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POLARIZED INFRARED SPECTRA OF CRYSTALLINE GLYCOSAMINOGLYCANS

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ABSTRACT

Polarized infrared spectra have been recorded for oriented, crystalline specimens of hyaluronates, chondroitin 4-sulfate and 6-sulfate, dermatan sulfate, and a cartilage proteoglycan, having different known chain conformations as determined by X-ray diffraction. The dichroism data for the vibrational modes of the amide and carboxyl groups have been interpreted with respect to the particular molecular structures.

INTRODUCTION

The glycosaminoglycans are widely distributed throughout mammalian connective tissue where it is thought they perform a structural role in conjunction with such proteins as collagen and elastin, as well as regulate the cellular micro-environment. These functions are likely to be intimately related to the chain conformations and side-appendage geometries adopted by the glycosaminoglycans in the various tissues. X-Ray diffraction investigations since 1971 have shown that these polysaccharides may adopt a variety of chain conformations and molecular shapes depending on such variables as type of counter-ion present, pH, ionic, strength, and degree of hydration[1]. For example, in the case of hyaluronate, two-fold, three-fold, and four-fold single-stranded, helical conformations have been reported[2–5]. One of the two different fourfold helical conformations was initially thought to consist of double-stranded helices[4], but single-stranded helices are now preferred[5]. The chondroitin sulfates and dermatan sulfate, cleaved off the protein core to which they

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are naturally covalently linked, exhibit various eight-fold, three-fold, and two-fold single-stranded, helical conformations[6-11]. More recently, the chondroitin 4-sulfate component of intact cartilage proteoglycan aggregate has been crystallized to give good quality X-ray fiber diffraction-patterns[12].

We have applied polarized infrared (i.r.) spectroscopy to oriented films of the glycosaminoglycans prepared in various crystalline forms. Measurements of dichroic ratios can be expected to indicate the orientation of the pendant acetamido, carboxyl, and, possibly, hydroxyl groups, as, for example, in chitin[13-14]. It has previously been shown that frequency and intensity differences in the vibrational spectra (in this case the Raman spectra) of V- and B-amylose can be used to identify the two polymorphic forms, and may be interpreted in terms of the differences in chain conformation[15].

The i.r. spectra of the glycosaminoglycans have been characterized by Orr[16] and Mathews[17], and i.r. spectroscopy remains one of the best techniques for distinguishing between the different isomers of chondroitin sulfate. Quinn and Bettleheim[18] reported polarized i.r. spectra for sodium hyaluronate samples, but their interpretation differed in some respects from the present results.

EXPERIMENTAL

In general, it has been found that samples used for X-ray studies are too thick to give useful information on a grating or prism spectrometer. Conversely, samples produced for i.r. studies require prohibitively long exposure-times in X-ray diffraction experiments. Thus, although Melvin and Taylor[19] were able to obtain i.r. dichroism data from very thin samples of hyaluronic acid, it was difficult to relate their results to specific chain conformations. This important step has been achieved by using the combination of a powerful, rotating-anode X-ray generator, and a Fourier-transform i.r. spectrophotometer with multiple scanning and signal-averaging capabilities.

Oriented film samples of the glycosaminoglycans were prepared as described by Atkins *et al.*[20]. The X-ray diffraction patterns of these specimens were recorded so that the subsequent i.r. spectra could be correlated with the appropriate molecular conformation. Specimens were prepared for both the extended and contracted, four-fold helical conformations of hyaluronate (axially projected disaccharide repeats of 0.90 and 0.84 nm, respectively). In addition, a three-fold helical conformation of chondroitin 4-sulfate[8] and eight-fold conformations for chondroitin 6-sulfate[10,11,21] and dermatan sulfate[7,9,11] were used. A specimen of pig laryngeal proteoglycan–hyaluronate aggregate (gift of T. Hardingham and H. Muir, Kennedy Institute of Rheumatology, London) was also examined; this specimen exhibited a two-fold conformation[12]. The X-ray fiber patterns of the samples investigated are illustrated in Fig. 1.

