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The Conformation of the Mucopolysaccharides

HYALURONATES

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X-ray-diffraction patterns of hyaluronate fibres from a variety of sources were obtained. Sodium hyaluronate gives well-defined patterns which index on a hexagonal unit cell with dimensions $a = 1.17 \pm 0.01$ nm and a fibre repeat-distance of 2.85 ± 0.03 nm. A further form of sodium hyaluronate is produced by annealing at 60°C in 75% relative humidity. This stable state indexes on a hexagonal unit cell of unchanged fibre repeat-distance but with $a = 1.87$ nm. The chain conformation is a threefold helix. Analysis of these diffraction patterns led to two tentative structures for sodium hyaluronate, involving different packing of the polysaccharide chains. The significance of side-chain interaction is discussed. Hyaluronic acid produces an X-ray pattern different from that obtained with the sodium salt. The fibre repeat-distance is 1.96 ± 0.02 nm and the unit cell appears to be monoclinic. The chain conformation is a twofold helix and conformational change between free acid and monovalent salt is discussed. These findings, together with model-building experiments, are interpreted as indicating a highly ordered structure, and the physical properties of hyaluronate solutions with regard to molecular shape and polyelectrolyte behaviour are rationalized.

Hyaluronic acid is a member of the connective-tissue mucopolysaccharide group of substances and has the particular ability of associating with large quantities of water to form a jelly-like matrix between cells. It is also a major constituent of vitreous humour, where it behaves as an optically transparent low-refractive-index gel which maintains the precise shape of the eyeball. In addition hyaluronic acid solutions are highly viscous and serve as a lubricant in synovial fluid.

Chemically hyaluronic acid is a glycosaminoglucuronan consisting of equivalent proportions of 2-acetamido-2-deoxy-D-glucopyranose and D-glucopyranosyluronic acid. Like the other mucopolysaccharides it is thought to be a regular, unbranched biopolymer with a repeating disaccharide unit of the type $[\text{-N-G-}]_n$ where, using trivial names, N is *N*-acetylglucosamine and G is glucuronic acid. The glycosidic linkages N to G and G to N are (1*eq*,4*eq*) and (1*eq*,3*eq*) respectively as shown in Fig. 1. It is reasonable to assume that both glucopyranose rings are in the C-1 chair conformation and so all substituent groups are equatorially positioned.

We have prepared oriented fibres and films of a number of mucopolysaccharides including hyaluronic acid together with various univalent and bivalent salts. The X-ray 'fibre' diffraction patterns obtained show that the material is in a highly crystalline state (Atkins & Sheehan, 1971, 1972).

Materials

Hyaluronic acid

Mesothelioma. The source was fluid secreted into the peritoneal cavity of a patient suffering from mesothelioma of the lower intestine, and was kindly given by Dr. J. E. Scott (M. R. C. Rheumatism Unit, Canadian Red Cross Memorial Hospital, Taplow, Berks., U.K.). The sample was stored at −18°C. Small portions were thawed, diluted with 0.5 vol. of 30 mM-sodium phosphate buffer, pH 6.0, containing 15 mM-EDTA and 15 mM-cysteine. Twice-recrystallized papain (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was added (1 mg/100 ml of solution) and the whole was incubated at 45°C for 16 h, after which trichloroacetic acid was added to a final concentration of 5% (w/v). The denatured protein precipitate was removed by centrifugation at 25000*g* for 0.5 h. Ethanol (3 vol.) was added to the clarified solution plus several drops of saturated sodium acetate and the solution was stored at 4°C for 5 h. The precipitate was collected by centrifugation at 10000*g* for 0.5 h, then it was dissolved in water and dialysed for 3 days against three changes of water (each 5 litres). The material was freeze-dried and stored at −18°C.

Vitreous humour. Fresh bovine eyes were cleaned from surrounding tissue. The posterior parts of the vitreous gel were removed by an equatorial cut. The gel was gently de-structured by a single passage

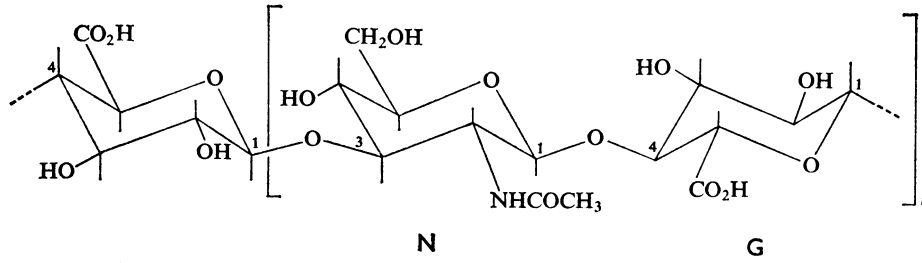


Fig. 1. *Repeating unit of hyaluronic acid*

N is 2-acetamido-2-deoxy-β-D-glucopyranosyl and G is β-D-glucopyranosyluronic acid. Notice the close proximity of the acetamido and carboxyl side groups on either side of the (1*eq*,4*eq*) glycosidic linkage. The chain conformation, as drawn, is close to the twofold screw structure found in the free acid.

through a Potter homogenizer equipped with a loose-fitting pestle. The free-flowing solution was centrifuged at 25000*g* for 0.5h and the solution treated with papain as described above.

