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Molecular modelling of secondary and tertiary structures of hyaluronan, compared with electron microscopy and NMR data. Possible sheets and tubular structures in aqueous solution

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Electron microscopy shows that hyaluronan (HA) forms sheets and tube-like structures in solution. Molecular modelling by Tartu plastic space-filling atomic models revealed that hydroxymethyl and carboxylate groups of HA anti-parallel chains can be joined by H-bonds. Using these bonds, HA molecules can be modelled as sheets and tubules. These tertiary structures have three kinds of lateral contact: (1) antiparallel chains stacked by hydrophobic patches; (2) parallel chains joined by both stacking interactions and H-bonds; and (3) crossing chains joined by H-bonds and stacking interactions. Sheet and tubular structures may explain some viscoelastic and biological properties of HA.

Keywords: space-filling molecular models; hydrogen bonds; glycosaminoglycans

Introduction

Supramolecular organization is the key to biological function, and the key to supramolecular organization is knowledge of the shapes of the participants [1]. Until recently only bulk properties of polymers in solution were available, that had to be interpreted in terms of unspecific shapes and mathematical abstractions. Now, electron microscopy-rotary shadowing (EMRS) outlines specific shapes and nuclear magnetic resonance (NMR) gives atomic resolutions. There is still a gap, in that EMRS cannot show the distribution of atoms, and NMR is not best suited to outline the shapes of large molecules. This gap can be bridged by modelling. If structures based on NMR data are modelled with space-filling atoms, producing shapes seen by electron microscopy, we assume that the models represent the 'truth', at all levels of resolution [2].

Models were used to elucidate biopolymer structures before the development of NMR and EMRS, with results being matched against X-ray, infra-red and other specific data. Stick models evolved into those using space-filling atoms, which incorporated a vast amount of crystallographic knowledge, and with these many successes in molecular biology were achieved. The evolution of stick to spacefilling models did not *per se* render stick models useless. They illustrate molecular skeletons or ground-plans that are difficult to see in models made of spacefilling atoms. Recently, computer graphics improved to the point where images of large numbers of atoms can be put together quickly. Given a fast processer, colour-coded models with appropriate highlights can be manipulated in real time to get a quasi-3-D feel for the structure. More powerful programs enable calculations of energies to be performed e.g. in assessing the influence of the solvent on secondary or tertiary structures [2].

Important though these improvements are, they do not render the manual models redundant. Most simulations are immutably 2-D, no matter how sophisticated the 3-D clues, needing expensive processers and programs to allow rapid repositioning of the model in 'space'. There is no substitute for having the model in the hand, in this respect. The physical feel of making the model contributes to one's understanding of steric hindrance and bond strain in a way that cannot be underestimated, nor quantified in a computer simulation. It is a built-in property of Courtauld models [3].

The programs that calculate energies are subject to fundamental problems. Some parameters are not well established, and calculations of entropies are currently

difficult or impossible, so that hydrophobic bonding energies are inaccessible. For the immediate future there are advantages in using both manual and computer-generated models in parallel.

The sophisticated Courtauld models are no longer made. They are bulky, and extended structures are unstable. Special arrangements are necessary to handle e.g. decasaccharides and larger chains. It is difficult to arrange docking and aggregation of two or more big molecules. Smaller scale versions are required for studying molecular recognition phenomena.

The Tartu models are of a suitably small scale, while permitting much detail of molecular structures to be seen. Models of quite large molecules are mechanically stable. We therefore examined secondary and tertiary structures of hyaluronan (HA), using these models. Results must be checked by physical or chemical tests, to be relevant to the real world [2], and this we have done by comparing predictions from the models with data from EMRS and NMR.

Hyaluronan

Hyaluronan (HA) is a polymeric anionic glycosaminoglycan (AGAG) consisting of repeating disaccharide units, containing alternating D-glucuronic acid (G) and 2-acetamido 2-deoxy D-glucose (*N*) residues. It is found in connective tissues, streptococcal culture media and synovial fluid. HA takes part in cell surface phenomena, interacting with receptors and proteoglycans with very high specificity [3].