Polarized and unpolarized i.r. spectra of these specimens were recorded by using a Digilab Fourier-transform, i.r. spectrophotometer, model FTS-14. The incident radiation was polarized by use of a gold wire-grid polarizer. The final spectra

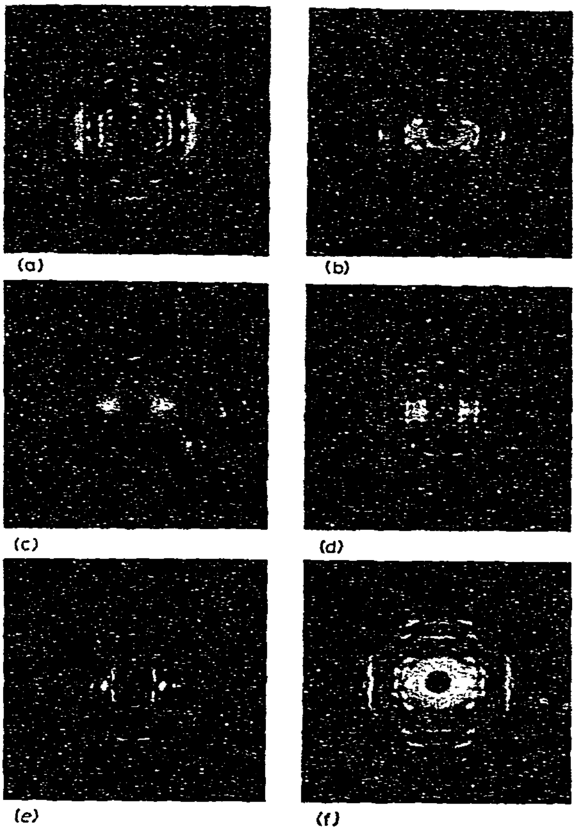

Fig. 1. X-ray fiber diffraction patterns of the glycosaminoglycans. (a) Sodium hyaluronate; contracted, four-fold helical conformation with projected axial rise per disaccharide (h) = 0.84 nm. (b) Sodium hyaluronate; extended four-fold helical conformation with h = 0.90 nm. (c) Chondroitin 6-sulfate; eight-fold (8/-3) conformation with h = 0.98 nm. (d) Chondroitin 4-sulfate; three-fold conformation with h = 0.95 nm. (e) Dermatan sulfate; eight-fold (8/-3) conformation with h = 0.93 mn (f) Proteoglycan–hyaluronate complex; two-fold conformation with h = 0.97 nm.

are the result of coaddition of 300 successive scans recorded at 2.0-cm^{-1} resolution. The frequencies were calibrated internally by a helium–neon reference laser, and the frequency scale was accurate to within ± 0.2 cm^{-1}.

RESULTS AND DISCUSSION

The polarized i.r. spectra from 2000–450 cm^{-1} for the six glycosaminoglycan specimens are shown in Figs. 2–7. The assignments and dichroisms for the various bands are listed in Table I, where a comparison is made between the hyaluronate, chondroitin 6-sulfate, chondroitin 4-sulfate, dermatan sulfate, and the proteoglycan–hyaluronate specimens.

Amide bands. — I.r. bands occurring at 1660–1650 cm^{-1} and 1560–1550 cm^{-1} are assigned to the Amide I and Amide II vibrational modes, respectively. Both modes

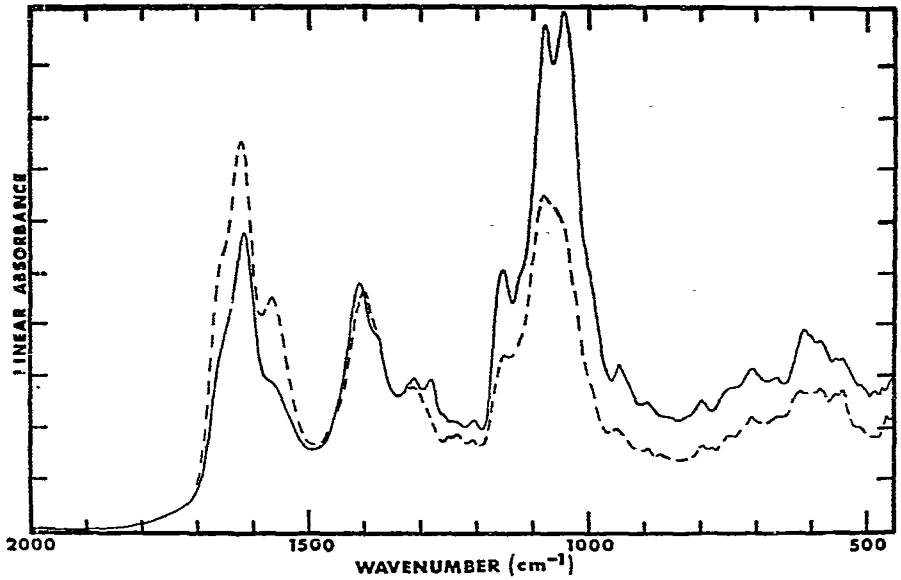

Fig. 2. Polarized-i.r. absorption spectrum obtained for contracted, four-fold conformation of sodium hyaluronate in the range 2000–450 cm^{-1}. This sample gave the X-ray diagram shown in Fig. 1(a). (———) $A_{\parallel}$, (– – –) $A_{\perp}$.

