Carbohydrate Research, 99 (1982) 1–11
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

SECONDARY STRUCTURE IN GLYCOSAMINOGLYCURONANS: N.M.R. SPECTRA IN DIMETHYL SULPHOXIDE OF DISACCHARIDES RELATED TO HYALURONIC ACID AND CHONDROITIN SULPHATE

FRANK HEATLEY,

Department of Chemistry, University of Manchester, Oxford Road, Manchester M13 9P (Great Britain)

JOHN E. SCOTT*,

Department of Medical Biochemistry, University of Manchester, Oxford Road, Manchester M13 9PT (Great Britain)

ROGER W. JEANLOZ, AND EVELYNE WALKER-NASIR**

Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Med. ine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114 (U S.A.)

(Received May 1st, 1981; accepted for publication, May 27th, 1981)

ABSTRACT

^{1}H-N.m.r. spectra for solutions in dimethyl sulphoxide-d_6 of disaccharides related to hyaluronate and chondroitin sulphate are compared with those of their methylated derivatives. All resonances, including those of HO and HN groups, have been assigned. The temperature and concentration dependences suggest that HO-4 of the hexosamine residue in hyalobiouronate (but not that in chondrosinate) is hydrogen-bonded to O-5 of the uronic acid residue. The resonance of HO-2 of the uronate residue of chondrosinate also shows anomalies that may arise from intra-residue hydrogen-bonding. These findings confirm the existence of some features previously suggested to be present in glycosaminoglycuronan polymers. The resonance of HO-4 of the uronate residue in the disaccharides and in sodium (methyl α-D-glucopyranosid)uronate behaves as though there was a hydrogen bond between the carboxylate group and HO-4.

INTRODUCTION

The acetamido group, like the peptide bond, can be simultaneously a donor and acceptor in a hydrogen-bonded system. In hyaluronate and chondroitin sulphate, carboxylate and hydroxyl groups are available to interact with the acetamido group[1]. Glycosaminoglycuronans thus occupy a position between the polypeptides, in which

*To whom reprint requests should be addressed.

**Amino Sugars 128 This is publication No. 874 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This work was supported by a research grant from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM-03564), National Institutes of Health, U.S Public Health Service.

extended, hydrogen-bonded sequences are possible, and the homoglycans, such as cellulose, in which intramolecular hydrogen-bonds occur, but only between singly bonded groups.

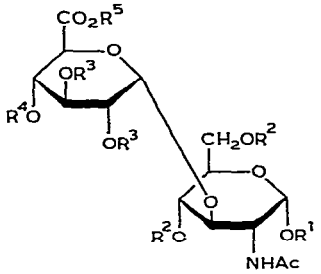
The role of the acetamido group in promoting long-range order in some glycosaminoglycuronans was first proposed to account for anomalously low rates of periodate oxidation[1]. Molecular model building with Courtauld space-filling units, constructed according to available X-ray data, supported the hypothesis[1]. Recently, computer calculations have reinforced the possibility[2]. Variations of the ^{1}H-n.m.r. chemical shift of the acetamido methyl group of glycosaminoglycuronans dissolved in D_2O were interpreted on the basis that the proposed hydrogen-bonded array was present in some polymers, but not in all[3].

There are important physiological implications. Polymers which possess acetamido-centred, hydrogen-bonded arrays should be stiffened, and the relevant glycosaminoglycuronans, hyaluronate and chondroitin 4- and 6-sulphates, do indeed behave in solution as very stiff chains[1]. Non-stiffened glycosaminoglycans should have a greater capacity for intermolecular interactions, since more of their hydrogen-bonding groups are free, and they can take up a greater range of polymer conformations. Molecular models suggest that dermatan sulphate and heparan sulphate are non-stiffened glycosaminoglycuronans, and they are seen to interact specifically with collagen fibres[4] and cell membranes.

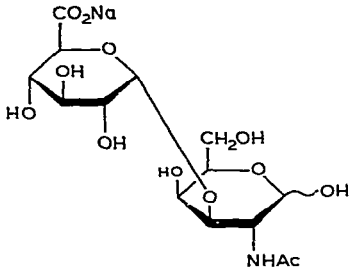
Although very suggestive, the evidence for the hydrogen-bonded structures is not direct. In principle, the n.m.r. spectrum should contain the necessary information, but only if the polymer is dissolved in a solvent in which the relevant protons do not exchange rapidly, thus rendering them unobservable. Because dimethyl sulphoxide was used successfully for this purpose in investigations of saccharides[5], we have applied it in the glycosaminoglycan field. The spectra of the constituent monomers (hexuronates, *N*-acetylhexosamines) have been interpreted[6], and we now present data at the next level of complexity, the disaccharides, in which inter-residue interactions may become visible.