Umbilical cord. Two grades of human umbilical cord were obtained from Miles Seravac Ltd. (Maidenhead, Berks., U.K.). Both samples were in the potassium salt form. Grade I contained 3% protein and 15% chondroitin sulphate, and grade II contained 2% protein, 3% chondroitin sulphate.

Methods

Preparation of hyaluronic acid and its derivatives

The free acid was prepared by slow passage of a 0.1% (w/v) solution of hyaluronate down a column (0.9cm × 10cm) of Amberlite Monobed resin (MB-3, analytical grade; Rohm and Haas Co., Philadelphia, Pa., U.S.A.). Solutions so prepared had a negligible ash content. The relevant hyaluronate solutions were prepared by titrating samples of the free acid with base to pH7.5 and were used as such. *N*-Acetylhexosamine was assayed by the Good & Bessman (1964) modification of the Morgan–Elson reaction. *N*-Acetylglucosamine (BDH Chemicals Ltd., Poole, Dorset, U.K.) was used as standard. Hexuronic acid was assayed by the method of Bitter & Muir (1962). Protein was determined by the biuret method as described by Gornall *et al.* (1949) with bovine serum albumin as standard. Sedimentation was observed in a Beckman model E analytical ultracentrifuge operating at 59780rev./min at 20°C. Schlieren optics were used and the sedimentation coefficients, calculated from the movement of the maximum ordinate, were corrected for solvent density and viscosity by standard procedures. The solvent was 10mM-sodium phosphate buffer, pH7.0, containing 100mM-NaCl. The chemical analysis of the material is given in Table 1.

Polysaccharide films and fibres

Specimens were prepared by allowing a viscous solution, usually sodium or potassium hyaluronate, to dry down on a clean glass surface. The film was cut into strips approx. 1–2mm wide which were then stacked together. The stacked specimen was then stretched under constant load at a relative humidity in the range 75–85% obtained by using saturated salt solutions. The specimen absorbed water and underwent a fairly rapid initial extension in the first few hours. The exact load used depended on the size of specimen, but a typical loading was 10g. The free-acid specimens were obtained by immersing a crystalline specimen of the univalent salt in ethanol–0.2M-HCl (4:1, v/v). Fibre densities were measured by the flotation method by using carbon tetrachloride and acetone mixtures.

X-ray diffraction

X-ray diffraction photographs were obtained by using CuKα radiation from either a Philips Fine Focus or an Elliott Rotating Anode X-ray generator. Various pin-hole collimators were used in the range 150–500μm diameter, with specimen-to-film distances typically 4–6cm. The X-ray photographs were obtained with the camera either filled with H_2 or evacuated to minimize scatter caused by air. Specimens were photographed in a number of orientations about the molecular axis, which were all identical, proving cylindrical symmetry.

Molecular model building and calculations

Molecular models were built of all the structures considered by using both CPK space-filling models and accurate wire models with a scale of 4mm ≡ 1nm. Standard bond lengths and angles were used to find the distance between chains which formed a linear hydrogen bond (CO···HN) between acetamido

Table 1. *Chemical analysis of various types of hyaluronic acid expressed as a percentage of the dry weight*

For experimental details see the text. Dry weights were determined on salt-free samples by drying for 16h at 105°C. Values are the means ± S.D., with the numbers of determinations in parentheses.

	Glucuronic acid (%)	Hexosamine (%)	Protein (%)
Hyaluronic acid (theoretical), assuming equimolar proportions of the two constituents	48.86	51.14	0.0
Mesothelioma hyaluronic acid	40.9 ± 1.7 (5)	49.7 ± 1.2 (5)	0.3
Vitreous-humour hyaluronic acid	45.8 ± 1.3 (7)	50.0 ± 2.7 (7)	0.3

groups. The models were checked by measurement and conformed to known stereochemical criteria. Preliminary structure-factor calculations are in agreement with the general features of the X-ray results, and precise calculations are in progress.

Results

Molecular-weight determinations

No detailed examination was undertaken of the molecular weight of the hyaluronate samples. The concentration-dependence of the sedimentation coefficients is shown in Fig. 2 for the two preparations used. The data are consistent with a molecular weight of 1.5×10^6 for the mesothelioma material and a lower molecular weight of 75000 for that from bovine vitreous humour.

X-ray-diffraction patterns

The material used came from various sources and also differed in the degree of purity. Table 2 relates the samples used with the approximate molecular weight and the quality of the X-ray pattern obtained.

