Ciba Foundation Symposium 143

THE BIOLOGY OF HYALURONAN

A Wiley-Interscience Publication

1989

JOHN WILEY & SONS

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Symposium on The Biology of Hyaluronan, held at the Ciba Foundation, London, 27–29 September 1988

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Introduction

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It is a pleasure for me to open this conference on the biology of hyaluronan. The idea of a Ciba Foundation symposium on hyaluronan came after the conference on the functions of the proteoglycans held in 1986 (Ciba Foundation 1986). Hyaluronan was then treated as an 'honorary proteoglycan'. Hyaluronan is, however, a unique polymer, as will be apparent from the discussions during this symposium, and I am glad that the Foundation acted positively on the proposal by Roger Mason and myself to devote a conference entirely to hyaluronan. I want to express our thanks to David Evered and his colleagues for all their efforts in organizing our meeting.

Karl Meyer described a polysaccharide isolated from the vitreous humour in 1934 (Meyer & Palmer 1934). He precipitated the polymer in acid conditions and the product was therefore an acid. It contained uronic acid and Meyer named the polysaccharide *hyaluronic acid* from *hyalos* (= glassy, vitreous) and uronic acid. At physiological pH all carboxyl groups on the uronic acid residues are dissociated and the polysaccharide should therefore be named *sodium hyaluronate* when sodium is the counter ion. It is, however, often difficult to specify the counter ion, for example in a tissue, and Balazs et al (1986) therefore suggested that the name *hyaluronan* should be used, when the polysaccharide is mentioned in general terms. This is in conformity with the accepted terminology that names of polysaccharides should end with -an. Hyaluronic acid and hyaluronate should be reserved to specifically indicate the acid and salt forms of the polymer, respectively.

During the 1930s and 1940s Karl Meyer and others isolated hyaluronan from a number of sources and larger amounts were found, apart from the vitreous body, in other soft connective tissues such as synovial fluid, umbilical cord, skin and rooster comb (see Meyer 1947). The polysaccharide was also isolated from certain strains of bacteria, such as streptococci (Kendall et al 1937). At the same time the hyaluronidases were also described, the first one as a 'spreading factor' in testicular extracts (Duran-Reynals 1942).

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The early chemical characterization of hyaluronan showed that it contained equimolar concentrations of glucuronic acid and *N*-acetylglucosamine. The complete structure was elucidated to a large extent by Karl Meyer and his co-workers in the 1950s. They isolated a crystalline disaccharide, hyalobiuronic acid, from polymer degraded with testicular hyaluronidase and acid hydrolysis (Rapport et al 1951). Structural studies on this disaccharide established the glucuronidic linkage in the polymer. By the use of enzymes and structural analyses on oligosaccharides obtained by enzymic digestion the glucosaminidic linkage could be similarly defined (see e.g. Brimacombe & Webber 1964). The polysaccharide is a linear polymer with the structure . . . (1-[β-4) D-glucuronic acid (1-β-3) *N*-acetyl-D-glucosamine (1-]$_n$-β-4) . . . (see Fig. 1). Subsequent studies on the conformation of the chain by X-ray diffraction and spectroscopy indicate that the molecule can take up helical conformations stabilized by hydrogen bonds. We shall get up-to-date information on the conformational work in John Scott's contribution.

The physicochemical characterization of the polymer was carried out in the 1950s and 1960s. It is notable that Blumberg and Ogston summarized the state of the art at a Ciba Foundation symposium in 1958. Hyaluronan is a linear polymer when visualized in the electron microscope (Fessler & Fessler 1966). It is polydisperse and has usually a weight-average molecular mass of the order of several millions. The chain behaves in solution as an expanded random coil with a diameter of the order of 500 nm. The molecular domain includes a large amount of solvent. The chains entangle already at concentrations in the order of 1 g/l and, as a consequence, at higher concentrations the solutions exhibit an extremely high but shear-dependent viscosity (see e.g. Laurent 1970).

The physical chemistry was used in the 1960s to define possible physiological functions of hyaluronan and other connective tissue polysaccharides (for a review see Comper & Laurent 1978). It was shown that the polysaccharides could regulate the water balance via osmotic pressure and flow resistance; interact with plasma proteins via sieve and exclusion effects; act as lubricants through their rheological properties; and stabilize structures by electrostatic and other interactions.

An important discovery was made by Hardingham and Muir in 1972 when they found that cartilage proteoglycans specifically interact with hyaluronan. Many proteoglycans bind to the same hyaluronan chain and form aggregates which are deposited between the collagen fibres. Hyaluronan thus has a central structural role in cartilage. A large amount of research during the 1970s has been centred on this interaction. Roger Mason will present some aspects of the role of hyaluronan in cartilage in this symposium, and I hope that Dr Hardingham will take an active part in the discussions.

Several developments during the present decade have enhanced our interest in hyaluronan:

