NUCLEAR MAGNETIC RESONANCE STUDIES OF HYALURONAN (HA): EVIDENCE OF COMPETITIVE INHIBITION OF INTERCHAIN ASSOCIATIONS BY PHOSPHOLIPIDS WHICH MAY RESULT IN DECREASED ANTI-INFLAMMATORY AND CARTILAGE PROTECTING PROPERTIES OF HA ✣

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Abstract - The chain flexibility of solutions of hyaluronan (HA) of different molecular weights was determined by ^{1}H-nmr spectroscopy in the absence and presence of the phospholipid Dipalmitoyl DL-α-phosphatidylcholine (DPC). Sonication of high or low molecular weight HA with DPC for periods up to 60 min markedly increased HA chain flexibility as determined by observing the spin-spin relaxation times for the glucosamine acetyl group protons. From these data it is proposed that DPC competes for those hydrophobic centres along the HA chain which are normally responsible for the inter and intra chain interactions and which confer stiffness to the HA molecule.

✣ Dedicated to Professor Alan Katritzky on the occasion of his 65th birthday.

Hyaluronan (hyaluronic acid, HA) is the most abundant glycosaminoglycan in mammalian tissues. It is particularly rich in connective tissues, such as skin (~ 200 μg/ml), vitreous humour (140 - 340 μg/ml), cartilage (~ 1.2 mg/ml) and umbilical cord (~ 4.1 mg/ml), where it mainly serves a space filling function.[1]

The largest single reservoir of HA in mammals, however, is in the synovial fluid (SF) of their diarthrodial joints where concentrations of 1.4 - 3.6 mg/ml of HA are achieved.[1] The high concentration of HA in SF is essential for normal joint function, since HA confers exceptionally high viscosity, elasticity and lubricating ability to SF. These rheological properties minimise wear and attrition of articular cartilage during load bearing.[2,3] In addition, the high exclusion volume of aqueous solutions of HA restricts the entry into SF of the large blood-borne proteins circulating in the synovial capillaries, but facilitates the transport of water and small molecular weight ions through SF to articular cartilage.[4,5] These selective effects on solute diffusion through HA are important for the nutrition of articular cartilage and the elimination of metabolites and noxious substances from the joint space.

The remarkable physical properties of aqueous solutions of HA arise from its unique macromolecular structure. It is an extremely long linear polyelectrolyte consisting of a repeating disaccharide unit (hyalobiuronic acid) which is composed of β-D-gluconyl residues linked glycosidually via their 4 positions to the 1-position of *N*-acetyl-β-D-glucosamine (Figure 1).

Hydroxyl oxygens at the glucuronyl-4-position and *N*-acetyl-β-D-glucosamine 3-position are the sites for further polymerisation of hyalobiuronic acid. The linear HA chains so arising are of variable length (polydispersed) but in SF the HA is extremely large with molecular weights (MWs) in the order of 6 - 10 x 10^6 Da.[2,6,7] In dilute aqueous solution HA is considered to adopt an extended random coil conformation but at high concentrations (> ~ 1 mg/ml) the chains are thought to become entangled to form an extended three-dimensional network.[8-11]