Sodium hyaluronate crystallizes in two distinct well-defined forms, the X-ray diffraction photographs of which are shown in Plates 1(*a*) and (*b*). The reflexions in Plate 1(*a*) index on a hexagonal unit cell with dimensions $a = 1.17 \pm 0.01$ nm and c (fibre axis) = 2.85 ± 0.03 nm. The X-ray pattern shown in Plate 1(*b*) indexes on a larger hexagonal unit cell with $a = 1.87 \pm 0.01$ nm, and c (fibre axis) = 2.85 ± 0.03 nm. Potassium hyaluronate also crystallizes in two forms identical in cell dimensions with those of the sodium salt.

The X-ray diffraction pattern of the free acid (Plate 1*c*), however, is quite different. We have not yet been able to index this pattern unequivocally, since some reflexions belong to a superimposed lattice. This probably involves some intermediate between the hexagonal sodium salt lattices. However, what is certain is that the layer plane spacing decreases to

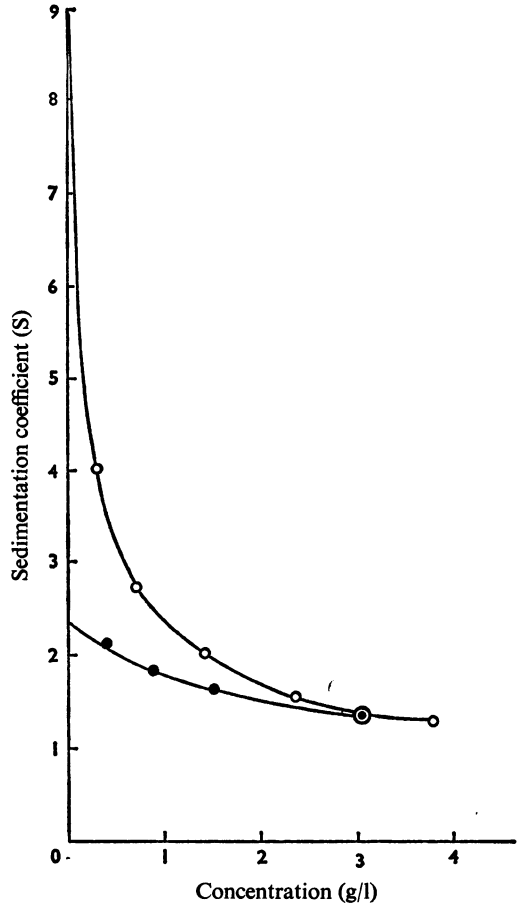


Fig. 2. *Sedimentation coefficient of hyaluronic acid as a function of concentration*

Estimates: bovine vitreous humour hyaluronate (●) mol. wt. 75000; human mesothelioma hyaluronate (o) 1500 000. Analytical runs were performed at 20°C in 100mM-NaCl–10mM-sodium phosphate buffer, pH7.0, at 59780rev./min and were corrected for density of solvent.

Table 2. *Relation of diffraction pattern to the source of hyaluronate*

The quality of the diffraction pattern obtained from the different sources of hyaluronate is correlated. The samples were prepared under identical conditions.

Source of hyaluronate	Molecular weight	Quality of X-ray diffraction pattern
Purified mesothelioma, sodium salt	1×10^6	Good
Impure human umbilical (3% protein, 15% chondroitin sulphate), potassium salt	$\sim1\times10^6$	Good
Pure human umbilical (2% protein, 3% chondroitin sulphate), potassium salt	1×10^6	Good
Dialysed raw vitreous humour, sodium salt	~150000	Fair
Purified vitreous humour, sodium salt	~75000	Poor

1.96±0.02nm and that the unit cell is likely to be monoclinic. Only when better X-ray photographs are obtained will the exact lateral dimensions be certain. The meridional reflexions occur on even-layer planes only, which is consistent with a twofold helix. The fibre densities of sodium hyaluronate were measured to be 1.47g/cm^3.

Discussion

Solid-state studies

To date remarkably little information has been forthcoming on the structure of the glycosaminoglycans. Sylven & Ambrose (1955) prepared fibres of hyaluronic acid which were birefringent. Bettelheim (1959) reported a disaccharide periodicity of 1.198nm for such fibres, but this cannot be taken seriously, since such a model is sterically impossible. Such fibres were prepared from low-molecular-weight material. Attempts to produce oriented fibres from high-molecular-weight material gave only amorphous X-ray diffractograms (Laurent, 1957). Some gel-forming polysaccharides could be oriented and crystallized. For instance sodium pectate has hexagonal symmetry, with $a = 1.62$nm and c (fibre axis) = 1.31nm (Palmer & Hertzog, 1945). The two component polyuronic acids of alginic acid crystallize in orthorhombic unit cells (Atkins *et al.*, 1970, 1971) giving good-quality X-ray 'fibre' diffraction patterns. The sulphated polysaccharide seaweed κ- and ι-carrageenans, more similar to the mucopolysaccharides with their [-A-B-]$_n$ repeat, crystallize in the form of double-stranded helices (Anderson *et al.*, 1969). It was likely therefore that hyaluronic acid should both orientate and crystallize, unless the chemical results were incorrect and we were dealing with either a non-linear polymer or a polymer with an extremely irregular sequence. This turns out not to be the case, as shown by the good quality of the X-ray diffractograms obtained.

