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Secondary and tertiary structures of hyaluronan in aqueous solution, investigated by rotary shadowing–electron microscopy and computer simulation

Hyaluronan is a very efficient network-forming polymer

John E. SCOTT,*§ Christine CUMMINGS,† Andy BRASS‡ and Yuan CHEN‡
*Chemical Morphology, †Medical Biophysics and ‡Biochemistry and Molecular Biology, University of Manchester, Manchester M13 9PL, U.K.

1. Hyaluronan from mesothelioma fluid, rooster comb and streptococci was examined by rotary shadowing and electron microscopy. All preparations showed extensive branched networks, but high-viscosity hyaluronan networks were essentially infinite, with no individual 'molecules' that were not integrated via multiple branched points into the meshwork. Low-viscosity hyaluronan, recovered after papain digestion of mesothelioma fluid, showed occasional single filaments that were independent of the main aggregates, some of which were themselves independent of other aggregates. 2. Hyaluronan is a polymer with a very marked capability to form meshworks at very low dilution (< 1 μg/ml). The longer the hyaluronan molecule, the more branching is potentially possible, and the more extensive and coherent is the network, with every hyaluronan molecule in contact with every other in the solution, via the network. This behaviour accounts for the mechanical properties of the soft tissues (e.g. vitreous humour) and fluids (e.g. synovial fluid) of which hyaluronan is a major component. 3. The hyaluronan twofold helix, previously demonstrated to be present in solution [Heatley & Scott (1988) Biochem. J. **254**, 489–493] was shown by computer simulation and energy calculations to be sterically capable of extensive duplex formation, probably driven by interactions between the large hydrophobic patches on alternate sides of the tape-like polymer, forming stable aggregates at biological temperatures in water. This 'stickiness' is postulated to be the basis of the network-forming and laterally aggregating behaviour of hyaluronan. 4. The tertiary structures formed by hyaluronan may not be possible in the case of chondroitin 4-sulphate.

INTRODUCTION

Hyaluronan (HA) is one of a family of glycosaminoglycuronans, including chondroitin sulphate (dermatan sulphate) [CS (DS)] and heparan sulphate, that occur in connective tissues. Although HA closely resembles CS (DS) and heparan sulphate in possessing a polymeric structure of repeating disaccharide units, consisting of alternating hexuronic acid and hexosamine (glucuronic acid and 2-acetamidoglucose in HA), it differs fundamentally in other respects. Thus it is probably not linked to a protein or polypeptide to form a proteoglycan, and it is not sulphated. It is synthesized at the cell membrane and extruded directly to the exterior, in contrast with the others, which are formed in the Golgi apparatus and excreted along more complicated paths. Consequently, it does not undergo post-polymerization modifications, as do the other glycosaminoglycuronans (for a review see Scott, 1989).

The glycosaminoglycuronans perform important structural and mechanical functions, often based on their marked tendency to occupy large domains in aqueous solution. CS (DS) and heparan sulphate proteoglycans are often associated with tissue collagen fibrils, sometimes in highly specific complexes (Scott, 1988). It is characteristic of these structures that collagen is quantitatively overwhelmingly predominant, and the tissues are solid or hard. HA, on the other hand, is present in at least three tissues, vitreous humour of the eye, rooster comb and Wharton's jelly of the umbilical cord, in which the collagen fibril matrix is sparse. HA is quantitatively important and the tissues are soft. Moreover, HA is the characteristic component of the (completely liquid) synovial fluid, conferring non-Newtonian viscosity and other mechanical properties essential for the proper functioning of a joint lubricant. No other glycosaminoglycuronan has such a function. The features of the HA structure that specifically match it to the requirements of these soft or liquid 'tissues' have not been identified.

The above roles depend largely on the bulk properties of HA in solution. In addition, HA interacts with proteoglycans, cell membranes, and receptors with very high specificity (for reviews see Evered & Whelan, 1989), on a molecule-to-molecule basis. It is remarkable that such a simple chemical structure should show such specificity and versatility. The potential of the basic structure might be amplified by entering into secondary and/or tertiary structures in aqueous solution (Scott, 1989).

The solution properties of HA have been investigated for about 50 years, without a clear consensus emerging. It is said to be a random coil, with considerable local stiffness, and quite large persistence length (for a review see Wik, 1979). N.m.r. studies demonstrated an extended hydrogen-bonded system, a twofold helix with elements of co-operativity that included water bridges between neighbouring sugar residues (Scott, 1989). This structure seemed to account for chain stiffness and also for the observation that the high viscosity of HA reversibly and dramatically decreased at high pH. Another feature was the presence of large hydrophobic patches, each extending over three saccharide units, repeated on alternate sides of the molecule throughout its length. It was suggested that these patches could be the basis of interactions with lipid membranes and with proteins, and also of self-aggregation (Scott, 1989).