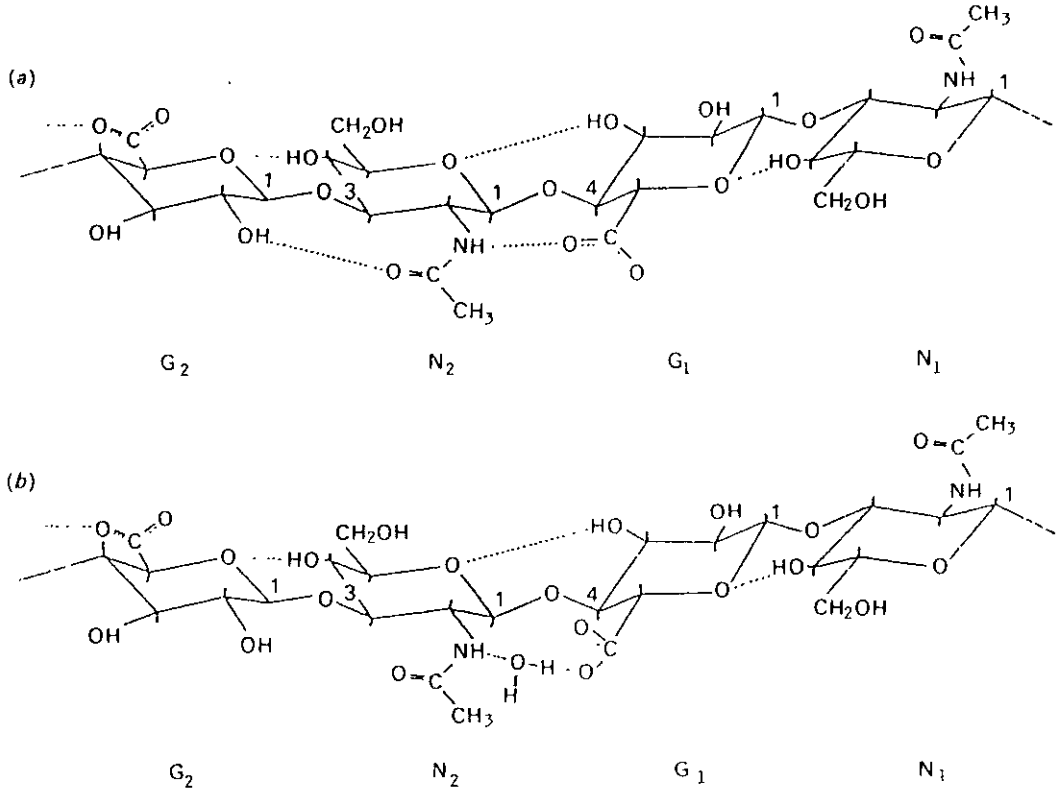


Figure 1 - Primary structure of hyaluronan (HA) showing hydrogen bonds in (a) non-aqueous solvent (dimethyl sulfoxide) and (b) dimethyl sulfoxide containing water. The hydrogen bonds between the *N*-acetylglucosamine (N) (2-acetamido-2-deoxy-β-D-glucopyranosyl) and glucuronic acid (G) (β-D-glucopyranosylgluronic acid) rings reduce rotation about the glycosidic linkages and confer some stiffness to the chains. [Taken from reference (15) with permission].

While the flexibility of the HA chains is determined in part by the restricted rotation about the various glycosidic linkages,[9,12] the conformational freedom of the molecule is also determined by other factors. Hydrogen-bonding between the glucuronyl carboxyl group and the adjacent acetamido hydrogen of the *N*-acetyl hexosamine ring has been reported to contribute to chain stiffness[10,13] (Figure 1). However, at solution concentrations of HA which allow chain-chain overlap, tertiary structures may form. On the basis of viscoelastic measurements, nmr relaxation techniques, rotary-shadowing electron microscopy and computer modelling, it has been suggested that HA undergoes intra- and

interchain associations.[10,14-16] Although the nature of the bonding in these chain overlap regions has not been resolved, it has been proposed that coupling occur between hydrophobic patches which are present at regular intervals along the HA chain[13,15] (Figure 2).