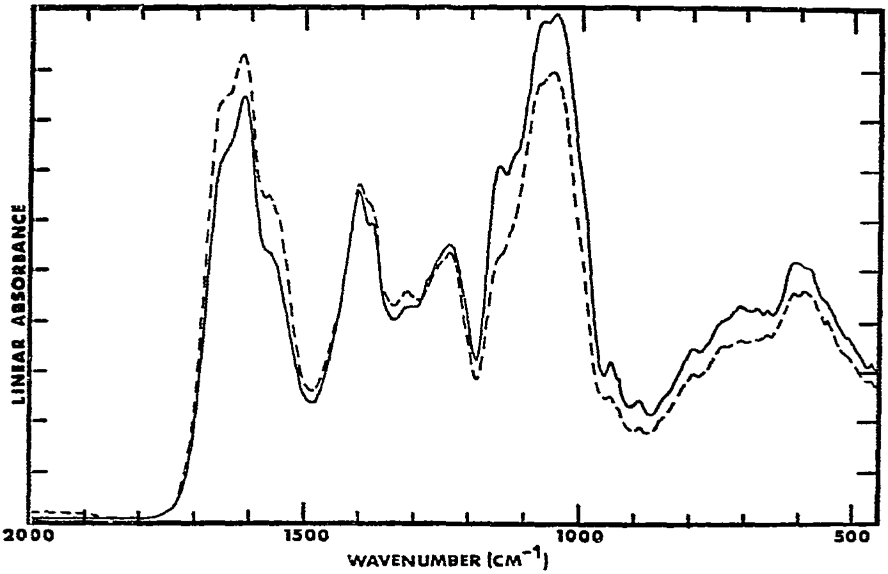

Fig. 3. Polarized-i.r. absorption spectrum obtained for the extended, four-fold conformation of sodium hyaluronate; the specimen contained up to 15% of chondroitin sulfate as an impurity. The spectrum is similar to that shown in Fig. 2 except for the 1300–1200-cm^{-1} region, which contains the vibrational modes of the sulfate groups. This sample gave the X-ray diagram shown in Fig. 1(b). (———) $A_{\parallel}$, (– – –) $A_{\perp}$.

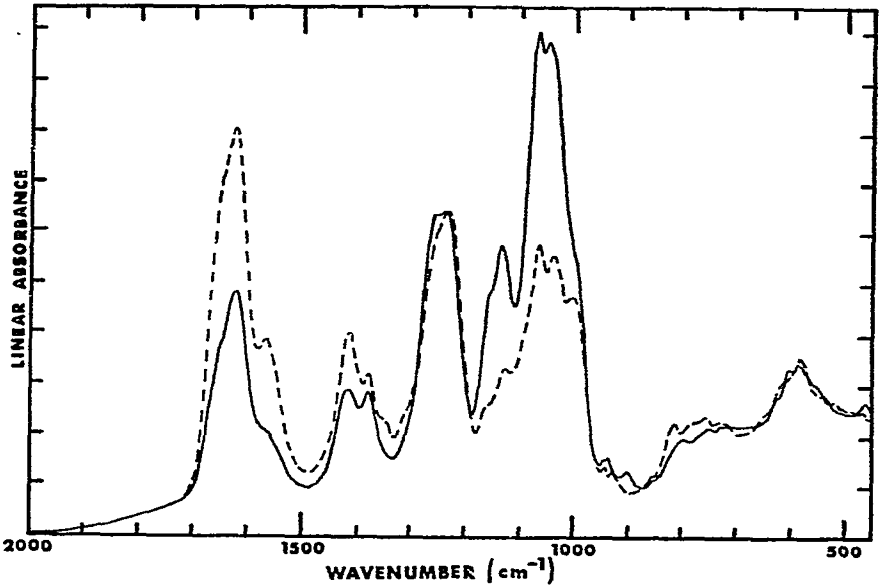

Fig. 4. Polarized-i.r. absorption spectrum obtained for the eight-fold conformation of chondroitin 6-sulfate. This sample gave the X-ray diagram shown in Fig. 1(c). (———) $A_{\parallel}$, (— — —) $A_{\perp}$.