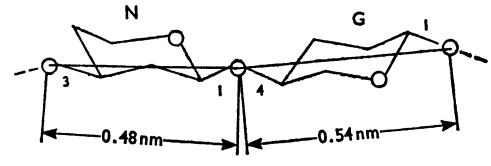
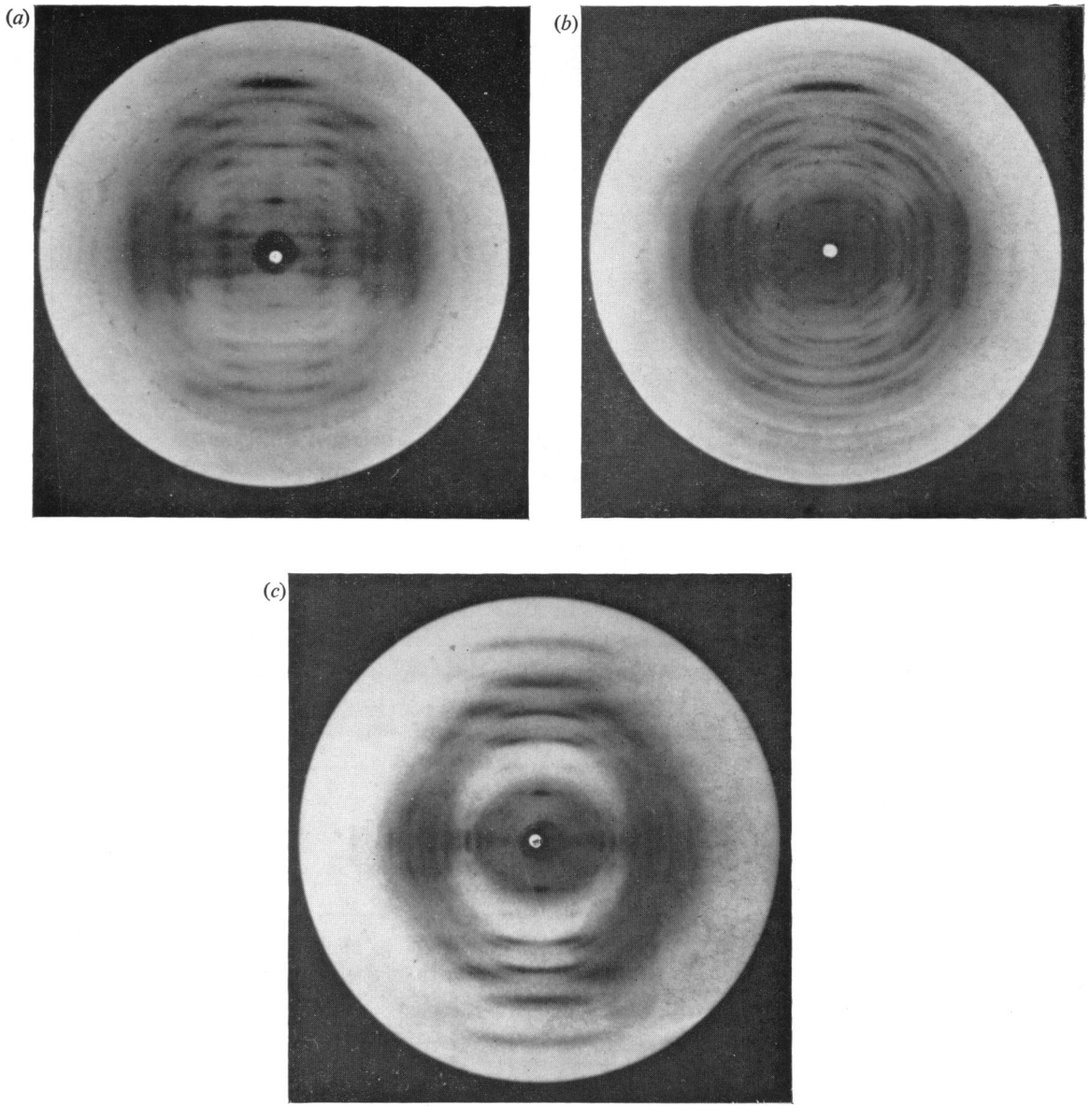


Fig. 3. *Dimensions of the disaccharide repeat-distance*

The maximum possible extension of a (1*eq*,3*eq*–1*eq*,4*eq*)-linked polysaccharide is the sum of the two monomer vectors. Thus all disaccharide repeats must be ⩽1.02nm.