In the present paper we describe results obtained by rotary

Abbreviations used: HA, hyaluronan; CS, chondroitin sulphate; DS, dermatan sulphate.

§ To whom correspondence should be addressed.

shadowing and electron microscopy, with which we observed HA from different sources, of various molecular masses, at low concentrations. Our findings demonstrate self-aggregation of a specific kind. Computer simulation and molecular graphics suggest that aggregation is explicable in terms of the twofold helical secondary structure previously observed by n.m.r. in HA fragments of low molecular mass.

Our results provide a rationale for the role of HA in the soft tissues and liquid environments described above.

EXPERIMENTAL

Materials

HA was obtained from mesothelioma fluid by the method of Scott (1960), involving papain digestion at 65 °C, precipitation by cetylpyridinium chloride and recovery of the sodium salt by precipitation from ethanol/sodium acetate solution. The reduced viscosity was 692 ml/g, corresponding to a molecular mass of 3.5×10^5–4.0×10^5 Da (Scott *et al.*, 1990). This preparation was compared with the original mesothelioma fluid, which contained about 4.0 g of HA/l and about 40 g of plasma proteins/l, predominantly albumin. The HA in this fluid was of much higher (> 5-fold) intrinsic viscosity than that of the papain-digested material (see, e.g., Preston *et al.*, 1965). The lower viscosity of papain-digested HA was due to cleavage of the glycan chain, caused by free radicals generated by EDTA and traces of heavy-metal ions in the digestion mixture (Ogston & Sherman, 1959). Commercial HA in the form of Healon, of high intrinsic viscosity, used for clinical replacement of vitreous humour, was from rooster comb. A preparation of streptococcal HA of high intrinsic viscosity was generously donated by Dr. Peter Prehm from the University of Munster, Munster, Germany.

Rotary shadowing of HA

The techniques used were those described previously (Scott *et al.*, 1990) for the investigation of proteoglycans and carbohydrate polyanions. A 5 μl drop of solutions of HA in 0.5 M-ammonium acetate buffer, pH 7.2, was sandwiched between 2 cm^2 freshly cleaved mica, freeze-dried and rotary-shadowed with platinum/tungsten at a glancing angle of 4°. Carbon replicas were made and examined in a JEOL 1200 EX electron microscope. Concentrations of HA varied from 1 μg/ml to 1 mg/ml.

Computer simulations of HA

Simulations were performed on HA molecules in water and *in vacuo*. The dynamic behaviour of these molecules was modelled by using the technique of molecular dynamics (McCammon & Harvey, 1987).

From a knowledge of the potential functions describing the intramolecular interactions, the force on every atom in the molecule at time t can be calculated. By using Newton's equations of motion it is possible to calculate the acceleration on every atom and then to integrate iteratively the equations of motion to obtain the position of every atom at time $t+\delta t$, where δt is typically of the order 1 fs. By performing 10^4–10^5 iterations the motion of a molecule over 10–1000 ps can be monitored. The simulations of HA described below used molecular-dynamics routines, monosaccharide potentials and descriptions from the CHARMM (Brooks *et al.*, 1985) package (version 20) and the QUANTA display program (all from Polygen Corporation) and were run on a Silicon Graphics 4D/240GTX graphics workstation.

The HA molecules used in the simulations were built by linking together the appropriate number of monosaccharide units (between four and 32) in a twofold helical conformation. The initial co-ordinates of the atoms in the twofold helix were from Atkins *et al.* (1980). In all the simulations the charge on the carboxylic acid group was set to -1. This takes no account of screening by counterions that occurs in solution, probably therefore underestimating the stability of the structures that we describe. The simulations of HA in water were performed by placing the molecule in a 3.0 nm × 3.0 nm × 3.0 nm cube filled with water molecules. To simulate the effect of an infinite water bath, periodic boundary conditions were applied. Using periodic boundary conditions is equivalent to modelling a system of infinitely repeating 3.0 nm × 3.0 nm × 3.0 nm cubes, thereby eliminating boundary effects (but not finite-size effects because the periodic repeats of the 3.0 nm × 3.0 nm × 3.0 nm cube impose unphysical correlations).

