Infrared spectroscopy of heparins suggests that the region 750–950 cm⁻¹ is sensitive to changes in iduronate residue ring conformation

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By careful definition of polymer environment, heparin i.r. spectra were examined in a region (750–950 cm⁻¹) in which sulphate half-ester absorptions occur. Changes seen in this region when metal ion–heparin complexes are converted into heparinic acid, when heparin is carboxy-group-reduced and when various concentrations of Li⁺–heparin are examined are tentatively interpreted in terms of changes in the ring conformation of iduronate residues.

INTRODUCTION

The conformational flexibility of the iduronate residues of heparins, heparan sulphates and dermatan sulphates may permit versatility of ligand binding by these highly evolved glycosaminoglycans (Casu *et al.*, 1988). A ready interconversion of iduronate residue rings in ¹C₄ (chair) and ²S₀ (skew-boat) conformation has been proposed (Ragazzi *et al.*, 1986; Sanderson *et al.*, 1987). Previous reports suggest that, in Na⁺– and Ca²⁺–heparin complexes in ²H₂O solution (Gatti *et al.*, 1979; Boyd *et al.*, 1980) and in the solid state (Atkins *et al.*, 1974; Atkins & Nieduszynski, 1977; Nieduszynski *et al.*, 1977), the predominant (approx. 90%) conformer is the ¹C₄ form (or possibly a slightly distorted version of this). Casu *et al.* (1988) appear to interpret the results reported by Gatti *et al.* (1979) in terms of a ¹C₄/²S₀ ratio of 1.5:1. These estimates are based on average coupling constants derived from n.m.r. spectroscopy, which cannot resolve individual conformers undergoing fast exchange.

In contrast, i.r. vibrational spectroscopy not only deals with biologically relevant energy changes occurring on a short time scale (approx. 10⁻¹² s), but also enables small quantities of sample obtained from defined cell sources to be studied, allows study of solid, gel and dissolved samples, and permits the influence of such factors as counter-cation type and the molecular structure of water molecules associated with the polymer to be evaluated (Grant *et al.*, 1987a). Despite these advantages, and although there have been numerous studies of extracellular sulphated polysaccharides by vibrational spectroscopy (e.g. Orr, 1954; Suzuki & Strominger, 1960; Lloyd & Dodgson, 1961; Lloyd *et al.*, 1961; Cael *et al.*, 1973, 1976; Ovsepyan *et al.*, 1977, 1979; Cabassi *et al.*, 1978; Casu *et al.*, 1978; Bychkov *et al.*, 1981; Panov & Ovsepyan, 1984; Greer & Yaphe, 1984; Rochas *et al.*, 1986; Zablackis & Santos, 1986), the complex and variable nature of the spectra produced has particularly limited examination of the complex heparin and heparan sulphate glycosaminoglycans by this technique.

Although interpretation of spectra is facilitated by careful definition of polymer environment, and by use of glycosaminoglycan preparations that have been characterized chemically and enzymically (Grant *et al.*, 1987a, 1989), technical problems can still make spectral interpretation awkward: light-scattering by the polymer may complicate spectra in some cases, and the use

of an inert dispersant of suitable refractive index such as Nujol to avoid this causes superimposition of dispersant and polymer absorbances. Incorporation of samples into salt discs avoids this latter difficulty, but, for sulphated polysaccharides, is of limited use and can lead to irreproducible spectra. This problem has been attributed by Cabassi *et al.* (1978) to differences in the 'degree of order' induced in the solid specimens during sample preparation, but the spectra may also be influenced by cation substitution (Grant *et al.*, 1987a) and by cationic impurities in the salt disc. In order to overcome such technical difficulties, it has been suggested that solution-state spectra be used, the preferred solvent being ²H₂O (Casu *et al.*, 1978). ²H₂O absorptions are, however, superimposed on those of the solute and may be difficult to account for because of shifted solvent absorbance-band positions in the presence of the solute. It is also possible that, in ²H₂O solutions, the pattern of glycosidic-bond rotation and other conformational and configurational states may differ from those occurring in ¹H₂O, because of a stabilization by deuterium of bonding between adjacent carboxy and *N*-sulphonate groups (Grant *et al.*, 1987b). Finally, the use of ²H₂O precludes study of a region of the spectrum (750–950 cm⁻¹) thought to be sensitive to iduronate residue conformation. The present paper reports an i.r.-spectroscopic study of heparins under conditions allowing this region of the spectrum to be examined.

