increasing NaCl reservoir concentrations, and the slight $\hat{\gamma}_{\pm}$ decrease at higher NaCl concentrations may be due to experimental errors. Though a decrease of activity coefficients at higher NaCl concentrations is conceivable, as the association of mobile ions becomes more prevalent, the experimental errors prevent us from reaching such a conclusion unambiguously.

The Donnan equilibrium is a thermodynamic result providing little insight into molecular interactions. However, the Debye−Hückel and Manning theories are statistical mechanical in origin, and their applications can improve the understanding of molecular level interactions. To evaluate the ionic behaviors in the polyelectrolyte under study, the mean activity coefficients calculated from the general Donnan equilibrium were compared with those predicted by the two statistical mechanical theories.

The data were first compared with the Debye−Hückel activity coefficients for NaCl solutions calculated at concentrations corresponding to the counterion concentration in BNC measured by sodium NMR (Robinson and Stokes, 1955; Hamer and Wu, 1972; Vanysek, 1990). This comparison is shown in Figure 3a. At very low concentrations (where Debye−Hückel accounts only for noninteracting electrostatic potential fields around each ion), the NaCl solution activity coefficient tends toward the ideal ($[Na^+]_t = 0$) value of unity where there are no ion-pair-attractive interactions. This behavior was not observed in our experimental data where ion-pairing between fixed charges and counterions will likely become the dominant interaction at low concentrations, removing essentially all sodium ions from the mobile pool and, thus, decreasing the mean ion activity coefficient. At higher co-ion concentrations, the discrepancy between these two becomes much less profound, which is expected as the ratio of free Na^+ to polyion-interacting Na^+ increases.

The activity coefficients determined by the Donnan equilibrium were next compared with those predicted by Manning's theory. Such comparisons may lead to more specific information about the molecular interactions, such as ion condensation and polyion−polyion spacing. To calculate activity coefficients predicted by the Manning theory, we used eq 8, assumed the values of γ^{MM} to be the mean activity coefficients of the simple NaCl electrolytes corresponding to the concentrations of the co-ion in the polyelectrolyte solutions, and calculated γ^{PM} from eq 6 or 7. Preston et al. (1972) reported the mean intercharge spacing in proteoglycan to be 0.75 nm, which gives a value of 0.95 for ξ. At this ξ value, no ion condensation is expected to occur and eq 6 should be used to calculate γ^{PM}. The value for γ^{MM} was taken to be the Debye−Hückel activity coefficient of a NaCl aqueous solution at the co-ion concentration ($[Na]_t$ − FCD) calculated from the extended equation of Hamer and Wu (1972). These calculated values were also shown in Figure 3a (dashed lines). The values of the $\hat{\gamma}_{\pm}$ calculated from the Donnan equilibrium were observed to increase with increasing reservoir NaCl concentrations. This trend is predicted qualitatively

by Manning's theory. However, the quantitative agreement with the experimental data was poor.

Qualitatively, the data can be extrapolated to $X \rightarrow \infty$ ($[Na]_t$ = FCD) and are seen crudely to predict an activity coefficient of approximately 0.5. The limits ($X \rightarrow \infty$) of eqs 6 and 7 are $\hat{\gamma}_\pm \approx e^{-x/2}$ and $\hat{\gamma}_\pm = (1/\xi e)^{1/2}$ for $\xi < 1$ and $\xi > 1$, respectively. The extrapolated value of 0.5 gives rise to $\xi = 1.4$ and $\xi = 1.5$ in the two limiting cases; thus, eq 7 for $\xi > 1$ was used for γ^{PM}. We found better overall agreement and a best fit value of $\xi = 6.2$ corresponding to an intercharge spacing of 0.1 nm (Figure 3a, the solid line).