The same strategy was used for all HA simulations, whether *in vacuo* or in solution. After the molecule had been built and placed in the appropriate environment, the energy of the system was minimized by using either a steepest descents or an adopted basis Newton–Raphson minimizer to remove any strain energy in the system and to allow the solvent molecules (if present) to relax about the HA. Performing a potential-energy minimization can be thought of as cooling the system to 0 K, and therefore all the molecular-dynamics simulations were started at 0 K and slowly heated to 300 K in 10 ps. The simulations were run for a further 30 ps to allow equilibration at this temperature, and then for a further 20–30 ps, during which time structural and thermodynamic data on the molecules was collected. The time step used for all the simulations was 1 fs, and the routine SHAKE (van Gunsteren & Berendsen, 1977) was used to model the high-frequency hydrogen-bond stretch motions.

RESULTS

Rotary shadowing of HA

The characteristic appearance of high-molecular-mass HA is of a highly branched network, even at the lowest concentrations of 1 μg/ml. An irregular honeycomb structure was produced by all three high-molecular-mass preparations (Figs. 1*b* and 2). No single molecules, i.e. unconnected to other molecules, were observed. The technique is not likely to have been responsible for the structures observed, since (*a*) they were not given by polymers other than HA (Scott *et al.*, 1990) and (*b*) similar structures were seen by Gross (1948) and J. Engel (see discussion to Scott, 1989) after using quite different methods of preparation for metallic shadowing.

The lower-viscosity papain-digested preparation also gave rise to many highly branched aggregates, but these were not as extensive as those from the high-viscosity preparations, and occasional unbranched filamentous images were seen (Fig. 1*a* and Scott *et al.*, 1990).

The filaments making up the meshworks were often completely regular in thickness over the entire visible length, but this thickness varied from filament to filament, by 10-fold or more. There appeared to be a tendency to form thicker filaments at higher concentrations of HA. The thickest regular filaments were of the order of 30 nm wide. In many cases thick filaments were seen to fray into a number of thinner filaments (Fig. 2). The meshworks showed similar phenomena, with thick sectors of the net giving off several branches in succession at different points, becoming thinner in the process. Frequently a group of thinner filaments, fairly densely arranged, appeared as a node, from which a number of long straight filaments originated, joining up with other nodes of a similar kind (Figs. 1*b* and 2).

At the highest concentrations (1 mg/ml) neighbouring filaments sometimes overlapped to the point of being indis-