EXPERIMENTAL

Heparin, from which complexes with cations were prepared, was a pharmaceutical-grade preparation of *M_n* (number-average *M_r*) 15000 derived from pig intestinal mucosa (lot no. 008; Glaxo, Runcorn, Cheshire, U.K.). High-field ¹³C-n.m.r. spectroscopy (90.6 MHz) yielded a spectrum similar to those reported elsewhere for heparin preparations (Gatti *et al.*, 1979). Although the heparin was supplied as a sodium form, spark-source m.s. revealed the following additional elemental content (expressed as p.p.m.): Ca, 25000; Si, 5900; K, 2000; Mg, 1300; Fe, 1100; Cu, 730; P, 440; Ti, 390; Ba, 140; Zn, 80; Sr, 65; Cr, 30; B, 25; Ga, 20; Co, 8. X-ray powder diffraction analysis was consistent with the material being amorphous. The heparin was extensively dialysed against several changes of glass-distilled water.

N-Desulphonation involved incubation in dimethyl

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sulphoxide/ $^1\text{H}_2\text{O}$ (19:1, v/v) for 90 min at 50 °C (Nagasawa & Inoue, 1980). *N*-Desulphonation together with *O*-desulphation of heparin involved incubation in dimethyl sulphoxide/methanol (9:1, v/v) for 10 h at 100 °C (Nagasawa *et al.*, 1977; Nagasawa & Inoue, 1980). *N*-Resulphonation of *N*-desulphonated *O*-desulphated heparin involved use of a pyridine- SO_3 complex (Levy & Petracek, 1962; Lloyd *et al.*, 1964; Inoue & Nagasawa, 1973; Bruce *et al.*, 1985). Partially carboxy-group-reduced heparin was prepared by the methods of Taylor & Conrad (1972) and Taylor *et al.* (1980). *N*-Desulphonated *O*-desulphated *N*-acetylated heparin was prepared by the method of Nagasawa & Inoue (1980), with the use of acetic anhydride.

Heparan sulphates were extracted from surfaces of cultures of BHK-21 (C13) and polyoma-virus-transformed (PyY) counterpart cells by a procedure based on that of Underhill & Keller (1975), and involving trypsin treatment of cells and Pronase treatment, ion-exchange chromatography and chondroitin ABC lyase (EC 4.2.2.4) treatment of extracts.

Polymers were characterized by uronic acid determination (Blumenkrantz & Asboe-Hansen, 1973), acid-base titration, ^1H -n.m.r. and ^{13}C -n.m.r. spectroscopy and h.p.l.c. analysis of specific enzyme-derived oligosaccharide fragments. These procedures and the i.r. spectroscopy reported in this paper indicate the absence of protein and other glycosaminoglycans from the starting polymer. Further details of polymer modification and characterization were given by Moffat (1987). Polymers were converted into particular salt forms by passage through appropriate cation forms of Amberlite IR-120 cation-exchange resin. Spark-source m.s. showed a greater than 99% efficiency of the cation-exchange process.

For i.r. spectroscopy of solid samples, a Specac Ltd. (Orpington, Kent, U.K.) multiple-reflectance attachment (cat. no. 11030) for a Specac 25 reflection ATR optical accessory (no. 110300) was fitted to a Grubb Parsons mark III grating dispersive i.r. spectrometer. A 100 μg portion of glycosaminoglycan was deposited from aqueous solution on to an aluminium foil stretched tightly across the sample surface of the multiple-specular-reflectance sample-holder. Traditional i.r. solution cell techniques were unsuitable for recording reliable spectra of heparin solutions because of the chemical reaction of the glycosaminoglycan with the salt- (e.g. BaF_2)-based i.r. cell windows. Instead, spectra were obtained by enclosing a weighed quantity of solution in a cuvette made by stretching high-density polyethylene films of 2 μm thickness across a salt-disc sample-holder (part no. 2304; Specac Ltd.) A similar film, without sample, was placed in the spectrophotometer reference beam.

Assignments of i.r.-absorption bands at 937 cm^{-1} (Cael *et al.*, 1973), 1230 cm^{-1} (Orr, 1954; Cabassi *et al.*, 1978), 1430 cm^{-1} (Casu *et al.*, 1978) and 1635 cm^{-1} (Cael *et al.*, 1976) follow those of the authors noted. Bands in the 800–880 cm^{-1} spectral region of sulphated sugars have been attributed to S–O–C stretching (Orr, 1954). For heparin, a multiple band at about 800–820 cm^{-1} , tentatively ascribed to sulphate half-ester absorptions, has been reported (Bychkov *et al.*, 1981). In the present paper, this has been resolved into two principal components. Both remain after de-*N*-sulphonation, are removed after de-*O*-sulphation and are absent from spectra of de-*N*-sulphonated de-*O*-sulphated re-*N*-sulphonated and de-*N*-sulphonated de-*O*-sulphated *N*-acetylated heparins (D. Grant, W. F. Long & F. B. Williamson, unpublished work). One component, at 820 cm^{-1} , is ascribed to C–O–S stretching within glucosamine 6-*O*-sulphated residues (Orr, 1954; Suzuki & Strominger, 1960; Lloyd *et al.*, 1961). The other, at 800 cm^{-1} , we ascribe to C–O–S stretching within iduronate 2-*O*-sulphated residues in the reportedly predominant axial form (Sanderson *et al.*, 1987). A further band, very characteristic of heparin, occurs at about 890 cm^{-1} . It is decreased in intensity

upon de-*O*-sulphation and upon de-*N*-sulphonation, and is tentatively ascribed to sugar ring C–O–C stretching with some coupling component of C–O–S stretching.