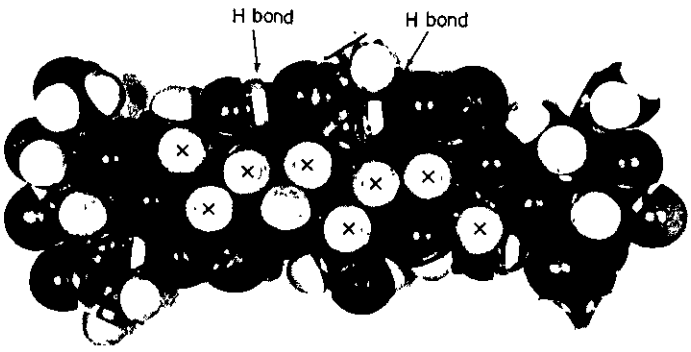


Figure 2 - Courtauld space-filling model of an oligosaccharide segment of hyaluronan (HA) with the conformation as predicted by Figure 1. This configuration exposes hydrophobic patches arising from the alignment of eight contiguous CH groups of the saccharide rings. These hydrogens are marked with a cross but could be augmented by the adjacent CH_2OH and methyl groups. The acetamido hydrogen bonds are also indicated. These hydrophobic patches are formed at regular intervals on alternate sides of the HA chain. The hydrophilic region of the HA molecules is on the reverse side of the model, as shown. (Taken from reference 15 with permission).

Intra- and interchain interactions of this nature are not uncommon in polysaccharide systems and occur especially during gel formation.[16-18] In addition, they have been reported to occur for HA as determined using a variety of physical techniques.[14,19,20] Using nmr spin-spin relaxation times, Darke et al.[14] demonstrated that aqueous solutions of HA consisted of two distinct domains. One domain had the mobility expected of a flexible linear polysaccharide chain, while the other regions (55 - 70 %) were stiff and indicative of strong interchain associations.[14] It was concluded that "the stiffer domains within HA were prevented from equilibrating with the flexible domains by some unknown covalent features". In a viscometric study, Welsh et al.,[16] showed that ordered intermolecular associations responsible for the high viscosity of HA solutions[17] could be

disrupted by the addition of HA fragments (~ 60 disaccharide units) which competed for the interchain coupling sites. Similar competitive inhibition of aggregation (gelling) has been reported for alginate, carrageenan and xanthan-galactosmannon in the presence of short chain fragments of the respective polysaccharides.[18]

Since HA contained repeating hydrophobic regions which were considered to be associated with intra and interchain associations[15] and phospholipids (PL) were known to be present in synovial fluid[21-24] and cartilage,[25] we formulated the hypothesis that hydrophobic molecules such as phospholipids (PL) could compete for the HA intra-interchain binding regions, thereby influencing the flexibility of HA in solution. We report here ^{1}H-NMR spin-spin relaxation studies of various HA preparations conducted in the presence and absence of PL which provides some support for our hypothesis.

EXPERIMENTAL

Highly purified HA preparations (10 mg/ml) of different molecular weight in 10 mM phosphate buffer containing 0.15 M NaCl, pH 7.3 were provided by Dr. Goren Smedegaard of Kabi-Pharmacia AB, Uppsala, Sweden. Healon® was a gift from Mr. Peter Chapman of Pharmacia Southseas Pty. Ltd., Sydney, Australia. HA as a purified lyophilised preparation isolated from synovial fluid of adult bovine stifle joints was a gift from Dr. D. Cullis-Hill (Arthropharm Pty. Ltd., Bondi Junction, NSW, Australia). Dipalmitoyl DL-α-phosphatidylcholine (DPC), *m*-phenylphenol, 2,4-diaminophenol dihydrochloride, dimethylaminobenzaldehyde and glucuronolactone were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ammonium heptamolybdate was a product of E. Merck AG, Darmstadt, Germany. All other chemicals used were of analytical grade or of the best quality available. Throughout this study double-distilled water was used to prepare all solutions and buffers.