Features of the HA structure that might explain the unique viscoelastic and physiological properties in tissues were not identified until recently. It was not clear how such a simple chemical structure could show such specificity and versatility. The elue must be in the HA secondary and tertiary structures.

NMR demonstrated a two-fold helix with an extended H-bonded system and water bridges which seemed to account for the unusual chain stiffness of HA [3–6]. There were extensive hydrophobic patches distributed on alternate sides of the tapelike molecule. Computer simulation and energy calculations suggested that these patches could stabilize duplex formation and lateral aggregation of HA molecules [4, 5], since they could easily interact intermolecularly, with possible H-bonding to add to the stability of the duplex [4]. X-ray diffraction of the HA crystalline phase showed the presence (albeit in non-physiological conditions) of antiparallel left-handed double-helical structures which might contribute to the special properties of HA in aqueous solutions [7, 8]. Examination of polysaccharides by rotary shadowing and electron microscopy showed that the thickness and clarity of the images given by HA usually surpass those of other AGAGs [4]. Preparations of HA revealed extensive branched networks in which filaments of various thickness and sheets of material were distinguished [5].

This investigation used molecular modelling to examine possibilities of hydrophilic and hydrophobic interactions between HA molecules, to gain insights into its behaviour in tissues.

Materials and methods

Molecular modelling used Tartu plastic space-filling atomic models having improved parameters and design [9, 10].

Rotary shadowing-electron microscopy of HA solutions was carried out as described by Scott *et al.* [4].

Results

Electron microscopy

Figure 1 shows that HA in solution forms sheets and tubules at physiological (1 mg ml^{-1}) concentrations.

Molecular modelling of HA secondary structure

Using published data [3, 6] molecular modelling was carried out for HA chains, with and without water bridges. To differentiate assembled models from each other they are provided with original designations.

Investigation of molecular models of HA fragments lacking water bridges (Fig. 2) indicated that there are two sterically possible conformations having the same type of H-bonding but differing in dihedral conformational angles near acetamido, glycol and carboxylate groups bound by H-bonds: (1) conformer N 45: $\angle$HN–C2H $\approx 45°$, $\angle HO_2$–

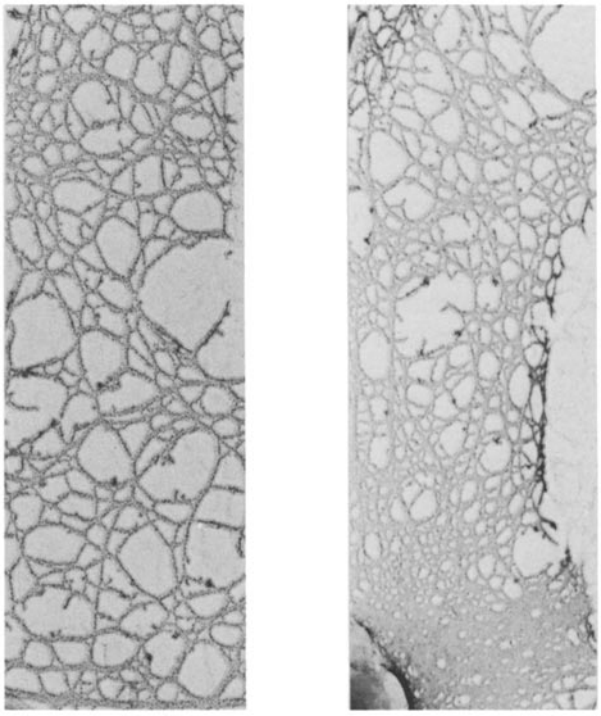

Figure 1. Electron micrographs showing HA strands merging into sheets and 'tubes' in aqueous solution (mg ml^{-1}). 11.5 mm ≡ 100 nm.

