Infrared spectroscopy of heparin–cation complexes

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Hydrated and partially hydrated films and aqueous solutions of heparin, heparans and *N*-desulphated preparations of these polymers were studied by near- and fundamental-region-i.r. spectroscopy in the presence of a range of counteranions. The results suggest that ion binding is not explicable solely in terms of simple electrostatic theory, and that specific cation effects, and the hydration pattern of the polymer–cation complex need to be taken into account.

INTRODUCTION

Heparins and heparans are glycosaminoglycans composed of substituted repeating disaccharide units of D-glucosamine and uronic acid. They possess many chemically distinct cation-binding groups, the stereochemical dispositions of which permit complex coordination chelation of a wide range of inorganic and organic cations. Possible modulations of heparin and heparan activities by metal ions *in vitro* and *in vivo* include effects of Na⁺ (Diakun *et al.*, 1978), K⁺ (Petersen & Jørgensen, 1983), Ca²⁺ (Long & Williamson, 1982), Zn²⁺ (Reyes *et al.*, 1983) and Cu²⁺ (Alessandri *et al.*, 1983). Heparans contain fewer sulphate groups, more *N*-acetyl groups, more glucuronate residues and fewer iduronate residues than do heparins. Further, heparins and probably heparans contain particular oligosaccharide sequences that bind specific proteins.

Results of studies of heparin–counteranion interactions in dilute solution have been interpreted either in terms of purely electrostatic interactions or in terms of additional chemical interactions resulting in cations binding to specific sites on the polymer (Liang & Chakrabarti, 1982). The Manning electrostatic theory (Manning, 1969*a,b,c*) predicts condensation of counterions when a critical density of charge along a linear charged polymer molecule is exceeded, and further suggests a critical counterion concentration, below which this occurs, and above which the counterions exist in an ion atmosphere. Ion condensation in this model is independent of the chemical nature of the polyelectrolyte charge site and, except for its charge, independent of the chemical nature of the counterion.

A dependence on Na⁺ concentration of the diffusion rates of the ions in aqueous heparin solutions lacking additional electrolyte (Ander & Lubas, 1981; Ander & Kardan, 1984), and an apparent polyanionic charge of heparin smaller than that predicted by electrostatic theory (Tivant *et al.*, 1983), suggest that all heparin–cation interactions may not be explained by simple electrostatic interactions. In the latter study, the possibility of the existence of ‘hydrodynamic interactions’ was discussed. Certainly any changes occurring in the hydration patterns of the polyanion during cation binding would need to be accounted for in the formulation of models of heparin–counterion interaction. Previously used experimental methods have not

allowed the occurrence of any such changes to be unequivocally assessed.

I.r. spectroscopy offers a method of directly assessing those molecular structures that may be involved in polyelectrolyte–cation interactions, and can be used to study very-short-lived species that might elude detection in n.m.r. studies. Near-i.r. spectroscopy can highlight any changes occurring in polymer hydration patterns during polyanion–cation interaction. We have briefly reported the i.r. spectra of several heparin–cation complexes (Grant *et al.*, 1983, 1984, 1985). Also, an i.r. and ¹³C-n.m.r. study of a range of heparin–metal cation complexes (Panov & Ovsepyan, 1984) accords with the present report.

The present paper reports a study of a selected range of such complexes by wide-range i.r. spectroscopy.

EXPERIMENTAL

Heparins and heparans

Heparin, from which complexes with cations were prepared, was a pharmaceutical-grade preparation of *M_n* (number-average *M_r*) 1.5×10^4 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 mass spectrometry 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 and then converted into different salt forms by passage through appropriate cation forms of Amberlite IR-120 cation-exchange resin. Spark-source mass spectrometry showed a greater than 99% efficiency of the cation-exchange process.

Heparans were prepared from bovine lung heparin by-products given by the Upjohn Co., Kalamazoo, MI, U.S.A., from rat livers (Pearce & Mathieson, 1967) and from cultured baby-hamster kidney (BHK) cells by the method of Underhill & Keller (1975). *N*-Desulphation of heparins and heparans was carried out by using the

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Fig. 1. I.r. spectra (fundamental band region) of heparin-cation complexes

Traces of original spectra are superimposed to facilitate comparison. Spectra are of complexes with heparin of (a) $(\text{CH}_3)_4\text{N}^+$, (b) Tl^+ , (c) K^+ , (d) Na^+ , (e) Li^+ (f) Ca^{2+} , (g) Ba^{2+} , (h) Zn^{2+} , (i) Cu^{2+} and (j) H^+ .

method of Nagasawa & Inoue (1980). Defined salt forms of heparans and of *N*-desulphated heparins and heparans were prepared on cation-exchange resin as described above.

Water content of the various polymer-cation preparations was determined by continuous-wave ^1H -n.m.r. spectroscopy.

