Carbohydrate Research, 244 (1993) 205–223
Elsevier Science Publishers B.V., Amsterdam

FABMS/derivatisation strategies for the analysis of heparin-derived oligosaccharides

Kay-Hooi Khoo [a], Howard R. Morris [a], Roy A. McDowell [a], Anne Dell [a,*], Marco Maccarana [b] and Ulf Lindahl [b]

[a] *Department of Biochemistry, Imperial College, London SW7 2AZ (United Kingdom)*
[b] *Biomedical Centre, Swedish University of Agricultural Sciences, S-751 23 Uppsala (Sweden)*

(Received August 20th, 1992; accepted November 27th, 1992)

ABSTRACT

Derivatisation/FABMS strategies applicable to the structure analysis of low microgramme quantities of heparin-derived oligosaccharides are described. Negative and positive FAB data from permethyl derivatives and positive FAB data from the products of subsequent methanolysis are reported for sulfated tetrasaccharides prepared by nitrous acid degradation of heparin. The preparation and FAB behaviour of acetylated derivatives of sulfated oligosaccharides are described for the first time, and the stability of the sulfate groups to base-catalysed acetylation is demonstrated. The acetylation/FABMS methodology, which yields high quality data, shows promise for the characterisation of a wide range of sulfated glycoconjugates.

INTRODUCTION

The proteoglycans are macromolecular constituents of extracellular matrices and cell surfaces, where they are immobilised through attachment of the core protein to the plasma membrane. The functional roles ascribed to proteoglycans are highly diverse[1]. They provide mechanical support, serve as scaffolds for sequestered enzymes or growth factors, modulate the activities of various enzyme systems, and influence such dynamic processes as cell adhesion and migration. Many of these effects depend on ionic interactions between glycosaminoglycan chains and proteins. The sulfated glucosaminoglycans, heparin and heparan sulfate, have attracted particular attention as ligands for a variety of proteins[1–3]. Such interactions seem to range from relatively nonspecific cooperative electrostatic binding to highly specific lock-and-key interaction, as best illustrated by the antithrombin-binding pentasaccharide sequence[4]. Analysis of these interactions at the molecular level relies on the availability of appropriate methods for saccharide

* Corresponding author.

sequence determination, usually involving identification of fragments generated by degradation of the saccharides with nitrous acid or with bacterial lyases[5,6]. An important requirement of such methods is applicability to the limited amounts of material generally recovered from biological specimens or cultured cells.

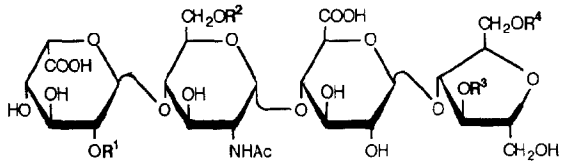
Deaminative cleavage of heparin/heparan sulfate with nitrous acid (low-pH procedure of Shively and Conrad[7]) converts *N*-sulfated GlcN residues into 2,5-anhydromannose residues (anhydromannitol, $aMan_R$, after reduction), with cleavage of the corresponding glucosaminyl linkage, whilst *N*-acetylated glucosamine residues are resistant. An exclusively *N*-sulfated saccharide sequence will therefore yield HexA–$aMan_R$ disaccharides, which are readily separated and quantified by anion-exchange HPLC (usually utilising the radioactive marker obtained on reducing the saccharides with $NaB[^3H]_4$ (refs 8–10)). The occurrence of isolated *N*-acetylated glucosamine residues will result in the formation of (mostly *O*-sulfated) tetrasaccharides with the general carbohydrate backbone structure, HexA–GlcNAc–GlcA–$aMan_R$. The identification of such components is essential for the overall elucidation of more extended saccharide sequences. Whilst the variously *O*-sulfated tetrasaccharide permutations are also separable by ion-exchange HPLC[10], the unambiguous identification of the various species usually involves cleavage of the tetrasaccharides (*N*-deacetylation by hydrazinolysis followed by deamination) followed by identification of the resulting disaccharides[10,11] and thus becomes fairly cumbersome. We have therefore explored the potential of fast atom bombardment mass spectrometry (FABMS) for identifying tetrasaccharide deamination products of heparin.

Previous FABMS studies of heparin-derived oligosaccharides[12–15] have yielded useful molecular weight information on native samples, but the full potential of the technique was not explored because no derivatives were analysed. It is now well established[16,17] that permethylation of sulfated oligosaccharides gives greatly enhanced sensitivity, allows unambiguous determination of sulfate heterogeneity, and enables sequence assignment either via negative ion fragmentation or via partial methanolysis monitored by FABMS. In this paper, we describe the first application of permethylation/FABMS protocols to heparin oligosaccharides. In complementary work, we show for the first time that heparin oligosaccharides can be converted into peracetyl derivatives without significant loss of sulfate. The peracetyl derivatives, which are simple to prepare, yield spectra of very high quality from microgramme quantities of material. The acetylation-based FABMS strategies described here, the first to be reported for sulfated oligosaccharides, are likely to have wide applicability to all classes of glycosaminoglycans as well as sulfated glycoproteins and glycolipids.

EXPERIMENTAL

Materials.—Unless otherwise stated, all reagents were obtained from Aldrich Chemical Co. Sulfated heparin tetrasaccharides based on the backbone structure

IdoA–GlcNAc–GlcA–aMan$_R$ (**1–3**) (where aMan$_R$ represents 2,5-anhydro-D-mannitol formed by reduction of the cleavage products) were obtained as described[11]. Briefly, heparin from pig intestinal mucosa was subjected to exhaustive, low-pH deaminative cleavage, and the resulting di- and oligo-saccharides were fractionated by gel chromatography. The tetrasaccharides were separated further by anion-exchange HPLC on a Partisil-10 SAX column (Whatman) into 5 major and a number of minor peaks. The structural characterisation of these components, involving *N*-deacetylation, deaminative cleavage, and identification of disaccharide products, was reported by Kusche et al.[11].