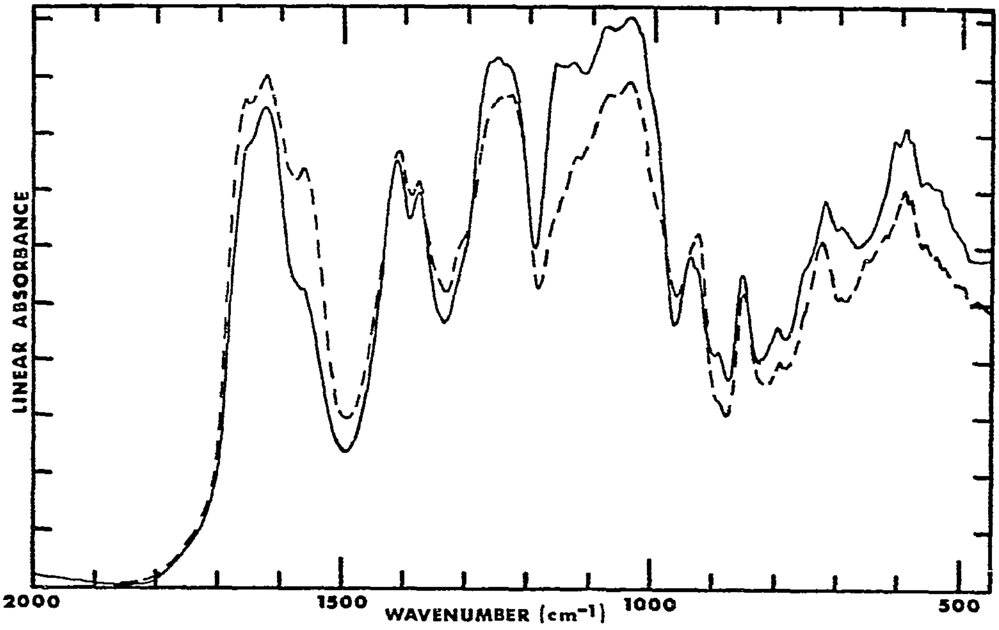

Fig. 5. Polarized-i.r. absorption spectrum obtained for the three-fold conformation of chondroitin 4-sulfate. This sample gave the X-ray diagram shown in Fig. 1(d). (———) $A_{\parallel}$, (— — —) $A_{\perp}$.

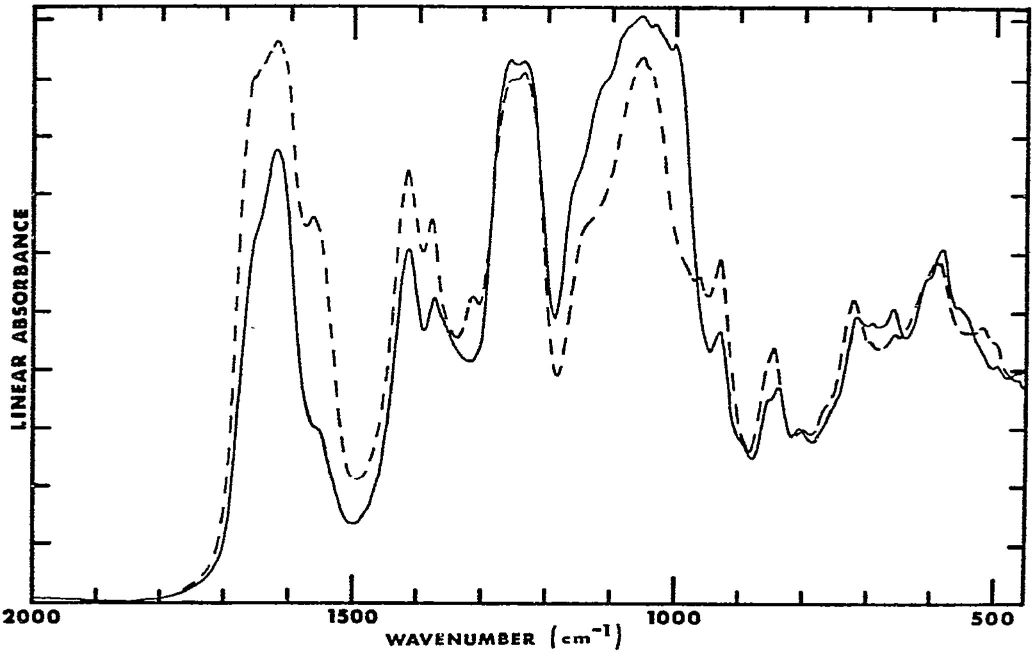

Fig. 6. Polarized-i.r. absorption spectrum obtained for the eight-fold conformation of dermatan sulfate. This sample gave the X-ray diagram shown in Fig. 1(e). (———) $A_{\parallel}$, (— — —) $A_{\perp}$.

