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Effect of calcium on structure and function of a hyaluronic acid matrix: Carbon-13 nuclear magnetic resonance analysis and the diffusional behavior of small solutes

(glycosaminoglycan/synovial fluid)

MARY A. NAPIER AND NORTIN M. HADLER*

Departments of Medicine and Bacteriology (Immunology), University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

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ABSTRACT **Natural-abundance ^{13}C NMR at 25.16 MHz has been used to study a 2.5% matrix of hyaluronic acid at various degrees of polymerization and at various ionic strengths. Peak assignment is facilitated by comparing proton-decoupled and off-resonance-decoupled spectra of a hyaluronidase-depolymerized matrix with spectra from relevant monosaccharides. In contrast to the spectrum following depolymerization, the spectrum for intact matrix has considerable broadening, particularly for peaks assigned to the *N*-acetylglucosamine moiety. This is most dramatic for the hydroxymethylene carbon. With the addition of Ca^{2+} above 5 mM these broadened peaks narrow and approach the sharpness observed for the hyaluronidase digest. There is no shift in resonance peak positions. These changes are quantitatively less impressive if Na^+ is substituted for Ca^{2+}.**

The data suggest the existence of a considerable degree of order in regions of the matrix at physiological concentrations of Ca^{2+}. Within such a matrix the translational movement of lysine and glucose is enhanced relative to that in a matrix of agarose. Further addition of Ca^{2+} abrogates not only matrix order, but the enhanced diffusivity as well.

Hyaluronic acid (HA) is a linear polymer of repeating disaccharide units of *N*-acetyl-D-glucosamine, $\beta(1\rightarrow4)$, and D-glucuronic acid, $\beta(1\rightarrow3)$. The molecules isolated from such tissues as rooster comb, Wharton's jelly, and synovial fluid are polydisperse, ranging in molecular weight between 10^4 and 10^7. In solution at physiologic concentrations, HA behaves as if enormous (200 nm) spherical domains overlap and intertwine, resulting in the viscoelastic and molecular sieving properties of the matrix (1).

Crystallographic analyses (2–5), nuclear magnetic resonance studies (6), and optical rotary dispersion/circular dichroism analysis (7,8) suggest a considerable degree of order in the HA matrix. We have recently reviewed this literature (9). It is likely that stiff segments are present in the primarily flexible chain of the polymer as it exists in solution (6). Furthermore, the alignment of these stiff segments could create regions in the matrix with the configuration of a corrugated sheet. Certainly such regions would contribute to the viscoelastic properties of the matrix. However, several recent observations suggest that the presence of a HA matrix facilitates the translational movement of a number of solutes (9–11). Such a property is highly germane to the biological function of HA and could also be ascribed to the presence of ordered regions within the matrix (9).

In the studies reported herein we have utilized natural abundance ^{13}C NMR to demonstrate orderedness in a HA matrix. Such orderedness can be ablated either by enzymatic depolymerization of the matrix or by the addition of calcium salts. Perturbing the matrix in this way reduces the translational diffusivity of lysine and glucose.

MATERIALS AND METHODS

Materials. Human umbilical cord HA, grade I, was purchased from Sigma (molecular weight 10^5–10^6). All samples purchased were of lot number 115C 0009. Analysis was listed as 3.7% K, 0.005% Na. Ovine testicular hyaluronidase, type II, 365 National Formulary units/mg, was purchased from Sigma. Radionuclides were purchased from New England Nuclear and Indubiose A-37 agarose from Gallard–Schlesinger.

Methods. ^{13}C NMR spectra were recorded at ambient temperature, 27°, on a Varian XL-100-12 spectrometer operating at 25.16 MHz in the pulsed Fourier transform mode (pulse angle, 23.3°). Spectra were collected with wide-band proton decoupling, pulse acquisition time of 0.4 sec, and exponential weighting time constant of −0.04 sec. The splitting pattern due to C–H coupling, but not the magnitude of coupling constants, is obtained by off-resonance decoupling. Line widths are determined by drawing a straight line through the center of the baseline noise and measuring peak width at half-height from this estimated peak base. The contribution to line width from instrumental sources is less than 3 Hz, the line width of the single sharp resonance peak of chloroform. A Varian 620-L computer with 16K core and Varian disk Fourier transform software no. 994120-B was used for data collection and Fourier transformation. The solvent deuterium resonance was utilized as a field-frequency lock and chemical shifts are expressed in parts per million (ppm) downfield from external tetramethylsilane. Sample solutions, 1.5 ml in 10-mm NMR tubes, contained hyaluronic acid in D_2O at 25 mg/ml.