α-L-IdopA-(1→4)-α-D-GlcpNAc-(1→4)-β-D-GlcpA-(1→4)-aMan$_R$

1 R^1=H and R^2=SO_3H or R^1=SO_3H and R^2=SO_3H, R^3 and R^4=H

2 R^1 and R^4=H, R^2 and R^3=SO_3H

3 R^1=H, R^2, R^3 and R^4=SO_3H

Permethylation.—Hakomori permethylation was carried out using a two-step procedure as described[18,19]. The monosulfated tetrasaccharide was eluted in the 35% acetonitrile fraction from the Sep-Pak® cartridge (Waters Ltd.) whilst di- and tri-sulfated tetrasaccharides were mainly collected in the 15% acetonitrile fraction. Typically, ca. 20 μg of sample was permethylated and 10% of the resulting Sep-Pak®-purified permethyl derivative was loaded onto the monothioglycerol matrix for FABMS analyses.

Methanolysis.—Time-course methanolysis of permethylated samples was performed as described by Dell[19]. Briefly, methanolic HCl was prepared by bubbling HCl gas into methanol, or deuteriomethanol, until the solution was hot to the touch[20] (ca. 0.1 M HCl). Although the concentration of acid will vary a little from one preparation to another, this has a negligible effect on the results because of the time courses used. Approximately 20 μL or less of the reagent was then added to the sample and aliquots (1 μL) were withdrawn at suitable time-points, prior to and after heating at 60°C and loaded directly onto monothioglycerol on the FAB probe for analysis. Methanolysis experiments were routinely performed on permethylated sulfated oligosaccharides after direct FABMS analyses in both negative and positive modes.

Peracetylation.—Base-catalysed peracetylation was performed with either anhyd pyridine–Ac_2O (1:1, v/v, 100 μL) at 80°C, 2 h or, 1-methylimidazole–Ac_2O (1:5, v/v, 30 μL) at room temperature for 1 h (deuterioacetic anhydride was used for deuterioperacetyl derivatives). The reagents were evaporated under nitrogen and

samples were redissolved in methanol for aliquoting into the FAB matrix. *m*-Nitrobenzyl alcohol (*m*-NBA) was the matrix of choice, particularly for more highly sulfated and/or salty samples, but monothioglycerol was also routinely used. Approximately 10 μg of the native sample was derivatised and 0.5–1 μg of the peracetylated sample was loaded for FABMS analyses.

FABMS.—FAB-mass spectra were obtained using a VG Analytical ZAB-HF mass spectrometer fitted with an M-Scan FAB gun operated at 10 kV. Spectra were recorded on oscillographic chart paper and manually counted. The negative-ion mode FAB spectra of the peracetyl derivatives were obtained using a ZAB-2SE FPD mass spectrometer fitted with a cesium ion gun operated at 25 kV or 19 kV.

RESULTS AND DISCUSSION

In preliminary experiments, 2–3 μg of each of the pooled fractions containing the major peaks was analysed directly without derivatisation by FABMS in the negative-ion mode. Good quality spectra were obtained, confirming the basic tetrasaccharide structure as well as defining the maximum degree of sulfation. In accord with previous studies[12–16,21], it was observed that the majority of the ion current is carried by the molecular ion cluster, together with fragment ions resulting from consecutive loss of sulfite moieties. Sequence-related ions were weak or absent at these sample loadings.

Permethyl derivatives.—As reported previously for heparan sulfate and dermatan sulfate oligosaccharides[16], full permethylation was achieved without loss of the sulfate groups using the short Hakomori procedure[18]. The permethyl derivatives afforded good quality negative FAB spectra, showing sequence ions in addition to the molecular ions and their related fragment ions formed by loss of sulfite moieties. Data obtained from di- and tri-sulfated samples (**2** and **3**, respectively, both derived from the antithrombin binding region[4]) are reproduced in Figs. 1a and 2a, respectively.

The disulfated tetrasaccharide **2** afforded molecular ions at m/z 1032 and 1054, corresponding to $[M - H]^-$ and $[M - 2H + Na]^-$ (where M corresponds to the molecular weight of fully protonated, fully permethylated disulfated tetrasaccharide **2**, Fig. 1a). Loss of sodium sulfite from m/z 1054 gave the signal at m/z 952. The trisulfated tetrasaccharide **3** yielded an abundant molecular ion $[M - 3H + 2Na]^-$ at m/z 1142, whilst loss of one and two sodium sulfite moieties afforded the signals at m/z 1040 and 938, respectively (Fig. 2a). In our earlier studies of heparan sulfate and dermatan sulfate[16], we observed cleavage on the reducing side of glycosidic oxygens, preferentially between the GlcNAc and the GlcA, resulting in a pair of ions whose masses defined the number of sulfate groups present on each side of the cleavage site. Analogous pairs of ions were reproducibly observed in all the permethylated heparin tetrasaccharides examined in the present study. For example, the disulfated tetrasaccharide **2** yielded a pair of signals at m/z 471 and 574 (Fig. 1a), corresponding to unsaturated GlcA–aMan$_R$(SO_3^-) and the