Glycosaminoglycan repeating-units are disaccharides of considerable chemical complexity, hitherto accessible only by enzymic or acid hydrolysis of the natural polymer. Appropriate enzymes are frequently not available, and products of acid hydrolysis require careful purification and modification (*e.g.*, by re-acetylation) before their relevance can be established. The best products from either procedure still pose difficulties in n.m.r. spectroscopy, since, in their reducing form, two different sets of signals, corresponding to the α and β anomers, are observed. Instead of $\sim$30 protons, close to 60 require assignment. In this paper, we make use of crystalline disaccharide glycosides, obtained either by synthesis (**2**, **4**) or by methanolysis of hyaluronic acid (**4**) or methylated hyaluronic acid (**5**). The n.m.r. spectra of these compounds were greatly simplified, and the chemical structures are not in doubt.

All of the protons have been assigned for disaccharides from the hyaluronate series, and for sodium *N*-acetylchondrosinate (**6**) prepared from chondroitin sulphate. Clear evidence for inter-residue hydrogen-bonding has been obtained, providing



1 R^1 = Allyl, R^2 = R^3 = R^4 = Ac, R^5 = Me
2 R^1 = Allyl, R^2 = R^3 = R^4 = R^5 = H
3 R^1 = R^5 = Me, R^2 = R^3 = R^4 = Ac
4 R^1 = Me, R^2 = R^3 = R^4 = R^5 = H
5 R^1 = R^2 = R^3 = Me R^4 = R^5 = H



6

direct support for some of the features in the previously proposed, secondary structures of the glycosaminoglycuronans

The crystalline allyl glycoside **2** was obtained by alkaline hydrolysis of allyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside[7] (**1**), the crystalline methyl glycoside **4** had been obtained by methanolysis of hyaluronic acid[8] or was obtained by alkaline hydrolysis of methyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside[9] (**3**), and crystalline methyl 2-acetamido-2-deoxy-3-*O*-(2,3-di-*O*-methyl-β-D-glucopyranosyluronic acid)-4,6-di-*O*-methyl-α-D-glucopyranoside (**5**) had been obtained by methanolysis of methylated hyaluronic acid[10].

EXPERIMENTAL

Methods. — ^{1}H-N.m.r. spectra were recorded at 300 MHz with a Varian SC-300 instrument. Chemical shifts were measured using the residual proton signal of the solvent as reference, assuming its chemical shift to be δ 2.5.

Materials. — 2-Acetamido-2-deoxy-D-glucose was a commercial sample. *N*-Acetylchondrosinate (**6**) was a gift from Dr. A. Olavesen. Methyl (methyl α-D-glucopyranosid)uronate (**9**) was prepared by Dr. B. Samuelsson (Stockholm University) and we are grateful to Professor B. Lindberg for a gift of this material. It was converted into the sodium salt by hydrolysis with aqueous Na_2CO_3 (50% excess) overnight at room temperature. The solution was neutralised with HCl, and freeze-dried. The disaccharides were titrated to pH 6.5, with 10mM sodium hydroxide. The crystalline compounds required ~90% of the theoretical amount of sodium hydroxide, whereas *N*-acetylchondrosinate (**6**) required ~40% of the theoretical amount of sodium hydroxide. The freeze-dried compounds dissolved easily in Me_2SO-d_6.

*Allyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucopyranoside* (**2**). — To a solution of allyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(methyl

2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside[7] (**1**, 75 mg) in anhydrous methanol (1 mL) was added 0.24M barium methoxide (4 mL) at 0°. The solution was kept at 0–4° overnight, and t.l.c. (65.25:4, chloroform–methanol–water) then showed total conversion of the starting material into a product having R_F 0.05. Water (5 mL) was added at 0°, and the solution, at room temperature, was passed through a column of Amberlite IR-120 (H^+) resin, which was eluted with 1·1 methanol–water (15 mL) and then water (5 mL). The combined eluate and washings were evaporated, and the residue was recrystallised from methanol–acetone, to give clusters of needles (40 mg, 81%), m.p. 195–198° (dec.), $[\alpha]_D^{20}$ +54° (*c* 0.14, methanol); t.l.c. (7.3 1-propanol–water): R_F 0.45; ν_{max}^{KBr} 3510, 3450, 3400 (OH), 3315 (NH), 1735 (CO_2H), 1665 (Amide I), and 1540 cm^{-1} (Amide II).

