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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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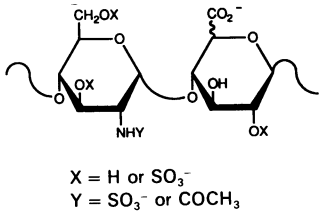
As part of our continuing studies on heparin, the present paper uses ^{13}C-n.m.r. spectroscopy to examine the acidity of heparin's uronic acid carboxylate groups. Heparin contains three different uronic acids. In porcine mucosal heparin these account for approx. 91, 7 and 2 mol % of the total uronic acid residues. These are α-L-idopyranosyluronic acid 2-sulphate, β-D-glucopyranosyluronic acid and α-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. D-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 carboxy carbon resonances were accomplished through the use of two-dimensional n.m.r. spectroscopy. Finally, application of this method to the simplest model compound, D-glucopyranosyluronic acid, permitted the determination of the pK_a of both its α- and β-anomers.

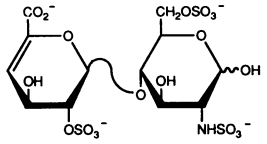
INTRODUCTION

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).

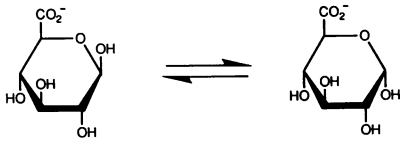
Heparin is a polydisperse, highly sulphated polysaccharide composed of repeating 1→4-linked uronic acid and glucosamine residues (Casu, 1985). Glycosaminoglycan heparin has an average



I

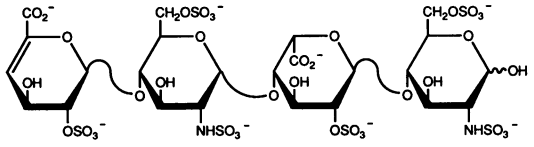


III

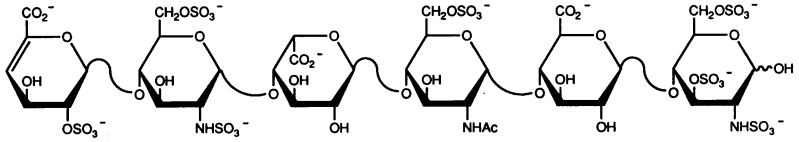


α β

II



IV



V

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 nucleus enhancement by polarization transfer; HMBC, heteronuclear multiple bond correlation spectroscopy; n.O.e., nuclear Overhauser effects; ΔUA2S, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulphate; IdoA2S, α-L-idopyranosyluronic acid 2-sulphate; IdoA, α-L-idopyranosyluronic acid; GlcA, β-D-glucopyranosyluronic acid.

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M_r of 10000 to 14000 [range 5000–40000 (Laurent *et al.*, 1978)]. Although the major repeating unit in heparin is a trisulphated disaccharide of the structure:

→4)-α-L-idopyranosyluronic acid 2-sulphate-(1→4)-2-deoxy-2-sulphamido-α-D-glucopyranose 6-sulphate-(1→

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 pK_a values ranging from 0.5 to 1.5 as measured by conductimetric titration (Casu & Gennaro, 1975). Less acidic are the carboxylate groups of the various unsulphated and monosulphated uronic acid residues, having pK_a 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 pK_a are incapable of distinguishing between the different carboxy groups within the heparin polymer. C.d. studies (Park & Chakrabarti, 1978) and ^{1}H-n.m.r. spectroscopy (Gatti *et al.*, 1979) afford an indirect measurement of pK_a. ^{13}C n.m.r. (Gatti *et al.*, 1979) provides a more direct method capable of differentiating between different carboxy groups. We report the application of ^{13}C n.m.r. to determine simultaneously the pK_a of multiple carboxy groups within heparin and other uronic-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-, tetra-, and hexa-saccharides are used as model compounds to determine the pK_a 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 Pharmacopeia) units/mg] 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. Spectropore 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) strong 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. ^2H_2O (99.96 atom% ^{2}H), NaO^{2}H solution (40 wt.% at 99+ atom% ^{2}H), ^{2}HCl (37 wt.% at 99+ atom% ^{2}H) and 3-(trimethylsilyl)propane-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 titrater 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-M_r cut-off dialysis membrane. After three changes of diffusate, 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 pK_a.