RESULTS AND DISCUSSION

Iduronate residue 2-*O*-sulphate groups occur in an axial position when the sugar ring exists in the $^1\text{C}_4$ form, and in an equatorial position when the ring is in the $^2\text{S}_0$ (or $^4\text{C}_1$) form. The spectral range over which C–O–S group absorptions occur is 802–880 cm^{-1} (Orr, 1954). Fig. 1 shows the i.r. spectra, between 750 and 950 cm^{-1} , of various solid-state complexes of heparin and its derivatives with counter-cations. The difference spectra (*h*), (*j*) and (*l*), derived by subtracting from spectra of native and chemically modified heparin the spectra of appropriate polymers from which *O*-sulphate groups had been removed, confirm that major absorbances related to *O*-sulphate groups centre at about 800, 820 and 890 cm^{-1} . Non-identity of the difference spectra shows that the state of nitrogen substitution at glucosamine position 2 affects the ratio A_{800}/A_{820} . The absorbance at 820 cm^{-1} is ascribed to the 6-*O*-sulphate group of the glucosamine residue, and that at about 800 cm^{-1} to the iduronate residue 2-*O*-sulphate group in the reportedly predominant axial position (Sanderson *et al.*, 1987).

Like the state of glucosamine substitution, identity of metal counter-cation affects the A_{800}/A_{820} ratio and the precise frequency of the putative iduronate residue 2-*O*-sulphate band [Fig. 1, spectra (*b*), (*c*) and (*d*)]. When the metal ion is replaced by H^+ , the absorbance at 800 cm^{-1} is replaced by one occurring at about 870 cm^{-1} [spectrum (*a*), and difference spectrum (*m*)]. We interpret this displacement of the absorption to a higher value to represent the displacement of the 2-*O*-sulphate group from an axial to an equatorial position. Neutralization of anionic charge, alteration of hydration and an increased hydrogen-bonding capacity of the carboxy group might be the cause of the putative iduronate residue conformational change.

Fig. 2 shows the i.r.-absorption spectra between 750 cm^{-1} and 950 cm^{-1} of heparin, carboxy-group-reduced (37%) heparin and carboxy-group-reduced (83%) heparin. All polymers were in the solid-state Na^+ complex form. The carboxy-group-reduced forms show, as expected, decreased absorbance at 1430 cm^{-1} (this absorbance, in the unmodified polymer, is due to symmetric stretching of the carboxy group), and altered absorbance due to asymmetric carboxy-group stretching and water bending at 1635 cm^{-1} (results not shown). During the modification, the number of *O*-sulphate groups remains the same. The frequency of the C–O–S band at 820 cm^{-1} , attributed to the glucosamine residue 6-*O*-sulphate group, remains largely unaltered, as does the frequency (about 890 cm^{-1}) of the absorption probably due to C–O–S and ring C–O–C coupling. The absorbance due to S=O asymmetric stretching, at about 1230 cm^{-1} , is also largely unaltered (results not shown). In contrast, an absorbance at about 800 cm^{-1} in the spectrum of the unmodified polymer is replaced by one at about 874 cm^{-1} in the spectra of the modified polymers. Like the similar change seen when Na^+ -heparin is converted into heparinic acid, this suggests that an iduronate residue conformational change from a $^1\text{C}_4$ to a $^2\text{S}_0$ form may occur as a consequence of the decrease in anionic charge at C-6 of the sugar ring and an increased hydrogen-bonding capacity of that part of the molecule.