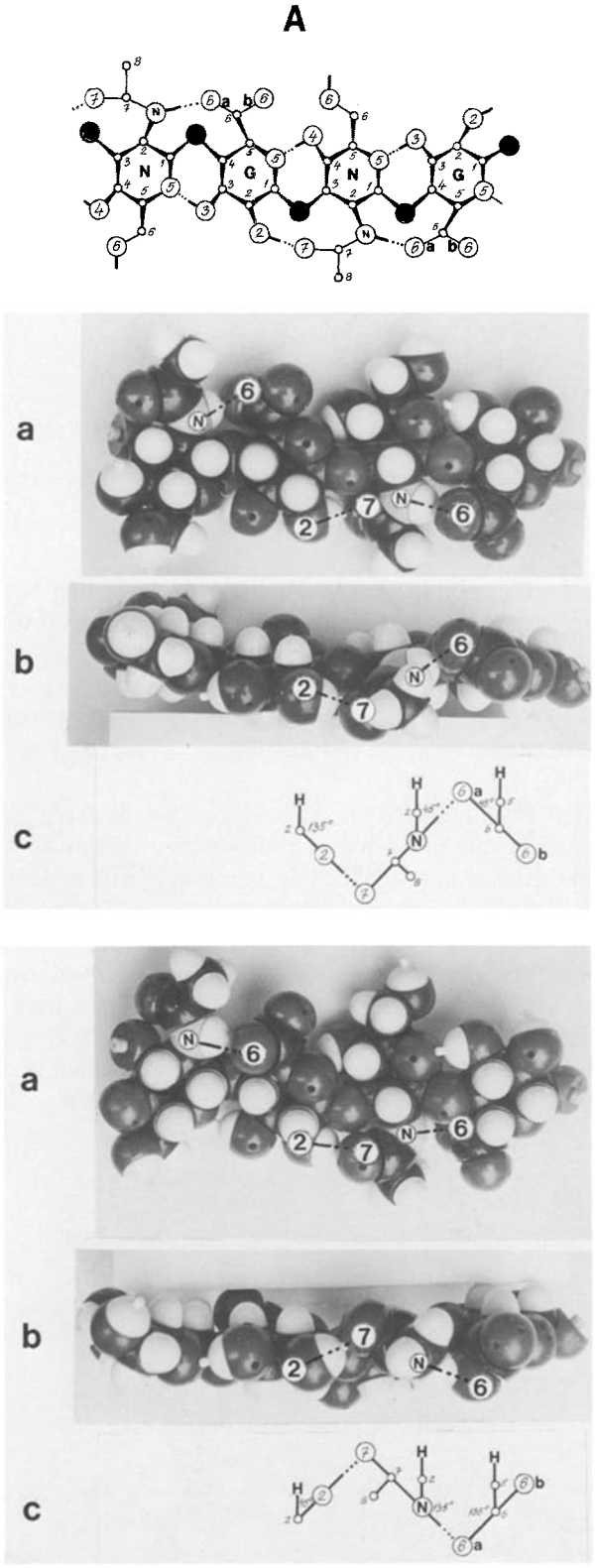

Figure 2. Molecular models of the secondary structure of HA molecules lacking water bridges: A: general chemical formula (small circles: C-atoms, large circles: O- and N-atoms, G: glucuronic acid residue, *N*: acetamidoglucose residue); B: conformer N 45 (a: frontal view, b: view to the lower side, c: diagram of the conformation); C: conformer N 135.

C2H ≈ 135°, ∠O⁻C–C5H ≈ 45°; and (2) conformer N 135: ∠HN–C2H ≈ 135°, ∠HO_2–C2H ≈ 45°, ∠O⁻C–C5H ≈ 135°. Due to these dihedral angles the planes of the above-mentioned atomic groups form a zigzag, being perpendicular to each other in the same conformer and to the planes of corresponding groups of the other conformer. Nevertheless, all glycose rings in both conformers are in the same plane.

