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INFRARED ABSORPTION AND RAMAN SCATTERING OF SULFATE GROUPS OF HEPARIN AND RELATED GLYCOSAMINOGLYCANS IN AQUEOUS SOLUTION

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ABSTRACT

The i.r. spectra for aqueous solutions of sulfated glycosaminoglycans and model compounds in the transmittance "window" region of the solvent (1400–950 cm^{-1}) are dominated by the strong and complex absorption centered at ~1230 cm^{-1} and associated with the antisymmetric stretching vibrations of the S=O groups. Primary and secondary *O*-sulfate groups absorb at somewhat higher frequencies (1260–1200 cm^{-1}) than *N*-sulfates (~1185 cm^{-1}). Each sulfate band lends itself to quantitative applications, especially within a given class of sulfated polysaccharide. Laser-Raman spectra of heparin and model compounds have been obtained in aqueous solution and in the solid state. The most-prominent Raman peak (at ~1060 cm^{-1}) is attributable to the symmetrical vibration of the S=O groups, with *N*-sulfates emitting at somewhat lower frequencies (~1040 cm^{-1}) than *O*-sulfates. The Raman pattern in the 950–800 cm^{-1} region (currently used in the i.r. for distinguishing between types of sulfate groups) also involves vibrations that are not localized only in the C–O–S bonds.

INTRODUCTION

Sulfated glycosaminoglycans (mucopolysaccharides) differ from each other in the type and distribution of sulfate groups that they contain. The *O*-sulfate group is encountered most commonly, and is located in a variety of positions and on different kinds of sugar residues, whereas *N*-sulfate (sulfoamino) substituents are found on aminodeoxyhexose residues of heparin and heparan sulfates[1,2].

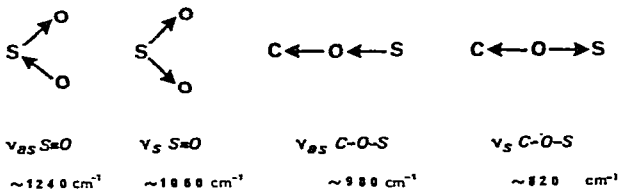
Due to the importance attributed to sulfate groups in determining the physicochemical and biological properties of glycosaminoglycans, methods for the characterization of such groups are of substantial interest. Infrared absorption spectroscopy of solid-phase samples has been used widely, based primarily on the analysis of weak bands in the 850–800 cm^{-1} region, attributable to C–O–S vibrations[3]. From studies of *O*-sulfated monosaccharides and chondroitin sulfates, bands at 850, 830, and 820

cm^{-1} have been assigned, respectively, to axial, equatorial, and primary (*eg.* CH_2O-S-) structures[4,5]. However, notable exceptions to these empirical correlations have been reported; variations in the physical condition of samples can produce significant differences in peak position, by as much as 20 cm^{-1} for a sample examined as a film *vs* a KBr pellet, or a Nujol mull[6,7]. Such variations arise, at least in part, from differences in the degree of order of the specimen.

In principle, solution spectra should eliminate this type of problem, although no attempts in this direction have been reported. Despite the strong i.r. absorption of water in most regions (and, to a lesser extent, of D_2O, which is the only other practical solvent for glycosaminoglycans in their usual ionized form)*, modern spectrophotometers are generally able to compensate for the absorption of polar solvents, especially in favorable regions. Among such regions, the transmittance "window" of water between 1400–900 cm^{-1} is of obvious interest for glycosaminoglycans, because the strongest band of sulfate groups is found there. Solution spectra of neutral mono-, oligo-, and poly-saccharides in this region have already been reported[8,9].

Fewer limitations should be encountered in Raman spectroscopy, because water and D_2O are poor Raman scatterers, advantages that have been realized recently in applications of laser-Raman spectroscopy to neutral oligo- and poly-saccharides[10,11], as well as to glycosamines and glucuronic acid[12].

