Determination of the $pK_a$ of glucuronic acid and the carboxy groups of heparin by $^{13}$C-nuclear-magnetic-resonance spectroscopy

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As part of our continuing studies on heparin, the present paper uses $^{13}$C-n.m.r. spectroscopy to examine the acidity of heparin's uronic acid carboxylate groups. Heparin contains three different uronic acids. In porcine mucosal heparin these account for approx. 91, 7 and 2 mol % of the total uronic acid residues. These are $\alpha$-l-idopyranosyluronic acid 2-sulphate, $\beta$-l-glucopyranosyluronic acid and $\alpha$-l-idopyranosyluronic acid. The $pK_a$ values of their carboxylate groups were determined as 3.13 (using heparin), 2.79 (using heparin) and 3.0 (predicted by using model compounds) respectively. $^{13}$C-n.m.r. spectroscopy, performed at various pH values, provided a convenient method of simultaneously determining the $pK_a$ of multiple carboxylate groups, of similar acidity, within heparin. $\beta$-Glucopyranosyluronic acid and heparin-derived di-, tetra- and hexa-saccharides were used as model compounds to determine $pK_a$ values of the different carboxy groups. These results suggested that molecular size had an effect on $pK_a$. Unambiguous assignment of carboxyl carbon resonances were accomplished through the use of two-dimensional n.m.r. spectroscopy. Finally, application of this method to the simplest model compound, $\beta$-glucopyranosyluronic acid, permitted the determination of the $pK_a$ of both its $\alpha$- and $\beta$-anomers.

INTRODUCTION

Despite its widespread use for over 50 years as an anticoagulant, heparin’s precise structure and certain of its physical, chemical and biological properties are still not well understood. Interest in heparin by the medical and scientific community has heightened in the past decade as a result of the discovery of a variety of new biological activities attributable to heparin (Linhardt & Loganathan, 1990; Lane & Lindahl, 1989). Heparin is a polydisperse, highly sulphated polysaccharide composed of repeating 1→4-linked uronic acid and glucosamine residues (Casu, 1985). Glycosaminoglycan heparin has an average

\[ \text{X = H or SO}_4^{2-} \]
\[ \text{Y = SO}_3^- \text{ or COCH}_3 \]

Abbreviations used: SP-, sulphopropyl; DSS, 3-(trimethylsilyl)propane-1-sulphonic acid sodium salt; SAX, strong anion exchange; COSY-45, correlation spectroscopy (45° pulse); INEPT, insensitive nuclear enhancement by polarization transfer; HMBC, heteronuclear multiple bond correlation spectroscopy; n.O.e., nuclear Overhauser effects; AUAS, 4-deoxy-$\alpha$-l-threo-hex-4-enopyranosyluronic acid 2-sulphate; IdoA2S, $\alpha$-l-idopyranosyluronic acid 2-sulphate; IdoA2S, $\beta$-l-glucopyranosyluronic acid.

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The heparin polymer has a high degree of structural variability (I). Biosynthetic studies suggest that not all of the possible structural variants occur in heparin (Lindahl et al., 1986), and oligosaccharide-mapping experiments confirm the presence of between eight and 12 different disaccharides (Linker & Hovingh, 1984; Merchant et al., 1985; Linhardt et al., 1988). Heparin represents an attractive, but challenging, target for scientific studies because of its structural complexity, including both its microheterogeneity and polydispersity.

The heparin polyanion is one of the most acidic molecules found in nature (Linhardt & Loganathan, 1990). Its biological activities result from the binding of various proteins to these anionic sites (Casu, 1985; Lane & Lindahl, 1989; Linhardt & Loganathan, 1990). Fairly recently it was reported that many heparin-binding proteins contain highly conserved peptide sequences. These consensus peptides are purportedly responsible for heparin binding to proteins (Cardin & Weintraub, 1989). Thus a better understanding of the acidity of heparin's anionic sites is necessary to develop fully structure-activity relationships.

There are three types of acidic functional groups in the heparin polymer. The sulphate monoesters and the sulphamido groups are both highly acidic, having pKₐ values ranging from 0.5 to 1.5 as measured by conductimetric titration (Casu & Gennaro, 1975). Less acidic are the carboxylic groups of the various unsulphated and monosulphonated uronic acid residues, having pKₐ values of between 2 and 4 as determined by titration (Casu & Gennaro, 1975). These weaker acids could exist as both protonated and non-protonated forms in certain physiological environments. They have also been shown to play a role in heparin binding to biologically important proteins such as antithrombin III (Van Boeckel et al., 1987).