I.r. spectroscopy

I.r. spectra were obtained with a Grubb Parsons Spectromaster mark 3 grating spectrometer. Polymer films (10–100 μm thickness) were cast on borosilicate glass and on high-density polyethylene over molecular sieves in a desiccator. Dehydrated films were prepared by using dry deoxygenated N_2 . They were subsequently handled in a dry glove bag, where they were mounted in cells with BaF_2 windows. Film opacity, when it occurred,

Fig. 2. Variation of carboxylate stretching frequency with radius of unhydrated cation

Values of cation radii are from C.R.C. Handbook of Chemistry and Physics, 47th edition (Chemical Rubber Co., Boca Raton), except for that of $(\text{CH}_3)_4\text{N}^+$, which was reported by Masterton *et al.* (1971).

was eliminated by treatment of films with a trace of Nujol. Solutions were studied with the use of BaF₂ or polyethylene spectrometer-cell windows. Band assignments were made with regard to previously published assignments (Cabassi *et al.*, 1978; Casu *et al.*, 1978). In addition assignment of bands at 1230 cm⁻¹, and in concentrated solutions at 1185 cm⁻¹, to *N*-sulphonate groups was made by comparing spectra of heparin, heparan, *N*-desulphated heparin and *N*-desulphated heparan, and of their cetylpyridinium complexes (Pearce & Mathieson, 1967).

RESULTS

Analysis of entirely hydrated films of heparin-cation complexes

Spectra in the i.r. fundamental region (400–1750 cm⁻¹) of heparinic acid and of nine heparin-cation complexes

Fig. 3. Variation of H-O-H bending vibration frequency with radius of unhydrated cation

Sources of values of cation radii are given in the legend to Fig. 2.

Fig. 4. Near-i.r. spectra of Na⁺-, Ca²⁺- and Zn²⁺-heparin complexes as films with different degrees of hydration

Traces of original spectra are superimposed to facilitate comparison. Spectra are of complexes with heparin of (a)–(d) Na⁺, (e)–(i) Ca²⁺ and (j)–(o) Zn²⁺. Number of water molecules present/disaccharide unit: (a) 10, (b) 5, (c) 3, (d) 1.5, (e) 4, (f) 2, (g) 1, (h) 0.5, (i) 0, (j) 8, (k) 6, (l) 4, (m) 3, (n) 1.5 and (o) 0.

are shown in Fig. 1. The frequency ($1410\text{--}1470\text{ cm}^{-1}$) at which the band due to the carboxylate carbonyl symmetric stretching absorption occurred varied systematically with the unhydrated radius of the cation (Fig. 2). Complexes of heparin with Mg^{2+} yielded hydrated films of insufficient transparency to permit direct spectroscopic examination. However, examination of Nujol mulls of these solids allowed the frequency at which the carboxylate carbonyl symmetric stretching band occurred to be determined (value included in Fig. 2). pH changes occurred during the formation of complexes between cations and heparin. For example, replacement of Li^+ by equivalents of Cu^{2+} or Ca^{2+} in aqueous solution at pH 7.0 resulted in a decrease in pH of 1.5 and 1.0 units respectively. Because such changes might have contributed to the observed frequency shifts of the carboxylate band, films were prepared from solutions at different pH. Variation in pH, in the range 4.5–10, did not affect the frequency at which this band occurred. This suggests that pH changes in this range have no major effect on the energy of the carboxylate groups.

In contrast with the effects observed on the carboxylate band frequency, there was little dependency on cation of the frequency (1230 cm^{-1}) at which the sulphate asymmetric stretching absorption occurred (Fig. 1). No separate band attributable to *N*-sulphonate was detectable under the conditions used. However, a pronounced cation-dependency of the frequency (around 1630 cm^{-1}) and intensity of the band arising from the water bending mode was observed (Figs. 1 and 3). A further observation suggesting an involvement of water in heparin-ion interaction was the broadening of a multiplicity of bands in the fundamental region (Fig. 1). This is reminiscent of spectral changes caused by strong hydrogen-bonding between metal ions, water molecules and the sulphonate groups of polysulphonates (Zundel & Metzger, 1968).

Under a variety of conditions of heparin film preparation, decreases in the band at 1230 cm^{-1} occurred. Control experiments suggested that this effect was due to the orientation of hydrated sulphate groups at polar surfaces. The effect was especially pronounced in films prepared from solutions at pH 7.0. This phenomenon was not dependent upon ion binding to the polymer and was not observed with films cast on non-polar high-density polyethylene.

Analysis of films of heparin-cation complexes of different water content

Because of the possible involvement of water in heparin-metal ion complexes, films of such complexes, from which water had been wholly or partially removed by controlled heating under dry N_2 , were analysed by near-i.r. spectroscopy (Fig. 4). A dependency on cation of the frequencies of the water overtone (around $6400\text{--}7000\text{ cm}^{-1}$) and combination (5200 cm^{-1}) bands has already been briefly reported (Grant *et al.*, 1983).