Conformational change between univalent salt and free acid

The X-ray diffraction photographs of the sodium salt shown in Plate 1(*a*) have meridional reflexions on layer planes with index $l = 3$, 6 and 9. We suggest that these reflexions are orders of the axially projected disaccharide repeat of 2.85/3 = 0.95nm. The distribution of layer-plane intensities corresponds to that of a helix with three disaccharide units per turn. The decrease in the layer-plane spacing in the case of the free acid having meridional reflexions on layer planes $l = 2$ and 4 suggests that the threefold hyaluronate helix has tightened into a twofold helix. The axially projected disaccharide repeat would be anticipated to increase slightly to the 1.96/2 = 0.98nm observed. Calculation shows that the maximum possible extension of a (1*eq*,3*eq*–1*eq*,4*eq*)-linked polysaccharide of this type (see Fig. 3) is 1.02nm (Rees, 1969) and model building shows that this value is close to that for a twofold-screw conformation. In this conformation the carboxyl group and acetamido groups lie in close proximity to each other on either side of the (1*eq*,4*eq*) glycosidic linkage (see Fig. 1). On formation of the univalent salt each disaccharide unit rotates away from its near neighbour to accommodate the larger



EXPLANATION OF PLATE 1

X-ray fibre diffraction photographs

(*a*) Obtained from sodium hyaluronate. The unit cell is hexagonal with $a = 1.17 \pm 0.01$ nm and a fibre repeat-distance of 2.85 ± 0.03 nm. There are two threefold helices per unit cell. (*b*) Obtained from sodium hyaluronate after annealing at 60°C and 75% relative humidity. Reflexions index on a hexagonal unit cell with $a = 1.87 \pm 0.01$ nm and a layer plane spacing of 2.85 ± 0.03 nm. There are six chains per unit cell. (*c*) Obtained from hyaluronic acid. The layer plane spacing is 1.96 ± 0.02 nm and meridional reflexions occur only on even-layer planes. There are certain forbidden reflexions which belong to a superimposed lattice.

substituent cation. Thus it is likely that most, if not all, of this rotation occurs about the (1*eq*,4*eq*) glycosidic linkage. This is consistent with the small change in the axially projected repeat. If a substantial part of the rotation occurred about the (1*eq*,3*eq*) glycosidic linkage we would expect a tilting of disaccharide units, causing a lowering in the axial projection as discovered for (1*eq*,3*eq*)-linked xylan by Atkins *et al.* (1969). That this rotation about the helix axis is exactly 60° (from the twofold helix) is probably due to the hexagonal environment of neighbouring chains rather than to some intrinsic property of an individual chain. Model-building trials indicate that the planar acetamido groups probably lie close to planes perpendicular to the helix axis. We have placed these groups with C=O *cis* to C(2)–H (Fig. 4) which conforms to the position found for poly-*N*-acetylglucosamine by Carlstrom (1957) and Blackwell (1969). There are indications that the left-hand helix is the more favourable conformation, since the carboxyl and acetamido groups rotate away from each other, providing space for the attendant cation. The separation of these side groups is noticeably less in the right-hand helix. It is noteworthy that the (1*eq*,4*eq*)-linked xylan prefers the left-handed threefold helix (Nieduszynski & Marchessault, 1971).

Threefold salt structures

When considering X-ray-diffraction patterns from helical structures one must be aware of possible families of intertwining multistrand models similar to those found for the (1*eq*,3*eq*) xylan (Atkins & Parker, 1969) and the carrageenans (Anderson *et al.*, 1969). There are, however, certain features of the hyaluronate X-ray patterns which do not favourably support such intertwined-chain models. The observed axial repeat of 0.95nm, only 0.07nm less than the maximum permissible extension, implies a nearly straight helix. It can be shown that a second chain may only intertwine (even if at all possible) by a relative twist along the helix axis by half (or nearly half) of the projected disaccharide repeat-distance. The diffraction pattern of such a structure would either have every odd-order layer plane disappearing or have a noticeable weakening of all odd-order layer planes. Such effects are not observed in the X-ray-diffraction photographs. Thus the structure consists of single-stranded, probably left-handed, threefold helices.

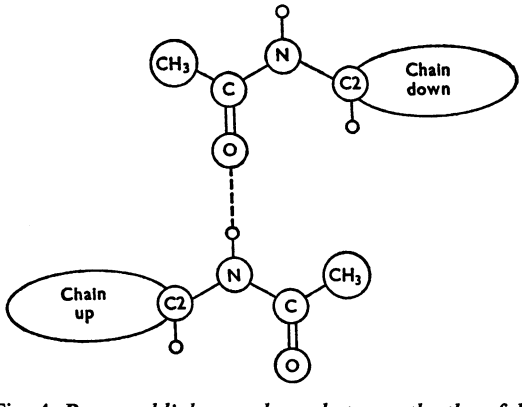


Fig. 4. *Proposed linkage scheme between the threefold chains*

The hydrogen bond formed between acetamido side-groups is only possible if the chains are antiparallel. Such an arrangement gives a calculated centre-to-centre distance close to the 0.67nm observed.