Standard titrimetric methods of measuring pKₐ are incapable of distinguishing between the different carboxy groups within the heparin polymer. C.d. studies (Park & Chakrabarti, 1978) and ¹H-n.m.r. spectroscopy (Gatti et al., 1979) afford an indirect measurement of pKₐ.¹³C n.m.r. (Gatti et al., 1979) provides a more direct method capable of differentiating between different carboxy groups. We report the application of ¹³C n.m.r. to determine simultaneously the pKₐ of multiple carboxy groups within heparin and other ionic acid-containing molecules. This approach makes use of modern pulsed-n.m.r. methods, including selective INEPT (insensitive nucleus enhancement by polarization transfer; Bax, 1984), two-dimensional COSY-45 (correlated spectroscopy (45° pulse); Nagayama et al., 1980) and HMBC (heteronuclear multiple bond correlated spectroscopy; Bax & Summers, 1986) to establish unambiguous spectral assignments. D-Glucopyranosyluronic acid and heparin-derived di-, tri-, and hexa-saccharides are used as model compounds to determine the pKₐ of rare but biologically important uronic acid residues in the heparin polymer.

**EXPERIMENTAL**

**Materials**

The sodium salt of heparin from porcine intestinal mucosa (157 USP (United States Pharmacopoeia) units/ml) was obtained from Hepar Industries, Franklin, OH, U.S.A. D-Glucuronic acid was from Sigma Chemical Co., St. Louis, MO, U.S.A. Bio-Gel P2 was from Bio-Rad, Richmond, CA, USA. Sulphopropyl (SP)-Sephadex-C50 was from Pharmacia, Piscataway, NJ, U.S.A. Spectrorepe 3500 dialysis membrane was from Spectrum Medical Industries, Los Angeles, CA, U.S.A. Spherisorb (5 μm particle size) 2.5 cm × 25 cm (semi-preparative) and 0.46 cm × 25 cm (analytical) strongly anion-exchange (SAX) h.p.l.c. columns were from Phase Separations, Norwalk, CT, U.S.A. NAoH (0.100 m) and standard pH solutions (pH 2, 4, 7 and 10) were from Fisher Scientific, Fair Lawn, NJ, U.S.A. H₂O (99.96 atom % ¹H), NaO⁻H solution (40 wt. % at 99+ atom % ¹H), HCl (37 wt. % at 99+ atom % ¹H) and 3-(trimethylsilyl)propene-1-sulphonic acid sodium salt (DSS) were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. The pH was measured on a Solution Analyzer (Model-450-3-A) from Amber Science, San Diego, CA, U.S.A., using an ultra-thin extra-long pH probe from Aldrich. U.v. spectroscopy was performed on a Shimadzu (Tokyo, Japan) model UV-160 spectrophotometer. Titrations were done on a Multi-Dosimat E415 titrator from Metrohm, Herisau, Switzerland.

**Methods**

**Preparation of heparin.** Heparin (6 g in 120 ml of water) was dialysed overnight against 20 vol. of distilled water using 3500-Mₜ cut-off dialysis membrane. After three changes of distilled, the sample was freeze-dried and stored at room temperature in the desiccator for further use.

**Preparation of oligosaccharide standards.** Heparin (5 g) was dissolved in 250 ml of 50 mM-sodium phosphate buffer, pH 7.0. Purified heparin lyase [4 units (Yang et al., 1985)] free of catalytic impurities (Linhardt et al., 1984) was added and the reaction mixture was incubated at 30 °C for 84 h. After the completion of the depolymerization reaction, the mixture was adjusted to pH 2.5 and passed through a column (15 cm × 0.5 cm) of SP-Sephadex to remove protein. After re-adjusting the pH to 7.0, the sample was desalted on a column (2.5 cm × 38 cm) of Bio-Gel P2, freeze-dried, and reconstituted at 100 mg/ml in distilled water. The initial purification of the oligosaccharide standards was performed by 75-100 mg injections on to a semi-preparative SAX h.p.l.c. column (2.5 cm × 25 cm) as described previously (Rice & Linhardt, 1989). Fractions were combined, freeze-dried and desalted, using the Bio-Gel P2 column, then freeze-dried again. The trisulphated disaccharide (1.75 g) was > 95% pure by analytical SAX h.p.l.c. (Linhardt et al., 1988) and gradient PAGE (Rice et al., 1987). The hexasulphated tetrasaccharide (150 mg) and heptasulphated hexasaccharide (110 mg) required an additional preparative SAX h.p.l.c. and desalting step to obtain 100 and 80 mg of tetrasaccharide and hexasaccharide, each of > 95% purity.