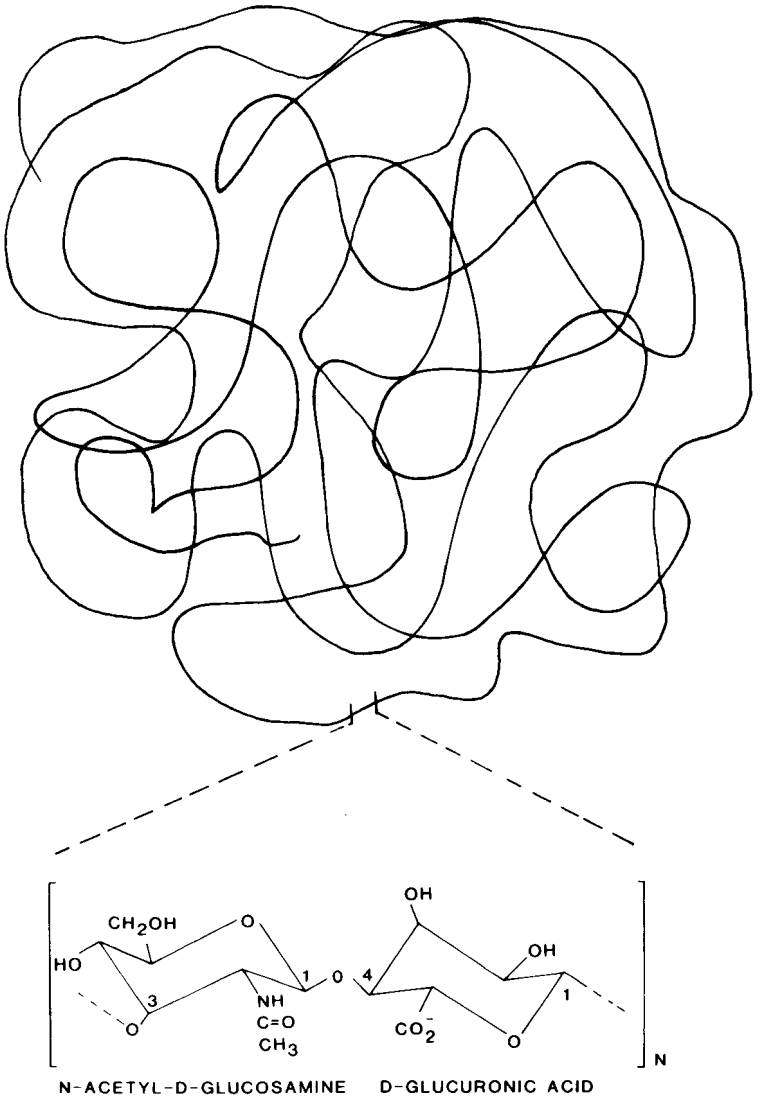

FIG. 1. The basic disaccharide unit of hyaluronan and its expanded random coil formation in solution.

(1) The discovery by Hardingham & Muir (1972) introduced proteins with specific affinity for hyaluronan and these have been used as analytical tools. We can now measure hyaluronan specifically in body fluids with a sensitivity which is 100–1000 times higher than that of previous techniques (Tengblad 1980).

The proteins have also been used to visualize hyaluronan histochemically, and several contributors to this symposium will address themselves to this subject.

(2) Fifty years after the discovery of the polysaccharide we have at last obtained information about the site where and the molecular mechanism by which the polymer is synthesized. We shall probably soon know a great deal more about the regulation of its synthesis. Peter Prehm and Nasi Mian, who have pioneered the field (Prehm 1983, Mian 1986), will introduce us to this fascinating subject.

(3) Similarly, there has been an advance in our knowledge of the turnover and catabolism of hyaluronan, as Robert Fraser and Lennart Rodén will tell us. The elucidation of the catabolic pathways (Fraser et al 1981) has led to interesting clinical applications, of which we shall hear more from Anna Engström-Laurent.

(4) The cell biological role of hyaluronan has been recognized for decades but interest has become focused on this aspect in the last few years. Most of the speakers at this symposium will deal with some type of hyaluronan–cell interaction. (a) There are many reports on the relation between cell growth and hyaluronan synthesis. The polysaccharide may be involved in the mitotic process (Brecht et al 1986). (b) Fibroblasts and other cells surround themselves with a coat of hyaluronan-containing material, which was ingeniously visualized by Clarris & Fraser (1968). The coat may be hyaluronan under synthesis or hyaluronan bound to specific receptors on the cell walls. Charles Underhill and Eva Turley will tell us about hyaluronan-binding proteins, which may act as receptors for hyaluronan and which may also, through this interaction, regulate the cellular functions. (c) The interaction between cells and hyaluronan may be especially important during embryonic and fetal development. Bryan Toole has pioneered the studies on the developmental role of hyaluronan and Bertrand Delpech will discuss its role in the developing brain. (d) Hyaluronan has been assigned interesting functions in for example malignant growth, the immune system, angiogenesis and wound healing. These topics will be covered by Warren Knudson, Theresa Whiteside, David West and Paul Weigel.

(5) Highly viscous hyaluronan preparations have been used as an aid in ophthalmic surgery and one can envisage the exploitation of its rheological properties for other purposes. Endre Balazs, who is the pioneer in the practical use of hyaluronan in what he calls viscosurgery (Balazs 1983), will also contribute to this symposium.

It is my hope that during the symposium we shall be able to shed some light on the biological function of hyaluronan. It is a compound which is ubiquitous and we do not know of any genetic disease in which hyaluronan is not synthesized. This indicates that hyaluronan is of fundamental importance in the animal organism and that mutations causing defects in hyaluronan synthesis are lethal. The participants in this symposium are well qualified

to produce new and original ideas about the functions of this interesting polysaccharide.

References

Balazs EA 1983 Sodium hyaluronate in viscosurgery. In: Miller D, Stegmann R (eds) Healon (sodium hyaluronate): a guide to its use in ophthalmic surgery. Wiley, New York, p 5–28

Balazs EA, Laurent TC, Jeanloz RW 1986 Nomenclature of hyaluronic acid. Biochem J 235:903

Blumberg BS, Ogston AG 1958 Physicochemical studies on hyaluronic acid. In: Chemistry and biology of mucopolysaccharides. Churchill, London (Ciba Found Symp) p 22–37

Brecht M, Mayer U, Schlosser E, Prehm P 1986 Increased hyaluronate synthesis is required for fibroblast detachment and mitosis. Biochem J 239:445–450

Brimacombe JS, Webber JM 1964 Mucopolysaccharides. Elsevier Science Publishers, Amsterdam

Ciba Foundation 1986 Functions of the proteoglycans. Wiley, Chichester (Ciba Found Symp 124)

Clarris B, Fraser JRE 1968 On the pericellular zone of some mammalian cells in vitro. Exp Cell Res 49:181–193

Comper WD, Laurent TC 1978 Physiological function of connective tissue polysaccharides. Physiol Rev 58:255–315

Duran-Reynals F 1942 Tissue permeability and the spreading factors in infection. Bacteriol Rev 6:197–252

Fessler JH, Fessler LI 1966 Electron microscopic visualization of the polysaccharide hyaluronic acid. Proc Natl Acad Sci USA 56:141–147

Fraser JRE, Laurent TC, Pertoft H, Baxter E 1981 Plasma clearance, tissue distribution and metabolism of hyaluronic acid injected intravenously in the rabbit. Biochem J 200:415–424

Hardingham TE, Muir H 1972 The specific interaction of hyaluronic acid with cartilage proteoglycans. Biochim Biophys Acta 279:401–405