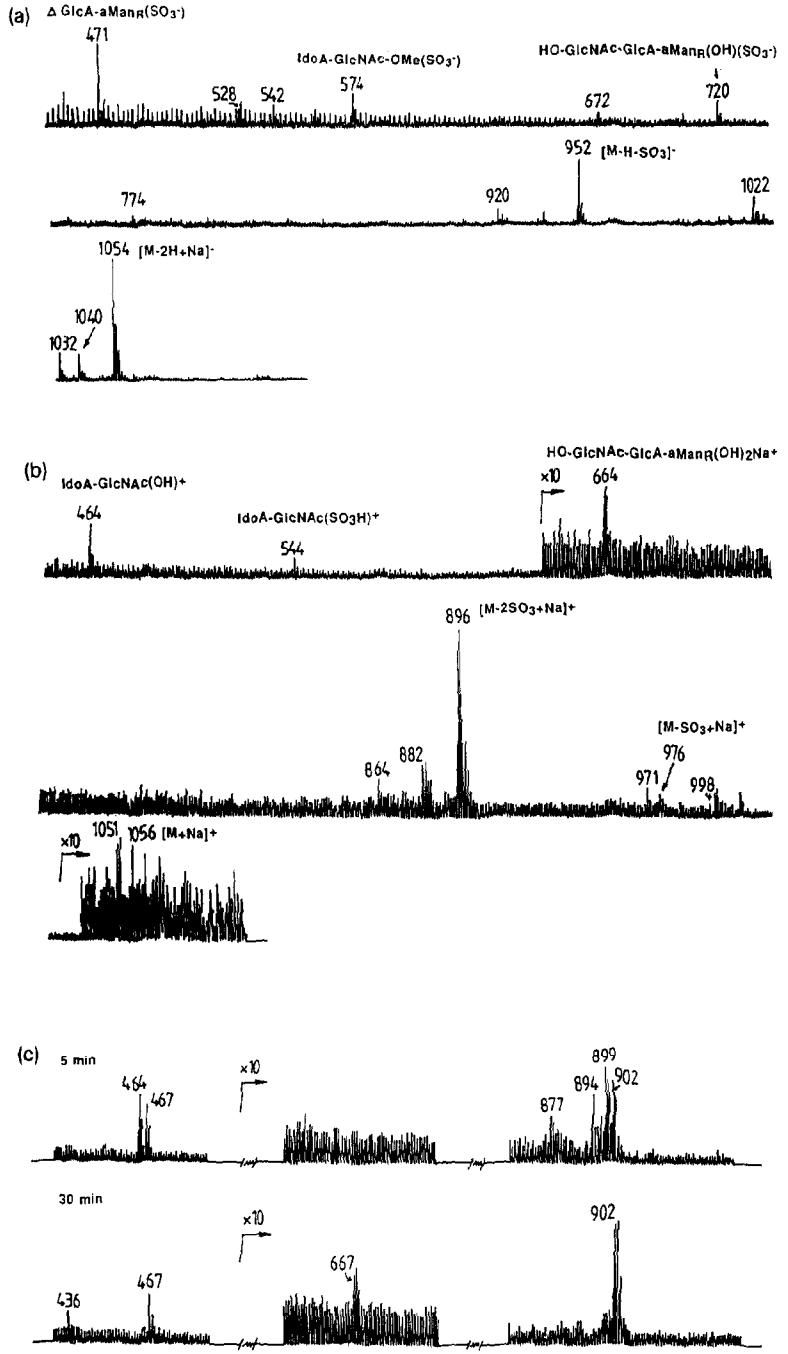


Fig. 1. FABMS analyses of permethylated disulfated heparin tetrasaccharide **2** in (a) the negative-ion mode; (b) the positive-ion mode; and (c) after methanolysis, for 5 and 30 min, in the positive-ion mode. Most signals are described in the text and are annotated. Signals 32 u lower than those assigned correspond to loss of methanol, whilst those 14 u lower correspond to undermethylation. Other signals are assigned as: $[M-H]^-$ (m/z 1032); $[M-SO_3+NH_4]^+$ (m/z 971); $[M-SO_3H+2Na]^+$ (m/z 998); and $[M+NH_4]^+$ (m/z 1051).