Estimation of the Protein and Phospholipid Contents of HA Samples

The lyophilised bovine HA preparation was dissolved in 10 mM phosphate containing 0.15 M NaCl, pH 7.3 (PBS) by gently stirring at 4 °C overnight and then made-up to a

concentration of 1 mg/ml in the same buffer. Other HA preparations were used directly. The total protein in the HA preparations was estimated by the Peterson method[26] whereby protein complexes with Cu^{2+} and phosphomolybdate to form a blue chromogen which was measured spectrophotometrically at 650 nm using a UV/VIS spectrophotometer (model SP8-100, Pye Unicam Ltd., Cambridge, England). The amount of phospholipid in HA samples was determined as their phosphorus content by using the method of Bartlett.[27] However prior to phosphorus analysis the aqueous solutions of HA (1 ml aliquots) were exhaustively dialysed against double-distilled water, by applying the Overall technique[28] to remove all free phosphate present in the sample buffer. The dialysis retentates were extracted in a chloroform/methanol mixture (2:1 by volume) by the method originally described by Folch et al.[29] The lower organic phase was separated and the chloroform removed by rotary evaporation under nitrogen gas. The non-volatile residue was assayed for the phosphorus content, as described previously.[27] In some experiments, the dialysis retentates were subjected to freeze-drying instead of solvent extraction, then assayed for phosphorus. *All glassware was soaked in 6N nitric for 24 h and then rinsed twice with* double-distilled water before use in these experiments.

Interaction of Hyaluronan (HA) Preparations with Dipalmitoyl DL-α-Phosphatidylcholine (DPC)

To a 2 ml solution of HA in PBS (1 mg/ml) was added an aliquot (20 µl) of DPC in 20 µl ethanol. The components were mixed for 15 - 60 min at 20 °C in an ultrasonicating waterbath (model G112SP1G, Laboratory Supplies Company Inc., Hicksville, NY, USA), as described by Hills.[22] The resulting opalescent solution showed no evidence of phase separation even after standing at 4 °C for several days.

Determination of the Effects of Ultrasonication and Ethanol on the Molecular Weight of HA

In order to determine if ultrasonication or the DPC vehicle, ethanol, affected the physical properties of HA, the protocol used above was repeated for up to 4 h in the absence of DPC. An aliquot (0.5 ml) of the resulting sonicate or unsonicated Healon® (1 mg/ml) in

phosphate buffer (pH 7.2) containing 20 µL of ethanol was then applied to a Sepharose CL-2B (Pharmacia Southseas Pty. Ltd, Sydney, Australia) gel-filtration column (30 cm x 1 cm) equilibrated with the same buffer. The column was eluted at 10 ml/h with PBS and the levels of HA in the fractions collected determined by hexuronate analysis using the Blumenkranze and Asboe-Hansen method.[30]

^{1}H-Nmr Spectroscopy

High resolution ^{1}H-nmr spectra (199.5 MHz) of samples were obtained using a JEOL FX-200 spectrometer (JEOL Ltd., Tokyo, Japan) operating in the Fourier transform mode. Samples were contained in 10 mm Wilmard™ glass tubes, together with a coaxial 5 mm tube containing tetramethyl silane in deuterium oxide. This was used as an external integration reference. The sample temperature was maintained at 37 °C. The water signal was irradiated to suppress that signal within the spectrum. All samples were degassed with argon prior to measurements. The conformational mobility (flexibility) of solutions of HA was determined using the method described by Darke et al.[14] This required the determination of the spin-spin relaxation time (T_2) for the N-acetyl protons of the glucosamine residues in the HA chains. Since T_2 is approximately proportional to the rate of motion of the molecule, protons in highly flexible regions of HA produce a sharp signal whereas those in rigid (less flexible) environments are seen as a broad band.[14] The percentage of flexible and rigid segments was assessed in duplicate from the line widths for the broad and narrow signals.[14]

^{13}C-Nmr Spectroscopy

^{13}C-Nmr proton decoupled spectra (50.1 MHz) of HA preparations (10 % w/v) dissolved in D_2O, pD 7.4 (pD = pH - 0.4) were obtained, using a JEOL FX-200 FT spectrometer. Samples were made-up in 10 mm Wilmard™ glass tubes in which a 5 mm coaxial tube containing acetonitrile as an external reference had been placed. The sample temperature was maintained at 37 °C and spectral widths of 10 KHz and 6K data points were used.