**Titration of glucuronic acid.** Aqueous glucuronic acid (5 ml at 1 M) was titrated with 1 M-NaOH standard in a Multi-Dosimat E415 titrator of 20 °C. The 79 data points obtained were analysed by the method of Gran (Gran, 1950; Boiani, 1986) to obtain the pKₐ.

**Preparation of samples for n.m.r. analysis.** Each sample was dissolved in H₂O (99.96 atom %) and freeze-dried. This exchange was performed three times, after which the sample was dissolved in H₂O (99.96 atom %), containing 42 mM-DSS, for n.m.r. studies. Samples were prepared either in 10 mm- or 5 mm-outerdiameter tubes at the following concentrations: heparin, 38, 19 and 9.5 mM (1600, 800 and 400 mg in 3 ml respectively); hepta-sulphated hexasaccharide (V), 106 mM (78 mg in 0.4 ml); hexa-sulphated tetrasaccharide (V), 81 mM (43 mg in 0.4 ml); tri-sulphated disaccharide (III), 262 mM (70 mg in 0.4 ml); and D-glucopyranosyluronic acid (II), 1.031 M (100 mg in 0.5 ml). The initial pH of each sample was measured using a pH probe by a
13C-n.m.r. determination of heparin's pKₐ values

Meter calibrated with an appropriate set of pH standards. (After each n.m.r. experiment the p\(^+\)H was again measured to ensure that it had not changed.) The p\(^+\)H measurement was made 5 s after immersing the probe into the sample. The initial p\(^+\)H (calculated from eqn. 1 below) of each sample was between 3.5 and 6.0. The p\(^+\)H was then adjusted downwards with \(\text{HCl} \) in \(\text{H}_2\text{O} \) (12 m) to the lowest p\(^+\)H value required. After n.m.r. analysis, the sample's p\(^+\)H was adjusted upwards with \(\text{NaOH} \) in \(\text{H}_2\text{O} \) (10 m) and the next spectrum was obtained. This process was repeated to obtain all the points on each titration curve. The volume change of any given sample throughout the data collection was < 5%.

N.m.r. spectroscopy. Spectra were obtained on three instruments: a Bruker NR/80 \((\text{H} \text{ at } 80 \text{ MHz} \text{ and } 13\text{C} \text{ at } 20.1 \text{ MHz})\) for the glucuronic acid titration curve, a Bruker W/M360 spectrometer \((\text{H} \text{ at } 360 \text{ MHz} \text{ and } 13\text{C} \text{ at } 90.56 \text{ MHz})\) for oligosaccharides and heparin and a Bruker WM 600 \((\text{H} \text{ at } 600 \text{ MHz} \text{ and } 13\text{C} \text{ at } 150.9 \text{ MHz})\) for assigning the carbon resonances of carboxy groups of the \(\alpha\) and \(\beta\)-anomeric form of d-glucopyranosyluronic acid. Broad-band-decoupled 13C n.m.r. spectra were measured for each sample at various p\(^+\)H values. All spectra were run at 29 °C, and the measurement time for each carbon spectrum ranged from 8 to 16 h. The three carboxy groups in the hexasaccharide (IV) were unambiguously assigned using selective INEPT spectroscopy (Bax, 1984). The two-dimensional COSY-45 spectrum (Nagayama et al., 1980) of the hexasaccharide (V) was obtained using standard Bruker software. The carboxy-carbon resonances of \(\alpha\) and \(\beta\)-anomers of d-glucopyranosyluronic acid (II) were assigned by two-dimensional HMBC spectroscopy (Bax & Summers, 1986) optimized for 14.3 Hz running standard Bruker software.