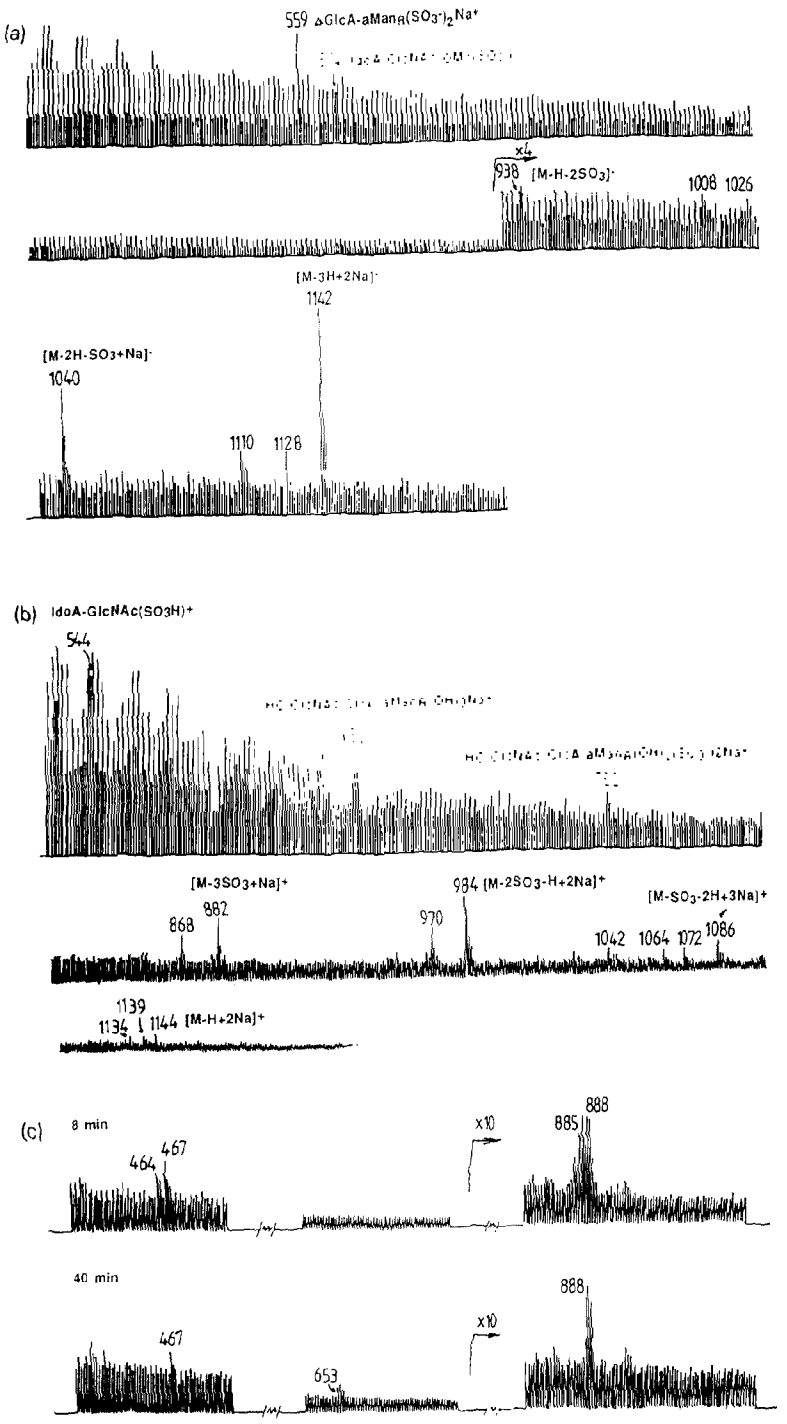
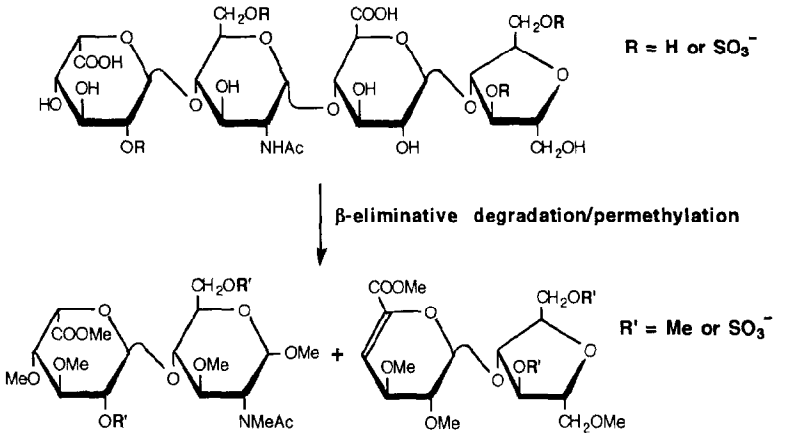


Fig. 2. FABMS analyses of permethylated trisulfated heparin tetrasaccharide 3 in (a) the negative-ion mode; (b) the positive-ion mode; and (c) after methanolysis, for 8 and 40 min, in the positive-ion mode. Signals at m/z 1134 and 1139 in (b) correspond to $[M-H+2NH_4]^+$ and $[M-H+NH_4+Na]^+$, respectively.



Scheme 1.

methyl glycoside of IdoA–GlcNAc(SO_3^-), respectively (to facilitate discussion of the data, we have translated HexA, as defined by FABMS, into IdoA or GlcA since the tetrasaccharide sequence was known), whilst the trisulfated tetrasaccharide **3** afforded signals at *m/z* 559 and 574 (Fig. 2a) corresponding to the unsaturated GlcA–aMan$_R$(SO_3^-)$_2$Na$^+$ and the methyl glycoside of IdoA–GlcNAc(SO_3^-), respectively. Thus, the additional sulfate group on **3** was localised to the GlcA–aMan$_R$ moiety, amply affirming the usefulness of this diagnostic pair of sequence-related ions. In our earlier work[16], where we observed glycosidic cleavage in front of HexNAc as well as HexA, we suggested that cleavage could be occurring during the FABMS process. It now appears more probable that the pairs of ions observed in the heparin experiments, all of which correspond to HexNac–HexA fission, are molecular ions of disaccharides formed by degradation occurring during the alkaline permethylation reaction via the well characterised β-eliminative degradation pathway[22] as shown in Scheme 1. Evidence supporting chemical cleavage during permethylation was provided by analysis of monosulfated heparin tetrasaccharides which eluted from the Sep-Pak in the 35% acetonitrile fraction, whilst their disaccharide degradation products were recovered in the 15% acetonitrile fraction. The presence of the disaccharides in the 15% fraction was unambiguously established by both positive and negative FABMS (data not shown). In contrast, in the Sep-Pak purification of the permethylated products from disulfated tetrasaccharide **2**, the monosulfated disaccharides co-eluted with the disulfated tetrasaccharides in the 15% acetonitrile fraction, giving the FAB data shown in Fig. 1a. In good quality spectra, a number of minor fragment ions predominantly originating from β-cleavages[23] were also observed. For example, in Fig. 1a, the signal at *m/z* 720 corresponds to GlcNAc–GlcA–aMan$_R$ with one sulfate and two free hydroxyl groups. One free OH group is produced by β-cleavage at the IdoA–GlcNAc glycosidic linkage whilst the other is formed by loss of a sulfite

moiety. These data are consistent with the presence of two sulfate groups in the GlcNAc–GlcA–aMan$_R$ trisaccharide. One of these sulfates must be located on the GlcNAc residue because each of the IdoA–GlcNAc and GlcA–aMan$_R$ disaccharides contains one sulfate as shown by the ion pair at m/z 574 and 471 (Fig. 1a).