Anal. Calc. for $C_{17}H_{27}NO_{12} \cdot 0.5\ H_2O$: C, 45.74; H, 6.32; N, 3.14; O, 44.80. Found: C, 45.51; H, 6.65; N, 3.15; O, 44.77.

Methyl 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-α-D-glucopyranoside (**4**). — A solution of methyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-glucopyranoside[9] (**3**, 20 mg) was treated with 0.24M barium methoxide (1 mL) at 0°, as described for **2**, to give, after crystallisation from methanol–acetone–ether, rosettes of needles (11 mg, 83%), m.p. 202–205° (dec.); lit.[8] m.p 207–210° (dec.); on admixture with the original sample[8], the melting point was not depressed, and both samples showed identical melting patterns; $[\alpha]_D^{20}$ +33° (*c* 0.06, methanol); lit.[8] $[\alpha]_D^{24}$ +32° (methanol); t.l.c. (7:3, 1-propanol–water): R_F 0.35; ν_{max}^{KBr} 3510, 3450, 3400 (OH), 3315 (NH), 1735 (CO_2H), 1650 (Amide I), and 1545 cm^{-1} (Amide II). This spectrum was similar to that of the original sample[8], except that the Amide-I band of the original sample, which crystallised with one molecule of water, appeared at 1630 cm^{-1}, an expected downward-shift of the C=O stretching, absorption frequency due to hydrogen bonding.

RESULTS

The crystalline disaccharides gave well-resolved, largely first-order spectra. The chemical shifts listed in Tables I and II were established by spin-decoupling, based on the unequivocal assignment of the lowest-field resonance at δ 7.82 to HN-2. The small differences between the chemical shifts of particular ring protons in the glycosides **2**, **4**, and **5** are understandable in terms of the difference in chemical structure. The coupling constants for the allyl glycoside **2** (Table I) were obtained from a first-order analysis. Values for $J_{3,4}$ and $J_{4,5}$ for the amino sugar residue are approximate, because these protons resonate at similar frequencies, and the spectrum shows significant second-order coupling effects. The coupling constants of the methyl glycosides **4** and **5** are essentially identical to those of **2**, and are therefore not reported separately. The values of <2 Hz assigned to $J_{H\text{-}4',HO\text{-}4'}$ is based on the observation that the HO-4′ resonance in **2** is a singlet with a line-width of 3 Hz.

A spectrum of sodium *N*-acetylchondrosinate (**6**) has been briefly described[6].

TABLE I

CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR A SOLUTION[a] OF THE SODIUM SALT OF ALLYL 2-ACETAMIDO-2-DEOXY-3-*O*-(β-D-GLUCOPYRANOSYLURONIC ACID)-α-D-GLUCOPYRANOSIDE (2) IN Me_2SO-d_6 AT 22°

Chemical shifts (δ)				*Coupling constants (Hz)*			
Amino sugar residue							
H-1	4 78			$J_{1,2}$	3.2		
H-2	3.76	HN-2	7.82	$J_{2,3}$	10 5	$J_{H-2,HN-2}$	7.3
H-3	3.65			$J_{3,4}$	~9		
H-4	3.26	HO-4	5.57	$J_{4,5}$	~9	$J_{H-4,HO-4}$	1.7
H-5	3.41						
H-6	3.65, 3.52	HO-6	4 63			$J_{H-6,HO-6}$	5.6
CH_3CO	1.85						
Uronic acid residue							
H-1′	4.35			$J_{1',2'}$	7.9		
H-2′	3.00	HO-2′	4.70	$J_{2',3'}$	8	$J_{H-2,HO-2}$	4.0
H-3′	3.12	HO-3′	4.95	$J_{3',4}$	8 5	$J_{H-3',HO-3'}$	4 5
H-4′	3.09	HO-4′	7.01	$J_{4',5'}$	9	$J_{H-4,HO-4'}$	<2[b]
H-5′	3 25						
Allyl group							
Allylic protons	3 93, 4.12						
Olefinic protons	5.17, 5.34, 5.90						

[a] ~1% w/w. [b] See text.