RESULTS

D-glucopyranosyluronic acid (II), a mixture of \(\alpha\) and \(\beta\)-anomers, is commercially available and was used to demonstrate the utility of 13C n.m.r. to determine pKₐ. Titration of II in water (1 m) at 20 °C gave a pKₐ of 2.96, slightly lower than the literature values of 3.28 (20 °C) and 3.20-3.33 (25 °C) (Kohn & Kovac, 1978) determined by potentiometric titration. Monoosaccharide II was \(\text{H}_2\text{O}\)-exchanged and dissolved at 1.03 m in \(\text{H}_2\text{O}\) in the presence of DSS as internal standard. The p\(^+\)H of the sample was measured using a pH-meter that had been calibrated with pH standards. The actual p\(^+\)H was calculated using eqn. (1) (Glaser & Long, 1960; Jencks, 1989):

\[
p\text{H} = p\text{H} \text{ (measured against pH standards)} + 0.4 \quad (1)
\]

After determining the p\(^+\)H of the sample, the 13C n.m.r. spectrum was obtained. The p\(^+\)H was again determined to ensure that it had not changed, and the sample was then adjusted to the p\(^+\)H required for the next point. Data collected for d-glucopyranosyluronic acid included the chemical shift of the signal corresponding to the carboxy carbon (C-6) for both the \(\alpha\)- and \(\beta\)-anomeric forms. The Henderson–Hasseit relationship of pKₐ to the concentration of ionized and non-ionized forms is given in eqn. (2):

\[
p\text{H} - p\text{K}_{\text{a}} = \log([\text{ionized form}]/[\text{non-ionized form}]) \quad (2)
\]

On the basis of this relationship, eqn. (3) can be written:

\[
p\text{K}_{\text{a}}(\text{H}_2\text{O}) = p\text{H} - \log([\text{p.p.m.}]-p\text{p.m.})/([\text{p.p.m.}]-p\text{p.m.})] \quad (3)
\]

In eqn. (3), p.p.m. is the shift in p.p.m. for the carboxy carbon of the sample at a given p\(^+\)H, p.p.m. is the shift for the carboxy carbon in its non-ionized form, and p.p.m. is the shift of the carboxy group in its ionized form. The p\(^+\)H of (H₂O) was calculated using eqn. (4):

\[
p\text{K}_{\text{a}}(\text{H}_2\text{O}) = p\text{K}_{\text{a}}(\text{H}_2\text{O}) - 0.6 \quad (4)
\]

Glucuronic acid was dissolved in \(\text{H}_2\text{O}\) and in \(\text{H}_2\text{O}\) and titrated with NaOH and NaO\(^+\)H respectively. The p\(^+\)Kₐ of \(\text{H}_2\text{O}\) was 0.62 units higher than the p\(^+\)Kₐ \(\text{H}_2\text{O}\), similar to differences reported by others for carboxylic acids (Glaser & Long, 1960; Bunton & Shinriner, 1961) and to the 0.6 unit difference predicted by eqn. (4). By using this approach, p\(^+\)Kₐ \(\text{H}_2\text{O}\) values at 29 °C of 2.83 and 2.93 were obtained for II. It was necessary to assign definitely the carboxy-carbon signals (and the pKₐ values determined for each of the \(\alpha\) and \(\beta\)-anomeric forms. First the following well-resolved

Table 1. Uronic acid residues in various samples and their pKₐ values determined using 13C n.m.r.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Saccharide residues</th>
<th>pKₐ (H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Glucuronic acid</td>
<td>α-D-GlcA</td>
<td>2.93 (+0.13, -0.05)</td>
</tr>
<tr>
<td></td>
<td>β-D-GlcA</td>
<td>2.83 (+0.10, -0.05)</td>
</tr>
<tr>
<td>Disaccharide</td>
<td>ΔUA2S</td>
<td>3.35 (+0.12, -0.15)</td>
</tr>
<tr>
<td>Tetrasaccharide</td>
<td>ΔUA2S</td>
<td>3.02 (+0.05, -0.05)</td>
</tr>
<tr>
<td>Hexasaccharide</td>
<td>ΔUA2S</td>
<td>3.24 (+0.05, -0.04)</td>
</tr>
<tr>
<td></td>
<td>α-1-IdoA</td>
<td>2.57 (+0.09, -0.11)</td>
</tr>
<tr>
<td></td>
<td>β-1-IdoA</td>
<td>2.35 (+0.13, -0.12)</td>
</tr>
<tr>
<td>Heparin</td>
<td>α1-IdoA2S</td>
<td>3.13 (+0.19, -0.19)</td>
</tr>
<tr>
<td></td>
<td>β-GLA</td>
<td>2.79 (+0.19, -0.23)</td>
</tr>
</tbody>
</table>