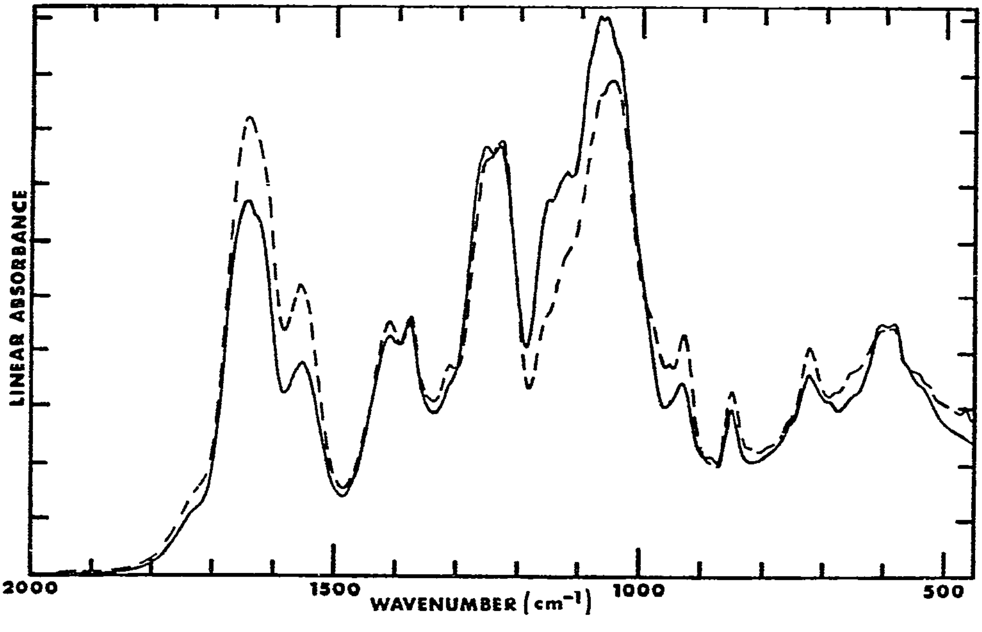

Fig. 7. Polarized-i.r. absorption spectrum obtained for the proteoglycan–hyaluronic acid complex. The major component (85%) of this specimen was chondroitin 4-sulfate, which had crystallized in the two-fold conformation and which gave the X-ray diagram shown in Fig. 1(f). The extra protein component in this preparation is apparent in the increase of intensity of the amide bands. (———) $A_{\parallel}$, (— — —) $A_{\perp}$.

TABLE I

OBSERVED VIBRATIONAL FREQUENCIES (cm^{-1}) AND ASSIGNMENTS

Hyaluronate	*Chondroitin 6-sulfate*	*Chondroitin 4-sulfate*	*Dermatan sulfate*	*Proteoglycan + hyaluronate*	*Assignments*
1655 ⊥	1650 ⊥	1650 ⊥	1644 ⊥	1645 ⊥	Amide I
1520 ⊥	1617 ⊥	1620 ⊥	1620 ⊥	1620 ⊥	CO_2 antisym. str.
1565 ⊥	1565 ⊥	1565 ⊥	1563 ⊥	1558 ⊥	Amide II
1410 ∥	1412 ⊥	1412 ∥	1415 ⊥	1411 ⊥	CO_2 sym. str.
1378 ∥	1378 ∥	1377	1377 ⊥	1379 ∥	C–H and O–H def.
	1347	1345 ⊥			
1320	1312 ⊥	1310 ⊥	1315 ⊥	1312 ⊥	
1284 ∥					
1256	1259 ∥	1262 ∥	1265 ∥	1262 ∥	SO_4^- related modes
1237 ⊥	1234 ⊥	1230 ⊥	1235 ⊥	1230 ⊥	
1206 ∥					C–O and C–C str.
1152 ∥	1163 ∥	1157 ∥	1155 ∥	1155 ∥	
1120	1131 ∥	1128 ∥	1130	1125 ∥	
1079 ∥	1083 ∥	1080 ∥			
1047 ∥	1069 ∥	1040 ∥	1040 ∥	1040	
	1025 ∥				
1000 ∥	1003	990	1000 ∥	990 ⊥	
			963 ⊥		
945 ∥	945 ∥	939 ∥	935		
935 ⊥	935	929 ⊥	930 ⊥	940 ∥	skeletal vibration
904 ⊥	905 ∥				
889 ∥		890 ∥	890 ∥	890 ∥	
855 ∥	857	851 ⊥	860	853 ⊥	
830 ∥			843 ⊥		
	818 ⊥	817 ∥		810 ⊥	
798	802 ∥	797 ∥	805 ∥		
	776 ⊥		770 ⊥		
755 ⊥	759	755 ∥		760 ∥	
740 ∥					
	724	724 ⊥		725 ⊥	
705 ∥	712		710 ⊥		
		692 ∥	693 ∥	690 ∥	
	675				
	659	660 ⊥	660 ∥	660 ⊥	
	638	625 ⊥			
615 ∥	617 ∥	610 ∥	610 ∥	610 ∥	
	598 ⊥	592 ∥			
585 ∥	584	580	585 ∥	585 ∥	
	552	558	550 ∥	550 ⊥	
542 ⊥	545	540 ∥			
		525	520 ⊥	520 ∥	
505 ⊥	505	500 ⊥	495 ⊥		
473 ⊥	470	472	465		