In the case of the sodium heparinate complex, the frequency of the first overtone hydroxy stretching band progressively shifted from 7000 cm^{-1} towards 6400 cm^{-1} as the less strongly hydrogen-bonded water was removed. The Na^+ -polymer-water complex appeared to be particularly resistant to dehydration. Complete dehydration of this complex was resisted even when it was heated under dry deoxygenated N_2 at 450°C . In contrast, dehydration of the Ca^{2+} and Zn^{2+} complexes

Fig. 5. I.r. spectra of Na^+ -heparin complex in water

Traces of original spectra are superimposed to facilitate comparison. Concentrations (% w/v) of complex were (a) 5, (b) 6, (c) 10, (d) 16, (e) 32 and (f) 40.

occurred at 50°C . Whereas the spectra of the Ca^{2+} form showed simple low-energy hydrogen-bonding, the Zn^{2+} form showed more complex behaviour, possibly due to altered co-ordination of the water molecules in an inner-orbital complex with the Zn^{2+} .

Analysis of aqueous solutions of sodium heparinate

Fig. 5 shows i.r. spectra in the $1150\text{--}1500\text{ cm}^{-1}$ region of aqueous solutions of heparinate for a range of polymer concentrations. In contrast with the results reported for the polymer-cation films, spectra of more

Fig. 6. I.r. spectra (fundamental band region) of heparin and heparan complexes

Spectra: (a) Na^+ -*N*-desulphated heparin; (b) Na^+ -heparin in Nujol; (c) Na^+ -*N*-desulphated heparin in Nujol; (d) subtraction of spectrum (c) from spectrum (b), giving position of N-S=O bands; (e) Ba^{2+} -heparin (bovine lung); (f) Na^+ -heparan (bovine lung); (g) cetylpyridinium-heparan (bovine lung); (h) Na^+ -heparan (rat liver); (i) Na^+ -heparan (BHK cells); (j) Na^+ -*N*-desulphated heparan from BHK cells; (k) cetylpyridinium-heparin; (l) cetylpyridinium-*N*-desulphated heparin; (m) cetylpyridinium chloride. Spectra, unless stated, were of transparent films.

dilute aqueous solutions revealed increased resolution of a shoulder at 1185 cm^{-1} , corresponding to the band attributed to *N*-sulphonate of heparin in $^2\text{H}_2\text{O}$ (Cabassi *et al.*, 1978). In agreement with these authors we found that solutions of polymers in $^2\text{H}_2\text{O}$ also showed enhanced 1185 cm^{-1} bands.

Analysis of films and aqueous solutions of heparans and *N*-desulphated polymers

I.r. spectra of heparans and of *N*-desulphated polymers were studied in order to allow assessment of the contribution of *N*-sulphonate groups to polymer-cation interactions (Fig. 6). In dilute aqueous solutions of heparins (polymer concentration less than 10%, w/v) a band at 1185 cm^{-1} was seen, corresponding to the shoulder previously assigned to the *N*-sulphonate group (Fig. 5). At higher concentrations (more than 10%, w/v; Fig. 5) and in hydrated heparin-cation films (Fig. 6), this band was hardly evident, being replaced by one at 1230 cm^{-1} . Neither of the absorptions at 1185 cm^{-1} and 1230 cm^{-1} , attributable to *N*-sulphonates, was observed in the spectrum of heparan that had been *N*-desulphated

(Fig. 6). Other bands due to the *O*-sulphate groups present in heparins (around 1260 cm^{-1}) were also evident.

Effects of interaction with an organic base

Cetylpyridinium chloride-polymer complex-formation was accompanied by a large increase in the 1185 cm^{-1} band attributable to *N*-sulphonates in heparin and heparan. Spectra of similar complexes formed after *N*-desulphation showed that the 1185 cm^{-1} band had been eliminated (Fig. 6).

The interaction of poly-L-lysine and poly-L-arginine with heparin was also accompanied, under conditions leading to precipitate formation, by a decrease in the frequency of the *O*-sulphate group band (results not shown).