Preparation of samples for n.m.r. analysis. Each sample was dissolved in ^2H_2O (99.96 atom %) and freeze-dried. This exchange was performed three times, after which the sample was dissolved in ^2H_2O (99.96 atom %), containing 42 mM-DSS, for n.m.r. studies. Samples were prepared either in 10 mm- or 5 mm-outer-diameter tubes at the following concentrations: heparin, 38, 19 and 9.5 mM (1600, 800 and 400 mg in 3 ml respectively); heptasulphated hexasaccharide (**V**), 106 mM (78 mg in 0.4 ml); hexasulphated tetrasaccharide (**IV**), 81 mM (43 mg in 0.4 ml); trisulphated 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 p^{2}H of each sample was measured using a pH probe by a

meter calibrated with an appropriate set of pH standards. (After each n.m.r. experiment the p^{2}H was again measured to ensure that it had not changed.) The p^{2}H measurement was made 5 s after immersing the probe into the sample. The initial p^{2}H (calculated from eqn. 1 below) of each sample was between 3.5 and 6.0. The p^{2}H was then adjusted downwards with ^{2}HCl in ^2H_2O (12 M) to the lowest p^{2}H value required. After n.m.r. analysis, the sample's p^{2}H was adjusted upwards with NaO^{2}H in ^2H_2O (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 (^{1}H at 80 MHz and ^{13}C at 20.1 MHz) for the glucuronic acid titration curve, a Bruker WM360 spectrometer (^{1}H at 360 MHz and ^{13}C at 90.56 MHz) for oligosaccharides and heparin and a Bruker WM 600 (^{1}H at 600 MHz and ^{13}C at 150.9 MHz) for assigning the carbon resonances of carboxy groups of the α- and β-anomeric form of D-glucopyranosyluronic acid. Broad-band-decoupled ^{13}C n.m.r. spectra were measured for each sample at various p^{2}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 by using standard Bruker software. The carboxy-carbon resonances of α- and β-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 α- and β-anomers, is commercially available and was used to demonstrate the utility of ^{13}C n.m.r. to determine pK_a. Titration of II in water (1 M) at 20 °C gave a pK_a 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. Monosaccharide II was ^2H_2O-exchanged and dissolved at 1.03 M in ^2H_2O in the presence of DSS as internal standard. The p^{2}H of the sample was measured using a pH-meter that had first been calibrated with pH standards. The actual p^{2}H was calculated using eqn. (1) (Glasoe & Long, 1960; Jencks, 1989):

$$p^2H = p^2H \text{ (measured against pH standards)} + 0.4 \quad (1)$$

After determining the p^{2}H of the sample, the ^{13}C n.m.r. spectrum was obtained. The p^{2}H was again determined to ensure that it had not changed, and the sample was then adjusted to the p^{2}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 α- and β-anomeric forms. The Henderson–Hasselbach relationship of pK_a to the concentration of ionized and non-ionized forms is given in eqn. (2):

$$pH - pK_a = \log([\text{ionized form}]/[\text{non-ionized form}]) \quad (2)$$

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

$$pK_a(^2H_2O) = p^2H - \log[(p.p.m._s - p.p.m._{ni})/(p.p.m._i - p.p.m._s)] \quad (3)$$

In eqn. (3), p.p.m.$_s$ is the shift in p.p.m. for the carboxy carbon of the sample at a given p^{2}H, p.p.m.$_{ni}$ is the shift for the carboxy carbon in its non-ionized form, and p.p.m.$_i$ is the shift of the carboxy group in its ionized form. The pK_a (^2H_2O) was calculated for each p^{2}H value. The pK_a(^1H_2O) was then calculated from pK_a (^2H_2O) by using eqn. (4):

$$pK_a(^1H_2O) = pK_a(^2H_2O) - 0.6 \quad (4)$$

Glucuronic acid was dissolved in ^1H_2O and in ^2H_2O and titrated with NaOH and NaO^{2}H respectively. The pK_a (^2H_2O) was 0.62 units higher than the pK_a (^1H_2O), similar to differences reported by others for carboxylic acids (Glasoe & Long, 1960; Bunton & Shriner, 1961) and to the 0.6 unit difference predicted by eqn. (4). By using this approach, pK_a (^1H_2O) 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 pK_a values determined for each) of the α- and β-anomeric forms. First the following well-resolved