are considered to be in-plane deformations of the amide group. The Amide I vibration results primarily from C=O stretching, with a minor contribution from C–N–H in-plane bending[22]. The resulting transition moment is disposed ~20° from the C=O bond toward the C–CH_3 bond (Fig. 8a)[23]. The Amide II vibration consists of the C–N stretching mode coupled to C–N–H in-plane bending[22], with a resulting transition-moment disposed at ~75° to the C=O bond toward C–CH_3 (Fig. 8b)[23]. Normal-coordinate calculations for α- and β-D-glucose[24,25], and for cellulose I (ref. 26), and V-amylose[27] have shown that vibrational modes occurring at frequencies higher than 1400 cm^{-1} are due primarily to single-group vibrations. However, below 1400 cm^{-1}, there is a high degree of vibrational coupling such that none of the modes arise from single-group vibrations. As a result, it is reasonable to expect the vibrations for both the Amide I and II modes to mix very little with other vibrations of the carbohydrate ring-atoms, thus allowing the use of dichroism data to establish the orientation of the acetamido side-chain relative to the molecular axis.

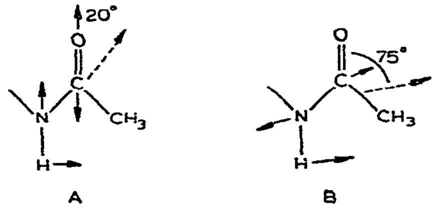

Fig. 8. Schematic representations of the Amide I (A) and Amide II (B) vibrations. The dotted arrows indicate the approximate direction of the transition moments.

From Figs. 2–7 it may be seen that all of the glycosaminoglycans exhibit perpendicular dichroism for both the Amide I and II absorption bands. Allowing for differences in sample orientation, thickness, and crystalline-to-amorphous ratio, the results indicate that the plane of the amide group is approximately perpendicular to the helix axis. In the spectra of the proteoglycan (Fig. 7), both Amide I and II bands have increased relative intensity because of the presence of the protein core (~10%).

For the contracted, four-fold helix of sodium hyaluronate (Fig. 2), the Amide I band has moderate perpendicular dichroism. The structure proposed by Guss *et al.*[5] for this form of hyaluronate was based upon an X-ray fiber diffraction-pattern similar to that illustrated in Fig. 1a. Thus, it is legitimate to compare the dichroism observed in the spectra shown in Fig. 2 with that predicted by their model. On the assumption that the Amide I and II vibrations have transition moments disposed ~20° and ~75°, respectively, from the C=O bond, strong perpendicular dichroism is predicted for both modes. The moderate dichroism observed for these bands is in qualitative agreement with the predictions; the discrepancy may be due to imperfect orientation of the specimen, although the X-ray fiber pattern for this material indicates a high degree of orientation. Alternatively, a particular type of hydrogen bonding for the amide groups could perturb the directions of the transition moment for both Amide I and II vibrations from the angles that we have assumed.

Carboxyl bands. — For the salt forms of the glycosaminoglycans, i.r. bands observed at ~1620 and ~1410 cm^{-1} are respectively assigned to the antisymmetric and symmetric stretching-modes of the planar carboxyl group[28]. Schematic representations for both vibrational modes are illustrated in Fig. 9. As discussed previously, it is reasonable to expect negligible mixing of these modes with other group vibrations of the disaccharide ring-atoms.

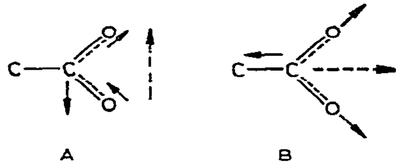

Fig. 9. Schematic representations of the antisymmetric (A) and symmetric (B) CO_2^- stretching-vibrations. The dotted arrows indicate the approximate direction of the transition moments.

For the symmetric carboxyl stretch (Fig. 9b), the direction of the transition moment is assumed to be approximately colinear with the C-5–C-6 bond, and it would thus be independent of the rotational conformation of the CO_2^- group about C-5–C-6. In addition, the transition moment lies in the plane of the carboxyl group, and the dichroic properties for this band should be sensitive to the backbone conformation of the polysaccharide. For the antisymmetric stretching-vibration of the carboxyl group (Fig. 9a), the transition moment is perpendicular to that for the symmetric stretching mode and approximately parallel to the O···O interatomic vector. As a result, the dichroic properties of this vibrational mode are a function of the rotational conformation of the CO_2^- group.