The pKₐ values were determined by calculating the Kₐ values point-by-point, averaging these and converting the average Kₐ into the average pKₐ. Each Kₐ value was also converted into a pKₐ value. The highest and lowest pKₐ values were subtracted from the mean and are given in parentheses (Albert & Sergeant, 1984).
proton signals in the one-dimensional spectrum of II were tentatively assigned as follows: 5.27 p.p.m. (J 3.6 Hz, H-1 α-anomer), 4.69 p.p.m. (J 7.9 Hz, H-1 β-anomer), 4.33 p.p.m. (J 10.0 Hz, H-5 α-anomer) and 4.01 p.p.m. (J 9.4 Hz, H-5 β-anomer). The nuclear-Overhauser-effect (n.O.e.) difference spectrum obtained by irradiating at 4.69 p.p.m. (H-1 β-anomer) showed a positive enhancement in the intensity of the signal at 4.01 p.p.m. (H-5 β-anomer) as expected from the 1,3-diaxial disposition of the H-1 and H-5 of the β-anomer. N.O.e. irradiation at 5.28 p.p.m. (H-1 α-anomer) did not show any enhancement of the H-5 signal of α-anomer at 4.33 p.p.m., but showed enhancement of a signal at 3.60 p.p.m. assignable to H-2 of the α-anomer. Having confirmed the assignment of the H-5 signals of the α- and β-anomers of II, an attempt was made to assign their carboxyl carbons (C-6) through the use of heteronuclear (C,H) two-bond scalar coupling. Selective INEPT studies performed at 360 MHz at coupling constants ranging from 2 to 9 Hz failed to correlate H-5 signals of α- and β-anomers to their carboxy-carbon signals. Two-dimensional HMBC spectroscopy (Fig. 1), however, gave a clear correlation, permitting the assignment of the upfield signal to the β-anomeric carboxy carbon and the downfield signal to the α-anomeric carboxy carbon.

Oligosaccharide standards (III, IV and V), of > 95% purity, were prepared enzymatically from heparin (Rice & Linhardt, 1989). These oligosaccharides contained the same uronic acid residues as those found in heparin. Disaccharide III contains an unsaturated uronic acid 2-sulphate (ΔUA2S) that is an artefact of the eliminative action of heparin lyase (Linhardt et al., 1986) at the -4)-deoxy-2-sulphamido-α-Glucopyranose 6-sulphate (1→4)-α-L-idopyranosyluronic acid 2-sulphate (1→ linkage. Although this ΔUA2S residue is not normally present in heparin, it
\[ ^{13}\text{C-n.m.r.} \text{ determination of heparin's pK}_a \text{ values} \]

\[ \text{Chemical-shift values are plotted against the measured pH for the IdoA2S residue in heparin. The concentration of heparin was 9.5 mm (■), 19 mm (○) or 38 mm (△).} \]

was necessary to assign the chemical shift of this carboxy carbon as it is also found at the non-reducing end of oligosaccharide standards IV and V. Tetrasaccharide IV contains both a ΔUA2S residue and an internal \( \alpha\)-l-idopyranosyluronic acid 2-sulfate (IdoA2S). By using the reported chemical-shift data of carboxy groups present in III and IV (Merchant et al., 1985), a stack plot of the \(^{13}\text{C}\) n.m.r. spectra at each pH (measured) was obtained for III and IV. From these plots, pK\(_a\) values were obtained for ΔUA2S and IdoA2S residues present in III and IV (see Table 1).