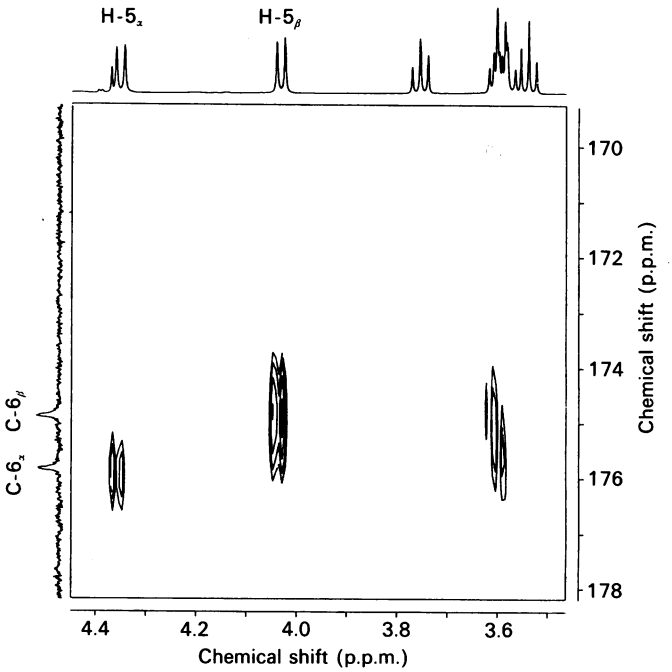

Fig. 1. HMBC spectrum of D-glucopyranosyluronic acid (II)

Assignments of H-5 and C-6 of α- and β-anomers are shown.

Table 1. Uronic acid residues in various samples and their pK_a values determined using ^{13}C n.m.r.

The pK_a values were determined by calculating the K_a values point-by-point, averaging these and converting the average K_a value into the average pK_a. Each K_a value was also converted into a pK_a value. The highest and lowest pK_a values were subtracted from the mean and are given in parentheses (Albert & Sergeant, 1984).

Sample	Saccharide residues	pK_a (H_2O)
D-Glucuronic acid	α-D-GlcA	2.93 (+0.13, −0.05)
	β-D-GlcA	2.83 (+0.10, −0.05)
Disaccharide	ΔUA2S	3.35 (+0.12, −0.15)
Tetrasaccharide	ΔUA2S	3.02 (+0.05, −0.05)
	IdoA2S	3.44 (+0.09, −0.05)
Hexasaccharide	ΔUA2S	2.24 (+0.05, −0.04)
	α-L-IdoA	2.57 (+0.09, −0.11)
	β-D-GLA	2.35 (+0.13, −0.12)
Heparin	α-L-IdoA2S	3.13 (+0.19, −0.19)
	β-D-GLA	2.79 (+0.19, −0.23)