Kendall FE, Heidelberger M, Dawson MH 1937 A serologically inactive polysaccharide elaborated by mucoid strains of Group A hemolytic streptococcus. J Biol Chem 118:61–69

Laurent TC 1970 Structure of hyaluronic acid. In: Balazs EA (ed) Chemistry and molecular biology of the intercellular matrix. Academic Press, London, vol 2:703–732

Meyer K 1947 The biological significance of hyaluronic acid and hyaluronidase. Physiol Rev 27:335–359

Meyer K, Palmer JW 1934 The polysaccharide of the vitreous humor. J Biol Chem 107:629–634

Mian N 1986 Analysis of cell-growth-phase-related variations in hyaluronate synthase activity of isolated plasma-membrane fractions of cultured human skin fibroblasts. Biochem J 237:333–342

Prehm P 1983 Synthesis of hyaluronate in differentiated teratocarcinoma cells. Biochem J 211:181–198

Rapport MM, Weissman B, Linker A, Meyer K 1951 Isolation of a crystalline disaccharide, hyalobiuronic acid, from hyaluronic acid. Nature (Lond) 168:996–997

Tengblad A 1980 Quantitative analysis of hyaluronate in nanogram amounts. Biochem J 185:101–105

Secondary structures in hyaluronan solutions: chemical and biological implications

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Abstract. Hyaluronan behaves as an unusually stiff polymer in solution. Periodate oxidation of the hexuronic acid moiety is severely hindered, as is that of chondroitin 4-sulphates and 6-sulphates. On the basis of X-ray and computer studies a secondary structure was proposed which accounted for the known facts. NMR data obtained subsequently in dimethyl sulphoxide were completely compatible with these proposals. Results obtained in H_2O suggested that the acetamido group could not be oriented in aqueous solution as it was in dimethyl sulphoxide solution. There is strong evidence that an H_2O bridge between the acetamido and carboxylate groups is involved in the secondary structure in H_2O. It is suggested that conversion of one structure (stable in the absence of H_2O) to the other (in aqueous solution) might occur during biosynthesis, as part of the driving force that results in shedding from the H_2O-poor environment of the membrane. The hydrogen-bonded secondary structures show quite large arrays of contiguous CH groups, giving a hydrophobic character to some parts of the polymer, which might be significant in self-association and for interactions with membranes.

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Hyaluronan (HA) is a molecule of paradoxes and contrasts. On the one hand, it is a homopolymer composed of simple disaccharide (hyalobiuronic acid) units (Fig. 1), endlessly repeated, present in some tissues (such as the cockscomb and vitreous humour) in large amounts, and doing very simple mechanical jobs as a space filler, or as a lubricant (in synovial joints). This is not the stuff of biological high drama. On the other hand, HA takes part in cell surface phenomena of great specificity, at very low dilution (see e.g. Laurent & Fraser 1986). It is probably a molecule of great evolutionary antiquity, since it is made by streptococci as well as by connective tissue cells of all animals. Some of the tricks it performs in the contemporary biosphere must therefore be relatively

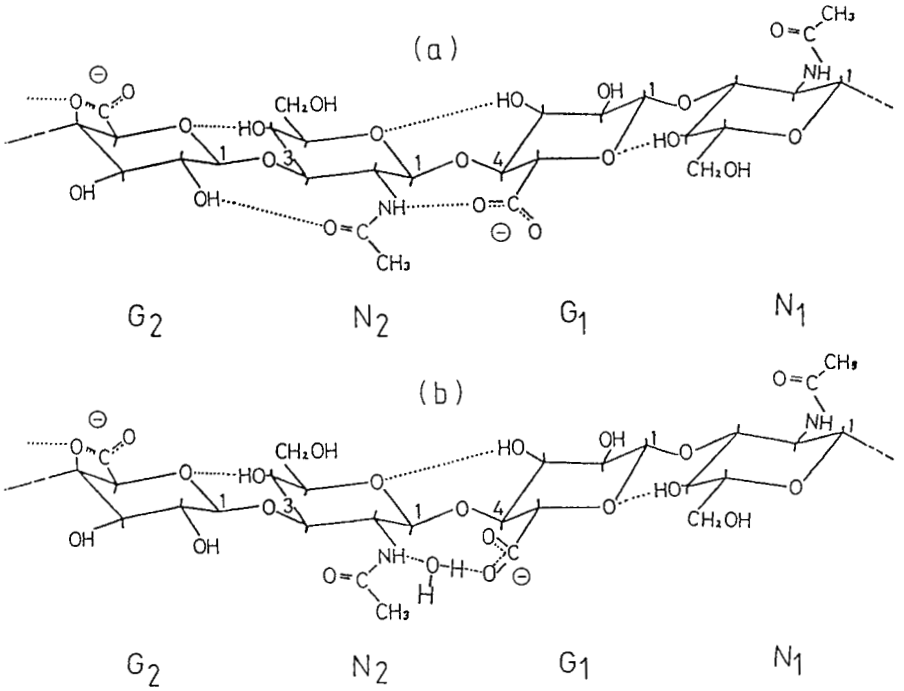

FIG. 1. Secondary structure of hyaluronan in (a) dimethyl sulphoxide and (b) dimethyl sulphoxide containing water. (From Heatley & Scott 1988.) The right-hand residue (N_1) is the reducing end in oligosaccharides produced from HA by hyaluronanase. Structures 1a and 1b are tetrasaccharides composed of two repeats of the fundamental disaccharide (hyalobiuronic acid) unit of HA.

new tricks, learnt during its evolutionary history. The simplicity of HA masks some important subtleties.

The structure of HA may contain atypical, quantitatively minor details that might be responsible for some of its specific biological properties. Claims that small amounts of amino acids and unusual sugars might be integral parts of the HA molecule have been made, but mainly discounted. Minor intrachain structures in other glycosaminoglycans, particularly the heparans, are functionally important, but these structures are made by sulphation, epimerization and deacetylation after the glycan chains are formed. HA is unique among the connective tissue glycosaminoglycuronans in that it does not undergo post-polymerization modifications—perhaps not unexpectedly, since it is extruded from the cell in the process of biosynthesis (Prehm 1984).