Investigation of molecular models of HA secondary structures containing water bridges (Fig. 3) revealed that such bridges can join acetamido and carboxylate groups in four different ways: (1) conformer N cis-short: cis NH/C2H conformation, H_2O bridge to atom O6a; (2) conformer N cis-long: cis NH/C2H conformation, H_2O bridge to atom O6b; (3) conformer N trans-short: trans NH/C2H conformation, H_2O bridge to atom O6a; and (4) conformer N trans-long: trans NH/C2H conformation, H_2O bridge to atom O6B (Fig. 4). Only conformer N trans-short contains glycose rings in the same plane, in other conformers these rings are in propeller-conformation. Despite this aplanarity all conformers include an H-bond between acetamido and glycol groups.

Tertiary structure of HA

The two-fold helix of HA features large clusters of contiguous CH groups on alternate sides of the molecule, forming patches of a highly hydrophobic character, which may not only interact with membranes, lipids and protein but also in self-association [3]. In addition to the hydrophobic interaction Scott *et al.* [5] considered stacking between carboxylate and acetamido groups as well as stacking between acetamido groups. Our models revealed that hydrophobic contacts are possible only between HA chains lacking water bridges in the secondary structure. Clusters of contiguous CH groups give equally good interactions in the cases of both HA conformers N-45 and N-135. Stacking between acetamido groups is possible, when conformers are N-45 or N-135. However, stacking between acetamido and carboxylate groups can be realized only when one of the partners is conformer N-45 and the other N-135.

Hydrophilic interactions in the tertiary structure of HA, carboxyl-carboxylate H-bonds (at low pH), ionic and water bridges were found in antiparallel double-helical structures [7, 8]. Because of the non-physiological conditions, and the evanescent nature of these structures, they were not modelled. We are mainly concerned with hydrophilic parts of HA molecules which can form extensive networks of H-bonds and at the same time enable hydrophobic patches to make good contact.

Observation of the peculiarities of the HA secondary structure (Figs 2 and 3) reveals that most polar groups can form intramolecular H-bonds. However, two groups are 'free': hydroxymethyl and an oxygen atom of the carboxylate group. These groups could mediate intermolecular