The diffusion coefficients for lysine, sucrose, glucose, and glutamic acid within the HA matrix were determined by modifications of the method employed to measure solute movement in synovial fluids (9, 10), which, in turn, were adapted from the technique devised by Redwood *et al.* (12) to measure the bulk diffusion coefficient of water in the erythrocyte membrane. Briefly, the hyaluronate solution is aspirated into an 8-cm length of polyethylene tubing (Intramedic, Clay–Adams) 0.086 cm in internal diameter. The tubing is fastened at one end in a custom-designed Lucite block and then equilibrated at 37° in a water-saturated atmosphere. The radiolabeled solute is applied to the free end of the column of HA in a volume of 1.0 μl. Following a timed incubation at 37°, the block is frozen at −70°. The tubing is sliced into 0.5-cm lengths by inserting a razor blade into the grooves machined in the

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Abbreviation: HA, hyaluronic acid.

* To whom reprint requests should be addressed.

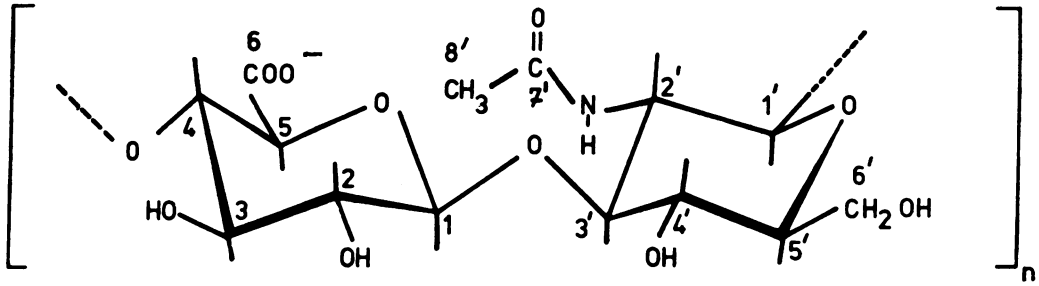

FIG. 1. Disaccharide repeat unit of hyaluronic acid. Numbers 1–6 designate the β-D-glucuronic acid (GlcUA) carbon atoms and 1′–8′ the *N*-acetyl-β-D-glucosamine (GlcNAc) carbon atoms. The glycosidic linkages GlcNAc to GlcUA and GlcUA to GlcNAc are 1→4 diequatorial and 1→3 diequatorial, respectively. The pyranose rings are in the chair conformation. The hydroxyl, carboxyl, hydroxymethylene, and *N*-acetylamino groups are equatorial.

block, perpendicular to the tubing axis. Each slice is incubated overnight in phase-combining scintillant (PCS, Amersham) and subjected to scintillation counting. A straight line is drawn by least squares relating the logarithm of percent of total solute at any distance (x) from application to x^2. For the data reported the correlation coefficient (R^2) describing nearness of fit of the data to the line drawn is >0.98. The diffusion coefficient D ($cm^2\ sec^{-1}$) is computed from the slope of the line, which equals $-1/4Dt$, in which t is incubation time in seconds.

RESULTS

The structure of hyaluronate and the numbering system we will employ are illustrated in Fig. 1. The proton-decoupled ^{13}C NMR spectrum of hyaluronic acid from human umbilical cord in D_2O is shown in Fig. 2*B*. Resonances are assigned to individual carbons of the disaccharide repeating unit, *N*-acetyl-D-glucosamine-β(1→4)-D-glucuronic acidβ(1→3), on the basis of comparison with the ^{13}C NMR spectrum of the enzyme digest with complete and off-resonance decoupling (Fig. 2*A* and 2*A*′), and by comparison with the spectra of monosaccharides (13, 14). Hyaluronidase cleaves glycosidic bonds, reducing the molecular weight and viscosity of hyaluronic acid (15). The off-resonance decoupled spectrum shows sharp peaks and clearly interpretable carbon–hydrogen (CH) coupling patterns.