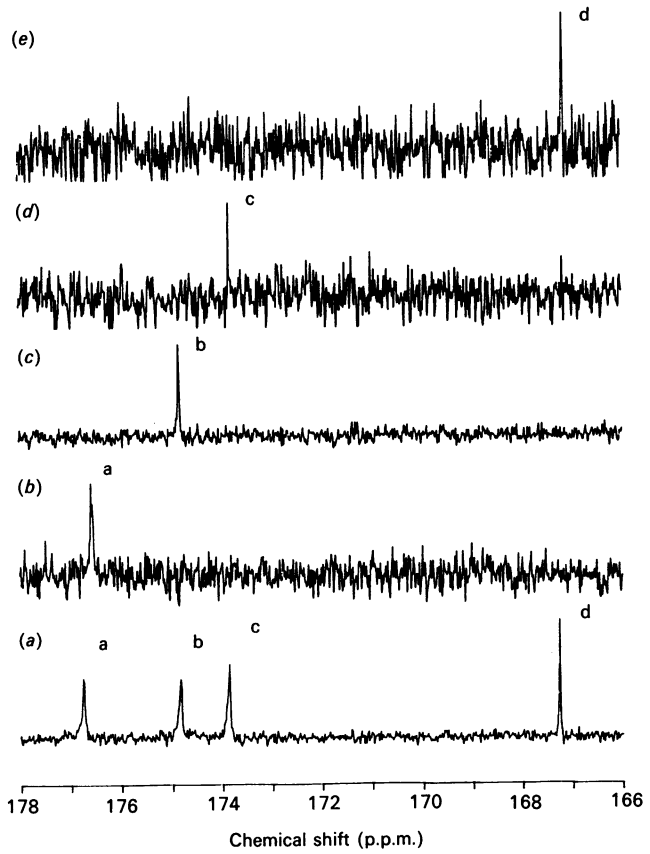

Fig. 2. Selective INEPT experiment performed on hexasaccharide (V) at 106 mM; p^2H (measured) 0.90

Bottom-most trace (*a*) represents the control ^{13}C n.m.r. spectrum. Proton signals irradiated at (*b*) 2.05 p.p.m. ($NH–COCH_3$); (*c*) 5.14 p.p.m. (H-5, IdoA); (*d*) 4.20 p.p.m. (H-5, GlcA); (*e*) 6.20 p.p.m. (H-4, ΔUA2S) resulted in the identification of carbon signals labelled as a, b, c and d respectively.

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 enzymically from heparin (Rice & Linhardt, 1989). These oligosaccharides contained the same uronic acid residues as those found in heparin. Disaccharide **III** contains an