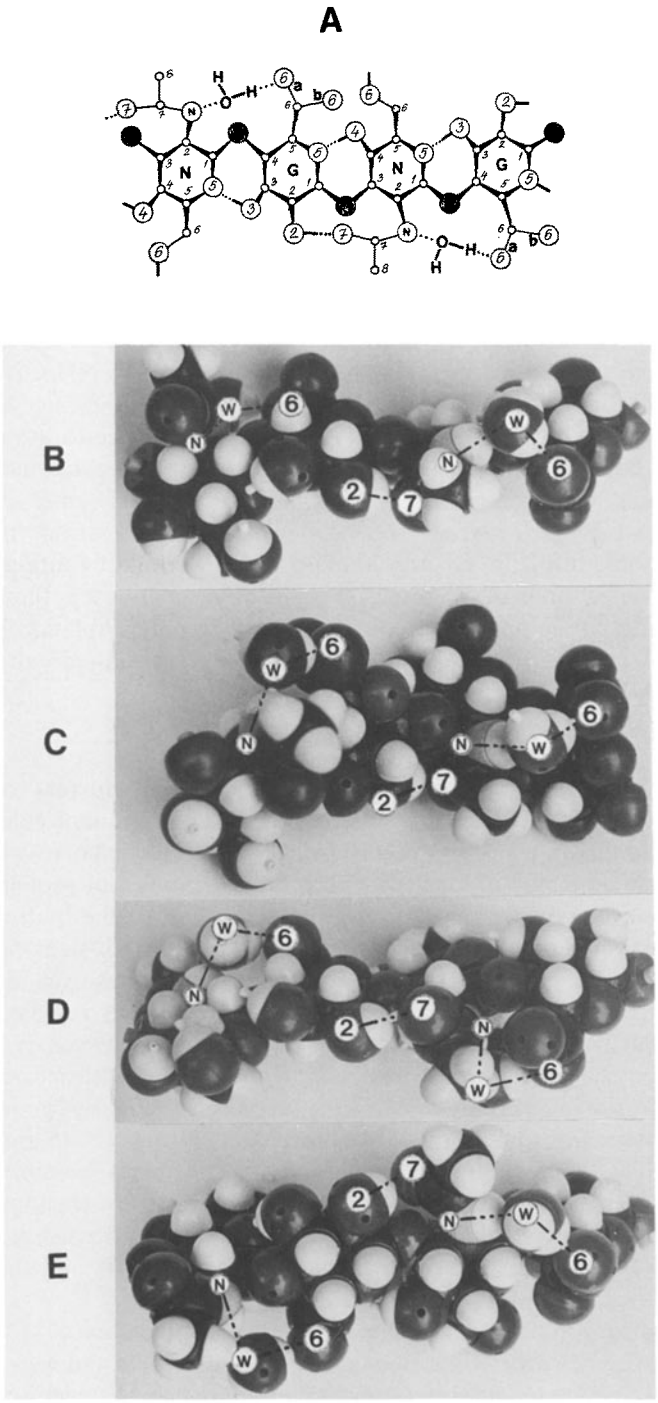

Figure 3. Molecular models of the secondary structure of HA molecules containing water bridges (W): A: general chemical formula; B: conformer N cis-short; C: conformer N cis-long; D: conformer N trans-short; E: conformer N trans-long.

hydrophilic interactions in assemblies containing large numbers of HA molecules.

Molecular modelling shows that H-bonds between hydroxymethyl and carboxylate groups are possible only between antiparallel HA molecules (Figs 4 and 5). Each

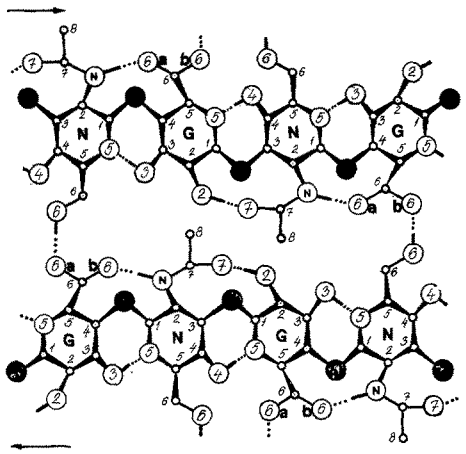

Figure 4. Hydrogen bonds between hydroxymethyl and carboxylate groups of HA antiparallel chains.

disaccharide residue can form two H-bonds, so that bonds on one side of the HA molecule alternate with analogous bonds on the other side. Such H-bonding can join antiparallel HA molecules into sheets which are planar or curved (Figs 6–8). The latter may form tubular structures that are closed along all circumferences, or are open to one side (Fig. 8).

Single HA molecular sheets held together by these polar interactions can be formed by chains with or without water bridges. However, a tight self-aggregation of HA molecular sheets is sterically possible only in the case of chains lacking water bridges. Due to the regularity of structural elements this aggregation is better between planar than curved sheets. There are three kinds of lateral contact, which may be formed by aggregation of HA molecular sheets (Fig. 7). First, the sheets cover each other so that each chain of one sheet is antiparallel to the corresponding chain of the

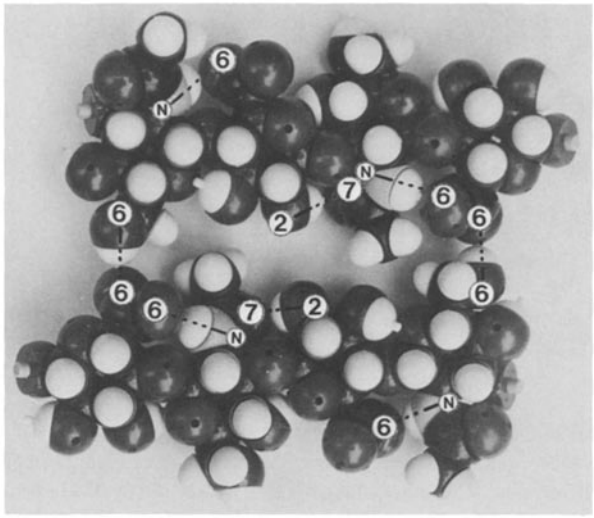

Figure 5. Molecular model of planar sheet formed by anti-parallel HA chains bound by H-bonds.