Vibrations associated with the C–O–SO_3^- group that have been investigated most extensively are represented below for the simplified model. Since these vibrations



are modified through coupling with vibrations of other bonds or groups, the corresponding absorption frequencies in the spectra of glycosaminoglycans may be substantially different** from each other. Furthermore, the four vibrations should not be equally active in i.r. and Raman spectroscopy; *i.e.*, asymmetrical vibrations of polar groups should give rise to strong i.r. bands, and symmetrical vibrations to weak ones, whereas the converse is true for Raman spectroscopy.

We now report that the i.r. and laser-Raman spectra of heparin and some related compounds bearing strongly polar sulfate groups are, indeed, different. Due to their complementary nature, together they provide a reliable method for characteriz-

*I.r. spectra of pyridinium salts of glycosaminoglycans can also be obtained from solutions in methyl sulfoxide (J. Boyd, personal communication).

**In principle, the extent of coupling may be evaluated by normal coordinate analysis, as has been attempted for glucose[10,11], but this is probably unwarranted as yet for such complex molecules as glycosaminoglycans.

ing this class of carbohydrate. Although each type of sulfate group has a different absorption and scattering pattern in the S=O stretching region, a given glycosaminoglycan produces vibrational "fingerprints", especially in the low-frequency region. In turn, this specificity reinforces the view that generalized correlations between vibrational frequency and the local geometry of sulfate groups are unreliable.

RESULTS

Infrared spectra. — Non-sulfated carbohydrates do not absorb appreciably in the region of the $\nu_{as}S{=}O$ band, as illustrated by a comparison of the spectra of aqueous solutions of D-glucose 6-sulfate and dextran sulfate with those of their neutral counterparts (Figs. 1a and 1b). This band is strong and broad, and comprises at least two major components in both instances. By contrast, the $\nu_{s}S{=}O$ band is barely observable (tentatively) as a shoulder on the complex group of bands (at lower frequency) attributable to various, strongly coupled, C–O–C vibrations. The $\nu_{as}S{=}O$ band for methyl α-D-glucopyranoside 2-sulfate is centered at a slightly lower frequency than that of the 6-sulfate, whereas the band for 2-deoxy-2-sulfoamino-D-glucose appears at even lower frequency (Fig. 1c).

In the spectrum of heparin (Fig. 1d), the $\nu_{as}S{=}O$ band corresponds roughly to a summation of all of the above bands, with the shoulder at 1180 cm^{-1} being attributed to the contribution of sulfoamino groups. This assignment is confirmed by the absence of an analogous shoulder in the accompanying spectrum of selectively *N*-desulfated heparin. An A-type heparin exhibits an appreciable extra absorption-band in the region of 1130 cm^{-1}, at which heparan sulfate* absorbs strongly (Fig. 1e); otherwise, the spectra of A- and B-type heparins show little difference.

Chondroitin sulfates A, B, and C give spectra (Fig. 1f) in which the $\nu_{as}S{=}O$ band is relatively weaker than for the compounds of higher sulfate content, and, overall, have slightly different profiles.

Frequencies at maxima for the $\nu_{as}S{=}O$ bands of these various compounds are given in Table I. Since the bands are usually not symmetrical, frequencies corresponding to the band centers are also listed. Data included for sodium sulfate show that this possible contaminant of glycosaminoglycan preparations should not interfere in analysis of organic sulfate.

Raman spectra. — Strong background fluorescence was found to interfere seriously with the Raman spectra of several materials in aqueous solution. However, acceptable spectra have been obtained for heparin, dextran sulfate, and two model monosaccharide sulfates.

As shown in Fig. 2, *O*-sulfation introduces prominent changes in the Raman spectrum of dextran; the latter spectrum is essentially indistinguishable from that reported by Vasko *et al.*[10]. Most notable is the appearance of a strong peak in the

*The ratio A_{1280}/A_{1225} is being used to evaluate the total sulfate/*N*-acetyl ratio in heparan sulfate samples[13].