The spectrum of a solid-state hydrated film of the Li^+ -heparin complex, like those of several other metal cation-heparin complexes, includes an absorbance at about 800 cm^{-1} (Grant *et al.*, 1987*a*) assigned to the axially positioned iduronate residue 2-*O*-sulphate groups. This is consistent with the notion that the iduronate residue rings exist predominantly in a $^1\text{C}_4$ chair

Fig. 1. I.r. spectra of solid-state complexes of native and chemically modified heparins

(a) H^+ -heparin; (b) Ca^{2+} -heparin; (c) K^+ -heparin; (d) Na^+ -heparin; (e) Na^+ -(de-*N*-sulphonated de-*O*-sulphated re-*N*-sulphonated heparin); (f) Na^+ -(de-*N*-sulphonated de-*O*-sulphated heparin); (g) Na^+ -(de-*N*-sulphonated de-*O*-sulphated *N*-acetylated heparin); (h) spectrum (d) minus spectrum (e); (i) Na^+ -(de-*N*-sulphonated heparin); (j) spectrum (i) minus spectrum (f); (k) Na^+ -(de-*N*-sulphonated *N*-acetylated heparin); (l) spectrum (k) minus spectrum (g); (m) spectrum (d) minus spectrum (a).

conformation under these conditions. In contrast, the spectrum of an aqueous 4 mM solution of the Li^+ -heparin complex shows little absorbance at 800 cm^{-1} and substantial absorbance in the 862 cm^{-1} region (Fig. 3). As the concentration of the complex is increased, absorbance at 800 cm^{-1} is progressively established, and at high concentration the spectrum resembles that of hydrated films of the complex. Absorptions centred around 890 cm^{-1} (due to coupling of C-O-S and ring C-O-C) and at around 937 cm^{-1} (ascribed to a vibration containing components of a C-O-C glycosidic bond and C-O-S stretching) also show concentration-dependent alterations in intensity. The absorption of the glucosamine residue 6-*O*-sulphate group remains at about 827 cm^{-1} as the concentration is increased. These results suggest

that the 2S_0 (skew-boat) iduronate residue ring conformer may predominate in solutions of Li^+ -heparin of low concentration, and that an increase in concentration results in the progressive production of the 1C_4 form. Complexes of several other metal cations with heparin do not exhibit these concentration-dependent spectral changes (results not shown). Of all cations, Li^+ is most similar to H^+ in size and in ability to form covalent bonds with oxygen atoms; it binds to heparin particularly strongly (Dais *et al.*, 1988).

Like the changes seen when Na^+ -heparin is converted into heparinic acid and when iduronate residues are chemically reduced to idose, the results suggest that a decrease in anionic charge at C-6 of the iduronate residue ring may stabilize the 2S_0

Fig. 2. I.r. spectra of solid-state Na^+ complexes of native and carboxy-group-reduced heparins

(a) Heparin; (b) carboxy-group-reduced (37%) heparin; (c) carboxy-group-reduced (83%) heparin. The base-line of spectrum (b), which was produced in a different experiment from the other two spectra, has been adjusted so as to allow plotting on the same axes.

conformational form of this sugar. In addition, Li^+ evidently elicits (perhaps through Li^+ -concentration-dependent hydrogen-bonding) a concentration-dependent alteration of the amounts of the different iduronate residue conformers present. These peculiarities may be clinically relevant: the rate of heparin-stimulated antithrombin III-thrombin interaction is apparently less in the presence of Li^+ than in the presence of Na^+ or K^+ ions in solutions of similar ionic strength (Petersen & Jorgensen, 1983).

Fig. 3. I.r. spectra of aqueous solutions of Li^+ -heparin

Concentrations of Li^+ -heparin complex were: (a) 4 mM; (b) 8 mM; (c) 17 mM; (d) 22 mM; (e) 45 mM; (f) 80 mM.

Fig. 4 shows the i.r.-absorption spectra of solid-state Na^+ complex forms of normal and transformed cell-surface heparan sulphates extracted and examined under identical conditions of pH (7.5) and ionic strength (5%, w/v, NaCl). The absorbance at about 820 cm^{-1} , attributed to the glucosamine residue 6-*O*-sulphate group, is decreased in intensity in the sample obtained from the surfaces of transformed cells. This accords with information obtained by conventional chemical analyses (Winterbourne & Mora, 1981). An absorbance that centres at about 790 cm^{-1} in the spectrum of the normal cell sample is less prominent in the spectrum of the transformed cell sample, in which increased absorption at about 880 cm^{-1} is seen. This change may reflect an alteration in the conformation of the

Fig. 4. I.r. spectra of solid-state Na^+ complexes of cell-surface heparan sulphates

(a) BHK cells; (b) PyY cells.

iduronate residue rings. N.m.r.-spectroscopic studies of model oligosaccharides suggest that structures having lowered *O*-sulphation possess a greater proportion of the $^2\text{S}_0$ conformer (Sanderson *et al.*, 1987). The lowered *O*-sulphation of transformed cell heparan sulphates may therefore be a contributory factor in producing an altered pattern of iduronate residue ring conformation.

These studies suggest that i.r.-spectroscopic analysis of carefully prepared well-defined glycosaminoglycan samples may provide valuable information about iduronate residue conformations that complements information provided by other more widely used techniques.

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