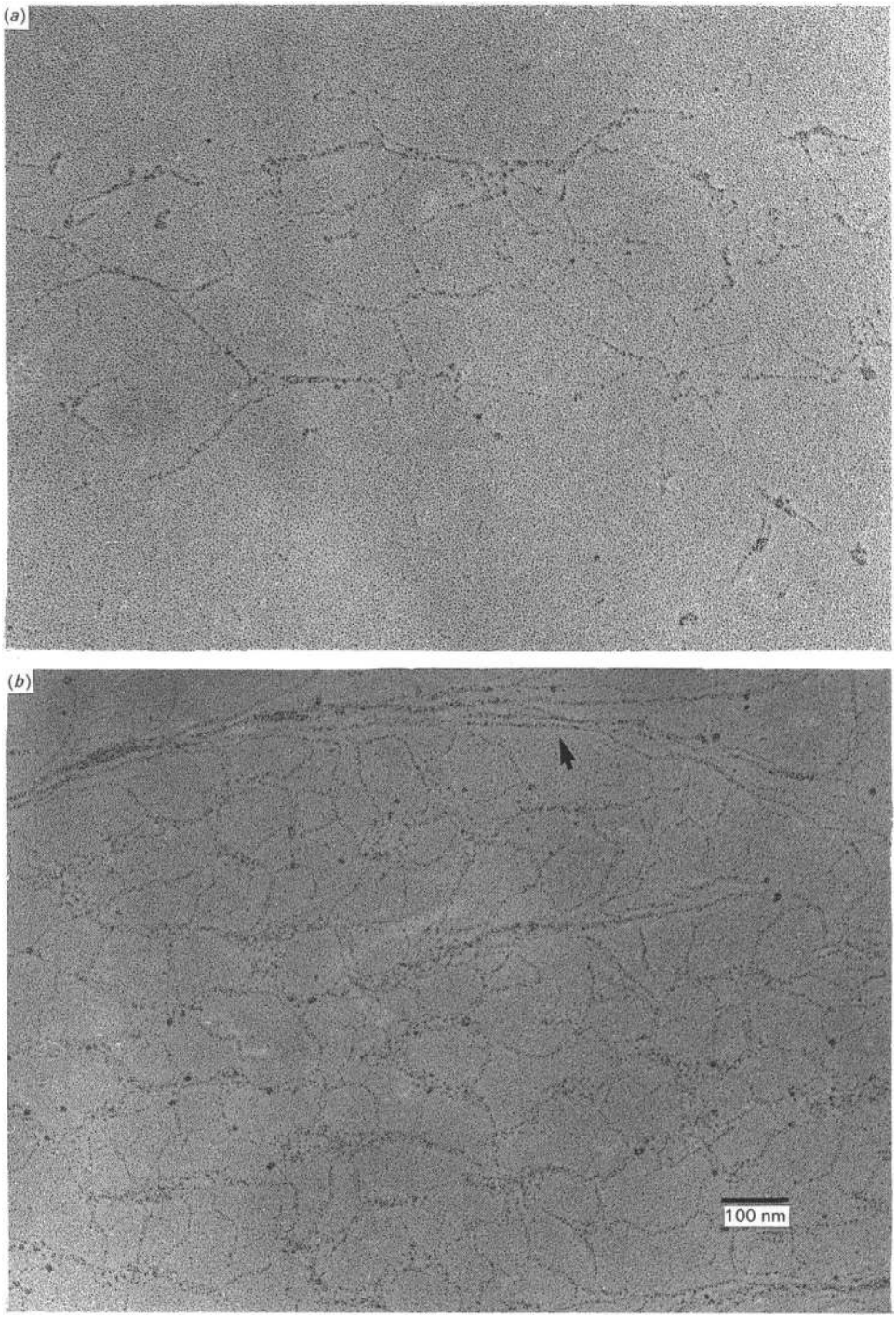

Fig. 1. Electron micrographs of rotary-shadowed HA from mesothelioma fluid, diluted with aqueous 0.5 M-ammonium acetate to a concentration of 5 μg/ml

For experimental details see the text. (*a*) HA recovered after papain digestion. Branched networks are visible, which are not as extensive as those in non-digested HA (*b*), where the network is essentially boundless, with nodes consisting of many filaments of HA, giving off clearly defined and quite thick uniform filaments, which must contain many molecules of HA in lateral array. In addition to these radiating nodes there are numerous simpler branching points, where a thin filament leaves a thicker filament at a Y junction. Note also the way in which several thick filaments appear to anastomose into one thicker filament (top, arrowed).

tinguishable, appearing as sheets of material (see also Gross, 1948). Even at these concentrations individual thick filaments surrounded by thinner ones were very frequently visible.

The cross-linking was not likely to be due to protein, since it was observed in papain-digested HA as well as in Healon, which contains only about 0.2% by weight of the HA as protein.

Molecular modelling of HA

Three possible arrangements of the HA molecules in a duplex were tried (see Fig. 3). Simulations were run of duplexes of 16-disaccharide-unit HA molecules *in vacuo* starting from these initial configurations. Fig. 4 shows the final structure obtained