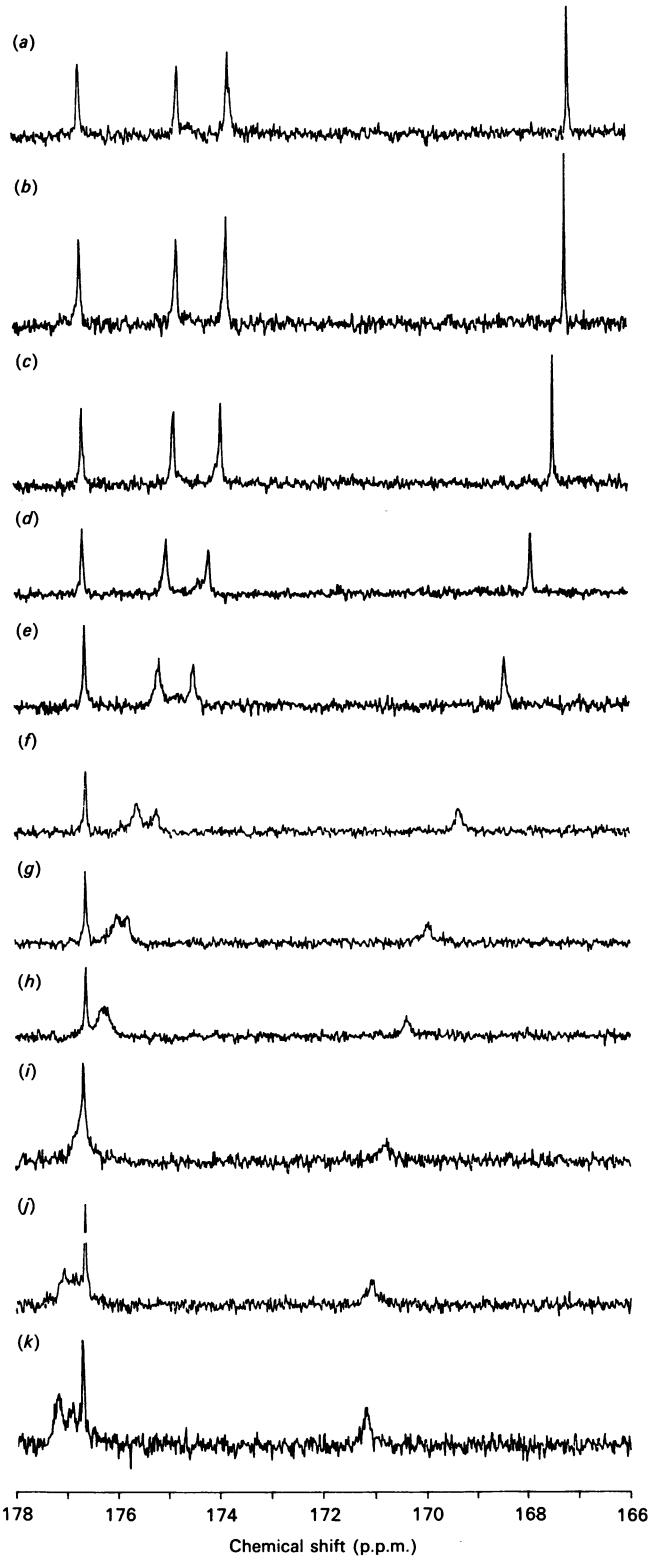

Fig. 3. Stack plot of broad-band decoupled ^{13}C-n.m.r. spectra of the hexasaccharide (V) obtained at p^2H (measured) values of (*a*) 0.55, (*b*) 0.90, (*c*) 1.36, (*d*) 1.84, (*e*) 2.19, (*f*) 2.56, (*g*) 2.86, (*h*) 3.13, (*i*) 3.52, (*j*) 4.27 and (*k*) 6.17

unsaturated uronic acid 2-sulphate (ΔUA2S) that is an artefact of the eliminative action of heparin lyase (Linhardt *et al.*, 1986) at the →4)2-deoxy-2-sulphamido-α-D-glucopyranose 6-sulphate-(1→4)α-L-idopyranosyluronic acid 2-sulphate(1→ linkage. Although this ΔUA2S residue is not normally present in heparin, it