The 1.17nm unit cell

The chemical repeat-unit has a molecular weight of 401 and the density ρ is given by $\rho = h \times 0.592\,g/cm^3$ where h represents the number of threefold helices in the unit cell. Thus if $h = 3$, $\rho = 1.78\,g/cm^3$, which we consider to be too high compared with the measured density of $1.47\,g/cm^3$, although measured densities of fibres are invariably less than those calculated. Since we know that hyaluronate associates with considerable quantities of water and since the material is crystallized under humid conditions it is reasonable to suspect that there is a considerable amount of water in the unit cell. We therefore believe it more likely that there are only two polysaccharide chains per unit cell and that the vacancy is filled with water. Thus for $h = 2$ and 30% water, $\rho = 1.58\,g/cm^3$. This appears to be a more reasonable model. There are additional indications for a two-chain hexagonal unit cell. First, we would expect a three-chain structure to give a very strong $11\bar{2}0$ reflexion and a weak $10\bar{1}0$ reflexion. This is contrary to what is observed. This may be seen by placing additional hyaluronate chains into the holes shown in Fig. 5. The 1.17nm hexagonal unit cell drawn in Fig. 5 would then have chains at each corner resulting in a higher density of chain centres along the $11\bar{2}0$ planes compared with the $10\bar{1}0$ planes. We appreciate that this argument will need confirming by precise structure-factor calculations. This packing arrangement gives a centre-to-centre distance between chains of 0.673nm. Secondly, when considering the packing of such threefold helices it is likely that the acetamido groups make hydrogen bonds between chains. As shown in Fig. 4, chains of opposite polarity are then able to make a linear hydrogen bond between acetamido groups and the distance between chain centres is very nearly 0.67nm. Since the acetamido groups project at angles 120° apart the packing arrangement

forms a network of threefold helices which hydrogen-bond through their acetamido groups.

Fig. 4 shows that the 'up' threefold helices act as donors for hydrogen-bond formation and the 'down' helices as acceptors. Thus in principle the conformation of the acetamido groups is different for 'up' and 'down' helices. Whether this difference will be detectable by detailed X-ray diffraction studies depends on the relative difference of atomic arrangements of the acetamido groups. If the planar acetamido groups are perpendicular (or nearly perpendicular) to the helix axis such differences are unlikely to be detected. No arrangement could be found in model-building trials for a scheme of parallel chains to hydrogen-bond in a similar manner. Also, any other linking mechanism between chains increased the distance between chains, leading to poorer agreement with X-ray-diffraction measurements. The space group of the packing arrangement shown in Fig. 5 is $P3_221$. Only when further detailed Fourier-transform calculations and polarized i.r.-spectroscopic results are obtained will we be able to confirm finally the antiparallel arrangement.

The 1.87 nm unit cell

On annealing at 60°C and in 75% relative humidity, the crystalline hyaluronate samples change to a new hexagonal unit cell with $a = 1.87$ nm, the fibre repeat-distance remaining constant (see Plate 1*b*). At intermediate stages in the annealing process both X-ray patterns are observed. The 1.87 nm unit cell is final and there is no appreciable change detected in the measured density. We have not found it possible to reverse the process. Maintaining the annealed sample in a vacuum of 10^{-2} mmHg (0.132 N/m^2) at 60°C for 2 weeks has no particular effect, the larger 1.87 nm unit cell still being preserved. Since the fibre repeat in both cases is the same we would expect the difference to lie in the packing of the threefold helices. The increase in volume by a factor of 2.54 would suggest five chains per larger unit cell. We are unable to conceive of a feasible packing arrangement for a five-chain system. Of course water may be absorbed during the annealing process and the number of chains per unit cell could fall to four or even three. This model would disagree both with the density measurements and, more important, with our experimental observation that no water can be made to leave on drying. This leads us to suggest that there are six chains per large unit cell, i.e. that water has been lost during the annealing process rather than absorbed. Although we should expect a small increase in density there is considerable evidence in favour of such a proposed six-chain model. It would be expected that six chains pack in a hexagonal manner. Having less water than the 1.17 nm unit cell it is not surprising that on drying the large unit cell persists. We are also able to suggest a scheme of rearrangement of chains to account for the size of the 1.87 nm unit cell. This packing scheme is shown diagrammatically in Fig. 6. The small 1.17 nm hexagonal unit cell represents an arrangement of high

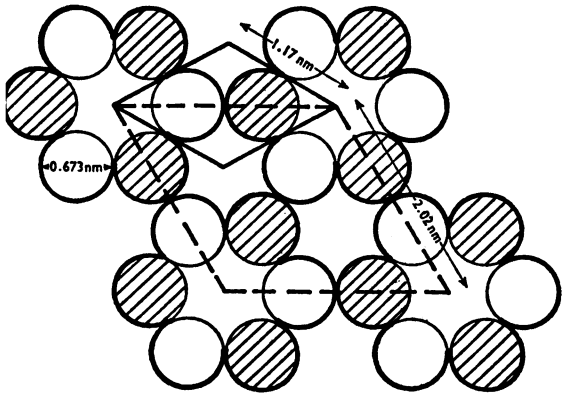


Fig. 5. *Proposed packing scheme of chains in the 1.17 nm hexagonal unit cell*

Each chain makes three links with its near neighbours. The open and shaded circles represent chains of opposite polarity. Certain rings of six chains are outlined to help understand the rearrangement that occurs on annealing. If such six-membered rings were individual entities the large 2.02 nm hexagonal unit cell would be observed.