Whilst a number of sequence-related ions are observed in the negative FAB spectra of permethylated, sulfated oligosaccharides, it is imperative to obtain as much corroborative data as possible in order to draw firm conclusions pertaining to the sequences of unknown samples and the location of the sulfate substituents. It is recommended that, whenever sample quantities permit, the permethyl derivatives should also be examined in the positive-ion mode to exploit the predictable fragmentation pathways that have yielded sequence-related fragment ions from a very wide range of complex carbohydrates[23]. As shown in Figs. 1b and 2b, for the permethylated di- and tri-sulfated tetrasaccharides, respectively, sample loadings of ca. 5 μg gave good quality positive spectra. As expected, the most intense ions were afforded by the mass spectrometric cleavage of sulfite moieties from the molecular ion. Thus, disulfated **2** yielded fragment ions at m/z 896 and 976 (Fig. 1b) corresponding to loss of two and one sulfite moieties, respectively, from the molecular ion $[M + Na]^+$ at m/z 1056, whilst trisulfated **3** afforded fragment ions at m/z 882, 984, and 1086 (Fig. 2b) corresponding to loss of 3, 2, and 1 sodium sulfite moieties, respectively, from the predicted molecular ion $[M - 3H + 4Na]^+$ at m/z 1188 (not seen). A weak disodiated molecular ion of **3** was observed at m/z 1144. In addition, A-type oxonium ions[23] were present at m/z 464 (loss of sulfite from m/z 544) (Fig. 1b) and 544 [IdoA–GlcNAc$(SO_3H)^+$] (Figs. 1b and 2b). These ions corroborate the data from the disaccharide pairs observed in the negative-ion mode. Specifically, the presence of the ion at m/z 544 in the positive spectrum of **3** (Fig. 2b) confirms that the additional sulfate group is on the reducing end GlcA–aMan$_R$ moiety. Similarly, β-cleavage ions were seen at m/z 664 (monosodiated GlcNAc–GlcA–aMan$_R$ with 3 free OH groups) for **2** (Fig. 1b) and m/z 650 (loss of sodium sulfite from m/z 752) and 752 (disodiated, monosulfated GlcNAc–GlcA–aMan$_R$ with 3 free OH groups) for **3** (Fig. 2b), confirming that the non-reducing end IdoA is not sulfated in either **2** or **3**.

Methanolysis.—When only limited quantities of material are available, direct analysis of permethylated derivatives in the positive-ion mode may not yield sufficiently good quality spectra for sequence-related fragment ions to be observed. Previously[16], we have advocated a subsequent step of acid-catalysed peracetylation of the permethylated sample on the basis that the sulfate substituents would be replaced by acetyl groups, making the sample more amenable to positive FABMS analysis. Although we have successfully applied this procedure in a number of instances, we now recognise that the removal of the sulfate groups can occur rather slowly under the mildly acidic conditions employed. Consequently, substitution of the sulfate groups by acetyl groups is frequently incomplete and may result in rather poor FAB data. To overcome this problem, we have explored an alternative approach to positive-ion analysis of permethylated heparin oligosaccha-

rides, namely partial methanolysis monitored by FABMS. Methanolysis has been widely used to partially and completely degrade carbohydrates. In particular, time-course methanolysis of permethylated oligosaccharides, coupled with direct FABMS analysis, has been successfully used to afford full or partial sequencing of oligosaccharides[24,25]. Typically, during partial methanolysis, the permethylated sample is gradually degraded by methanolysis of the glycosidic linkages. Methyl (or deuteriomethyl if deuteriomethanol is used) glycosides are produced at the released reducing ends whilst the methanolysed glycosidic oxygen becomes a free hydroxyl group. The number of free OH groups associated with each fragment observed is deduced from the mass data and used to define sequence and branching. Since different glycosyl and non-glycosyl substituents exhibit different susceptibility towards methanolysis, sample aliquots taken at early time-points invariably contain relatively intact oligosaccharide backbones with only the most labile substituents being methanolysed. We predicted, therefore, that the first substituents to be lost from the sulfated heparin oligosaccharides would be the sulfate groups, generating free hydroxyl groups at the positions formerly substituted with sulfates. This was confirmed when permethylated heparin tetrasaccharides were subjected to deuteriomethanolysis. The first aliquot was withdrawn immediately after adding the deuteriomethanolic-HCl, prior to any heating. The mono- and di-sulfated tetrasaccharides afforded acceptable spectra, showing weak molecular ions retaining the sulfates and strong fragment ions resulting from loss of sulfite(s) (data not shown). The spectrum of disulfated tetrasaccharide **2** at this early stage in the reaction is similar to that obtained prior to methanolysis (Fig. 1b). Trisulfated tetrasaccharide **3**, in contrast, did not yield meaningful data at the initial time-point, but gave good data once the sulfate groups had been removed by the reagent. Within the first 10 min of incubation at 60°C, some notable changes were observed, as typified by the mass spectra obtained after 5 min for **2** and 8 min for **3**, reproduced in Figs. 1c and 2c, respectively.

Firstly, any weak molecular ions observed at the initial time-point had disappeared. For tetrasaccharide **2**, new molecular ions were observed at m/z 899 and 902, which are, respectively, 3 and 6 mass units higher than m/z 896, which is the dominant ion observed in the high-mass region of the non-methanolysed material (Fig. 1b). The mass of this last ion corresponds to the monosodiated, nonsulphated molecular ion of **2** bearing two free hydroxyl groups. The "doublet" at m/z 899 and 902 is consistent with a tetrasaccharide from which both sulfates have been removed by the methanolysis reagent and whose methyl ester groups have been replaced by one and two deuteriomethyl groups, respectively. For tetrasaccharide **3**, molecular ions now appear at m/z 885 and 888 (Fig. 2c), analogous to those observed for **2** but 14 mass units lower, consistent with this oligosaccharide having three free OH groups in place of the three sulfate groups removed by methanolysis. Secondly, A-type oxonium ions were observed at m/z 464 and 467, corresponding to IdoA–GlcNAc$^+$ with one free OH group (the latter incorporating a single deuteriomethyl ester).