RESULTS

Analysis of Hyaluronan (HA) Samples and the Effects of Ultrasonication on Molecular Weight

The percentage of protein and phosphorus present in the various HA preparations used in these studies is shown in Table 1. Of the preparations examined it was found that the low molecular HA standards provided by Kabi-Pharmacia AB were of the highest purity; having undetectable phosphorus levels and low protein content. Healon® was also very pure (protein ~ 0.22 %) but contained detectable phosphorus levels. The HA prepared from bovine synovial fluid was the least pure sample tested.

Table 1 - Molecular weights (MW), total protein and phosphorus content of hyaluronan (HA) samples used

HA Sample	MW (x 10^6)	% Protein (w/w)	% Phosphorus (w/w)
Healon®	3.80 *	0.22	0.005
H9	0.88 *	0.14	N.D.
H5	0.54 •	0.20	N.D.
H4	0.35 •	0.21	N.D.
Bovine HA	3.04 #	0.47	0.020

N.D. = Not detectable; * = As determined by low-angle laser light scattering[31]; # = As determined by gel-exclusion chromatography.[32]

Ultrasonication of Healon® in the presence of ethanol for 4 h but absence of DPC produced only a small effect on HA molecular polydispersity. Some degree of depolymerisation of HA was evident as shown by the inclusion in the gel of a higher proportion of lower molecular species than unsonicated Healon®. However, sonication of Healon® for periods up to 60 min produced no observable change in the respective chromatographic profiles (data not shown).

Nmr Studies

The ^{13}C-nmr chemical shift values for Healon® and HA from bovine synovial fluid are shown in Table 2. As can be seen, the ^{13}C chemical shift values for the ring carbons of the *N*-acetylglucosamine and glucuronic acid disaccharide repeat unit of HA were similar for both preparations even though their purities and origins were different.

Table 2 - The ^{13}C-nmr chemical shift values for HA obtained from rooster comb (Healon®) and bovine synovial fluid (bovine HA).

Position #	Chemical Shift (ppm) •	
	Healon®	Bovine HA
A1	101.08	101.91
A2	54.84	54.91
A3	83.45	83.33
A4	69.31	69.04
A5	74.42	74.37
A6	61.55	61.24
A7-(CO)	175.64	175.27
A8-(CH_3)	23.14	23.12
U1	103.59	103.42
U2	76.97	76.64
U3	76.12	76.00
U4	80.53	80.36
U5	73.30	73.20
U6	174.25	174.10

• Relative to acetonitrile; #A = N-acetylglucosamine ring carbons, #U = Glucuronic acid ring carbons

The percentage of flexible segments in the various HA preparations, as determined from their spin-spin relaxation times were found to have varied widely (Table 3). With the low molecular weight HA standards (H4, H5, H9), which were free of phospholipid, the percentage of flexible segments was inversely related to their molecular weights. However,

with the high molecular weight preparations which contained phospholipid this relationship was not followed. With the bovine HA preparation, which contained 4 x the amount of phospholipid associated with Healon®, 75 % of the molecules favoured the flexible form (Table 3).

Table 3 - *The percentage of flexible segments within HA preparations as determined by spin-spin relaxation times of their acetamido proton signals.*

HA Sample	MW (x 10^6)	% Flexible Segments
H4	0.35 *	28
H5	0.54 *	19
H9	0.88 *	11
Healon®	3.80 *	40
Bovine HA	3.04 †	75

* Determined by low-angle laser-light scattering[31]; † Determined by gel-exclusion chromatography.[32]

Sonication of a high (Healon®) or a low molecular weight HA preparations (H4) with DPC markedly increased the proportion of flexible domains present. In the control experiments in which DPC was omitted, minimal changes in flexibility in the HA preparations was observed (Figure 3). It was shown that the effect of DPC on HA chain flexibility was concentration independent over the range 50 - 500 μg/ml of Healon® used (Table 4).