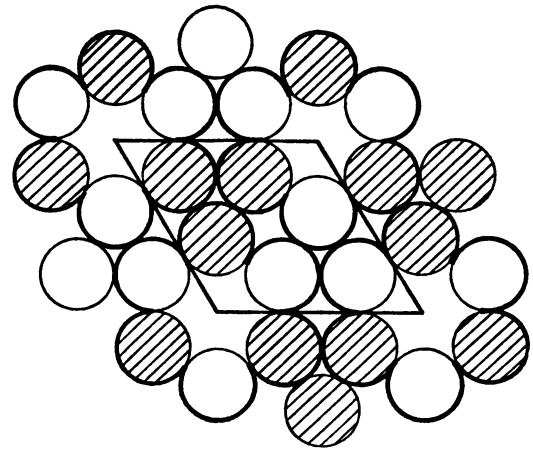


Fig. 6. *Proposed packing scheme for the annealed sample*

The rearrangement may be thought of as a rotation of six-membered rings relative to each other. Theoretically the unit cell shown has a side of 1.84 nm.

symmetry where each chain links with near neighbours in an identical environment. Why then should the crystal packing alter on annealing? The answer concerns the relationship between crystallization and lowest free energy. Although the packing scheme depicted in Fig. 6 has lower symmetry than the scheme shown in Fig. 5 each chain now makes four interchain links instead of three. The packing scheme postulated in Fig. 6 would require that one-third of the acetamido hydrogen bonds would break but that this would be more than compensated by interchain links (probably hydrogen bonds between hydroxyl groups) to two chains rather than one. If the bond energy of these new interchain links is greater than 50% of that of the broken acetamido hydrogen bond then the 1.87nm unit cell would have lower free energy and therefore greater stability than the 1.17nm unit cell. This free-energy difference cannot be too great, or the material would crystallize directly in the 1.87nm unit-cell arrangement.

The relationship between the two packing arrangements may be envisaged by concentrating on the six-membered groups outlined in Fig. 5. Although in this particular packing scheme they are arbitrarily chosen, examination of the packing scheme depicted in Fig. 6 shows how such six-membered groups may be rotated bodily by 30° to form a closer-packed system. Thus the hexagonal unit cell of side $a = 2.02$nm and containing six chains which is drawn in Fig. 5 would change to the hexagonal unit cell with $a = 1.84$nm as drawn in Fig. 6. This result is calculated on a fixed centre-to-centre distance between chains. It is likely that the new bonds formed by replacement of one acetamido group will not hold the chains so closely together. This would suggest that the six-membered groups are a little further apart, fitting the observed unit cell of 1.87nm better. One feature of the X-ray diffraction results which favours a relationship between unit cells of this type is the relative weakness of the $10\bar{1}0$ reflexion in the 1.87nm unit cell. Since the rearrangement of chains postulated involves mainly rotations of six-membered groups the observed strong equatorial reflexions should have approximately the same spacing in both packing arrangements. This is borne out by comparing the $11\bar{2}0$ reflexion in the 1.87nm unit cell with the $10\bar{1}0$ reflexion in the 1.17nm unit cell. Fig. 5 shows that both these reflexions represent similar sets of diffracting planes.

Molecular shape

Most studies of molecular shape have involved derivative deductions from experimental parameters obtained from solution studies. However, the electron micrographs obtained by Fessler & Fessler (1966) by surface techniques showed single strands of hyaluronate with an average strand length of 2.4μm.