Further incubation at 60°C resulted in complete conversion into deuteriomethyl esters with the *m/z* 464/467, 899/902, and 885/888 doublets becoming single peaks at *m/z* 467, 902, and 888, respectively, and the appearance of new ions at *m/z* 667 for **2** and 653 for **3** (Figs. 1c and 2c). The latter are molecular ions of methanolysis products and are analogous to the β-cleavage ions of the non-methanolysed material, which occur at *m/z* 664 (Fig. 1b) and 650 (Fig. 2b), respectively. The interval of 3, rather than 6, mass units between the β-cleavage ions and the products of deuteriomethanolysis, is consistent with the latter being reducing-end trisaccharides having a single HexA residue rather than non-reducing end trisaccharides containing two HexA residues.

It is significant that no data were obtained for the trisulfated tetrasaccharide prior to heating, but structurally informative spectra were obtained after the negatively charged substituents had been methanolytically removed, thus attesting to the enhanced positive-ion sensitivity achieved by the methanolysis procedure. The above data suggest that (*a*) when sufficient material is available, positive-mode analysis of the intact permethylated sulfated oligosaccharides will yield sequence-informative fragment ions, which complements the data obtained in the negative-ion mode; (*b*) when analysis in the positive-ion mode is not successful due to sample quantity limitations, a time-course methanolysis strategy of enhanced sensitivity due to the removal of the negatively charged substituents can be employed; (*c*) during methanolysis, the labile sulfate substituents are removed within the first few minutes of heating without any appreciable degradation of the rest of the molecule; in related experiments on permethylated glycoprotein-derived sulfated oligosaccharides, we have observed that sulfate groups are labile to prolonged incubation with methanolic-HCl at room temperature and their loss precedes the cleavage of acid-sensitive glycosidic bonds involving sialic acid and/or fucose residues (Khoo and Dell, unpublished data); (*d*) if deuteriomethanolic-HCl is used, the methyl ester of the HexA will eventually be replaced by a deuteriomethyl group and the resulting mass shift is helpful for sequence assignment.

Peracetyl derivatives.—The most effective FAB-based strategies for characterising complex carbohydrates from biological sources employ acetyl as well as methyl derivatives[23,26]. Advantages of acetylation include (*a*) relatively simple experimental manipulation and, unlike permethylation, sample purification is not essential prior to FABMS analyses, minimising the possibility of derivatised highly sulfated oligosaccharides being lost during the purification step by coelution with hydrophilic salt impurities; (*b*) peracetyl derivatives frequently fragment at glycosidic linkages that are refractory in the permethyl derivative, thereby affording complementary data; and (*c*) the acetyl groups can be readily replaced with methyl groups if subsequent linkage analysis is required. As noted above, acid-catalysed acetylation of permethyl derivatives has been helpful in some glycosaminoglycan structure assignments[16], but the method can suffer from low yields. Further, acid-catalysed acetylation of native sulfated oligosaccharides only affords data pertaining to the oligosaccharide structure, because desulfation occurs during the derivatisation. We

decided, therefore, to investigate alternative methods of preparing acetylated derivatives; in particular, we sought procedures which would give retention of the sulfate moieties. Since base catalysis appeared to be a viable means of achieving this objective (alkaline conditions are employed in many procedures for isolating glycosaminoglycan oligosaccharides without loss of sulfate), the products of reaction of a variety of heparin tetrasaccharides with acetic anhydride, catalysed by either pyridine or 1-methylimidazole, were examined by FABMS. To avoid potential ambiguities that might arise due to similarity in mass of a sodium sulfite loss (102 u) and the elimination of an acetic acid moiety (60 u) from an underacetylated (42 u) species, deuteriated acetic anhydride was used throughout with either pyridine or 1-methylimidazole as the base catalyst. The data which are described below revealed that peracetylation can, indeed, be achieved with little or no loss of sulfate and the resulting derivatives give excellent FAB data.

Figs. 3a and b show the negative FAB-spectra of the monosulfated heparin tetrasaccharide **1** after deuterioacetylation catalysed by pyridine and 1-methylimidazole, respectively. Both samples afforded a molecular ion $[M - H]^-$ at m/z 1203, corresponding to **1** in which all hydroxyl groups are deuterioacetylated and the sulfate moiety is retained without modification. Notably, the absence of a signal corresponding to the molecular ion of desulfated **1** (m/z 1168 for $[M - H]^-$) suggests no significant loss of the sulfate. Signals at m/z 1140 and 1077 correspond in mass to loss of one and two deuterioacetic acid moieties from the molecular ion. Because the intensities of m/z 1140 and 1077 were reproducibly higher than m/z 1203 in the spectra of pyridine-catalysed products, but were significantly less intense in the spectra of 1-methylimidazole-catalysed reactions (cf. Figs. 3a and b), it is probable that m/z 1140 and 1077 are, as least in part, quasimolecular ions of lactonised species formed during the derivatisation, and are unlikely to be solely fragment ions derived from consecutive elimination of deuterioacetic acid from m/z 1203. Supporting this assignment are the signals at m/z 1109 and 1172 in Fig. 3a, which are 32 u higher than m/z 1077 and 1140, respectively. These correspond to quasimolecular ions of methyl esters formed by ring opening of the lactones with the methanol which is used to dissolve the sample prior to analysis. Notably, this pair of signals grew in intensity each time the sample was redissolved for further FABMS analyses. This "methanol addition" was also observed in experiments where 1-methylimidazole was used as the catalyst (data not shown). In an attempt to confirm that lactones can be produced under the basic acetylation conditions, we carried out similar experiments on mono- and di-sialylated *O*-glycans. These gave data consistent with partial lactonisation of the sialic acid residues (data not shown). Although lactones are not expected to be stable at basic pH, lactone formation in the presence of acetic anhydride, which will form mixed anhydrides with the carboxyl groups, is perhaps not too surprising. In this regard, it is interesting that lactones of sialic acids have been observed after alkaline permethylation of glycophorin-derived oligosaccharides[27], suggesting that the organic reaction conditions that pertain during derivatisation may result in