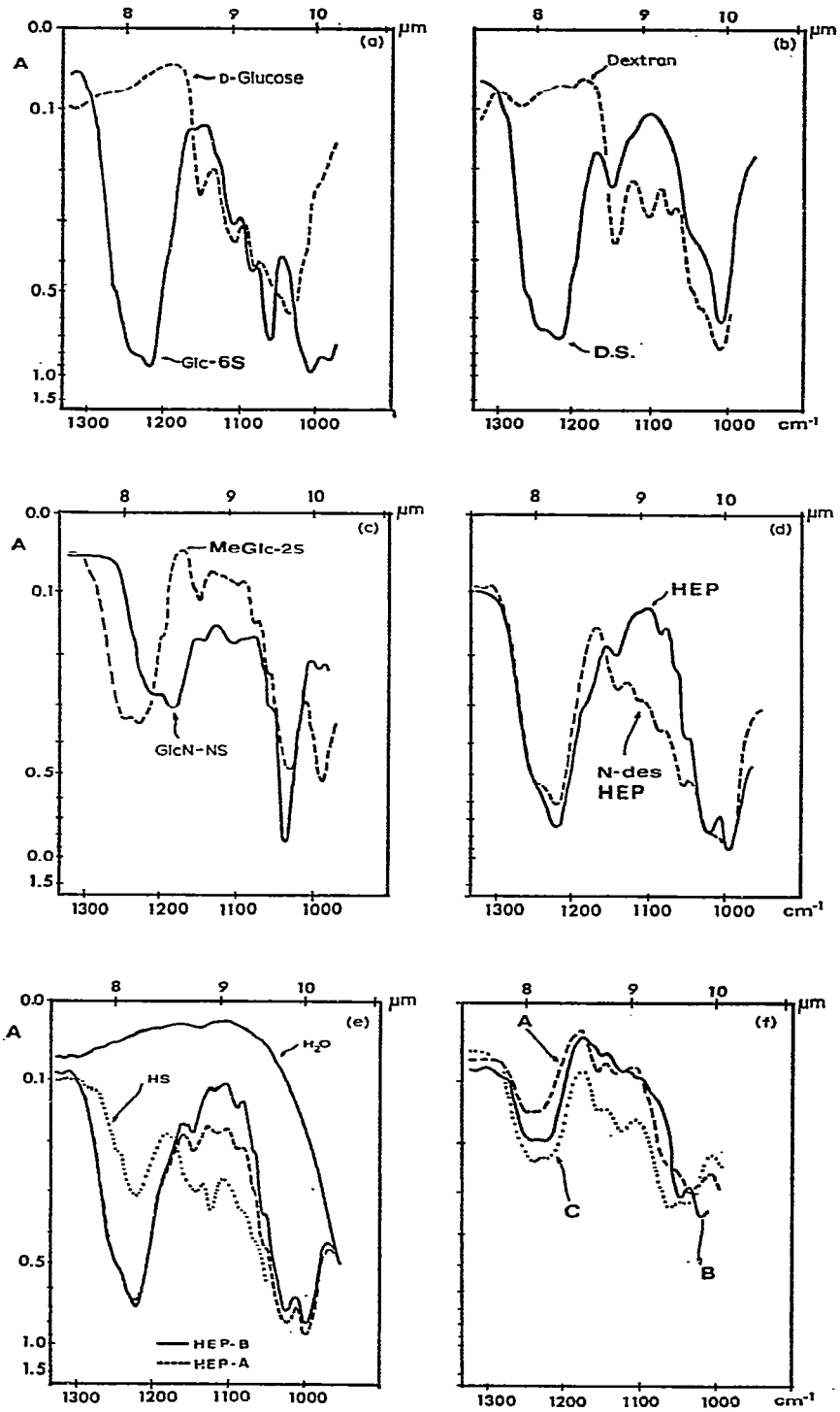

8
9
10
μm
(a)
A
0.0
0.1
0.5
1.0
1.5
D-Glucose
Glc-6S
1300
1200
1100
1000
(b)
Dextran
D.S.
cm⁻¹
(c)
MeGlc-2S
GlcN-NS
(d)
HEP
N-des
HEP
(e)
H₂O
HS
HEP-B
HEP-A
(f)
A
B
C