This paper will make the point that secondary structures, *in solution*, may be the bases of some of the properties of HA which would not be predicted

from its primary structure. Configurations of HA in the solid state have been investigated and reviewed (Atkins 1985, Arnott et al 1983) but will not be discussed here, except where the results are relevant to those obtained in solution.

Indications that the situation was not simple came with the observations that HA behaved in aqueous solution as an unusually stiff polymer (Laurent 1970) and that, in spite of the presence of a glycol group in the glucuronic acid moiety (Fig. 1), it was not oxidizable by periodate.

Periodate oxidation of hyaluronan and its analogues

The resistance of HA to periodate oxidation might have been due to the Donnan exclusion of IO_4^- anions from the polyanionic domain of HA. Even when this important factor was taken into account, consumption of IO_4^- by HA was still extremely slow, but nevertheless specific to the glucuronate glycol group (Scott & Harbinson 1969). In this respect, HA closely resembled chondroitin 4- and 6-sulphates but, remarkably, not the isomeric dermatan sulphate, which was oxidized very much faster (Scott 1968). We suggested that the glucuronate of HA and the chondroitin sulphates contained a trans-annular hydrogen bond, which might hinder oxidation, that was not sterically possible in the iduronate of dermatan sulphate. Later, detailed comparisons, with more precise kinetic measurements, between appropriate monomers and polymers demonstrated that it was not glucuronate *per se*, but the polymer environment around it, that inhibited oxidation (Scott & Tigwell 1978). By a process of elimination, the groups in HA that determined the IO_4^--resistant configuration were found to include carboxylate and acetamido groups (Scott & Tigwell 1978). X-ray studies on HA fibres (Atkins et al 1972) suggested that these groups were close together, and space-filling models based on this work disclosed that a hydrogen bond ($>NH \rightarrow O=C-O$) could form between them, plus an additional hydrogen bond from the glucuronate C2OH to the O=C of the acetamido group (Scott & Tigwell 1978) (Figs. 1 and 4). This structure, repeated throughout the polymer, could account for the stiffness and resistance to IO_4^- oxidation.

A computer simulation gave greater precision to the model, and showed two more hydrogen bonds, between ring oxygens and hydroxyl groups (Atkins et al 1980).

NMR studies

To obtain unequivocal information about these structures, Frank Heatley and I did a series of nuclear magnetic resonance (NMR) studies starting from simple monosaccharides and working up to the complete polymer. Benito Casu pointed out the advantages of working in deuterated dimethyl sulphoxide, in which protons do not exchange with the aprotic solvent, in principle allowing all

structural hydrogen atoms to be 'seen' in the spectrum (Heatley et al 1979). A key compound, the repeating disaccharide unit of HA (hyalobiuronic acid), was chemically synthesized by Drs Roger Jeanloz and E. Walker-Nasir, and the fundamental NMR spectrum of HA was thus established (Heatley et al 1982). Comparison of the spectrum of hyalobiuronic acid with that of the tetra-, hexa- and octasaccharides prepared from HA by hyaluronanase digestion revealed a new NH resonance in the higher oligosaccharides, well down field (9.2 p.p.m.) of those at about 7.8 p.p.m. in *N*-acetylglucosamine, hyalobiuronic acid, and other simple *N*-acetyl compounds (Fig. 2). The new resonance was in addition to a 'normal' one at 7.8 p.p.m. It increased in amplitude as the oligosaccharide

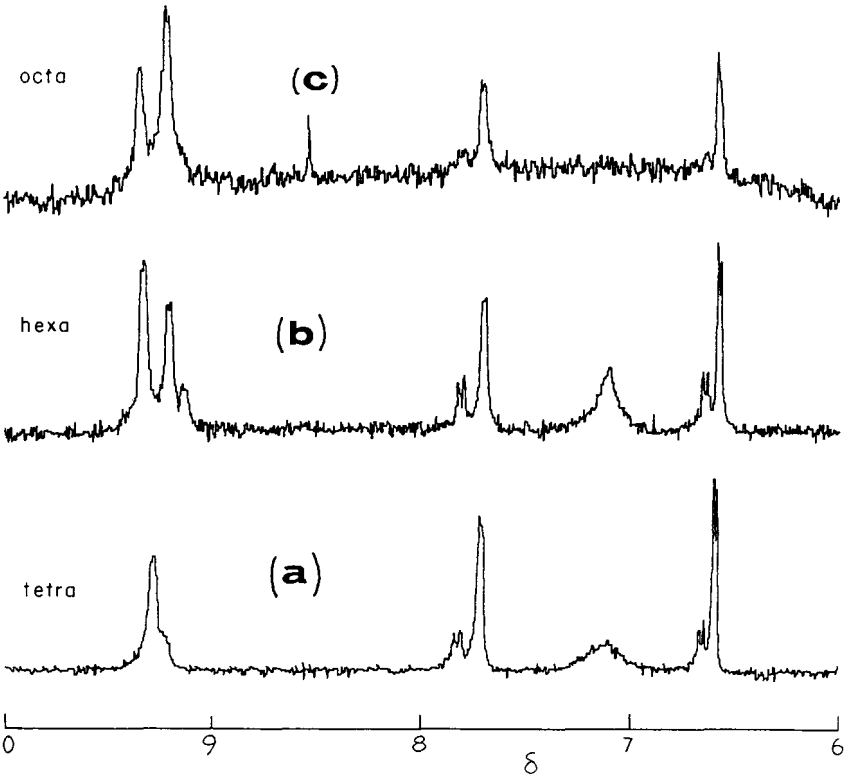

FIG. 2. Part of the spectra at 300 MHz in [$^2H_6$]dimethyl sulphoxide of the (a) tetra-, (b) hexa- and (c) octasaccharides from hyaluronan, showing the HN resonance region, approx. 9.2 and 7.8 p.p.m. The ratio of the integrated signals 9.2:7.8 increases in the proportion 1:2:3 as required by the structure in Fig. 1a. The signal at 9.2 is the only HN resonance visible in the spectrum of highly polymerized HA, and that at 7.8 is the only HN resonance in the spectrum of the disaccharide, or *N*-acetylglucosamine. (Taken from Scott et al 1984.)

series was ascended. The new resonance had properties characteristic of a hydrogen-bonded NH; that is, on deuteration, or with a change in temperature, this resonance differed from the 'normal' NH resonance (Scott et al 1984).