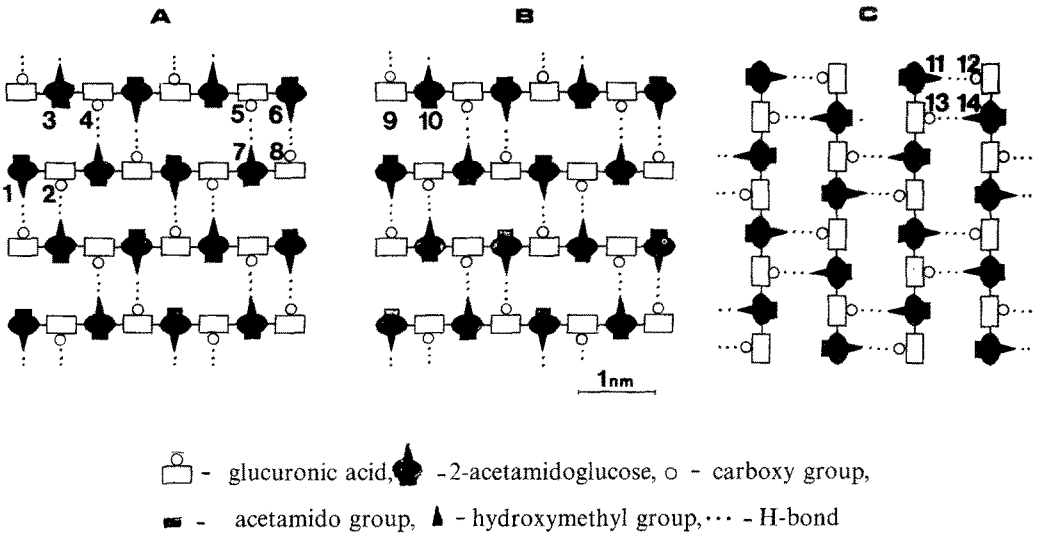

Figure 6. Tertiary structure of HA in planar sheets. If sheet B staggered by one molecular chain is placed directly above the sheet A so that residue 19 and 10 cover 1 and 2, respectively, all carboxylate and acetamido groups will be stacked to each other (see Fig. 7a). If sheet B is placed above the sheet A so that residues 9 and 10 cover 3 and 4, alternate carboxylate and acetamido groups are stacked, alternate hydroxymethyl and carboxylate groups can form intersheet H-bonds replacing analogous intrasheet H-bonds (see Fig. 7b). If sheet C is placed above the sheet A at 90° so that atomic groups 11, 12, 13, 14 cover 5, 6, 7, 8, respectively, all intrasheet H-bonds can be replaced by analogous intersheet bonds and stacking occurs between acetamido groups (see Fig. 7c).

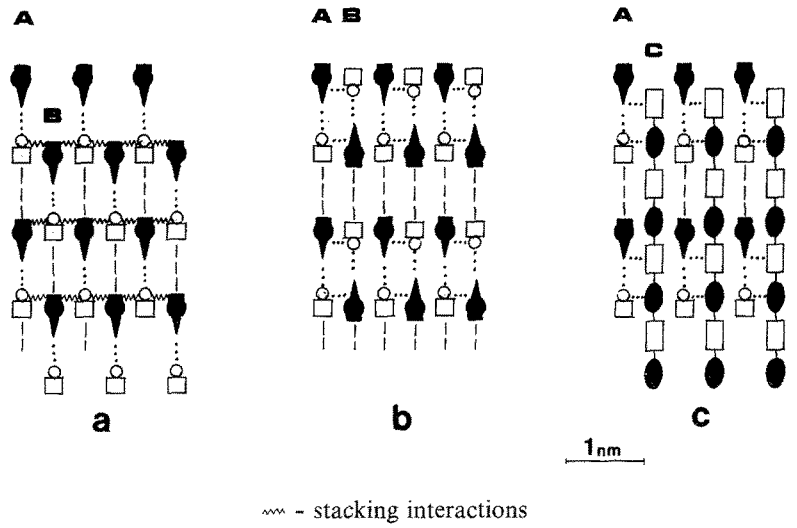

Figure 7. A view of stacked and hydrogen-bonded HA planar sheets (cf. Fig. 6) along the axis of molecular chains.