TABLE I

I.R. FREQUENCIES (CM^{-1}) OF $\nu_{as}S{=}O$ BANDS[a] OF GLYCOSAMINOGLYCANS AND MODEL COMPOUNDS IN SOLUTION (Na SALTS IN H_2O)

Compound	*Frequency (cm^{-1})*			*Band barycenter*
Methyl α-D-glucopyranoside 2-sulfate	1245	*1226*		(1240)
D-Glucose 6-sulfate	1245	*1216*		(1230)
2-Deoxy-2-sulfoamino-D-glucose		1210	*1182*	(1190)
Dextran sulfate	*1240*			(1240)
Chondroitin 4-sulfate	*1240*	1230		(1240)
Dermatan sulfate	1240	*1225*		(1230)
Chondroitin 6-sulfate	*1240*	1220		(1230)
Heparin	1245	*1222*	~1115 (sh)	(1230)
Heparan sulfate	1245	*1220*		(1220)
N-Desulfated heparin	1245	*1225*		(1235)
COOH-reduced heparin	1245	*1220*		(1230)
Sodium sulfate			1095	

[a]The strongest peaks are shown in *italics*.

TABLE II

RAMAN FREQUENCIES (CM^{-1}) ASSOCIATED WITH SULFATE GROUPS IN GLYCOSAMINOGLYCANS AND MODEL COMPOUNDS

Compound	$\nu_s S{=}O$	$\nu_{as}C{-}O{-}C$[a]		
In aqueous solution:				
Methyl α-D-glucopyranoside 2-sulfate	1050	905	845	
2-Deoxy-2-sulfoamino-D-glucose	1040	920 (br)	850 (br)	
Dextran sulfate	1069	934	860	
Heparin	1062	893	827	
Heparin-OD	1060	897	833	
In solid phase:				
2-Deoxy-2-sulfoamino-D-glucose	1046	—	867	
Chondroitin 4-sulfate	1067	—	847	
Dermatan sulfate	~1065	866		808
Chondroitin 6-sulfate	~1065	[b]	[b]	
Heparin	1065	888	855	820
Heparin sulfate	~1055	[b]	[b]	
Keratan sulfate	1066	[b]	[b]	

[a]Tentative assignment. The vibration is thought to be strongly coupled with other vibrational modes.
[b]Data not obtained, because of weakness of the signals and strong fluorescence background.

Fig. 1 *(page 4)*. I.r. spectra, in aqueous solution, of (*a*) D-glucose, D-glucose 6-sulfate (Glc-6S); (*b*) dextran, dextran sulfate (D.S.); (*c*) methyl α-D-glucopyranoside 2-sulfate (MeGlc-2S), 2-deoxy-2-sulfoamino-D-glucose (GlcN-NS); (*d*) B-type heparin (HEP), *N*-desulfated heparin (N-des HEP); (*e*) B-type and A-type heparin (HEP-B, HEP-A), heparan sulfate (HS); and (*f*) chondroitin sulfates A, B (dermatan sulfate), and C (A, B, and C); $c = 10\%$ w/v, except for D-glucose (6.6%), dextran and D.S. (6%), MeGlc-2S and GlcN-NS (7%), and A, B, and C (4%); $l = 0.050$ mm.