The increase in the ratios of the integrated intensities of the 'new' to the 'normal' resonance to 1:1, 2:1 and then 3:1 in the tetra-, hexa- and octasaccharides, respectively (Fig. 2), is as expected from the hydrogen-bonded acetamido→carboxylate structure (Fig. 1). In highly polymerized HA, only the downfield (9.2 p.p.m.) resonance was observed (Scott et al 1984). The 'normal' (7.8 p.p.m.) resonance is due to the non-hydrogen-bonded reducing terminal acetamido glucose (Fig. 1a), and the contributions of the hydrogen-bonded residues, towards the non-reducing end, increase linearly with the number of repeating units in the oligosaccharide. Precisely similar arguments established that G2C2OH, N1C4OH and G1C3OH were hydrogen bonded to the acetamido carbonyl oxygen and the ring oxygens, respectively. This proved the existence of the fully hydrogen-bonded structure (Fig. 1a) (Scott et al 1984), predicted by molecular models and computer calculations based on IO_4^- oxidation kinetics in aqueous solution.

Proof that this was the structure in aqueous solution was lacking, although studies of the acetamido methyl group of HA oligosaccharides in D_2O provided evidence for close approach of $-COO^-$ and acetamido groups in the higher homologues (Scott & Heatley 1979). (The labelling of the di-, tetra- and hexasaccharides in Fig. 4 of that paper was incorrect; they should have been labelled tetra-, hexa- and octasaccharides, respectively.)

The protons of all except one of the groups involved in the hydrogen bonds could not be 'seen' in spectra obtained from H_2O solutions, because of rapid exchange with H_2O protons. The exception was the NH proton, which Cowman (1985) examined. Neither the chemical shifts nor the coupling constants of the putative hydrogen-bonded protons differed significantly from those of the non-hydrogen-bonded NH protons, in the series of oligomers from two to four disaccharides, in contrast to what was found in dimethyl sulphoxide. The new coupling constant (>8 Hz) implied that the acetamido group was not oriented with the NH at 90° to the C2H of the glucose ring, and the upfield chemical shift suggested that it could not be directly hydrogen bonded to the $-COO^-$ group, as required by the structure in Fig. 1a.

We therefore re-examined models of that structure to see whether interactions of H_2O with the NH and $-COO^-$ groups, resulting in a *trans* NH:C2H conformation (as required by the coupling constant), could be included while allowing some or all of the rest of the structure (Fig. 1a) to persist, to account for the results obtained with periodate. Indeed, a very favourable H_2O bridge, $-NH...OH...^-O_2C$ was possible, in which the acetamido NH orientation was *trans* to C2H, with the rest of the structure essentially as in Fig. 1a (see Fig. 1b) (Heatley & Scott 1988). Since the NH therein interacts with an H_2O molecule,

as does the reducing terminal, N2NH, no difference in their chemical shifts would be expected.

Two additional lines of evidence strongly support the structure in Fig. 1b.

(1) Addition of H_2O to the dimethyl sulphoxide solution of the HA oligosaccharides results in an upfield movement of the NH resonance. Assuming that the observed chemical shift is an average of two chemical shifts, due respectively to the H_2O-free and the H_2O-binding form, the

$$>NH + H_2O \rightleftharpoons NH...OH_2$$

results were consistent with the binding of one water molecule to the NH group (Heatley & Scott 1988).

(2) Saturation transfer experiments, in which the H_2O frequency was strongly irradiated, showed that the N2NH behaved differently from the N1NH (the reducing terminal, not H_2O bridged) (Heatley & Scott 1988). It is relevant that X-ray diffraction studies showed a H_2O molecule in this position in HA fibres (Mitra et al 1983).

The structures in Fig. 1 show elements of cooperativity, in which the presence of a hydrogen bond in one position implies the existence of hydrogen bonds in other positions. There is evidence of reversible 'melting' on raising the temperature, as seen from changes in circular dichroism (J. E. Scott & P. M. Bayley, unpublished results quoted in Scott 1976) and from the Arrhenius plot of log k (the second-order reaction constant of IO_4^- oxidation of HA) against T^{-1}, but only at high temperatures (Scott & Tigwell 1978) (Fig. 3).

Changes in structure Fig. 1b may be possible, short of complete melting. The transition in the Arrhenius plot at about 37 °C may signify such a change. This transition had no parallel in the analogous case of chondroitin sulphate (Fig. 3). Thus, it may involve the hydrogen bond across the glucuronate→glucosamine glycosidic link, which is not present in chondroitin sulphate. An Arrhenius plot of log viscosity against T^{-1} showed no comparable transition (J. E. Scott, unpublished work), nor did the change in circular dichroism with temperature (J. E. Scott & P. M. Bayley, unpublished work), suggesting that neither the solvent-draining properties of the polymer nor the acetamido group was affected at the transition. The possible effect of configurational changes with temperature should be considered when specific interactions are investigated.

Biological significance of secondary structures in hyaluronan

Stiffness

The obvious consequence of the array of hydrogen bonds and water bridges, present throughout the hyaluronan molecule, is to reduce configurational