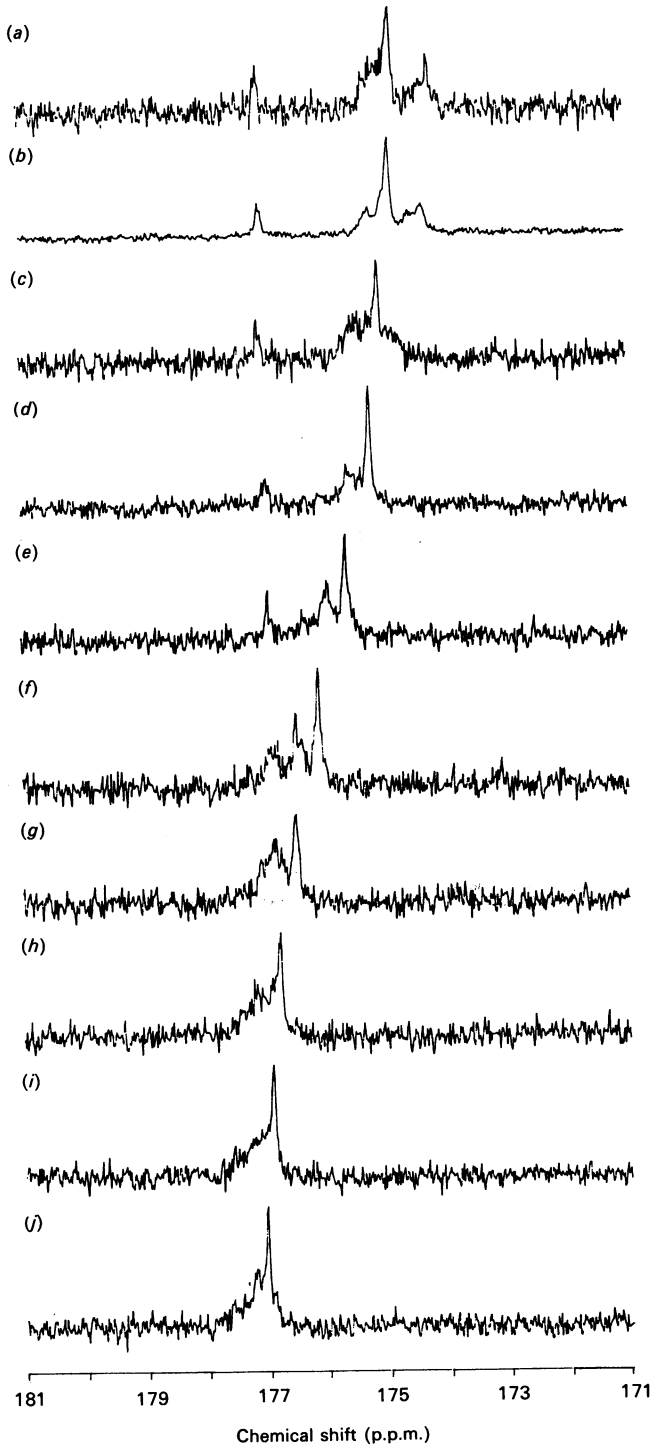

Fig. 4. Stack plot of broad-band decoupled ^{13}C-n.m.r. spectra of heparin obtained at different p^{2}H (measured) values of (*a*) 1.12, (*b*) 1.65, (*c*) 2.12, (*d*) 2.72, (*e*) 3.16, (*f*) 3.60, (*g*) 4.04, (*h*) 4.42, (*i*) 4.78 and (*j*) 5.65

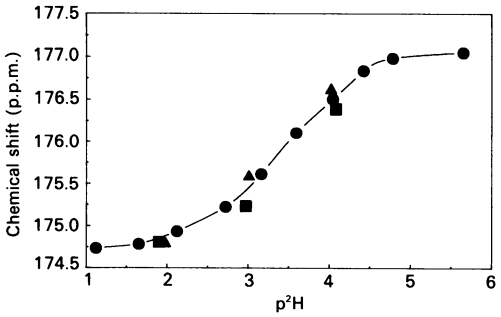

Fig. 5. Determination of the pK_a of uronic acid residues in heparin

Chemical-shift values are plotted against the measured p^{2}H 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 α-L-idopyranosyluronic acid 2-sulphate (IdoA2S). By using the reported carbon chemical-shift data of carboxy groups present in **III** and **IV** (Merchant *et al.*, 1985), a stack plot of the ^{13}C n.m.r. spectra at each p^{2}H (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 α-L-idopyranosyluronic acid (IdoA) and β-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 *et al.*, 1980) and selective INEPT experiments (Bax, 1984). The ^{1}H n.m.r. (360 MHz) spectrum of **V** (106 mM, p^{2}H 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 β-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$_3$), 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}C n.m.r. spectra of **V** at each p^{2}H 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 mol% of the total uronic acid in a typical porcine mucosal heparin (Linhardt *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}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 *et al.*, 1979). These assignments agree with those made for oligosaccharide model compounds **IV** and **V**. The ^{13}C n.m.r. spectrum of heparin at each measured p^{2}H 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}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}C n.m.r. spectroscopy to determine the pK_a of carboxylic acids present in small molecules was previously

reported (Spillane & Thomson, 1977). More recently, Gatti *et al.* (1979) reported using this method to determine the pK_a of the IdoA2S residue in heparin. This approach should, however, be capable of determining the pK_a of multiple carboxy groups within a single heparin molecule.

The pK_a of acidic sugars such as D-glucopyranosyluronic acid (**II**) are typically determined by standard titration methods (Kohn & Kovac, 1978). This method of pK_a measurement is only useful when there is a single carboxy group present, as it is inherently incapable of resolving two acidic groups having a pK_a 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 pK_a determined by standard titration methods provides only a weighted average of the pK_a values of both anomers. ^{13}C n.m.r. afforded two pK_a 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 pK_a 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 polyanions 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; Atha *et al.*, 1984). The anionic sites within heparin may be responsible for its specific interactions with various proteins (Casu, 1985; Cardin & Weintraub, 1989).