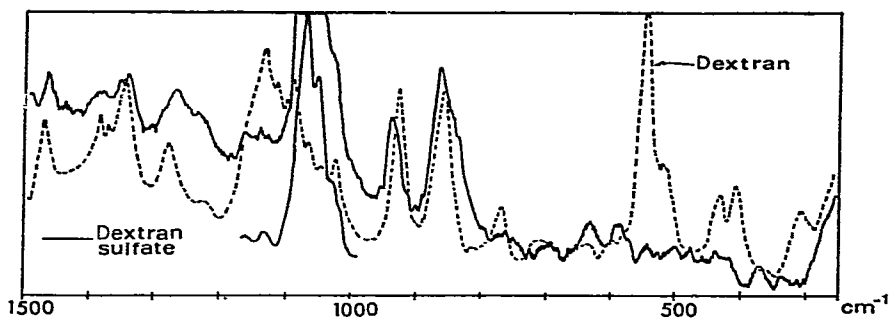


Fig. 2. Raman spectra of dextran and dextran sulfate in aqueous solution (c = 10% w/v).

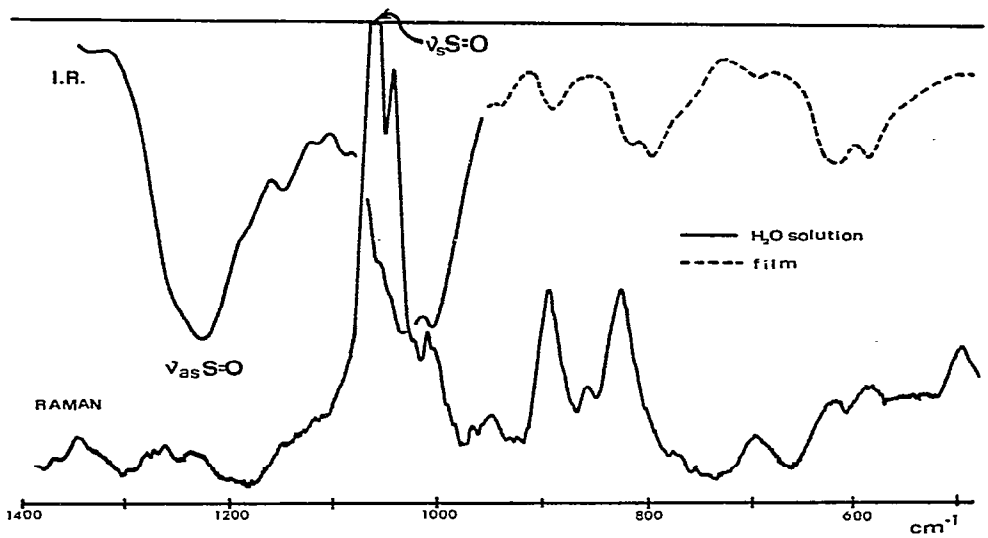


Fig. 3. I.r. and Raman spectra of heparin (B-type) in aqueous solution (c = 10% w/v for i.r., 20% w/v for Raman). At frequencies below the solvent cut-off (~950 cm^{-1}), the i.r. spectrum obtained was from a solid film.

1060 cm^{-1} region, and the disappearance of the medium-to-strong peak at 540 cm^{-1}. Both the sulfated and the unsubstituted polymer show two peaks of medium intensity between 950 and 800 cm^{-1}.

Fig. 3 compares the Raman spectrum of an aqueous solution of heparin with the corresponding i.r. spectrum in the "transmittance window" region*. As would be anticipated, the strong i.r. band due to the ν_{as}S=O band is virtually absent in the Raman spectrum. By contrast, the strongest Raman absorption band near 1060 cm^{-1}

*At frequencies lower than 950 cm^{-1} (the solvent cut-off), the i.r. spectrum was run in the solid phase, to allow for a rough comparison with the Raman peaks.

finds only a weak counterpart in the i.r. spectrum and, accordingly, may be designated $\nu_{as}S{=}O$. This assignment is also supported by the fact that the band is not appreciably affected by *O*-deuteration, whereas the frequencies and intensities of most other peaks are altered relative to those for aqueous solutions. Furthermore, the strongest peak in the Raman spectra of all of the *O*-sulfates examined lies in the 1070–1050 cm^{-1} region (Table II).