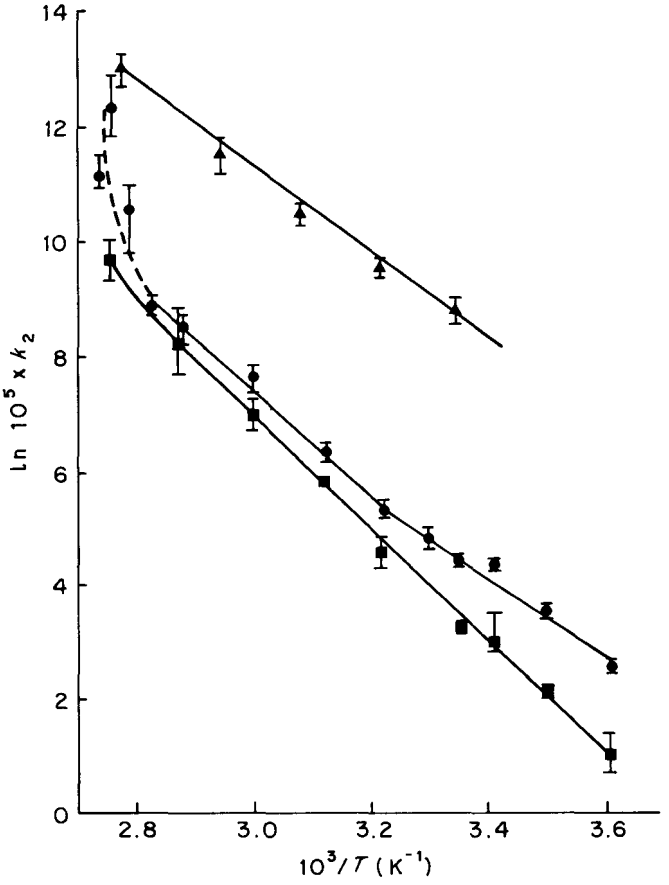

FIG. 3. Arrhenius plot of the reaction of periodate with glycol-containing compounds, methyl 4-*O*-methyl-α-D-glucopyranoside (▲), chondroitin 6-sulphate (■) and hyaluronate (●). k_2, second-order rate constant; T, absolute temperature. (Taken from Scott & Tigwell 1978.) The points are arithmetical averages with spreads of values indicated by vertical lines. The transition in the HA plot, which is not present in the other plots, corresponds to a temperature of about 37 °C.

flexibility—that is, to increase 'stiffness'. This would be significant in the space-filling roles of HA, exploited in the vitreous humour, Wharton's jelly of the umbilical cord, and the cockscomb. Probably all tissues in which HA is present in considerable amounts (such as young skin and tendon) make use of it in keeping fibrils apart, thereby forming channels of gel through which water-soluble molecules can diffuse.

Diminished capacity to interact with other molecules

It is necessary that the aqueous channels in the pericellular matrix should contain polymers which offer few opportunities for strong interactions with the generality of molecules which must pass through them, to and from cells. A large proportion of the polar groups in HA are involved in intramolecular interactions, which thus limits the capacity to interact intermolecularly.

As a corollary, the remaining non-hydrogen-bonded groups offer fewer opportunities for multipoint attachments, which must therefore be of higher specificity.

Hydrophobic bonding

A feature of HA (in the form shown in Fig. 1) that has not attracted attention previously is the presence of large clusters of contiguous CH groups, forming patches of a highly hydrophobic character, repeated at regular intervals on alternate sides of the molecule (Fig. 4). The significance of these patches, which span several sugar units, is not clear in functional terms, but interactions with membranes and hydrophobic proteins, such as the link protein, are obvious possibilities, as is self-association.

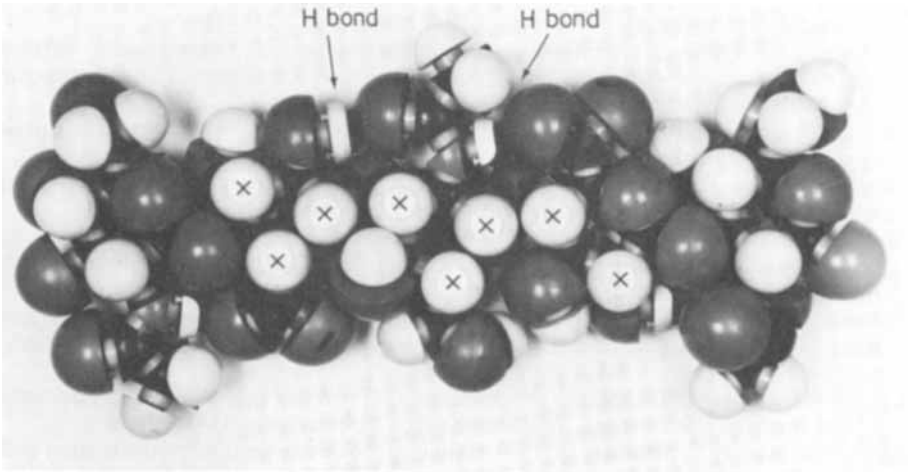

FIG. 4. Model of a hyaluronan oligosaccharide constructed from Courtauld space-filling units, based on the structures shown in Fig. 1. The acetamido hydrogen bonds indicated are those in Fig. 1a. The H atoms marked with a cross are part of a hydrophobic chain consisting of eight CH groups. The CH_2OH and methyl groups can be oriented in such a way that the hydrophobic character of this segment is enhanced. There is then a marked amphiphilic character to this segment, with the hydrophilic part being on the other side of the molecule.

Alternative configurations as a driving force in hyaluronan biosynthesis

The demonstration that there are at least two alternative configurations for HA, one stable in non-aqueous environments and the other stable in H_2O, has implications for the biosynthesis of HA, which appears to take place at or in a cell membrane (Prehm 1984). It is not clear whether H_2O is absent at the point of formation of the structure shown in Fig. 1a but, if so, the conversion of that structure to the structure in Fig. 1b in the extracellular aqueous environment could provide the energy for the extrusion of completed HA from its membranous origin.

Acknowledgement

My thanks are due to Dr F. Heatley for helpful discussion.

References

Arnott S, Rees DA, Morris ER 1983 Molecular biophysics of the extracellular matrix. Humana Press, Clifton, NJ

Atkins EDT 1985 Topics in structure and morphology. VCH, Weinheim, FRG

Atkins EDT, Phelps CF, Sheehan JK 1972 The conformation of the mucopolysaccharides. Hyaluronates. Biochem J 128:1255–1263

Atkins EDT, Meader D, Scott JE 1980 Model for hyaluronic acid incorporating four intramolecular hydrogen bonds. Int J Biol Macromol 2:318–319

Cowman MK 1985 Preparation and characterisation of enzymatically derived oligosaccharides and segments from glycosaminoglycans. In: Crescenzi V (ed) Industrial uses of polysaccharides. Gordon & Breach, New York, p 233–253

Heatley F, Scott JE 1988 A water molecule participates in the secondary structure of hyaluronan. Biochem J 254:489–493

Heatley F, Scott JE, Casu B 1979 ^1H NMR spectra of glycosaminoglycan monomers and dimers in methyl sulphoxide and water. Carbohydr Res 72:13–23