Figure 3 - The effects of sonication on the flexibility of high molecular weight (Healon®) (●, ○), and low molecular weight (H4) (■ , □) hyaluronan (HA) in the absence of (○ ,□) and presence (●, ■) of DPC at 37 °C, as determined by ^{1}H-nmr spin-spin relaxation times for acetyl group protons (see text). As can be seen sonication in the presence of DPC increase the flexibility of hyaluronan. Points shown are means ± SEM.

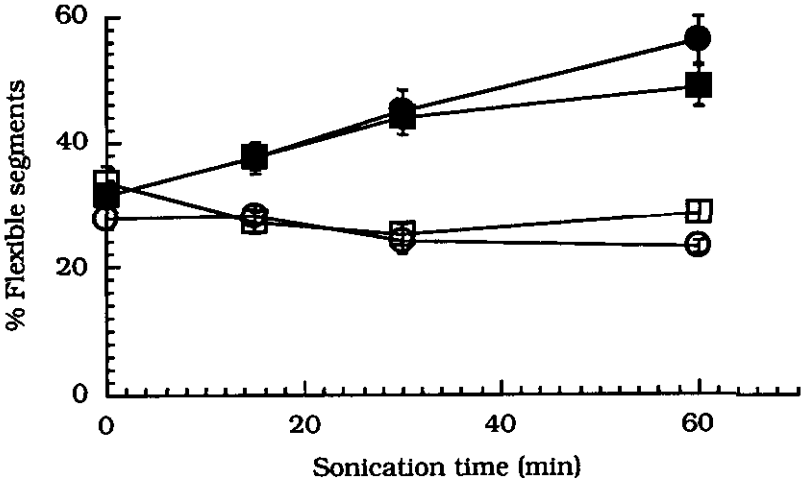


Table 4 - The % of flexible segments in HA chains (Healon®) at various concentrations when sonicated in the presence of DPC (1 mg/ml) for 60 minutes. Values shown are means ± SEM.

Healon® Concentrations (μg/ml)	% Flexible Segments
500	44.2 ± 2.7
250	45.7 ± 3.2
100	47.0 ± 3.1
50	47.5 ± 3.0

DPC = Dipalmitoyl DL-α-phosphatidylcholine

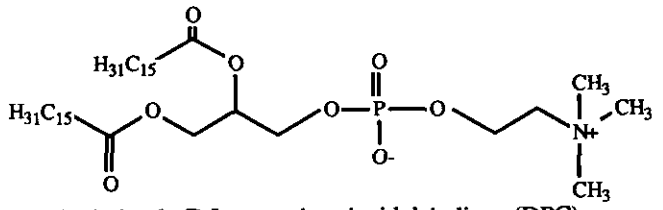
DISCUSSION

In the present study, data has been obtained to show that the interaction of DPC with HA modifies the flexibility of the latter in aqueous solution. The interactions which occurred appeared to be independent of the concentration or molecular weight of the HA used but were dependent on the sonication time employed to mix the components.

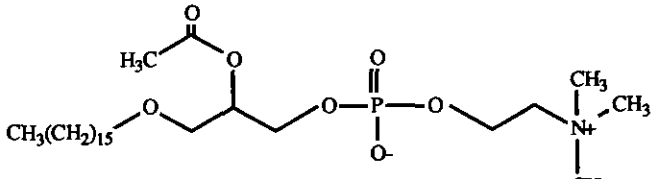
HA is reported to behave as a random coil with unusual stiffness in aqueous solution.[1,8,9] It has been proposed that this stiffness is due to co-operative interactions between certain regions within the HA chains.[14-17] Furthermore, these interactions were reported to be sufficiently strong to resist the effect of alterations in solution ionic strength, temperature or the addition of denaturants, such as urea.[14] The finding that the enhanced segmental motion of the HA molecules resulting from 60 min sonication with DPC was independent of the HA concentration (up to 0.5 mg/ml) (Table 4) and molecular weight (Figure 3), supports our hypothesis that: the association between DPC and HA was due to intramolecular interactions within the HA chains rather than non-specific intermolecular entanglements.