Hexasaccharide V contains a ΔUA2S residue and internal \( \alpha\)-l-idopyranosyluronic acid (IdoA) and \( \beta\)-D-glucopyranosyluronic acid (GlcA). Unambiguous assignment of carbon signals for the two internal carboxy groups in V was required. This was accomplished by the combined use of COSY-45 (Nagayama \textit{et al}., 1980) and selective INEPT experiments (Bax, 1984). The \(^1\text{H}\) n.m.r. (360 MHz) spectrum of V (106 mm, pH 0.9) exhibited well-resolved signals of N-acetyl methyl protons (2.05 p.p.m.), H-4 (6.20 p.p.m.) of the ΔUA2S residue and H-5 (5.14 p.p.m.) of the internal IdoA residue. Application of two-dimensional COSY-45 spectroscopy confirmed the above assignments and, in addition, identified H-5 (4.20 p.p.m.) of the internal \( \beta\)-D-GlcA residue. A series of selective INEPT experiments were then carried out by irradiating the proton resonances at 2.05 p.p.m. (NH-CO-CH\(_2\)), 5.14 p.p.m. (H-5, IdoA), 4.20 p.p.m. (H-5, GlcA) and 6.20 p.p.m. (H-4, ΔUA2S) that clearly identified the respective carbon signals at 176.73, 174.84, 173.85 and 167.25 p.p.m. through long-range coupling (Fig. 2). A stack plot of the \(^{13}\text{C}\) n.m.r. spectra of V at each pH value measured is shown in Fig. 3.

The IdoA2S, IdoA and GlcA residues found in oligosaccharide standards IV and V are the same as those found in heparin. These residues account for 91, 7 and 2 \text{ mol}\% of the total uronic acid in a typical porcine mucosal heparin (Linhardt \textit{et al}., 1988; Linhardt \& Loganathan, 1990). The pK\(_a\) (H\(_2\)O) for the carboxy group in each uronic acid is given in Table 1.

Once the pK\(_a\) of the major uronic acid residues present in heparin had been established using oligosaccharide model compounds, it was possible to begin studies on heparin. The \(^{13}\text{C}\) n.m.r. spectrum of heparin has been reported and the carboxy carbons of IdoA2S and GlcA residues have been assigned on the basis of chemical shifts (Gatti \textit{et al}., 1979). These assignments agree with those made for oligosaccharide model compounds IV and V. The \(^{13}\text{C}\) n.m.r. spectrum of heparin at each measured pH is shown in Fig. 4. By using eqns. (1), (3) and (4), the pK\(_a\) (H\(_2\)O) values for the two major uronic acids found in heparin were determined (Table 1). To test the effect of sample concentration on pK\(_a\), the pK\(_a\) of the IdoA2S residue in heparin was determined by \(^{13}\text{C}\) n.m.r. at concentrations of 9.5, 19 and 38 mm. The pK\(_a\) (H\(_2\)O) values were 3.31, 3.13 and 3.14 respectively (Fig. 5).

**DISCUSSION**

The use of \(^{13}\text{C}\) n.m.r. spectroscopy to determine the pK\(_a\) of carboxylic acids present in small molecules was previously...
reported (Spiliane & Thomson, 1977). More recently, Gatti et al. (1979) reported using this method to determine the pKₐ of the ΔUA2S residue in heparin. This approach should, however, be capable of determining the pKₐ of multiple carboxy groups within a single heparin molecule.

The pKₐ of acidic sugars such as D-glucopyranosyluronic acid (II) are typically determined by standard titration methods (Kohn & Kovac, 1978). This method of pKₐ measurement is only useful when there is a single carboxy group present, as it is inherently incapable of resolving two acidic groups having a pKₐ within ±0.3–0.4 pH units of each other (Albert & Sergeant, 1984). The measurement of even a simple monosaccharide such as D-glucopyranosyluronic acid (II) is limited by this method. Both the α- and β-anomeric forms are present in an aqueous solution of II; thus, the pKₐ determined by standard titration methods provides only a weighted average of the pKₐ values of both anomers. ¹³C n.m.r. afforded two pKₐ values, one for each anomeric form, thus yielding more information on the physical-chemical properties of the molecule. Assignments of the signals for each carboxy group were made using n.O.e. difference spectroscopy and HMBC spectroscopy (Fig. 1). In the case of D-glucopyranosyluronic acid (II) the difference in the pKₐ of the α- and β-anomers is slight, with the β-anomer being more acidic. This approach becomes even more valuable in the study of polysaccharides containing multiple carboxy groups.

Heparin is a polyanionic complex polysaccharide that has been under continuous study since its introduction over 50 years ago. Interest in this drug has recently been heightened by the discovery of a specific pentasaccharide-binding site responsible for its anticoagulant activity (Lindahl et al., 1983; Althå et al., 1984). The anionic sites within heparin may be responsible for its specific interactions with various proteins (Casu, 1985; Cardin & Weintraub, 1989).