TABLE II

CHEMICAL SHIFTS FOR SOLUTIONS[a] OF THE SODIUM SALTS OF METHYL 2-ACETAMIDO-2-DEOXY-3-*O*-(β-D-GLUCOPYRANOSYLURONIC ACID)-α-D-GLUCOPYRANOSIDE (4) AND METHYL 2-ACETAMIDO-2-DEOXY-3-*O*-(2,3-DI-*O*-METHYL-β-D-GLUCOPYRANOSYLURONIC ACID)-4,6-DI-*O*-METHYL-α-D-GLUCOPYRANOSIDE (5) IN Me_2SO-d_6 AT 22°

Compound 4				*Compound* 5			
Amino sugar residue							
H-1	4 64			H-1	4.46		
H-2	3.75	HN-2	7.82	H-2	3.94	HN-2	8.01
H-3	3 64			H-3	3.94		
H-4	3.3	HO-4	5.58	H-4	3.08		
H-5	3 4			H-5	3.64		
H-6	3.64, 3 51	HO-6	4 64	H-6	3 51		
CH_3CO	1.84			CH_3CO	1.82		
CH_3O	3.26			CH_3O	3.27, 3 30, 3.46		
Uronic acid residue							
H-1′	4.34			H-1′	4.64		
H-2′	3.02	HO-2′	4.70	H-2′	2 69		
H-3′	3.12	HO-3′	4.96	H-3′	2.90		
H-4′	3.12	HO-4′	6 98	H-4′	~3 24	HO-4′	7.18
H-5′	~3 5			H-5′	~3 24		

[a] ~1% w/w.

TABLE III

CHEMICAL SHIFTS AND SELECTED COUPLING CONSTANTS FOR A SOLUTION[a] OF SODIUM *N*-ACETYLCHONDROSINATE (6) IN Me_2SO-d_6 AT 22°

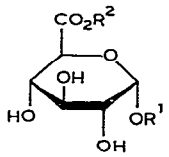
Chemical shifts (δ)				*Coupling constants (Hz)*			
I α-D Anomer							
Amino sugar residue							
H-1	5.05	HO-1	6.45	$J_{1,2}$	~2.5	$J_{H-1,HO-1}$	3.8
H-2	4.04	HN-2	7 69			$J_{H-2,HN-2}$	7.5
H-3	~3.5						
H-4	3.86?	HO-4	4.68?			$J_{H-4,HO-4}$	4.5
H-5	3.96?						
H-6	~3.5	HO-6	4 68			$J_{H-6,HO-6}$	~5.5
CH_3CO	1.82						
Uronic acid residue							
H-1′	4.39			$J_{1',2'}$	7.5		
H-2′	3.02	HO-2′	4.57			$J_{H-2',HO-2'}$	2.6
H-3′	~3.1	HO-3′	4.91			$J_{H-3',HO-3}$	~2.5
H-4′	~3.1	HO-4′	6 95				
H-5′	~3.5						
II β-D- Anomer							
Amino sugar residue							
H-1	4.45	HO-1	6 56	$J_{1,2}$	7.5	$J_{H-1,HO-1}$	6.2
H-2	3.71	HN-2	7.79			$J_{H-2,HN-2}$	7.8
H-3	~3.5						
H-4	3.82?	HO-4	4.61?			$J_{H-4,HO-4}$	~4 5
H-5	~3.5						
H-6	~3 5	HO-6	4 68				
CH_3CO	1.82						
Uronic acid residue							
H-1′	4.29			$J_{1',2'}$	7.5		
H-2′	3.02	HO-2′	4.42			$J_{H-2',HO-2'}$	2.3
H-3′	~3.1	HO-3′	4 91			$J_{H-3',HO-3'}$	~2.5
H-4′	~3.1	HO-4′	6 95				
H-5′	~3 5						

[a] ~1% w/w.