A spacing of 0.1 nm is physically difficult to reconcile and less than a previous result, 0.75 nm (Preston et al., 1972), where extracted proteoglycan from BNC was studied. Cartilage glycosaminoglycans consists of almost equimolar proportions of chondroitin and keratan sulfate, the former containing two negatively charged groups per disaccharide unit while the latter contains only one. Qualitatively, however, our experimental results from BNC are in good agreement with the findings of Wells (1973a,b) who fit Preston's data for extracted proteoglycan to the modified Manning theory. Thus, our findings support the hypothesis that proteoglycans predominantly determine the ion behavior in normal BNC. Though the fit to the data in Figure 3a is poor, a smaller spacing likely reflects, in part, the fact that proteoglycan in cartilage is confined to a volume created by the collagen matrix which is significantly less than the natural hydrodynamic radius of the proteoglycan. The spatial confinement will reduce the physical separation of charges.

It has been shown that the $\hat{\gamma}_\pm$ increases with decreasing FCD by Maroudas who measured $\hat{\gamma}_\pm$ ranging from 0.67 to 0.72 *m* in femoral head articular cartilage which has a lower FCD (0.105−0.178 *m*) compared to the FCD of BNC, 0.25−0.35 *m* (Maroudas, 1968). We reduced the FCD of the BNC by protonating the polyion sites in pH 3 saline. Figure 3b shows that the discrepancy between the experimental results and Manning's theory is small even for low bath concentrations. This finding suggests that in the acid-neutralized matrix, the assumption that polyionic interactions are negligible is valid. The predictions of the Debye−Hückel and the modified Manning theory for $\xi < 1$ are shown as in the case of normal BNC except the FCD value used was 0.205 *m*. Using the modified Manning theory for $\xi > 1$, a best fit value of $\xi = 3.1$ was obtained corresponding to an intercharge space of 0.22 nm. The larger distance between charges is consistent with the protonization of the polyelectrolyte fixed charges.

Though the qualitative agreement between the BNC data and the polyelectrolyte theory is good, the predicted intercharge distances are clearly too small in both cases. The likely reasons responsible for the discrepancy are twofold: first, the sensitivity of ξ on the dielectric coefficient of the medium should be noted. The large dielectric coefficient (of pure water) used in our calculation is not necessarily applicable to the polyelectrolyte matrix. Any reduction in the medium's polarizability would result in a corresponding increase in the calculated charge separation. And, second, the theory of Manning was intended as a limiting law based on infinite line charge distributions, which is an oversimplified model for the system we are studying. The highly branched and folded proteoglycan structure no doubt gives rise to unaccounted fixed group interactions which would increase the system's internal energy.

Conclusions and Summary

We studied sodium sorption by the polyelectrolyte matrix of bovine nasal cartilage using ^{23}Na NMR microscopy. The sodium ion electrostatic interactions with the dense polyelectrolyte cartilage matrix were probed and the results of activity coefficient determinations indicated that at high bath concentrations, ideal Donnan equilibrium exists and the degree of polyion−mobile ion interaction is probably on the same order as the ion−ion interaction in a simple electrolyte. At low bath concentrations, however, interactions between the fixed charge groups may dominate sodium ion interactions.

The following major conclusions and observations emerge from our study:

(1) Reductions in cartilage sodium concentration brought about by reductions in the FCD by protonization of polyanionic sites or enzymatic digestion were detectable by NMR microscopy as a reduction in matrix signal intensity compared with a reservoir of known salt concentration. This methodology may be translated into noninvasive ^{23}Na imaging of human tissues such as cartilage whereby physiological reservoirs of constant sodium concentration such as the synovial fluid may be imaged adjacent to articular cartilage. In this way, changes in FCD associated with diseases might be detected.

(2) The equilibrium concentration of sodium ions and thus fixed charge groups in the matrix of normal bovine nasal cartilage is in the range of 0.25−0.35 *m* under physiological conditions. The variance is likely due to biological variability in the sample due to such things as the age and anatomical position of each sample.

(3) The mean activity coefficient of NaCl in cartilage shows a dependence on the reservoir salt concentration and was observed to decrease with decreasing salt concentration. This trend is consistent with the predictions of modified Manning theory which accounts for interactions between counterion and matrix fixed charges and is not consistent with Debye−Hückel theory or ideal Donnan theory. Thus, in dense connective tissue such as cartilage, electrostatic interactions between ions and matrix fixed charges are significant and important in understanding mobile ion behavior and their colligative and thermal properties.

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