Solid-state Raman spectra of some of the glycosaminoglycans, undoubtedly because fluorescence and solubility were less limiting, were of somewhat better quality than those for solutions. Although, even then, only the most intense peaks were readily observable, such spectra furnished data suitable for comparative purposes. These data are included in Table II, together with corresponding values for heparin, which gave a good quality, solid-phase spectrum that differed little from that in aqueous solution.

Quantitative aspects. — Since i.r. absorption in the $\nu_{as}S{=}O$ region is due almost exclusively to sulfate groups, background contributions by the carbohydrate backbone are easily allowed for by a base-line trace between 1320 and 1180 cm^{-1} (1100 cm^{-1} for heparins). Fig. 4 shows that the absorbance of the $\nu_{as}S{=}O$ band of dextran sulfate or heparin is linearly proportional to the concentration of sulfated polysaccharide. To see if the absorbance value measured at the band maximum could serve as a measure of the content of sulfate groups in an unknown sample, the absorbance for a given concentration of polysaccharide (10% w/w) was plotted against the SO_3^- content as determined by conductimetric[14] analysis. Fig. 5 demon-

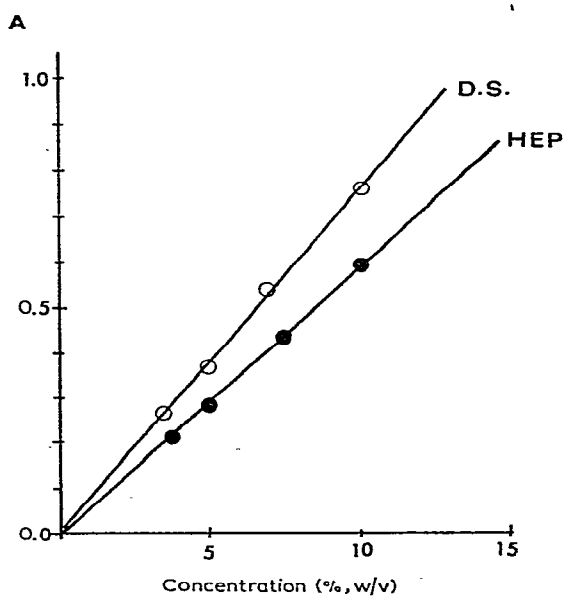


Fig. 4. Absorbance–concentration relationship for the i.r. $\nu_{as}S{=}O$ band of dextran sulfate (D.S.) and heparin (HEP) in aqueous solution.

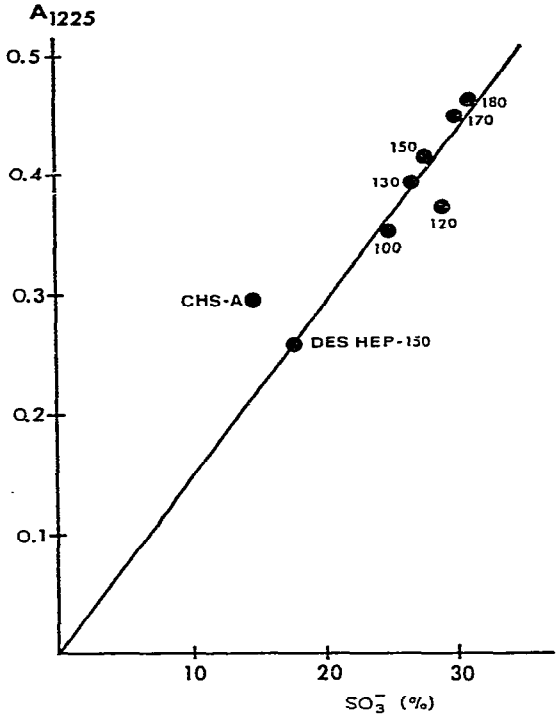


Fig. 5. Absorbance of the i.r. $\nu_{as}S{=}O$ band as a function of the content of sulfate groups for a number of heparins and *N*-desulfated heparin; a value for chondroitin sulfate A is also shown; $c = 10\%$ w/v, $l = 0.05$ mm. The SO_3^- content was determined by conductimetry[14].

strates a reasonably good relationship for several heparins and an *N*-desulfated heparin, whereas the value for chondroitin sulfate is somewhat higher than expected.