The chemical shifts of the individual carbons of the glucuronate and *N*-acetylglucosamine correlate with the observed chemical shifts of the analogous hexose (16, 17) except for differences associated with the introduction of the carboxyl and *N*-acetylamino groups. The resonance peaks are assigned as follows: *N*-acetylamino and carboxyl carbonyl (singlets in off-resonance-decoupled spectrum, 177.3 and 176.5 ppm, respectively); anomeric carbons C_1 and $C_{1'}$ (doublets, 105.5 and 102.8); C_5 (85.1); C_3 (82.4); $C_{4'}$ (70.8); ring atoms $C_{3'}$–$C_{5'}$ (85.1) and C_2–C_5 (70.8); C_2, C_4, $C_{3'}$, and $C_{5'}$ (78.7–44.9); methylene side-chain, $C_{6'}$ (triplet, 62.9); and *N*-acetylamino methyl, $C_{8'}$ (quartet, 24.8). The decreased intensity of $C_{7'}$ versus C_6 reflects carbonyl bonding to a nitrogen atom (18). These assignments are consistent with those previously proposed for hyaluronic acid (6).

The room temperature ^{13}C spectrum of a 2.5% solution of hyaluronic acid (Fig. 2*B*) exhibits remarkable detail considering its extremely high viscosity. The carbonyl carbon and methyl carbon resonances are quite sharp. The resonance positions appear unshifted relative to the enzyme digest material. The major changes observed, relative to the depolymerized HA, are broadening and decrease in resolution of the side-chain methylene carbon ($C_{6'}$) and the ring carbons (C_1–C_5 and $C_{1'-C5'}$).

Titration of HA with Ca^{2+}. Changes in the ^{13}C NMR spectra induced by the addition of $CaCl_2$ are illustrated in Fig. 3. At high calcium concentration (0.1 M), the resonance peaks narrow considerably, but no shifts in resonance positions are observed. The narrowing of the resonance peaks is most easily seen for the single sharp component of the methyl carbon ($C_{8'}$) resonance peak. The peak width at half-height (corrected for instrumental contributions) decreases from 29 Hz at 0.005 M Ca^{2+} to 17 Hz at 0.1 M Ca^{2+}. The most dramatic changes in peak morphology are apparent for carbon atoms of the acetylglucosamine unit; $C_{2'}$ and to a lesser extent $C_{6'}$, $C_{1'}$, and the *N*-acetyl carbonyl, $C_{7'}$ (Fig. 3). There is very slight narrowing of the C_1 of the glucuronate in contrast to the striking increase in resolution of $C_{1'}$. These changes argue for greater increase in flexibility at the glucosamine residue when compared to the glucuronate subsequent to calcium titration.

No change in peak morphology is observed in buffered relative to nonbuffered solutions (i.e., 0.01 M sodium phosphate, pH 7.0 or 0.01 M sodium acetate, pH 7.0). Spectra essentially equivalent to Fig. 3*A* were observed for 0.04 M and 0.1 M KCl. The addition of 0.5 M NaCl, 0.01 M sodium phosphate buffer, pH 7.0, to HA results in a spectrum comparable to 3*B*. Therefore the change in peak morphology with the addition of Ca^{2+} in Fig. 3 cannot be ascribed to pH. Furthermore, ionic strength effects are not sufficient to explain the Ca^{2+} effect, because concentrations of Na^+ and K^+ much greater than the ionic equivalent of Ca^{2+} are required to achieve these changes.

Effect of Ca^{2+} Titration on Small Solute Diffusivity. The apparent diffusion coefficients (D) for lysine, glucose, sucrose, and glutamate in a 2.5% HA matrix in different solvents are presented in Table 1. It is clear that, in the presence of as little

Table 1. Diffusion coefficients* (D, $10^{-5}\ cm^2\ sec^{-1}$) at 37°

		Solute			
Matrix	Solvent	Lysine	Sucrose	Glucose	Glutamate
2.5% HA	H_2O	2.03	0.44	1.91	0.69
	0.01 M Ca^{2+}	0.53	0.44	0.48	0.56
	0.10 M Ca^{2+}	0.57	0.46	0.54	0.55
	H_2O†	2.19	0.50	2.16	0.69
	0.15 M NaCl†	2.11	0.52	1.99	0.69
2.5% Agarose	H_2O	0.85	0.68	0.91	0.96
	0.01 M Ca^{2+}	0.94	0.75	0.92	0.98
	0.10 M Ca^{2+}	0.96	0.78	0.96	1.00
	0.15 M NaCl†	0.94	0.78	0.94	0.97

* All data were derived from codiffusion experiments measuring [^{14}C]lysine/[^3H]sucrose and [^{14}C]glucose/[^3H]glutamate together. The solute is applied in a concentration <0.5 mM. The mean of a minimum of three experiments is tabulated. The standard errors averaged 8% of the respective means.