Non-specific chain entanglement would be expected to increase as the molecular weight and concentration of HA were elevated.[11,13,15] The amount of phospholipid associated with the HA chains (or not removed during its purification) would therefore appear to be an important determinant of the intra and inter-chain interactions in solution and thus its flexibility. Phosphorous analysis of the HA preparations employed for these studies showed that the HA isolated from bovine synovial fluid had the highest phospholipid content (Table 1). As predicted by our hypothesis, this preparation had the highest segmental flexibility in aqueous solution (Table 3). The possible contribution of bound protein to chain-chain interactions is not totally excluded from the present experiments, however, the observation that the protein content of the four Pharmacia preparations (H9, H5, H4, and Healon®) were similar (Table 1) but their flexibilities were quite different (Table 3) suggests that the contribution of bound proteins to these molecular interactions was small or non-existent. Consideration of the structure of DPC (Figure 4) provides some insight into the manner by which this molecule and other phospholipids could efficiently interact with HA. Any ionic interaction of the quaternary ammonium head of DPC with the glucuronyl carboxyl anions of HA would be expected to be augmented by the association of the palmitoyl fatty acid chains with the hydrophobic "patches" which are

Figure 4 - Structural formulae of (A) dipalmitoyl-DL-α-phosphatidyl choline (DPC) and (B) 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor, PAF).



(A) Dipalmitoyl - D,L - α - phosphatidylcholine (DPC)



(B) Platelet Activating Factor (PAF)

exposed on alternate "sides" of the HA chain[15] (see Figure 2). The length and flexibility of the palmitoyl side chains of DPC would facilitate their orientation about the HA molecule permitting optimum interaction with the hydrophobic "patches".[15]

Darke et al,[14] using HA derived from rooster combs and umbilical cord suggested, on the basis of nmr relaxation measurements, that in aqueous solution two types of domains existed in the HA chains. 55 - 70 % of the chains were reported to be present in a "stiff" form, while the remainder exhibited mobility expected of a flexible chain polymer. It was further noted by these authors, that the "stiff" domains of the HA chains were prevented from equilibrating with the flexible form by "some unknown covalent features".

We consider that in the absence of phospholipids, these stiff regions within the HA structure arise from intra and interchain hydrophobic interactions involving the hydrophobic "patches" reported to be exposed at repeating sites along the chains.[15] Scott et al,[13,15] identified these patches as potential sites for HA self-aggregation and associations with proteins and lipid membranes, however the possibility that phospholipid impurities could compete for these hydrophobic sites has not been previously reported.

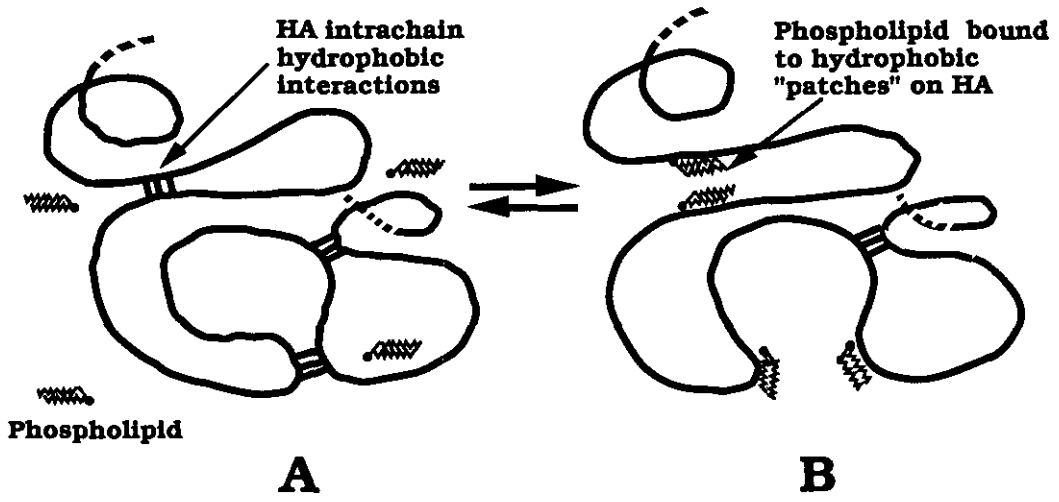
Our explanation is also consistent with the observed increase in chain flexibility after pure preparations of HA were sonicated with DPC for periods up to 60 min (Figure 3). Furthermore, HA preparations devoid of phospholipids were stiffer than higher molecular weight preparations which contained this impurity. Our proposed model for competitive interaction of a phospholipid with HA is illustrated schematically in Figure 5. While only part of a single HA chain is shown in Figure 5 other HA chains could also participate and in the absence of a competing species, such as phospholipid, could link up to produce an extended interlocking HA network. Such a network would be expected to have high viscosity.