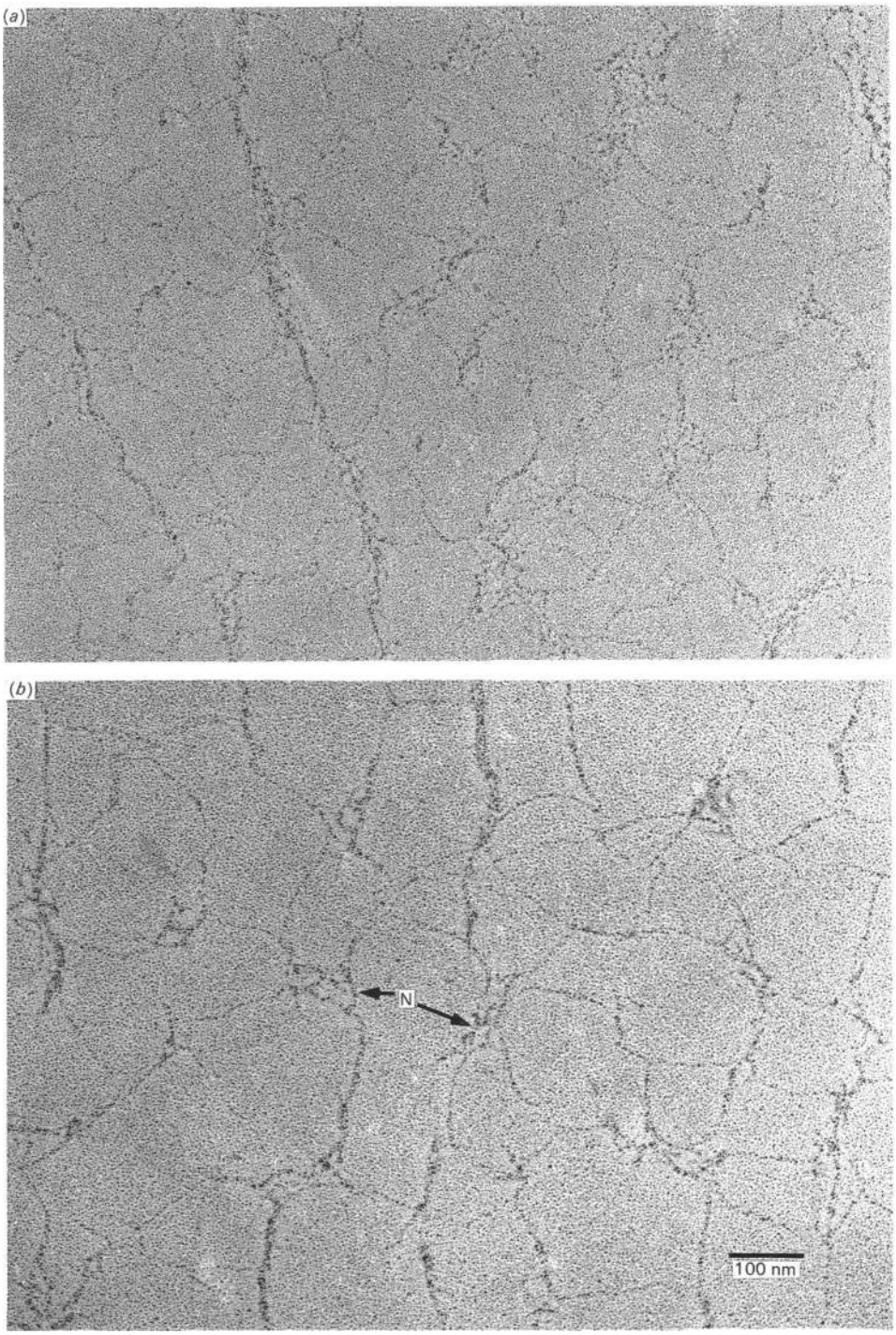

Fig. 2. Electron micrographs of HA from rooster comb (*a*) and streptococci (*b*), at concentrations of 10 and 5 μg/ml respectively, in aqueous 0.5 M-ammonium acetate

For experimental details see the text. The limitless meshworks appear much the same as in Fig. 1(*b*), with similar nodes (N) and innumerable simpler branching points.

from one of these. In all cases duplexes were found to be geometrically possible, HA molecules being flat and flexible enough to allow good contacts to be made along the entire length of the duplex. The binding energy per monosaccharide unit for each of the three complexes was calculated and is shown in Table 1. It appears that duplexes in which the acetamido groups are staggered (for either parallel or anti-parallel arrangements of the two molecules) have a larger binding energy than duplexes in which the acetamido groups are placed on top of one another.

To check the validity of the molecular-dynamics models, simulations were run of HA in water and compared with the results obtained using n.m.r. (Scott, 1989). Fig. 5(*a*) shows an instantaneous conformation obtained from a molecular-dynamics simulation of a four-disaccharide-unit HA molecule in water. The structure obtained agreed well with the n.m.r. data, in that the molecule was twofold helical and water bridges between the carboxy and acetamido groups were as seen in the n.m.r. structure for HA in water (Heatley & Scott, 1988).