neighbouring sheet, all carboxylate groups are stacked to acetamido groups but no intersheet H-bonds are formed. Second, molecular chains of one sheet are parallel to chains of neighbouring sheets with some carboxylate groups stacked to acetamido groups, but intersheet H-bonds join carboxylate and hydroxymethyl groups. Third, sheets cross each other at 90°, hydrophobic stacking interactions are weak but all intrasheet H-bonds between carboxylate and hydroxymethyl groups may be replaced by analogous intersheet H-bonds.

Discussion

Molecular modelling by Tartu space-filling atomic models [9, 10] confirmed that H-bonds were present in the HA secondary structure, reducing configurational flexibility and increasing stiffness of this molecule [3, 6].

Further investigation revealed two different conformations of HA chains without water bridges and four conformations in chains containing such bridges. These conformations are important from the point of view of HA stacking (hydrophobic) interactions. Effective stacking

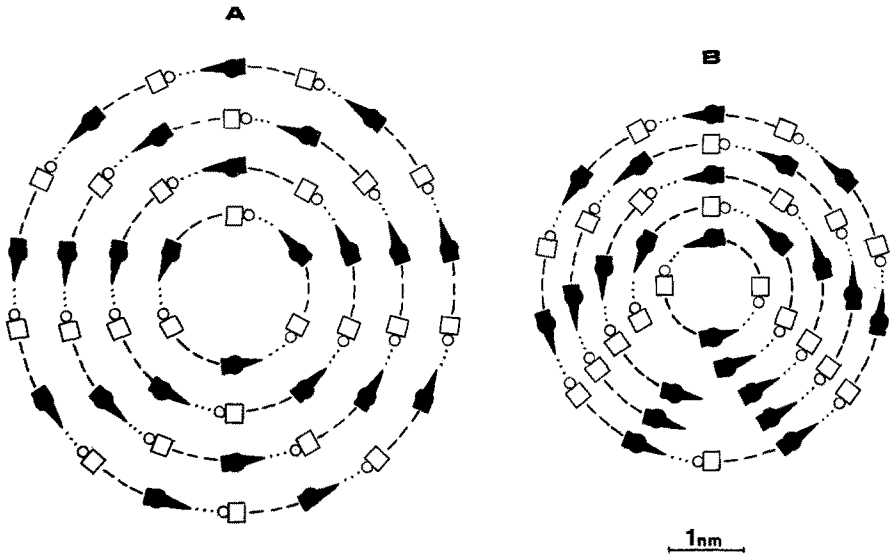

Figure 8. Tertiary structure of HA in curved sheets (a view along the axis of molecular chains, cf. Figs 6 and 7). A, all sheets closed but not stacked; B, all sheets stacked but only some of them closed.

interactions were found only between chains lacking water bridges. Some hydrophobic contacts are tight between analogous molecular chain conformers, others need different conformations (Results).

The main finding was that of H-bonding between antiparallel HA molecules. These bonds are similar to the polar interactions between hydroxymethyl and glycol groups of cellulose chains and between hydroxymethyl and acetamido groups of chitin molecules [11–15]. By contrast with cellulose, where intermolecular H-bonds are formed in the course of biosynthesis. HA H-bonded sheets may form at high concentrations as a self-aggregation process.