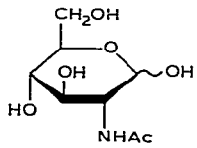
We subsequently found that the sample contained the equivalent of 40% of unneutralised acid, which led (see below) to the removal (by rapid exchange) of the HO-4′ signal from the spectrum and to shifts in several other peaks. On full neutralisation, proton exchange was suppressed and the HO-4′ resonance appeared at δ ~6.95. A full assignment of the spectrum was not possible previously, but both α and β anomers were present in appreciable amounts. The same was true of the fully neutralis-

ed sample, the α:β ratio being ~3:2. Further spin-decoupling experiments have established the almost complete assignments in Table III.

The spectra of methyl (methyl α-D-glucopyranosid)uronate (**9**) and the sodium salt **7** were compared, and details are given in Table IV. The line-width of the HO-4′ resonance in the sodium salt was ~25 Hz, due to exchange broadening, and the H-4′,HO-4′ coupling was not resolvable. If $J_{H\text{-}4',HO\text{-}4'}$ were large enough to produce resolvable splittings (≥3 Hz), comparable exchange-broadening effects would be expected in the H-4′ resonance. However, no such effects were observed, implying that $J_{H\text{-}4',HO\text{-}4'}$ is <2 Hz.



7 R^1 = H, R^2 = Na^+
8 R^1 = Me, R^2 = Na^+
9 R^1 = R^2 = Me



10

All individual HO and NH resonances were observed, indicating relatively slow proton-exchange with other sugar molecules or with the small amount of unavoidable water impurity. With three exceptions, the exchange was so slow that spin–spin coupling was clearly resolved. The exceptions were the HO-4′ signals for the sodium salt of the methyl glycoside **4**, sodium *N*-acetylchondrosinate (**6**), and sodium

TABLE IV

CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR SOLUTIONS[a] OF METHYL (METHYL α-D-GLUCOPYRANOSID)URONATE AND SODIUM (METHYL α-D-GLUCOPYRANOSID)URONATE

Chemical shifts (δ)				*Coupling constants (Hz)*			
Methyl ester							
H-1	4.61	MeO-1	3 29	$J_{1,2}$	3.5		
H-2	3.24	HO-2	4.88	$J_{2,3}$	[b]	$J_{H-2,HO-2}$	6
H-3	3.40	HO-3	4 96	$J_{3,4}$	[b]	$J_{H-3,HO-3}$	4
H-4	3.36	HO-4	5 28	$J_{4,5}$	9.5	$J_{H-4,HO-4}$	5 5
H-5	3.85	MeO-6	3.67				
Sodium salt							
H-1	4.63	MeO-1	3 27	$J_{1,2}$	3.5		
H-2	3.20	HO-2	4.79	$J_{2,3}$	[b]	$J_{H-2,HO-2}$	6
H-3	3.35	HO-3	4.81	$J_{3,4}$	9	$J_{H-3,HO-3}$	5
H-4	3 09	HO-4	6.74	$J_{4,5}$	10	$J_{H-4,HO-4}$	<2[c]
H-5	3.45						

[a] ~1% w/w. [b] Insufficiently resolved. [c] See text.

TABLE V

TEMPERATURE COEFFICIENTS (Hz/DEGREE) OF NH AND OH CHEMICAL-SHIFTS FOR SOLUTIONS[a] OF MONO- AND DI-SACCHARIDES IN Me_2SO-d_6 AT 300 MHz

Proton	*Compound*						
	2	**5**	**6**	**7**	**8**	**9**	**10**
HO-1′				2.9	2.0	2.0	
HO-2′	2 0		0 8	2 3			
HO-3′	2 3		1.3	2 4	2.0	1.9	
HO-4′	0 11	0.5	0.24	0.45	0 55	1.63	
HO-1			0.9				1 3
HN	1.3	1 2	0.9[b]				2.1
HO-3							1 6
HO-4	0.9		1.0				1.6
HO-6	1 8		1 0				1.8

[a] ~ 1% w/w. [b] 0.4 for β-D anomer

TABLE VI

CONCENTRATION DEPENDENCE[a] OF NH AND OH CHEMICAL-SHIFTS FOR SOLUTIONS OF MONO- AND DI-SACCHARIDES IN Me_2SO-d_6 AT 300 MHz

Proton	*Compound*						
	2	**5**	**6**	**7**	**8**	**9**	**10**
HO-1			−5				0
HN	0	+9	+1				0
HO-3							0
HO-4	−22		−14				0
HO-6	−9 5		−14				0
HO-1′				−99			
HO-2′	−7		−14	−54	−21	−2	
HO-3′	−15		−9	−48	−26	−2	
HO-4′	+50	+37	+38	+99	+52	−2	

[a] Shift in Hz for a four-fold dilution of a solution originally ~ 1% (w/w). Positive shifts are to higher frequency (lower field).