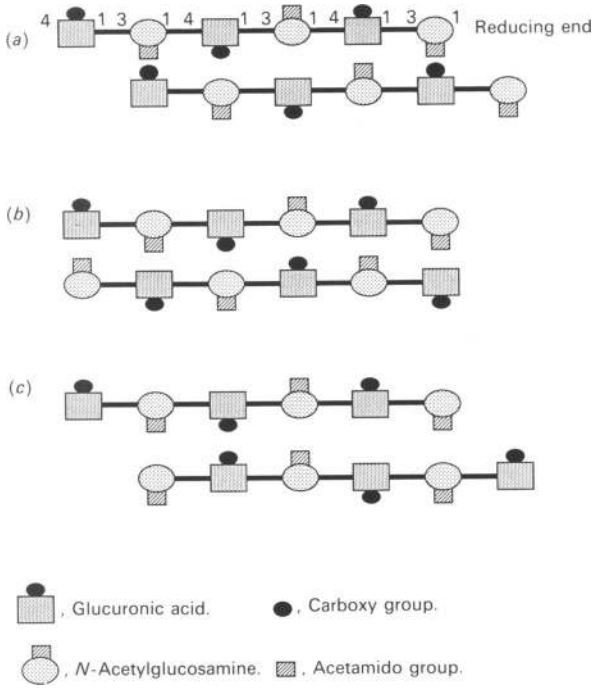

Fig. 3. Scheme of the three different duplex geometries that were modelled

The first chain in each pair should be visualized as lying directly above the second. The numbers refer to the positions of the glycosidic bonds. In (*a*), the chains are parallel, with alternate carboxy group–acetamido group pairs stacked above each other. In (*b*), the chains are anti-parallel, with all carboxy group–acetamido group pairs lying above each other. In (*c*), the chains are anti-parallel, but staggered by one sugar unit compared with (*b*), so that acetamido groups are stacked above acetamido groups, and carboxy groups are *trans* to each other. There are extensive overlaps between hydrophobic 'patches' on opposing molecules in the duplexes in all three structures.

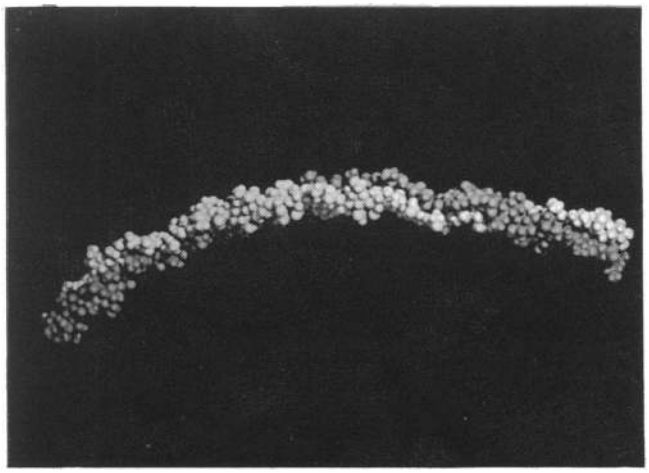

Fig. 4. Space-filling representation of an HA duplex obtained after a run of 70 ps *in vacuo* at 300 K

Two 16-disaccharide-unit HA units, configured in a twofold helix, as in Fig. 5(*a*), were initially aligned anti-parallel, as in Fig. 3(*b*). Each of the HA molecules in the duplex is a different shade of grey. They appear to twist around each other, as did also the two HA molecules in a parallel duplex (i.e. as in Fig. 3*a*).

Table 1. Binding energy per subunit of three different duplex models

The binding energy was defined to be the difference between the average internal energy of two isolated HA chains *in vacuo* (calculated from a molecular-dynamics simulation) and the average internal energy measured over the molecular-dynamics simulation of the various duplex models.

Structure	Binding energy/ monosaccharide unit (kJ/mol)
Parallel (as in Fig. 3*a*)	68.6
Anti-parallel (as in Fig. 3*b*)	71.1
Anti-parallel (as in Fig. 3*c*)	37.2

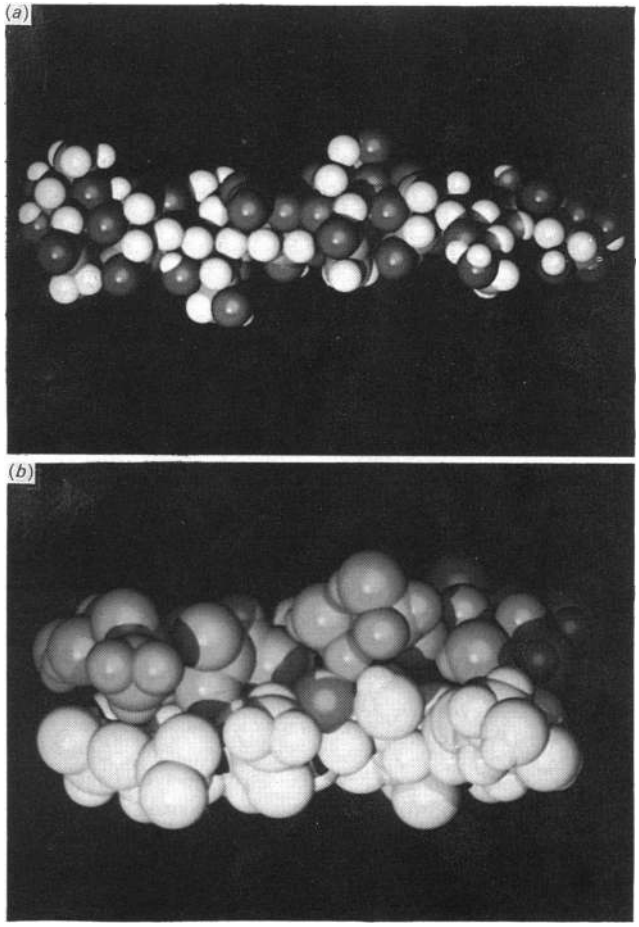

Fig. 5. Space-filling representations of instantaneous configurations of HA units in water from 70 ps molecular-dynamics simulations run at 300 K in a 3.0 nm × 3.0 nm × 3.0 nm water box with periodic boundary conditions