In the process of self-aggregation polar interactions between HA chains are apparently supplemented by hydrophobic contacts. The best stacking interactions occur when chains of one sheet are antiparallel to the chains of the neighbouring sheet. The binding potential per monosaccharide unit is somewhat smaller when chains are parallel [5]. Sheets crossing each other at 90° probably have lowest binding energy. On the contrary, intersheet H-bonds are absent between antiparallel sheets, but they are numerous between parallel sheets and most frequent between crossing sheets.

There are two kinds of sheets which are one molecule thick: (a) in which the tapelike molecules aggregate face-to-hydrophobic-face, with the surfaces of the sheet showing their hydrophilic groups to the outside; and (b) in which interaction between molecules is as shown in Figs 4–6, with the sheet surfaces exposing their hydrophobic aspects to the environment. Sheet (b) has 'oily' surfaces which may be of value in lubrication. In H_2O it would be surprising if (b) were preferred, since the H-bonds would be weak, in competition with those to H_2O, and there would be a strong tendency to pack the hydrophobic surfaces together, in extensive hydrophobic bonding, to produce structures similar to those in Figs 7 and 8. When sheets (a) and (b) undergo further aggregation by, respectively, H-bonding between antiparallel (a) sheets, or by hydrophobic stacking of (b) sheets, the final multi-sheet aggregates are very similar, no matter which was the original nucleus.

The combination of hydrophilic and hydrophobic interactions gives highly-ordered structures which may be responsible for the unique visco-elastic and physiological properties of HA in tissues. Self-aggregated planar sheets may give rise to filaments of varied thickness and sheets of material, as seen by EMRS (Fig. 1). H-bonds between crossed sheets could serve as a basis for extensive branched networks. Curved sheets of HA molecules might form channels through which e.g. water-soluble molecules could diffuse [1].

Because all HA molecular sheets contain antiparallel chains it is easy to see how in certain conditions (i.e. at low pH) left-handed antiparallel helical structures may be formed [7, 8].

We emphasize that these models define only the spatial *possibilities* inherent in the HA two-fold helix. Energies in H_2O will need to be calulated, to probe the stabilities of the proposed structures. Unfortunately, there are problems in computing hydrophobic bonding energies, which could indicate what kinds of interaction may be strong.

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References

1. Scott JE (1992) *FASEB J* **6**:2639–45.
2. Scott JE, Chen Y, Brass A (1992) *Eur J Biochem* **206**:675–80.
3. Scott JE (1989) In *The Biology of Hyaluronan* (Ciba Foundation Symposium 143), (Evered D, Whelan J, eds) Chichester: Wiley, pp. 6–20.
4. Scott JE, Cummings C, Greiling H, Stuhlsatz HW, Gregory JD, Damle SP (1990) *Int J Biol Macromol* **12**:180–84.
5. Scott JE, Cummings C, Brass A, Chen Y (1991) *Biochem J* **274**:699–705.
6. Scott JE, Heatley F, Hull WE (1984) *Biochem J* **220**:197–205.
7. Sheehan JK, Gardner KH, Atkins EDT (1977) *J Mol Biol* **117**:113–35.
8. Arnott S, Mitra AK, Raghunathan S (1983) *J Mol Biol* **169**:861–72.
9. Mikelsaar R-H, Bruskov VI, Poltev Vi (1985) New precision space-filling atomic-molecular models. Pushchino.
10. Mikelsaar R-H (1986) *Trends Biotechnol* **4**:162–63.
11. Gardner KH, Blackwell J (1974) *Biopolymers* **13**:1975–2001.
12. Woodcock C, Sarko A (1980) *Macromolecules* **13**:1183–87.
13. Mikelsaar R-H, Kusnetsova N (1992) In *Ligno-cellulosics: Science, Technology, Development and Use* (Kennedy JF, Phillips GO, Williams PA, eds.) New York, London, Toronto, Sydney, Tokyo, Singapore: Ellis Horwood Ltd, pp. 479–83.
14. Gardner KH, Blackwell J (1975) *Biopolymers* **14**:1581–95.
15. Minke R, Blackwell J (1978) *J Mol Biol* **120**:167–81.