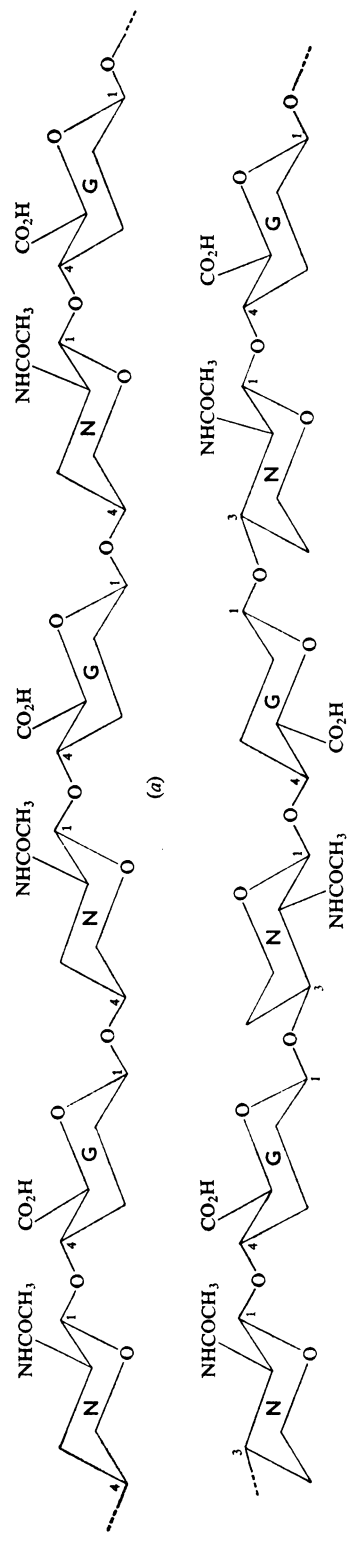


Fig. 7. *Schematic diagram for hyaluronic acid*

(*a*) is drawn using the hypothetical (1*eq*,4*eq*) glycosidic linkage G to N, which positions the main substituent groups on one side only. (*b*) shows the correct model, the main substituent group being distributed equally on both sides of the chains.

This is equivalent to a weight-average molecular weight of 1×10^6–2×10^6 assuming single chains. These pictures both confirmed the chemical evidence that hyaluronate is unbranched and supported the molecular-weight conclusions based on hydrodynamic data.

Polyelectrolyte behaviour

Since the polymer carries one carboxyl group per disaccharide unit and this group is dissociated at physiological pH values the polysaccharide behaves as a polyanion in solution.

The (1*eq*,4*eq*)-linked polyuronic acids (Atkins *et al.*, 1970, 1971) distribute their carboxylate groups equally on either side of the chain in the twofold helix, and any untwisting from this position distributes such groups more evenly over the surface of a cylinder. We consider that this is a requirement for the formation of linear polyelectrolytes. If therefore we consider a polyelectrolyte such as hyaluronic acid, but with all (1*eq*,4*eq*) glycosidic linkages, we would arrive at a situation depicted by Fig. 7(*a*). All the important substituent side-groups are positioned on one side of the polymer only. Even after allowing some rotation about the glycosidic bonds we would still have a predominantly polar polyelectrolyte. Such a chain is stereochemically feasible and there is no particular difference in flexibility of such a structure compared with the true structure shown in Fig. 7(*b*). This model has the alternate (1*eq*,3*eq*) and (1*eq*,4*eq*) glycosidic linkages. The conformational map of a (1*eq*,3*eq*)-linked polysaccharide again has a single stereochemically-allowed region, but the mean position is near a slightly bent chain with successive monomers in the same orientation to each other. The combination of alternating linkages automatically distributes the substituent groups equally on both sides of the polymer. The twofold screw conformation represents the mean conformation of the polymer. Any rotation around the glycosidic linkages distributes the ionic charges even more uniformly over the surface of the long, thin cylindrical molecule.

Influence of ionic environment on shape

Solutions of hyaluronic acid display non-Newtonian viscosity, and measurements of the reduced viscosity must therefore be extrapolated to zero shear gradient. When this is done, hyaluronic acid displays a sensitive response to both pH and ionic strength. A detailed study of the rheology of hyaluronic acid from umbilical cord has been made by Gibbs *et al.* (1968), where the viscous and elastic components of the polymer were studied as a function of pH, ionic strength and temperature. At low-strain frequencies a sharp transition, which was strongly pH-dependent, to elastic behaviour occurred. At pH2.5 particularly the solution showed an elastic behaviour, and it was proposed that carboxyl–carboxyl hydrogen bonding might stiffen the chains.

All these findings hint at some stiffening by non-covalent cross-links of a structure which is predominantly a random coil. The acetamido groups may only hydrogen-bond between antiparallel chains in the threefold sodium salt structure and in the manner indicated in Fig. 4. This is an essential feature of the packing arrangement shown in Fig. 5. When the pH is lowered sufficiently to form the free acid the polysaccharide helix tightens to form the twofold conformation. In this conformation the acetamido groups are able to act as both donors and acceptors (as in chitin), and hydrogen-bond to both parallel and antiparallel chains. In other words the requirements imposed by a threefold hydrogen-bonded network would be relaxed. This could possibly double the interchain hydrogen-bond density of acetamido groups and may explain the sharp transition to elastic behaviour at low pH values observed by Gibbs *et al.* (1968).

Conclusion

The discussion shows that hyaluronic acid has a subtle ability to cross-link in a variety of ways. We consider that we have made a start in the understanding of the molecular conformation of such a polymer. The variety of conformations we find exciting, since we wish to monitor the molecular shape as a function of environment. Structure for its own sake is interesting crystallographically, but it is the relationship between molecular structure and shape on the one hand and biological function and mechanical properties on the other that is of more importance.

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