For experimental details see the text. For clarity, the water molecules are not displayed. (*a*) A four-disaccharide-unit HA unit in water. The lightest spheres are hydrogen atoms, and the darker spheres represent carbon, nitrogen and oxygen atoms. This structure agrees well with that inferred from n.m.r. investigations (Heatley & Scott, 1988). (*b*) A duplex made up of two two-disaccharide-unit HA molecules, each having the configurations shown in (*a*). The two molecules in the duplex are shaded differently.

These water bridges were not present in HA in dimethyl sulphoxide, where there is a hydrogen bond directly between the acetamido and carboxy groups (Scott *et al.*, 1984). That we see a twofold helical structure in water is not a trivial result, since simulations of single HA molecules *in vacuo* always produced HA molecules with a random-coil configuration (having regions

that were two-, three- and four-fold helical). The twofold helix seen in water is presumably stabilized by hydrogen-bonding networks in the water shells arranged round the carboxy and acetamido groups of the HA.

These results demonstrate that the computer model of HA accurately reproduces the structure established by n.m.r. and other experimental methods (Scott, 1989), and validates the method for probing HA duplexing processes. However, it is stressed that our intention was not to prove the actual structure of HA complexes, but rather to test the feasibility of a number of HA duplex models.

Simulations were also performed of complexes in water of HA molecules made up of two disaccharide units. Fig. 5(*b*) shows the duplex structure obtained after a run of 70 ps at 300 K. Although the binding energy of this complex has not been calculated, it is stable in water at biological temperatures.

Thus computer simulations of HA in water are potentially useful in elucidating mechanisms that stabilize the HA twofold helix in water, and in showing that HA duplexes are geometrically and energetically possible.

DISCUSSION

The rotary-shadowing results leave no doubt that HA is fundamentally a network-forming polymer, with marked capacity to form branch points with other molecules, re-linking at other points to form three-dimensional honeycomb-like structures of enormous dimensions, even at very low (< 1 μg/ml) concentrations. The images are incompatible with entangled random coils or overlapping stiff rods, in view of the absence of observable free ends (Figs. 1 and 2). HA forms thick lateral aggregates of a uniform diameter extending for hundreds of nanometres, containing scores of HA molecules, side-by-side. This 'stickiness' provides the adhesion between HA molecules at the branching points.

Previous electron-microscopic studies, which apparently showed little evidence of branching (Fessler & Fessler, 1966), were done on HA in cytochrome *c* films, and it is possible that interactions with the cytochrome affected HA–HA association. If this was so, investigations of complex-formation between biopolymers in cytochrome and similar (e.g. cationic detergent) films will need to be re-assessed. The first electron-microscopic study of HA (Gross, 1948), using metallic shadowing, found 'anastomosing fibrous processes' (see also J. Engel in discussion to Scott, 1989), and it is noteworthy that Ogston and co-workers, after careful investigations on several sorts of HA by most of the relevant physical-chemical techniques then available (light-scattering, viscosity, osmometry and sedimentation) concluded tentatively in favour of 'a branched structure, with partial cross-linking to form a "cage" within which most of the branches remain mobile' (Preston *et al.*, 1965).

Proposals for aggregated HA in aqueous solution have been made, and rejected (for a discussion see Scott, 1989). With the elucidation of the fully detailed secondary structure of HA oligosaccharides in solution by n.m.r., the recognition of large hydrophobic patches, repeated regularly and alternately on opposite faces of the tape-like HA molecule, provided a base for possible self-aggregation, based on hydrophobic interactions (Scott, 1989). Preliminary observations of branched and possibly duplexed structures in low-viscosity HA, coupled with a marked discrepancy between the observed rotary-shadowed size of this HA and that calculated from its reduced viscosity, led to the suggestion that self-aggregation to form duplexes by HA was usual, and probably extremely stable (Scott *et al.*, 1990).

Starting from the structure established by n.m.r. (Heatley & Scott, 1988; Scott, 1989), computer simulation showed that duplex formation, based on interaction between the hydrophobic patches on two participating molecules, was sterically possible and energetically likely, over very long stretches of HA (see the Results section and Fig. 4). It is particularly relevant that duplex formation does not exhaust the possibilities of self-aggregation by HA, since the two molecules in the duplex present essentially similar surfaces to a third and a fourth HA molecule as they did to each other in forming the duplex. It is not possible, without further extensive modelling, to predict where this process of accretion might stop, but it is clear from the electron micrographs that it could be very extensive.