† These samples were buffered at pH 7.0 with 0.01 M sodium phosphate.

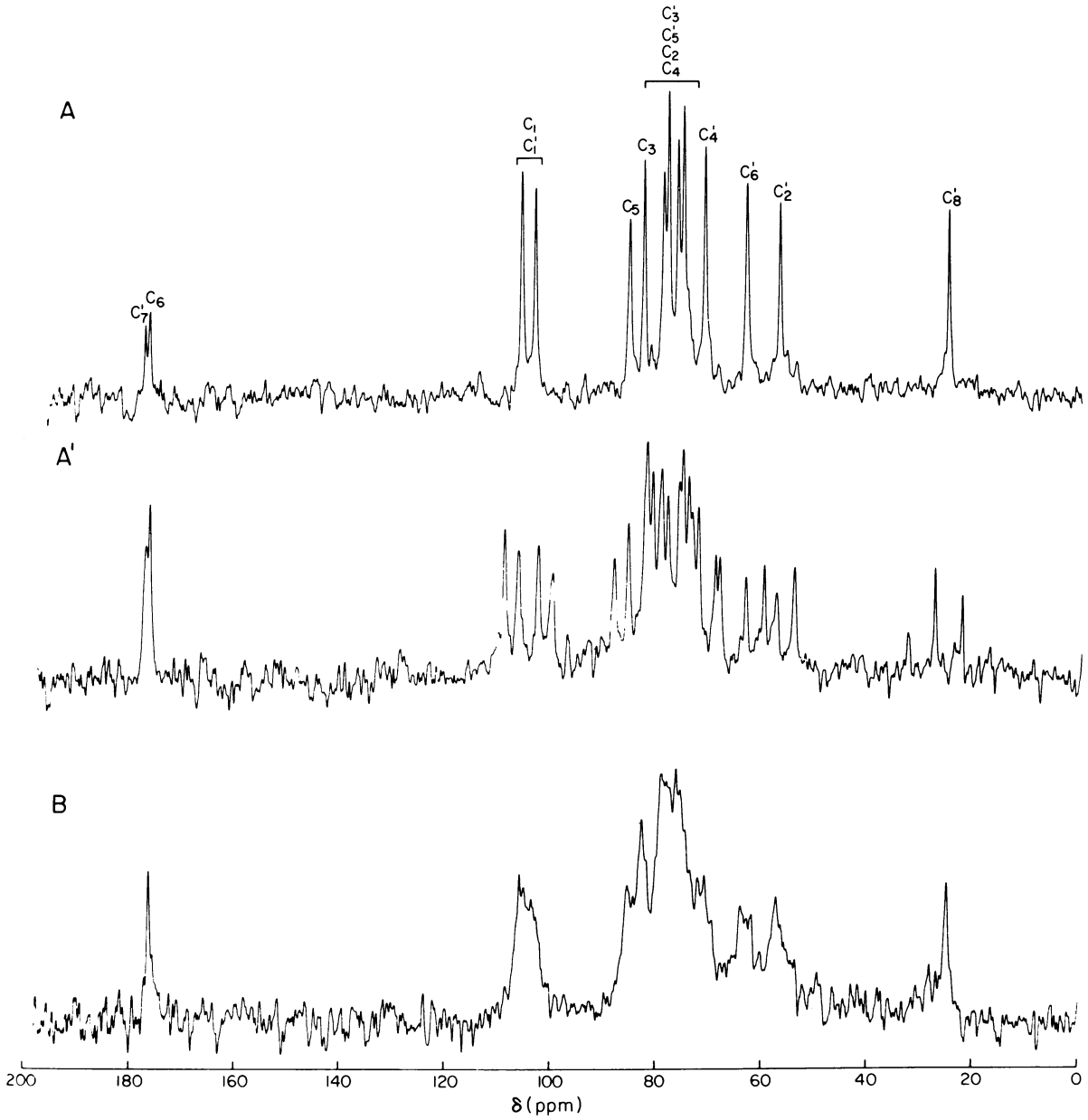

FIG. 2. ^{13}C NMR spectra (25.16 Hz) at ambient temperature. For spectrum *A*, a 2.5% HA matrix was digested with hyaluronidase (0.03% wt/wt, 37° overnight). *A* is the proton-decoupled spectrum and *A'* the off-resonance decoupled spectrum. *B* is the spectrum for the undigested matrix of 2.5% HA. Spectra were obtained after 120,299 (*A*), 66,560 (*A'*), and 118,921 (*B*) transients of 0.4-sec sweep time. For resonance peak numbering refer to Fig. 1.