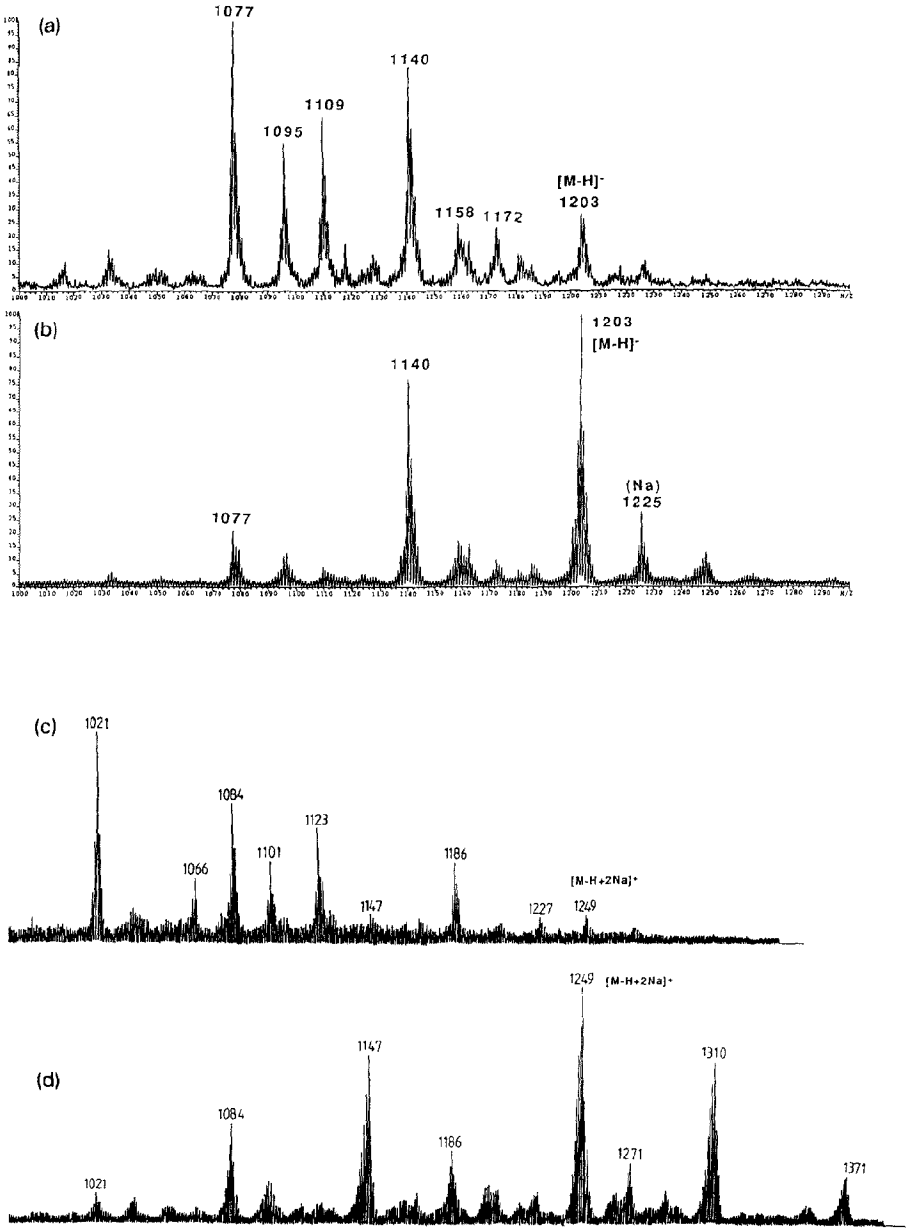


Fig. 3. Quasimolecular ion regions of the spectra of deuterioacetylated monosulfated heparin tetrasaccharide **1** in (a) the negative-ion mode, pyridine catalysis; (b) the negative-ion mode, 1-methylimidazole catalysis; (c) the positive-ion mode, pyridine catalysis; and (d) the positive-ion mode, 1-methylimidazole catalysis. The unusual isotope pattern observed in these spectra is due to the batch of deuterioacetic anhydride used for these experiments being incompletely deuteriated in a portion of the molecules. The monosodiated form of the ion at m/z 1123 is observed at m/z 1101. The signal at m/z 1271 corresponds to $[M-2H+3Na]^+$. Signals at m/z 1095 and 1158 in (a) correspond to underdeuterioacetylated quasimolecular ions. Other signals are described in the text.

products that would not be expected to survive in an aqueous environment at similar pH.