DISCUSSION

In the i.r.-spectral region accessible with water as the solvent, carbohydrate *O*-sulfates are characterized by a strong $\nu_{as}S{=}O$ band, at $\sim$1230 cm^{-1}. For degrees of sulfation greater than $\sim$1.5, the intensity of this band is higher than that of the familiar "carbohydrate band" in the 1050–1000 cm^{-1} region. Differences in the shape of the $\nu_{as}S{=}O$ band for different mono- and poly-saccharide sulfates may be attributed to variations in the complexity of vibrations that give rise to this band. Even if the SO_3^- group is considered independently of the carbohydrate to which it is attached, it represents a system of three vibrationally coupled S–O bonds. These, in turn, are affected by changes in pseudosymmetry and local environment associated with different types of *O*-sulfate, as well as with *O-vs-N*-sulfate. Moreover, the contribution of one sulfate group to absorption in the 1230 cm^{-1} region is expected

to be influenced in polymers by interaction with other sulfate groups present on contiguous units along the chain.

Despite these limitations, the data in Table I suggest that the overall shape of the $\nu_{as}S{=}O$ band (as evidenced by the main absorption maxima and the approximate frequency of the band barycenter) is useful for distinguishing the type of sulfate group contributing to the absorption. Whereas the difference between primary and secondary *O*-sulfates is too small to distinguish between these groups in sulfated polysaccharides, the presence of *N*-sulfate should be detectable from the band (or inflexion) at ~ 1180 cm^{-1}. The observed shift of ~ 50 cm^{-1} relative to *O*-sulfate is attributable to the mesomeric effect of the α-amino group, which diminishes the double-bond character of the S=O bond. Accordingly, also, the $\nu_{s}S{=}O$ frequency, corresponding to the most intense peak in the Raman spectra, is lower for the *N*-sulfated than for the *O*-sulfated models; in the heparin spectrum, this peak is probably hidden in the strong non-resolved S=O peak.

The i.r. $\nu_{as}S{=}O$ band lends itself to quantitative applications. However, because of the complex nature of this vibration and differences in the chemical environment of sulfate groups, each glycosaminoglycan requires its own calibration curve of absorbance *vs* degree of sulfation. Possibly, more-generalized calibration curves could be obtained by using areas instead of absorbance values, and statistically sulfated polysaccharides (such as heparan sulfates) as standards; work along these lines is in progress[13].

In the region of the $\nu_{as}C{-}O{-}S$ vibration (950–800 cm^{-1}), not easily accessible in the i.r. because of severe solvent absorption, Raman spectra for solutions in water (and D_2O) show a number of medium-intensity peaks, two of which are usually prominent. However, no apparent relationship was found between these maxima and the type or orientation of sulfate group, which stresses the need for caution in using i.r. frequencies in this region for correlation purposes.

In conclusion, the i.r. and Raman spectra of glycosaminoglycans appear to be useful for characterizing these polysaccharides, the i.r. spectra being more indicative of the type and content of sulfate groups, and the Raman spectra more characteristic of the specific backbone structure. The non-destructive nature of the two approaches is an attraction for this class of biopolymers. As to sample requirements, i.r. examination using a standard cell requires at least 0.2 ml of a 5–10% solution; however, a reduction by ten- to fifty-fold can be realized if a microcell and a high-class (high-energy) instrument are used. Raman spectra may be obtained with only a droplet of a concentrated solution of the glycosaminoglycan; as pointed out in the Experimental, microsampling increases, rather than decreases, the detectability of Raman peaks. Thus, when routine methods are worked out for the elimination of fluorescent impurities, Raman spectroscopy of aqueous solutions promises to be a valuable technique for the qualitative and quantitative characterization of glycosaminoglycans. In this respect, both i.r. and Raman should supplement ^{1}H- and ^{13}C-n.m.r. spectroscopy, which only indirectly furnish information about sulfate groups in these biopolymers.