as 0.01 M Ca^{2+}, the translational diffusivity of both lysine and glucose is markedly reduced. Furthermore, this effect is not apparent if Na^+ is substituted for Ca^{2+}. In an agarose matrix (2.5% Indubiose) there is no significant effect of ionic strength or pH on the movement of these solutes.

The *D* for lysine and glucose in the HA matrix in the absence of Ca^{2+} is markedly enhanced compared to that in the agar matrix regardless of buffer. The presence of an HA matrix facilitates the movement of these two solutes but not sucrose or glutamic acid. It is this enhanced diffusivity in the HA matrix that is abrogated by the addition of Ca^{2+}.

DISCUSSION

Crystallographic studies of hydrated HA (2–4) have demonstrated that direct interchain H-bonding occurs exclusively at the $C_{6'}$ methylene hydroxyl group. Stereochemical models indicate that such bonding would impede the flexibility of the *N*-acetylglucosamine relative to the glucuronate moiety. Reducing this interchain bonding would allow greater motion of the polymer as a whole with a greater relative freedom for *N*-acetylglucosamine units.

The effect of Ca^{2+} on the natural-abundance ^{13}C NMR spectrum of HA suggests that interchain stabilization in a 2.5% matrix at 0.005 M Ca^{2+} or less occurs in a fashion similar to that seen in the putty studied crystallographically. As the matrix is titrated to 0.01 M Ca^{2+}, marked narrowing of the $C_{8'}$ resonance peak is seen (Fig. 3). At this and higher concentrations of calcium, definite sharpening of all carbon resonances assigned to the *N*-acetylglucosamine moiety is manifest. At 0.10 M Ca^{2+} the entire spectrum approaches that of the hyaluronidase digest (Fig. 2*A*). These observations suggest that the flexibility of the entire polymer increases subsequent to the addition of calcium.