The pKₐ of the sulphamido, sulphate monooester and carboxylate groups in heparin had been previously examined by standard titration methods (Fransson et al., 1978) and by i.r. spectroscopy (Casu et al., 1978), ¹H n.m.r. (Gatti et al., 1979) and c.d. (Park & Chakrabarti, 1977) spectroscopy. These methods, however, failed to distinguish between the pKₐ of similar anionic groups: within different sugar residues, at different positions within a residue or at a particular position in a residue present within different sequences (i.e. having different neighbouring sugars). A set of oligosaccharide standards (III, IV and V) was prepared to determine the pKₐ of the different carboxy groups within heparin (I). The three oligosaccharides chosen were prepared from heparin in the milligram quantities required for ¹³C n.m.r. analysis. The structure of the oligosaccharide standards III, IV and V were established by chemical (Merchant et al., 1985), enzymic (Linhardt et al., 1990) and spectroscopic methods (Merchant et al., 1985; Linhardt et al., 1986; Maling et al., 1989; Loganathan et al., 1990). Their purity was confirmed by S.N.S. h.p.l.c. (Linhardt et al., 1988) and gradient PAGE (Rice et al., 1987).

Definitive assignments of carbon resonances of carboxy groups in disaccharide III and tetrasaccharide IV have been reported (Merchant et al., 1985). For the hexasaccharide V, unambiguous assignments of carboxy-carbon resonances were made in the present study using two-dimensional COSY-45 and selective INEPT experiments. This study represents a new application of a selective INEPT technique to assign the ¹³C n.m.r. spectra of heparin-derived oligosaccharides.

The pKₐ of the ΔUA2S residue present at the non-reducing end of each of the three oligosaccharide standards (III, IV and V) demonstrated a surprising decrease with increased oligosaccharide size (Table 1). Although the pKₐ of each oligosaccharide was determined at slightly different weight and molar concentrations, the observed trend in pKₐ could not be ascribed to a concentration effect. The tetrasaccharide (IV), having the lowest molar and weight concentration, demonstrated an intermediate pKₐ value. Nor could the difference in pKₐ be ascribed to ionic strength derived from contaminating salt, as each oligosaccharide sample was salt-free. One factor that may be responsible for this effect is the difference in the net charge of each molecule. Although it was expected that increased molecular charge should decrease the acidity of the ΔUA2S residue at the non-reducing end of each oligosaccharide, this was not the case. At pH values above the carboxylate pKₐ oligosaccharide III, IV and V have net charges of −4, −8 and −10, whereas the pKₐ (H₂O) of the ΔUA2S carboxylate groups in III, IV and V are 3.35, 3.02 and 2.24 respectively. The pKₐ of IdoA₂S in the tetrasaccharide IV was 3.44 (Table 1). This is the predominant uronic acid residue in the heparin polymer. ¹³C n.m.r. studies of hexasaccharide (V) over a range of pH values (Fig. 2) resulted in a pKₐ (H₂O) of 2.35 and 2.57 for GlcA and IdoA respectively. Because of the differences observed in the pKₐ of the ΔUA2S residue in oligosaccharides III, IV and V (discussed above), it may not be possible to compare directly the pKₐ values obtained for the other uronic acid residues found in these model compounds. However, if the relative differences between the pKₐ values of uronic acid residues within a given molecule are constant, some predictions can be made. In tetrasaccharide IV the pKₐ (H₂O) of IdoA₂S is 0.42 unit greater than that of ΔUA2S. In hexasaccharide V the pKₐ (H₂O) of IdoA and GlcA is 0.33 and 0.11 units greater than that of ΔUA2S. Thus, if all of these residues occurred within a single molecule, we would predict their relative acidities to be:

ΔUA2S > GlcA > IdoA > IdoA₂S

Unfortunately, such a model compound is not currently available.

The presence of a 2-sulphate group in the IdoA residue increases the pKₐ of its carboxyl group (Table 1). This second, charged, group makes this saccharide residue a di-anion, thus destabilizing it and increasing the carboxy pKₐ. The slight difference between the pKₐ of GlcA and IdoA (Table 1) is more difficult to rationalize. This difference might be attributable to: (1) the influence of neighboring saccharide units; (2) the configurational differences at the C-5 position of these uronic acids; or (3) known differences in the conformational flexibility of these two uronic acids (Ferro et al., 1990).