(methyl α-D-glucosid)uronate (**8**), which showed significantly enhanced broadening, resulting in line-widths at half-height of ~25 Hz. In comparison, the HO-4′ signals in the allyl glycoside **2** and the methyl tetra-*O*-methylglycoside **5** showed line-widths of only 3 Hz. In view of the similar structures and solution conditions of the four samples, it is difficult to attribute the variation in line-width to anything other than the presence of small amounts of adventitious acidic or basic impurities.

To investigate association effects, including hydrogen bonding, the temperature and concentration dependences of the chemical shifts were studied. The results for the disaccharide HO and HN signals are given in Tables V and VI together with

results for the corresponding monomeric sugars. The ^{1}H-n m.r. spectrum of 2-acetamido-2-deoxy-D-glucose (**10**) in dimethyl sulphoxide-d_6 has been published[11], but not the temperature and concentration dependences. The chemical shifts of CH protons were essentially independent of changes in temperature and concentration.

DISCUSSION

The chemical shifts and coupling constants in Tables I–III are consistent with those reported elsewhere for similar compounds. In particular, the coupling constants indicate $^4C_1$(D) conformations of the rings in all the compounds.

The principal interest in this work is the behaviour of the OH and NH chemical shifts, which reflect the extent of hydrogen bonding. It is well known that increased strength of hydrogen bonding leads to a downfield shift of the proton involved, and hence, in general, the temperature and concentration dependences of the chemical shifts given in Tables V and VI indicate the existence of hydrogen bonding involving all OH and NH protons. This is not surprising in view of the undoubted occurrence of hydrogen bonding to the highly polar dimethyl sulphoxide solvent. However, there are several significant differences between the behaviours of certain protons, which point to specific hydrogen-bonded structures other than general sugar–solvent interactions.

The temperature coefficient of the chemical shift for the HO-4′ proton in the disaccharides **2**, **5**, and **6** and in sodium D-glucuronate (**7**) is much less than that for other hydroxyl-group protons, and this peak moves downfield with decrease in concentration, whereas all others move upfield. For sodium D-glucuronate, the HO-4 shift is consistent with an equilibrium of rapidly exchanging monomers and dimers[6]. Since dilution decrease dimerisation, the upfield shift of HO proton signals other than that for uronic HO-4 indicates that, for the dimer, these protons are involved to some extent in hydrogen bonds that are stronger than those for the monomer. The contrasting downfield-shift of the uronic HO-4 signal indicates that the hydrogen bonding in the monomer is stronger than in the dimer. Since no dilution shifts were observed for 2-acetamido-2-deoxy-D-glucose or the methyl ester **9**, it is clear that the carboxylate group plays a central role in the association phenomenon. It is suggested that the sugar aggregation occurs chiefly *via* strong hydrogen bonds between hydroxyl-group protons and the carboxylate group, possibly supported by weaker bonds between pairs of hydroxyl groups that are not sufficient in themselves to produce aggregation, but which may be formed once the principal bond has drawn the molecules together. The upfield shift of proton signals other than that of uronic HO-4 on dilution can therefore be understood in terms of the replacement of the strong, carboxylate hydrogen bond by weaker solvent-interactions. The downfield shift of uronic HO-4 on dilution must then arise from the development of an intramolecular hydrogen-bond to the carboxylate group in the monomer. This intramolecular bond is similar in structure to the well-known example in salicylic acid. However, because of the aliphatic ring structure, the geometry is not entirely favourable, and the intra-

molecular carboxylate bond will form only when the intermolecular bonds are disrupted The low value (<2 Hz) of $J_{H\text{-}4',HO\text{-}4'}$ in the sodium salts of compounds **2** and **8** is consistent[12] with the dihedral angle of ~90° needed to form this intramolecular bond. In comparison, the value of 5.5 Hz for $J_{H\text{-}4,HO\text{-}4}$ in the methyl ester is consistent[12] with an essentially freely rotating hydroxyl group. The occurrence of the HO-4 signal at much lower field for the sodium salt (δ 6.74) than for the methyl ester (δ 5.28) is also indicative of the formation of a hydrogen bond with the carboxylate group.