Functional implications of the honeycomb structures

In principle, there is a connection from every HA molecule in aqueous solution to every other, via the meshwork. Thus, dissolved HA represents an integral structure, not requiring any other participant to hold it together. This accounts for the properties of HA solutions, which at concentrations of 0.5–1.0 g/l behave like a weak and elastic gel. With minimal reinforcement from sparse collagen meshworks, such gels would be expected to have the properties of soft tissues such as vitreous humour and Wharton's jelly, which elastically maintain a shape against moderate deformational forces.

Long HA molecules may form more cross-links than short molecules, and the meshwork could be stronger at a given concentration. Our results suggest that low-viscosity HA forms meshworks of limited extent compared with 'native' HA. Thus relatively few cleavages of HA chains would weaken the meshwork, progressing through semi-liquid to liquid, as their number increased. This effect may underlie several pathological conditions where a modest decrease in HA size has been observed, e.g. the decreased rigidity of vitreous humour with age (Swann, 1987). Similarly, the non-Newtonian viscosity would be expected to decrease in magnitude with damage to HA that resulted in shortening, and a concomitant decrease in the total cross-linking in the HA domain. The effect on the lubricant properties of synovial fluid would be expected to be more damaging than the limited chemical changes would imply.

The marked and reversible decrease of viscosity of HA solutions at high pH is more readily explicable on the basis of disaggregation of the meshwork structures caused by simultaneous disruption of the secondary and contingent tertiary structures than by the effects on the secondary structures alone, as previously proposed (Scott, 1989).

CS has a secondary structure that resembles that of HA, lacking only the hydrogen bond between the C-4 hydroxy group of the hexosamine residue and the ring oxygen atom of the neighbouring uronic acid residue (Scott *et al.*, 1983). The geometry of duplex formation ought therefore to be very similar for CS and HA. Examination of the duplexes formed by HA (Fig. 3) suggests that a similar structure for CS might be less likely, since there would necessarily be close approach between the charged groups, which are twice as numerous in CS as in HA. Rotary shadowing showed that large branched aggregates were very uncommon in chondroitin 4-sulphate preparations (Scott *et al.*, 1990). It remains to be seen whether unsulphated chondroitin has similar properties to those of HA.

REFERENCES

Atkins, E. D. T., Meader, D. & Scott, J. E. (1980) Int. J. Biol. Macromol. **2**, 318–319

Brooks, B. R., Brucceroli, R. E., Olafson, D. J., States, S., Swaminathan, S. & Karplus, M. (1985) J. Comput. Chem. **4**, 187–217

Evered, D. & Whelan, J. (eds.) (1989) Ciba Found. Symp. **143**

Fessler, J. H. & Fessler, L. I. (1966) Proc. Natl. Acad. Sci. U.S.A. **56**, 141–147

Gross, J. (1948) J. Biol. Chem. **172**, 511–515
Heatley, F. & Scott, J. E. (1988) Biochem. J. **254**, 489–493
McCammon, J. A. & Harvey, S. C. (1987) Dynamics of Proteins and Nucleic Acids, Cambridge University Press, Cambridge
Ogston, A. G. & Sherman, T. (1959) Biochem. J. **72**, 301–305
Preston, B. N., Davies, M. & Ogston, A. G. (1965) Biochem. J. **96**, 449–471
Scott, J. E. (1960) Methods Biochem. Anal. **8**, 145–198
Scott, J. E. (1988) Biochem. J. **252**, 313–323
Scott, J. E. (1989) Ciba Found. Symp. **143**, 6–20
Scott, J. E., Heatley, F., Jones, M. R. N., Wilkinson, A. & Olavesen, A. H. (1983) Eur. J. Biochem. **130**, 491–495
Scott, J. E., Heatley, F. & Hull, W. E. (1984) Biochem. J. **220**, 197–205
Scott, J. E., Cummings, C., Greiling, H., Stuhlsatz, H. W., Gregory, J. D. & Damle, S. P. (1990) Int. J. Biol. Macromol. **12**, 180–183
Swann, D. A. (1987) Bull. Soc. Belge Ophtalmol. **223**, 59–72
van Gunsteren, W. F. & Berendsen, H. J. C. (1977) Mol. Phys. **34**, 1311–1327
Wik, K. O. (1979) Acta Univ. Ups. Abstr. Uppsala Diss. Fac. Sci. no. 334

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