Heatley F, Scott JE, Jeanloz RW, Walker-Nasir E 1982 Secondary structure in glycosaminoglycuronans: N.M.R. spectra in dimethyl sulphoxide of disaccharides related to hyaluronic acid and chondroitin sulphate. Carbohydr Res 99:1–11

Laurent TC 1970 Structure of hyaluronic acid. In: Balazs EA (ed) Chemistry and molecular biology of the intercellular matrix. Academic Press, London & New York, p 703–732

Laurent TC, Fraser JRE 1986 The properties and turnover of hyaluronan. In: Functions of the proteoglycans. Wiley, Chichester (Ciba Found Symp 124) p 9–29

Mitra AK, Arnott S, Sheehan JK 1983 Hyaluronic acid: molecular conformation and interactions in the tetragonal form of the potassium salt containing extended chains. J Mol Biol 169:813–827

Prehm P 1984 Hyaluronate is synthesized at plasma membranes. Biochem J 220:597–600

Scott JE 1968 Periodate oxidation, pKa and conformation of hexuronic acids in polyuronides and acid mucopolysaccharides. Biochim Biophys Acta 170:471–473

Scott JE 1976 Periodate and biopolymers: new aspects of a familiar reagent. In: Robert AM, Robert L (eds) Burkitt lymphoma, hemostasis and intercellular matrix. Karger, Basel, p 176–191

Scott JE, Harbinson RJ 1969 Periodate oxidation of acid polysaccharides. II. Rates of oxidation of uronic acids in polyuronides and acid mucopolysaccharides. Histochemie 19:155–161

Scott JE, Tigwell MJ 1978 Periodate oxidation and the shapes of glycosaminoglycuronans in solution. Biochem J 173:103–114

Scott JE, Heatley F 1979 ^{1}H nuclear-magnetic-resonance spectra of the methyl group of the acetamido moiety and the structure of acid glycosaminoglycans in solution. Biochem J 181:445–449

Scott JE, Heatley F, Hull WE 1984 Secondary structure of hyaluronate in solution. A ^{1}H-n.m.r. investigation at 300 and 500 MHz in [$^{2}H_6$]dimethyl sulphoxide solution. Biochem J 220:197–205

DISCUSSION

Weigel: In your NMR spectra of the various hyaluronan oligosaccharides in deuterated DMSO I noticed a signal at about 7.1 p.p.m. with the hexasaccharide which was missing in the octasaccharide spectrum (Fig. 2).

Scott: That is due to the end group *external* GC4 hydroxyl which, when it is internal, i.e. G1C4, is hydrogen bonded to a ring oxygen. It has a very broad 'hump' and its resonance behaves differently in the spectra of different compounds (see e.g. Heatley et al 1979, Scott et al 1984). It may be hydrogen bonded to C6 carboxylate. This is the only hydroxyl in the hyaluronan structure which behaves inconsistently in NMR.

Weigel: It seems to undergo a considerable transition with the addition of the extra disaccharide.

Scott: In the glucuronate monomer, that hydroxyl resonance undergoes transitions with small changes in pH, and concentration (Heatley et al 1979). Possibly it is sensitive to the presence of slight amounts of impurity, such as copper.

Torvard Laurent: You mentioned a break in the HA melting curve at around 37 °C, which sounds biologically interesting. Can you speculate on the kind of change in conformation that causes the break?

Scott: Since we don't see that break with chondroitin sulphate (see Fig. 3), it may relate to the NC4 hydroxyl. The glucosamine C4 hydroxyl would be able to hydrogen bond to the uronate ring oxygen. The galactosamine C4OH could not. Perhaps the hydrogen bond from glucosamine C4 to the glucuronate ring oxygen is being slightly rearranged in space. I don't think it breaks. Perhaps there are alternative angles which it could make, with little difference to the rest of the structure.

Warren Knudson: Does hyaluronan in water also have a hydrophobic patch, as it does in DMSO?

Scott: The only difference detected between the water form and the DMSO (or water-poor) form is the change at the acetamido group; the hydrophobic patch will be unaffected by that change.

Engel: How far do your results agree with the view of Torvard Laurent (1970) and others that hyaluronate is a random coil in water? This is based on its physical properties measured in solution (reviewed by Phelps 1984). What can one conclude from the NMR data about possible double helical structures that have been demonstrated by fibre diffraction (reviewed by Arnott & Mitra 1984)? Does the polymer in solution contain regions of order interrupted by disorder, or does it assume a coiled conformation with some residual structure?

Scott: The random coil polymer in water is stiff. The persistence length of the polymer is longer than if it were like alginate, where there is little stiffness at the repeating unit level. Bob Cleland (1977) showed the persistence length of hyaluronan to be quite long. HA is a large molecule with plenty of room for twists and turns, but the intrinsic springiness of the backbone is much increased by the hydrogen bonds that I discussed. I am not suggesting that there are regions of order, except at the level of the repeating unit. At that level there is a 'stiffness' which translates into the physical behaviour of the entire random coil. I cannot suggest any order beyond the three- or four-saccharide level, but that does make for a greater persistence length.

Weigel: Pursuing the observation about the insertion of a water molecule in an existing intramolecular hydrogen bond, have you done any modelling with the basic unit of four hydrogen bonds? You might expect that a protein could come close enough to insert amino groups or hydroxyl groups, and could do the same thing in all four hydrogen bonds in the right geometry. This could therefore generate a specific binding interaction in an otherwise mundane-looking region of the polymer. There might be some distortion, but the energetics would be very reasonable and, if there were several sites of interaction like that between hyaluronan and a protein, one could obtain the kind of binding affinities that have been found for many hyaluronan-binding proteins.

Scott: The structure does present these potentialities. I sorted out regions which I think would interact—for example, through hydrophobic bonding. The hydroxyl groups which are linked to the ring oxygen of the neighbouring sugar probably can be ruled out of any intermolecular interactions. The acetamido and carboxylate groups, and the GC2 hydroxyl group in the water bridge system, have possibilities which were not present in the water-poor structure that might be present in a cell membrane.

Hardingham: Do the structures that you are suggesting via hydrogen bonding lead to a useful understanding of the high and low pH changes in the properties of hyaluronan that have been reported?