Because of its commercial, therapeutic and biological importance, heparin provides the most interesting application of this methodology. The only resonances assignable to the carboxy groups in heparin are those of IdoA₂S and GlcA, corresponding to 91 and 7 mol% of the uronic acid residues present in a typical porcine mucosal heparin (Linhardt et al., 1988; Linhardt & Loganathan, 1990). Tentative assignments of these two residues, on the basis of their chemical shift, have been reported (Gatti et al., 1979). This study, using hexasaccharide (V) and tetrasaccharide (IV) as model compounds, confirm these assignments. The ¹³C n.m.r. of heparin through a range of pH values (Figs. 4 and 5) was used to calculate the pKₐ of heparin's major uronic acid residues (Table 1). The IdoA₂S residue in the heparin polymer was more acidic than the same residue found within tetrasaccharide standard (IV). Several possible explanations for this difference include: (1) a higher net negative charge for heparin (approx. −80); (2) a polyelectrolyte effect (Gatti et al., 1979); (3) the effect of neighbouring saccharide residues on pKₐ; and (4) the effect of secondary structure on pKₐ. A polyelectrolyte effect resulting in a concentration-dependence of carboxylate pKₐ observed by Gatti et al. (1979) was explained as being the result of chain-chain interaction, resulting in a concentration-depen-
\( ^{13}\text{C-n.m.r.} \) determination of heparin's \( p K_a \) values

dence of carboxylate \( p K_a \). Three concentrations of heparin were used in the present study, but only a slight concentration-dependence of \( p K_a \) was observed (Fig. 5). This suggests that maximum chain-chain interaction is taking place at the high concentrations required for \(^{13}\text{C} \) n.m.r. spectroscopy. The nearest-neighbour saccharide residues are identical in both the tetrasaccharide standard (IV) and heparin polymer, suggesting that this effect is also an unlikely explanation for the observed differences in \( p K_a \). Differences in the secondary structure may play a role in the observed differences in \( p K_a \). The heparin polymer can adopt a helical coil, whereas the smaller oligosaccharide model compounds have no such secondary structure.

The \( p K_a \) of the GlcA residue in the heparin polymer, although the same as the \( p K_a \) of \( \beta\)-glycopyranosyluronic acid \( (\text{II}) \), is substantially less acidic than the GlcA found within hexasaccharide \( (\text{IV}) \). Again, a plausible explanation for this difference in \( p K_a \) might involve differences in the secondary structures of hexasaccharide \( (\text{V}) \) and the heparin polymer. Finally, because of its low abundance in the heparin polymer, the resonance for the IdoA carboxylate group could not be directly assigned (Fig. 4). This residue is particularly important, as it lies next to heparin's antithrombin III-binding site and gives conformational flexibility to this site. Data obtained from the oligosaccharide standards suggest that the carboxylate group within IdoA may be a more acidic site than the more common sulphated residue. The IdoA carboxylate group may also be slightly less acidic than the GlcA residue found within the antithrombin III-binding site. On the basis of the oligosaccharide model compounds we predict a \( p K_a \) of 3.0 for the IdoA residue of heparin.

In conclusion, we have demonstrated that it is possible to
determine the \( p K_a \) of acidic carboxylic acids using \(^{13}\text{C} \) n.m.r. spectroscopy. These measurements required from 20 mm (in wide-bore tubes) to 100 mm (in narrow-bore tubes) sample concentrations and overnight acquisition times for each pH value. An
dynamic \( p K_a \) could be determined with as few as five spectra if the
PH points were chosen carefully, thus representing one week of
data collection for each \( p K_a \) determined. Sample size requirements or acquisition times might be substantially reduced using
newer proton-detected \(^{13}\text{C} \) n.m.r. spectroscopy methods such as
HMBC (this method requires the use of \(^{1}\text{H}_2\text{O} \) as demonstrated in the
present study using \( \beta\)-glucuronic acid). Reduced experimental times might make it possible to examine other factors that could affect the \( p K_a \) of carboxylic groups determined by this method. These factors include examining: (1) wider sample concentration ranges; (2) solutions containing salt, to simulate physiological conditions; and (3) the effect of temperature on \( p K_a \), to understand the influence of conformational flexibility.

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