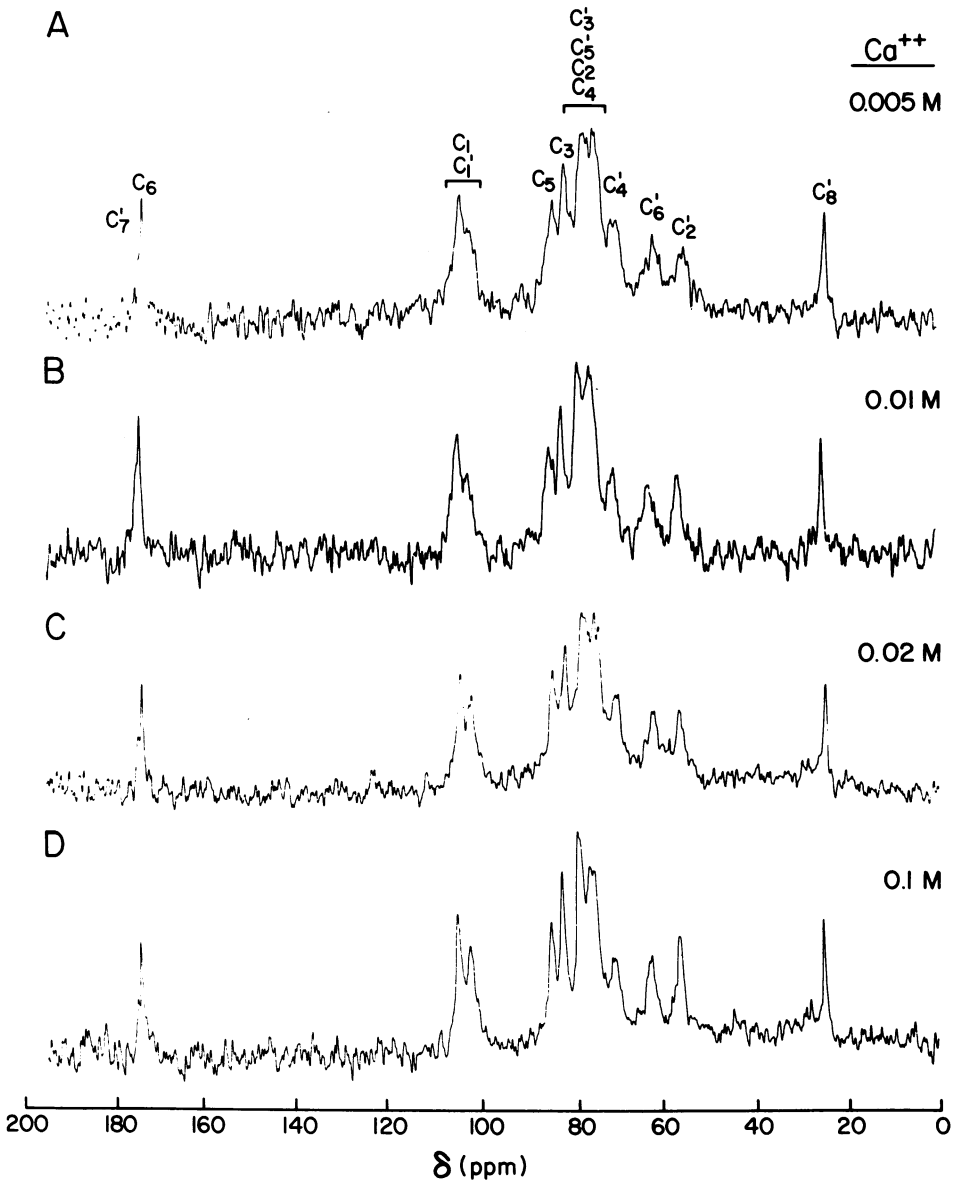

FIG. 3. ^{13}C NMR spectra (25.16 Hz) at ambient temperature of 2.5% HA at the calcium concentrations indicated in D_2O. Spectra were obtained after 118,357 (*A*), 119,236 (*B*), 117,310 (*C*), and 119,400 (*D*) transients of 0.4-sec sweep time. For resonance peak numbering refer to Fig. 1.

Because the peak narrowing is more dramatic for glucosamine relative to glucuronate carbons, it is likely that the increased flexibility of the entire polymer primarily reflects interference with interchain stabilization mediated by H-bonding at $C_{6'}$. The dramatic sharpening of all carbon peaks that follows hyaluronidase depolymerization suggests as well that sizable chain lengths are required for interchain interactions of this nature.

One predictable consequence of loss of stiff segment interaction in a matrix of HA would be a decrease in intrinsic viscosity. It has long been known that both hyaluronidase depolymerization and the addition of calcium salts can effect such a decrease in solutions of synovial mucin (19). We have demonstrated an exquisite sensitivity of isolated HA viscosity to calcium concentration (D. Gabriel, M. A. Napier, and N. M. Hadler, unpublished observations). Why is the translational diffusivity of lysine and glucose in the HA matrix dependent on calcium concentration? The movement of both solutes is enhanced relative to their movement in a matrix of agar at physiologic Ca^{2+} concentrations. For a cationic species such as lysine, enhanced movement in the polyanionic matrix of HA could in part be explained by Donnan effects (11, 20). However, such an explanation could not pertain to glucose.

We have previously suggested several hypotheses to explain such aberrant or facilitated movement within an HA matrix (9). One hypothesis borrows heavily from Metzner's considerations of diffusive movement in structured media (21). It is postulated that the molecular "packing" of the solvent adjacent to the polymer is different from that in the bulk solvent phase. This could provide a region with low resistance to mass transport analogous to the mechanism of conduction of electricity in disperse systems or diffusion in crystalline solids. It is the concept of adsorption of the solute to a surface with high intrinsic conductance.

With the ^{13}C NMR analysis we have demonstrated orderedness consistent with the presence of interacting segments of adjacent chains in regions of the HA matrix at physiologic concentrations of calcium. At higher calcium concentrations such regions become disordered and, coincidentally, the ability of the matrix to enhance the translational diffusivity of glucose

and lysine is lost. It is possible that these regions of interacting segments retain the configurations documented in hydrated HA studied by X-ray diffraction (3). In that case, these regions could have the configuration of corrugated sheets with highly structured solvation shells and might well provide a high-conductance surface at physiologic concentrations of Ca^{2+}.

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