Scott: Yes. Benito Casu pointed this out (in unpublished discussion at the Biochemical Society Carbohydrate Group, Bedford, April 1979). In NaOH solution, hyaluronan becomes much less viscous. This is reversible, as David Swann showed (1970). Dr Casu thought our proposed structure with the

hydrogen bonds was compatible with the ^{13}C NMR spectra that Welti et al (1979) showed at that meeting.

Hardingham: Would the hydrogen-bonded structures predict the unusual transition at low pH to a more gel-like state?

Scott: I am not sure. I haven't seen any spectra that would help us there. With a change from neutral to acid pH there is the possibility of dimerization of HA, and of higher-order structures arising. Two papers describe circular dichroism (CD) studies of hyaluronate (Staskus & Johnson 1988a,b). In conditions of low pH, when ethanol was added, the CD changes were consistent with dimerization. These authors thought that HA in neutral conditions was 'unordered'. One way to interpret that statement is that they didn't think dimers were present. I don't believe that HA is unordered, in the sense that I discussed in my paper.

Hardingham: An antiparallel double helix of HA has been reported from a fibre crystal analysis (Sheehan et al 1977, Arnott et al 1983). Could that play a part in the solution properties?

Scott: We don't have evidence for a long length of chain, but, for the octasaccharide (the biggest we have looked at), we found no evidence from NMR for the association in DMSO of one HA molecule with another. The geometries, the chemical shifts and the behaviour with increased temperature were inconsistent with dimerization, so double helices would not be likely, up to about ten sugar units. John Sheehan claimed that the sodium salt of hyaluronan did dimerize; but Månsson et al (1985), disagreeing, said that sodium hyaluronate in aqueous solution was essentially a single chain. Apart from in conditions of low pH and the presence of ethanol, I don't know of convincing evidence for aggregation in dilute solution. In highly concentrated HA solutions, we wouldn't be able to look at the NMR spectrum with good resolution.

Torvard Laurent: There is conflicting evidence from rheological data on interactions between HA chains. However, Drs Morris and Rees and their collaborators (Morris et al 1980) find that at high concentrations there seems to be some kind of interaction which is not only entanglement. To follow up Tim Hardingham's point, would your model accommodate a double helix, so that two strands are wound round each other and are held together by the hydrophobic surfaces on each chain, and could this add to the interaction at high concentrations?

Scott: One assumes that the hydrophobic regions would be favoured points at which mutual interactions would be expected. The hydrophilic part of the molecule should not be as keen to form associations with other hydrophilic parts. I have not had access to a computer programme that would enable me to model this.

Toole: Continuing on this theme of the hydrophobic patches, I have sometimes been asked, when talking about hyaluronic acid–cell receptor interactions, about the improbability of a highly negatively charged molecule

like hyaluronic acid being able to approach the highly negatively charged surface of the cell closely enough to interact with a plasma membrane-bound protein of a not particularly large size. I don't have a good feeling for the molecular distances involved here, but would these hydrophobic patches help in this process of interaction, or is the whole idea not valid because the negative charge repulsion would be a problem anyway?

Scott: The hydrophobic patches would be obvious places for hyaluronan to attach to a fatty membrane. It might even be that the HA molecule could orient with one side pointing out to the aqueous medium and the other pointing inwards. So I can accept the hydrophobic interaction as favouring HA–membrane interactions. The charge–charge repulsion is difficult to speculate

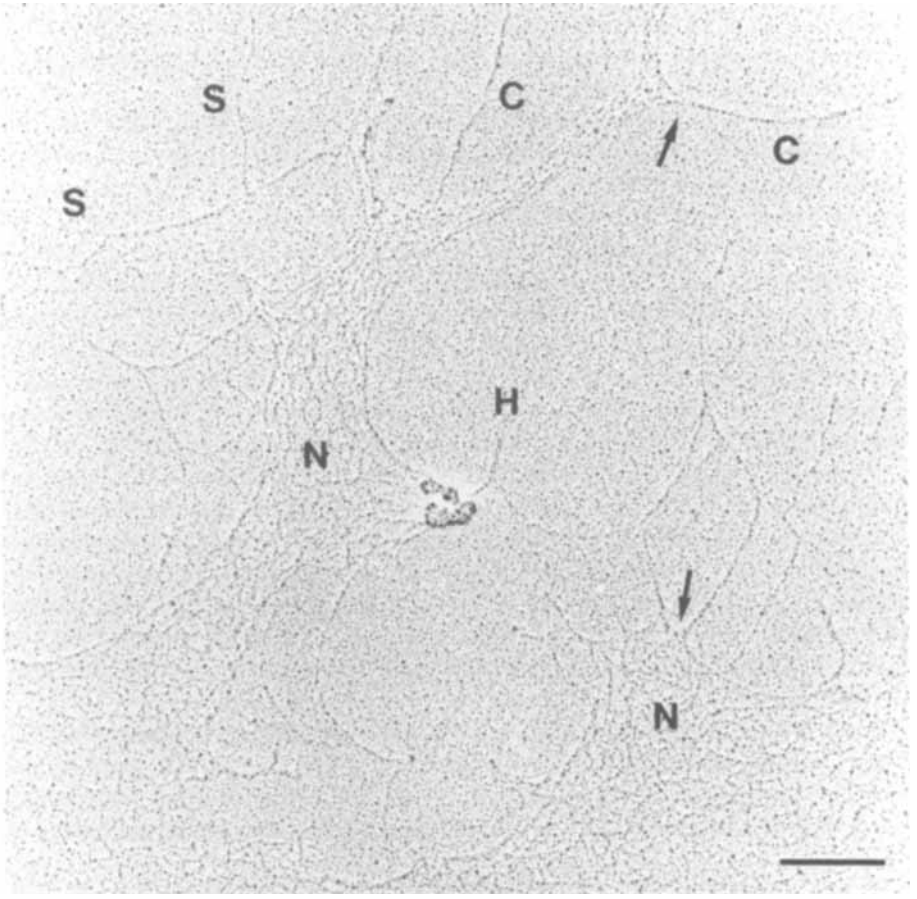

FIG. 1 *(Engel)* Electron micrograph of high molecular mass hyaluronate isolated from umbilical cord. See text for explanation of abbreviations. Bar, 100 nm.