From Figs. 2–7 it may be seen that the band due to antisymmetric carboxyl stretching has perpendicular dichroism for all of the glycosaminoglycan specimens. However, for the symmetric stretching-mode, chondroitin 6-sulfate, dermatan sulfate, and the proteoglycan–hyaluronate complex exhibited perpendicular dichroism at ~1410 cm^{-1}, whereas, for chondroitin 4-sulfate and both hyaluronate specimens, parallel dichroism was observed. The dichroism for the symmetric, carboxyl-stretching band may be correlated with the rise per disaccharide residue for each of the glycosaminoglycans. Of the specimens examined, the eight-fold helix of chondroitin 6-sulfate and the two-fold helix of the proteoglycan–hyaluronate complex are the most extended structures, with an axially projected rise per disaccharide residue of 0.98 and 0.97 nm, respectively. However, for the three-fold conformation of chondroitin 4-sulfate and both four-fold conformations of hyaluronate, the rise per disaccharide residue is 0.95, 0.90, and 0.84 nm, respectively. These observations indicate a transition from perpendicular to parallel dichroism for the symmetric carboxyl-stretching band with helix contraction, corresponding to a decrease in the angular projection of the C-5–C-6 bond on the molecular axis.

An exception to this correlation occurs with dermatan sulfate, where the C-5–C-6 bond is disposed axially to the carbohydrate ring (not equatorially as, for example, in chrondroitin 4-sulfate). The covalent repeats of chondroitin 4-sulfate and dermatan sulfate differ in the uronic acid residue of the disaccharide repeat: β-D-glucopyranuronic acid in the former corresponds to the 5-epimeric α-L-idopyranuronic acid residue in the latter, where the possibility of the ${}^{1}C_{4}$ conformation also needs to be considered. When the α-L-idopyranuronic acid residues of dermatan sulfate have the ${}^{1}C_{4}$ conformation, the C-5–C-6 bond is disposed equatorially, and such a structure (rise per disaccharide residue 0.93 nm) is more likely to display parallel dichroism for the symmetric carboxyl-stretching band. Conversely, α-L-idopyranuronic acid-${}^{4}C_{1}$ residues would have the C-5–C-6 bond attached axially to the pyranose ring. Inspection of molecular models indicated that, for this structure, the C-5–C-6 bond in the α-L-idopyranuronic acid-${}^{4}C_{1}$ residue would be approximately perpendicular to the helix axis. Therefore, the perpendicular dichroism observed for the carboxyl symmetric stretch favors α-L-idopyranuronic acid ${}^{4}C_{1}$-chair conformation, in accordance with the X-ray diffraction data[7,9,11].

The structure for the four-fold helix of sodium hyaluronate proposed by Guss *et al.*[5] predicted strong perpendicular dichroism for the 1620-cm^{-1} band and no dichroism for the 1410-cm^{-1} band. The perpendicular dichroism observed for the 1620-cm^{-1} band is in qualitative agreement with their model, but the slight parallel dichroism observed for the 1410-cm^{-1} band is less consistent. As for the Amide I and II vibrational modes, the discrepancy between the observed and predicted dichroisms for the antisymmetric and symmetric stretching-vibrations may be due to incomplete orientation of the sample, or to perturbation of the direction of the transition moments through nonequivalent coordination of the sodium cation to the carboxyl oxygen atoms.

The 1400–1200 cm^{-1} region. — Based on normal-coordinate calculations for cellulose I (ref. 26) and V-amylose[27], i.r. absorption bands in the 1400–1200-cm^{-1} region can be assigned primarily to coupled vibrations of the C–C–H, O–C–H, and C–O–H groups. In addition, for the sulfated glycosaminoglycans, the strong absorption bands at ~1255 and ~1230 cm^{-1} are assigned as sulfate-related modes, and can probably be attributed to antisymmetric and symmetric stretching of the sulfate group, respectively. An analysis for relating the orientation of the sulfate side-chain to the dichroism data can be established in a way similar to that used for the acetamido and carboxyl groups. However, the particular vibrational modes for this group may be expected to mix with other group vibrations of the carbohydrate ring-atoms (such as C–C–H and C–O–H deformations), thereby restricting the use of such an interpretation. The i.r. absorption bands occurring at ~1255 and 1230 cm^{-1} generally exhibit parallel and perpendicular dichroism, respectively.

As hyaluronate does not contain sulfate groups, it shows more detail than the other glycosaminoglycans in this region of the infrared. The two spectra of hyaluronate shown in Fig. 2 and 3 correspond to Grade I and Grade II material purchased from Miles–Serevac Ltd., London. Grade II hyaluronate contains ~15 percent of impurities, mainly in the form of chondroitin sulfate, and this fact explains the difference between these two spectra in the 1300–1200 cm^{-1} region.