EXPERIMENTAL

Materials. — Samples of "M.P.S." glycosaminoglycans (chondroitin sulfates, keratan sulfate, heparan sulfate, and heparin) were supplied from the collection at the University of Chicago by J. A. Cifonelli. Other heparin samples were furnished by Upjohn (Kalamazoo, Mich., U.S.A.) (B-type[15], from beef lung), and by Syntex (Buenos Aires, Argentina) and L.D.O. (Milan, Italy) (A-type[15], from hog intestinal mucosa). *N*-Desulfated heparins were prepared from the L.D.O. A-type sample by treatment[16] with 0.04M HCl, and from the B-type sample as described previously[15]. Carboxyl-reduced heparin was prepared by M. Vincendon, using borohydride–carbodiimide[17]. Dextran was obtained from Fluka (Buchs, Switzerland), dextran sulfate (S, 16%) from Schuchardt (Munich, Germany), and D-glucose 6-sulfate from Miles Laboratories (Elkhart, Ind., U.S.A.). Methyl α-D-glucopyranoside 2-sulfate and 2-deoxy-2-sulfoamino-D-glucose were prepared by published procedures[18,19].

Measurement of spectra. — The i.r. spectra were recorded with a Perkin–Elmer 337 spectrophotometer. The i.r. spectrum of Fig. 3 was obtained with a Perkin–Elmer 180 spectrophotometer. Solutions were prepared in water (*c*, 3–10%) and filtered through Millipore membranes (0.45 μm). Cell windows were of IRTRAN-2 or KSR-5 (1, 0.050 mm). Due to the high refractive index of these materials, an interference fringe pattern is usually superimposed on the absorption spectrum, especially with new cells. Such a drawback was eliminated by gently scratching the inner surfaces of the windows with fine sand-paper before assembling the cells. Although windows of BaF_2 should be preferable, because they lose less energy through refraction, their use was avoided when it was observed that glycosaminoglycans are partially desulfated by exchange with the fluoride; such windows soon become coated with a layer of $BaSO_4$. The spectra of aqueous solutions were run either with a cell filled with H_2O in the reference beam, or (as for the spectra in Fig. 1e) with a beam attenuator inserted to compensate for the background absorption of water. The instrumental conditions were set for work with higher energy and gain than are ordinarily needed when using non-absorbing solvents. The solid-phase spectrum of heparin (Fig. 3) was recorded under normal instrumental conditions, from films cast on KRS-5 plates from aqueous (~1%) solutions.

Raman spectra were recorded with a Jarrel–Ash 25–300 spectrometer, using the 4880 Å (Ar) or 6471 Å (Kr) excitation lines from a coherent radiation 52MG Ar/Xenon laser. Most of the spectra were obtained with aqueous solutions, in a borate-glass capillary inserted in the focus of a microsampling optics system; the sample required was a single droplet of a concentrated (5–40%) solution in H_2O or D_2O. A few spectra were also recorded for the same solutions with normal (1 ml) cells and optics. In both cases, the spectra were obtained after a period of exposure of the solution to the laser beam, necessary for quenching the background fluorescence of the samples. Such a quenching was achieved, presumably, through photochemical degradation of unsaturated or aromatic trace-impurities in the samples, and was more efficient with the capillary cell–micro-optics arrangement than with the normal cells

and optics. In fact, fluorescence usually levelled-off to a satisfactory value in ~30 min when using the micro mode; with the normal mode, more than 4 h were generally required to reduce the fluorescence to a workable, though much less satisfactory, level. This slower decrease in the fluorescence background in the normal cells was probably due to continuous diffusion of fresh solution into the area exposed to the laser beam. Solid samples were run as powders, in a micro-pellet holder, using the microsampling optics; a satisfactory quenching of background fluorescence was usually achieved in 0.5 h.

The conductimetric analyses[14] were performed with a Derritron Model E conductimeter.

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