Competitive interactions of the type described for HA have been noted for other polysaccharides. Gel formation by interchain association occurs for solutions of carrageenan, polymeric xanthangalactomannan and alginate.[18] However, gel formation (stiffness) can be prevented or modified by the addition of a structurally related small molecular weight species to a solution of the polysaccharide.[18] Significantly, the addition of HA fragments (~ 60 disaccharides) to solutions of purified HA has been observed to uncouple the intermolecular associations present.[16]

As would be expected, lipophilic molecules other than DPC can interact with HA. We have shown using equilibrium gel-filtration chromatography, that platelet-activating factor (PAF) and cortisol are also capable of forming complexes with highly purified HA.[32]

PAF (Figure 4) is an inflammatory mediator with a wide-range of biological effects including promoting chemotaxis of neutrophils and monocytes, and increasing vascular permeability (see Camussi[33] for collected references). The similarities between its biological activities and those described for tumour necrosis factor have led to the suggestion that PAF has a participating role in the pathogenesis of rheumatoid arthritis.[34] The ability of HA to bind PAF and other structurally related amphilytes in the manner described herein, could account for the reported anti-inflammatory properties of purified preparations of HA (see Ghosh[35] for collected references). Furthermore, as the

Figure 5 - Proposed model of reversible phospholipid (PL) binding to HA in which the polar cationic head of PL (represented by a solid circle) interacts with the anionic glucuronyl carboxyl group and the hydrocarbon fatty acid chains (depicted by wavy lines) orient themself in such a manner that they can interact with the hydrophobic pockets present along the HA molecule.[15] Upon binding to PL, the intra-chain coupling within the HA polymer A (as shown by parallel bars) is disrupted to give B. This process allows the closed loops to open resulting in increased flexibility of the HA chains. While only part of one HA chain is shown the coupled loops could enclose loops of other HA chains resulting in an extensive three-dimensional network in solution.



phospholipid content of synovial fluid is substantially elevated in inflamed joints,[24,36] it is highly likely that in chronic disease, the binding capacity of the joint HA could be exceeded. When this occurs the effectiveness of HA to function as "a scavenger of inflammatory mediators" would be compromised. In addition, the viscosity of synovial fluid would be significantly reduced. Both events could lead to accelerated cartilage destruction.[35]

Intra-articular administration of highly purified high molecular weight HA into joints is now widely used for the treatment of arthritic disorders,[35,37-39] even though a rational explanation for its rapid relief of symptoms has not been provided.

It is clear, on the basis of the present study, that HA possess the capacity to bind phospholipids and other molecules which may enter the joint during synovial

inflammation but these binding sites may become saturated. Therefore the replacement of the endogenous joint synovial fluid of arthritic patients by a HA preparation which was free of any interchain competing species could temporarily restore, not only the diminished viscoelasticity the synovial fluid, but also its ability to retain pro-inflammatory mediators within its network.

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