The observation that all OH and NH signals show a dilution shift suggests that any hydroxyl-group proton may be involved in hydrogen bonding to the carboxylate group, though the magnitude of the dilution shift does not necessarily reflect the degree of such participation, since the extent of hydrogen bonding with other hydroxyl protons and with the solvent may also vary from site to site.

The relative temperature coefficients of the OH chemical-shifts support the above proposals. An increase in temperature decreases the extent of intermolecular hydrogen-bonding, but leaves intramolecular bonding essentially unaffected. Hydroxyl signals other than that of uronic HO-4 therefore move upfield with increase in temperature, since their interactions in both monomer or dimer are largely intermolecular. The uronic HO-4 signal is relatively unaffected by warming, because the anticipated, upfield, temperature shift is balanced by the downfield shift consequent on disrupting the dimer, and forming an intramolecular hydrogen-bond.

A further aspect of this work concerns the possible formation of intramolecular hydrogen-bonds between sugar residues. Concerning HO-4 of the hexosamine residue, two findings are relevant: (*a*) the low value of the temperature coefficient of the chemical shift in the allyl glycoside compared to those for HN and HO-6, and (*b*) the low value (1.7 Hz) of $H_{H\text{-}4,HO\text{-}4}$, which indicates[12] a dihedral angle of 90–120°. These data are consistent with the formation of a hydrogen bond between the HO-4 and the ring O-5′ of the uronic acid residue, as previously suggested[2]. It is significant that, in sodium *N*-acetylchondrosinate (**6**), the temperature coefficient of the HO-4 proton signal is the same as those of other hydroxyl group protons, and the value (4.5 Hz) of $J_{H\text{-}4,HO\text{-}4}$ is much closer to that expected for free rotation of the hydroxyl group. In sodium *N*-acetylchondrosinate (**6**), HO-4 is axial, as opposed to equatorial in the allyl glycoside, and the proposed inter-residue hydrogen-bond is therefore not possible.

With regard to HO-2′, it is relevant that the coupling constant ($J_{H\text{-}2',HO\text{-}2'}$) in sodium *N*-acetylchondrosinate is 2.3 Hz, compared with 4 Hz in the allyl glycoside of sodium hyalobiouronate. The lower value shows a preference for conformations with a dihedral angle of 60–120°, whereas the higher value indicates relatively unrestricted rotation. It is possible that the difference arises from the formation in *N*-acetylchondrosinate (**6**) of a weak hydrogen-bond between HO-2′ and the acetamido C=O group. Molecular models show that this bond requires a dihedral angle of ~90°, but it cannot be formed simultaneously and efficiently along with the HO-4 bond mentioned above.

The evidence for the existence of these hydrogen bonds provides support for the proposed secondary structures in certain glycosaminoglycuronans[1].

REFERENCES

1 J. E. SCOTT AND M. J. TIGWELL, *Biochem. J*, 173 (1978) 103–114.
2 E. D. T. ATKINS, D. MEADER, AND J. E. SCOTT, *Int. J. Biol Macromol.*, 2 (1980) 318–319.
3 J. E SCOTT AND F. HEATLEY, *Biochem. J.*, 181 (1979) 445–449.
4 J. E. SCOTT, *Biochem. J.*, 187 (1980) 887–891.
5 B. CASU, M. REGGIANI, G. G. GALLO, AND A. VIGEVANI, *Tetrahedron*, 22 (1966) 3061–3083.
6 F. HEATLEY, J. E. SCOTT, AND B. CASU, *Carbohydr. Res.*, 72 (1979) 13–23.
7 E. WALKER AND R. W. JEANLOZ, *Carbohydr. Res.*, 68 (1979) 343–363.
8 R. W. JEANLOZ AND D. A. JEANLOZ, *Biochemistry*, 3 (1969) 121–122.
9 H. M. FLOWERS AND R. W. JEANLOZ, *Biochemistry*, 3 (1969) 123–124.
10 R. W. JEANLOZ, *Carbohydr. Res.*, 99 (1982) 51–58.
11 T. J. SCHAMPER, *Carbohydr. Res.*, 36 (1974) 233–237.
12 R. R. FRASER, M. KAUFMAN, P. MORAND, AND G. GOVIL, *Can J Chem*, 47 (1969) 403–409.