DISCUSSION

Interaction of a range of cations with heparin affected the frequency of the main carboxylate carbonyl symmetric stretching. This suggests the direct involvement of

Fig. 7. Variation of carboxylate stretching frequency with cation polarizing power

Polarizing powers were calculated from the following equation (Wait & Janz, 1963):

$$\text{Polarizing power} = (z/r)(5z^{1.27}/r^{0.5}I)$$

where z is ionic charge, r is ionic radius in Å (0.1 nm) and I is ionization potential in volts; these data were obtained from CRC Handbook of Chemistry and Physics, 47th edition (Chemical Rubber Co., Boca Raton).

the carboxy groups of heparin in cation interaction, and is in agreement with results of ^{23}Na , $\text{Gd}^{3+}/^{13}\text{C}$, ^1H and ^{13}C n.m.r. and chiroptical studies of the interaction of heparin with various metal ions (Herwats *et al.*, 1978; Casu *et al.*, 1975; Boyd *et al.*, 1980; Liang & Chakrabarti, 1982). The systematic variation of the vibrational energy of this group with the unhydrated radius of the cation is not predicted by simple Manning theory, and this suggests that the change in the carboxylate group associated with ion binding involves interactions in addition to those that are purely electrostatic, at least under the moderately high polymer concentrations reported in the present study.

When the absorption maxima used to obtain Fig. 3 were plotted against the polarizing power of the cation, perturbation of the stretching energy of the carboxylate groups was shown to be greater for the Cu^{2+} complex, and less for the Ca^{2+} , Ba^{2+} and Tl^{+} complexes, than is predicted by a linear dependence of band frequency on the polarizing power of the cations (Fig. 7).

Under the experimental conditions used here, little cation-dependent change was observed in the ionic sulphate asymmetric stretching frequency at 1230 cm^{-1} for most heparin-cation complexes (Fig. 1). However, Cu^{2+} and Tl^{+} were notable exceptions, with shifts in frequency of the order of 10 cm^{-1} .

A discrete band attributable to *N*-sulphonate was not evident in spectra of hydrated and partially dehydrated films of heparin-cation complexes. However, analysis of heparans, of preparations of heparin and heparans which had been *N*-desulphated and of aqueous solutions of heparins and heparans showed that frequency shifts as great as 45 cm^{-1} occurred when the *N*-sulphonate environment was changed.

In dilute aqueous solutions of heparin and heparan at low ionic strength (Fig. 5), when quaternary amino salts were added to heparin (results not shown), when heparin was precipitated with cetylpyridinium chloride [Fig. 6, spectra (g), (k) and (l)] and in solutions of heparin in $^2\text{H}_2\text{O}$ (Cabassi *et al.*, 1978; confirmed, but not shown, in the present study) an *N*-sulphonate band appeared at 1185 cm^{-1} . In the present study, in addition to the effect of the cation on the frequency at which the carboxylate carbonyl symmetric stretching absorption band occurred, a pronounced increase in the intensity of absorption of this band was observed. In contrast, in spectra of more concentrated solutions of heparin and heparan at higher ionic strength, and in hydrated and partially hydrated heparin films, no *N*-sulphonate band at 1185 cm^{-1} was observed. Under these conditions this group presumably absorbed at a frequency of 1230 cm^{-1} and was therefore obscured by the *O*-sulphate bands in the case of heparin, but not in the case of heparan.

Although cation-dependency on the frequency of the carboxylate band was observed in circumstances under which *N*-sulphonate groups absorbed at 1230 cm^{-1} , a pronounced change in the intensity of absorbance at this frequency did not occur. However, in these circumstances, a cation-dependent broadening of a multiplicity of bands in the fundamental region was observed, suggesting the possibility of the presence of strong hydrogen-bonding similar to that involved in polysulphonate-water-metal ion interaction (Zundel & Metzger, 1968). Also, a cation-dependent variation in the frequency (around 1630 cm^{-1}) of the water bending mode and in the frequency (around 6400 cm^{-1}) of the overtone bands was observed. These results suggest a general involvement of water molecules in heparin-cation interaction.

It is likely that *O*-sulphate groups are involved in this putative heparin-water-metal ion interaction because of the demonstrated correspondence between *O*-sulphate content of sulphated polysaccharides and the amount of water binding to the polymer (Atkins *et al.*, 1974; Gekko & Noguchi, 1974). In agreement with the results reported by Gekko & Noguchi (1974), we have demonstrated a decreased tendency for cation-dependent broadening of fundamental bands, for cation-dependent variation of the water bending mode and for cation-dependent variation in overtone bands in glycosaminoglycans of lower *O*-sulphate content than heparin (results not shown).

Although the details of heparin-cation interaction mechanisms remain to be elucidated, the present study suggests that considerations of simple electrostatic effects, devoid of hydration and specific cation effects, are likely to be inadequate.

We gratefully acknowledge the continuing financial support of the Cancer Research Campaign and of the Nuffield Foundation. We thank Mr. Colin Moffat for preparation of the thallium salt of heparin, Dr. Allan Ure and Dr. John Bacon of

the Macaulay Institute for Soil Research, Aberdeen, for carrying out the spark-source mass spectrometry, and Dr. Ian Sadler and Dr. David Reid of the Science and Engineering Research Council high-field n.m.r. service in the University of Edinburgh for carrying out the n.m.r. spectroscopy.

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Received 3 November 1986; accepted 9 February 1987