The 1200–1000 cm^{-1} region. — The region of the i.r. from 1200 to 1000 cm^{-1} is associated mainly with C–C–C, C–C–O, and C–O–C stretchings of the pyranose ring structures. In general, there are four major absorption-bands for each spectrum, occurring at ~1150, 1130, 1065, and 1040 cm^{-1}, and which, in most cases, exhibit parallel dichroism. These vibrational modes can probably be related to those found in the extended, two-fold helix of cellulose, which likewise show strong parallel dichroism[29,30].

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REFERENCES

1 E. D. T. Atkins, D. H. Isaac, I. A. Nieduszynski, C. F. Phelps, and J. K. Sheehan, *Polymer*, 15 (1974) 263–271.
2 E. D. T. Atkins and J. K. Sheehan, *Nature (London), New Biol.*, 235 (1972) 253–254.
3 E. D. T. Atkins, J. H. Brown, J. M. Landsdall, I. A. Nieduszynski, and J. K. Sheehan, *J. Polym. Sci.*, C42 (1973) 1513–1520.
4 I. C. M. Dea, R. Moorhouse, D. A. Rees, S. Arnott, J. M. Guss, and E. A. Balazs, *Science*, 179 (1973) 560–562.
5 J. M. Guss, D. W. L. Hukins, P. J. C. Smith, W. T. Winter, S. Arnott, R. Moorhouse, and D. A. Rees, *J. Mol. Biol.*, in press.
6 E. D. T. Atkins, R. Gaussen, D. H. Isaac, V. Nandanwar, and J. K. Sheehan, *J. Polym. Sci.*, B10 (1972) 863–865.
7 E. D. T. Atkins and T. C. Laurent, *Biochem. J.*, 133 (1973) 603–605.
8 D. H. Isaac and E. D. T. Atkins, *Nature (London), New Biol.*, 244 (1973) 252–254.
9 E. D. T. Atkins and D. H. Isaac, *J. Mol. Biol.*, 80 (1973) 773–779.
10 S. Arnott, J. M. Guss, D. W. L. Hukins, and M. B. Mathews, *Science*, 180 (1973) 743–745.
11 S. Arnott, J. M. Guss, D. W. L. Hukins, and M. B. Mathews, *Biochem. Biophys. Res. Commun.*, 54 (1973) 1377–1383.
12 E. D. T. Atkins, T. E. Hardingham, D. H. Isaac, and H. Muir, *Biochem. J.*, 141 (1974) 919–921.
13 D. Carlstrom, *J. Biophys. Biochem. Cytol.*, 3 (1957) 669–683.
14 S. E. Darmon and K. M. Rudall, *Discuss. Faraday Soc.*, 9 (1950) 251–260.
15 J. J. Cael, J. L. Koenig, and J. Blackwell, *Carbohydr. Res.*, 29 (1973) 123–134.
16 S. F. D. Orr, *Biochim. Biophys. Acta*, 14 (1954) 173.
17 M. B. Mathews, *Nature (London)*, 181 (1958) 421.
18 F. R. Quinn and R. A. Bettelheim, *Biochim. Biophys. Acta*, 69 (1963) 544–551.
19 J. L. Melvin and P. H. Taylor, Project Report, University of Bristol, England, (1973).
20 E. D. T. Atkins, C. F. Phelps, and J. K. Sheehan, *Biochem. J.*, 128 (1972) 1255–1263.
21 M. Fleming, M. Sc. Thesis, University of Bristol, England (1973).
22 T. Miyazawa, in G. D. Fasman (Ed.), *Poly-α-amino Acids*, Marcel Dekker, New York, 1967.
23 I. Sandeman, *Proc. R. Soc. London Ser. A*, 232 (1955) 105–113.
24 P. D. Vasko, J. Blackwell, and J. L. Koenig, *Carbohydr. Res.*, 23 (1972) 407–416.
25 J. J. Cael, J. L. Koenig, and J. Blackwell, *Carbohydr. Res.*, 32 (1974) 79–91.
26 J. J. Cael, K. H. Gardner, J. L. Koenig, and J. Blackwell, *J. Chem. Phys.*, 62 (1975) 1145–1153.
27 J. J. Cael, J. L. Koenig, and J. Blackwell, *Biopolymers*, 14 (1975) 1885–1903.
28 F. S. Parker, *Applications of Infrared Spectroscopy in Biochemistry, Biology, and Medicine*, Plenum Press, New York, 1971.
29 C. Y. Liang and R. H. Marchessault, *J. Polym. Sci.*, 39 (1959) 269–278.
30 J. Blackwell, P. D. Vasko, and J. L. Koenig, *J. Appl. Phys.*, 41 (1970) 4365–4379.