The pK_a of the sulphamido, sulphate monoester 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 pK_a 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 pK_a of the different carboxy groups within heparin (**I**). The three oligosaccharides chosen were prepared from heparin in the multimilligram quantities required for ^{13}C 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; Mallis *et al.*, 1989; Loganathan *et al.*, 1990). Their purity was confirmed by SAX 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 ^{13}C n.m.r. spectra of heparin-derived oligosaccharides.

The pK_a 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 pK_a of each oligosaccharide was determined at slightly different weight and molar concentrations, the observed trend in pK_a could not be ascribed to a concentration effect. The tetrasaccharide (**IV**), having the lowest molar and weight concentration, demonstrated an intermediate pK_a value. Nor could the difference in pK_a 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 pK_a, oligosaccharide **III, IV** and **V** have net charges of −4, −8 and −10, whereas the pK_a (H_2O) of the ΔUA2S carboxylate groups in **III, IV** and **V** are 3.35, 3.02 and 2.24 respectively. The pK_a of IdoA2S in the tetrasaccharide **IV** was 3.44 (Table 1). This is the predominant uronic acid residue in the heparin polymer. ^{13}C n.m.r. studies of hexasaccharide (**V**) over a range of pH values (Fig. 2) resulted in a $pK_a(H_2O)$ of 2.35 and 2.57 for GlcA and IdoA respectively. Because of the differences observed in the pK_a of the ΔUA2S residue in oligosaccharides **III, IV** and **V** (discussed above), it may not be possible to compare directly the pK_a values obtained for the other uronic acid residues found in these model compounds. However, if the relative differences between the pK_a values of uronic acid residues within a given molecule are constant, some predictions can be made. In tetrasaccharide **IV** the pK_a (H_2O) of IdoA2S is 0.42 unit greater than that of ΔUA2S. In hexasaccharide **V** the $pK_a(H_2O)$ 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:

$$\Delta UA2S > GlcA > IdoA > IdoA2S$$

Unfortunately, such a model compound is not currently available.

The presence of a 2-sulphate group in the IdoA residue increases the pK_a of its carboxyl group (Table 1). This second, charged, group makes this saccharide residue a dianion, thus destabilizing it and increasing the carboxy pK_a. The slight difference between the pK_a of GlcA and IdoA (Table 1) is more difficult to rationalize. This difference might be attributable to: (1) the influence of neighbouring 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 ^{13}C n.m.r. of heparin through a range of p^2H values (Figs. 4 and 5) was used to calculate the pK_a 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 pK_a; and (4) the effect of secondary structure on pK_a. A polyelectrolyte effect resulting in a concentration-dependence of carboxylate pK_a observed by Gatti *et al.*, (1979) was explained as being the result of chain–chain interaction, resulting in a concentration-depen-

dence of carboxylate pK_a. Three concentrations of heparin were used in the present study, but only a slight concentration-dependence of pK_a was observed (Fig. 5). This suggests that maximum chain–chain interaction is taking place at the high concentrations required for ^{13}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 pK_a. Differences in the secondary structure may play a role in the observed differences in pK_a. The heparin polymer can adopt a helical coil, whereas the smaller oligosaccharide model compounds have no such secondary structure.

The pK_a of the GlcA residue in the heparin polymer, although the same as the pK_a of D-glucopyranosyluronic acid (**II**), is substantially less acidic than the GlcA found within hexasaccharide (**IV**). Again, a plausible explanation for this difference in pK_a might involve differences in the secondary structures of hexasaccharide (**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 pK_a of 3.0 for the IdoA residue of heparin.

In conclusion, we have demonstrated that it is possible to determine the pK_a of acidic carbohydrates having multiple carboxy groups of comparable acidity using ^{13}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 accurate pK_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 pK_a determined. Sample size requirements or acquisition times might be substantially reduced using newer proton-detected ^{13}C n.m.r. spectroscopy methods such as HMBC (this method requires the use of ^2H_2O) as demonstrated in the present study using D-glucuronic acid. Reduced experimental times might make it possible to examine other factors that could affect the pK_a of carboxy 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 pK_a, to understand the influence of conformational flexibility.

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