Similar patterns of quasimolecular ions were present in the positive spectra of deuterioacetylated **1** (Figs. 3c and d for pyridine- and 1-methylimidazole-catalysis, respectively). A weak disodiated molecular ion $[M - H + 2Na]^+$ was present at *m/z* 1249, accompanied by signals corresponding to the disodiated mono- and di-lactonised species at *m/z* 1186 and 1123, respectively. As in the negative spectra, these latter ions were most prominent in the spectrum of the pyridine-catalysed deuterioperacetylated sample (Fig. 3a). In addition, a strong signal corresponding to the monosodiated dilactonised product was observed at *m/z* 1101, consistent with the loss of a negative charge through lactonisation. As expected, loss of sodium sulfite from the quasimolecular ions is a major fragmentation pathway in the positive-ion mode, resulting in signals at *m/z* 1021, 1084 and 1147. These mass-spectrometric fragment ions are distinguishable from quasimolecular ions of possible products of chemical desulfation because the latter will carry an additional deuterioacetyl group at the site of sulfation. Finally, in the spectra of the 1-methylimidazole-catalysed perdeuterioacetylated sample (Fig. 3d), strong signals were observed at *m/z* 1310 and 1371, one and two increments of 61 u, respectively, above the disodiated molecular ion at *m/z* 1249. When acetylation was carried out with non-deuteriated reagents, the corresponding signals were observed 60 and 120 u above the disodiated molecular ion. These data are consistent with the presence of a monosodiated molecular ion bearing a single protonated methylimidazole (MW, 82) adduct, i.e., $[(M - H)^- + Na^+ + MeImidazoleH^+]$, and a molecular ion having two protonated methylimidazole adducts, i.e., $[(M - H)^- + 2MeImidazoleH^+]$. The one mass-unit difference between the normal and deuterioacetylated products can be rationalised as deuterium incorporation, probably through deuteriation of the imine nitrogen giving deuteriated methylimidazole, i.e., $MeImidazoleD^+$. As would be expected, these cation adducts were not seen in the negative-ion mode (Fig. 3b).

In addition to the fragment ions resulting from loss of sodium sulfite, a number of sequence ions were also observed in the positive-ion mode. The relevant region of the positive spectrum of the 1-methylimidazole-catalysed deuterioacetylated **1** is reproduced in Fig. 4a. A-type oxonium ions present at *m/z* 582 (monosodiated IdoA–GlcNAc$(OH)^+$) and 662 (monosodiated, monosulfated IdoA–GlcNAc$^+$) suggest that the single sulfate group in this sample is located on either the IdoA or the GlcNAc residue, whilst oxonium-type cleavage between the GlcA and $aMan_R$ residues produced the ion at *m/z* 848 corresponding to monosodiated IdoA–GlcNAc–GlcA$(OH)^+$. Related ions at *m/z* 537 and 803 were 45 u lower than signals at *m/z* 582 and 848, respectively, consistent with their bearing one additional free OH group as a result of under-deuterioacetylation. Alternatively, since the parent molecule was almost completely deuterioacetylated with no or very weak −45-u signals (Figs. 3b and d), they could also be rationalised as resulting from glycosidic cleavage of the lactonised parent molecule, retaining the

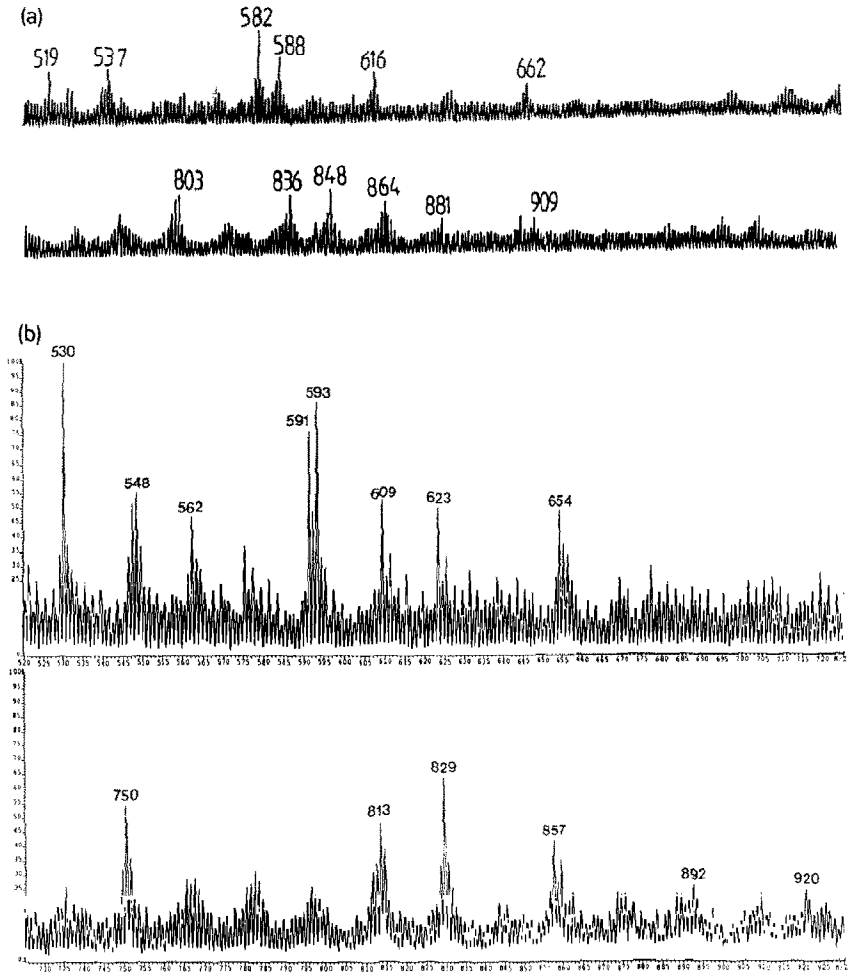
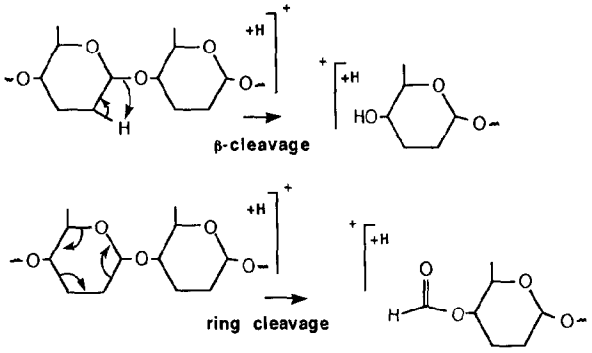


Fig. 4. Regions of the spectra of deuterioacetylated I which show sequence-related fragment ions, (a) positive-ion mode and (b) negative-ion mode. These are the lower mass regions of the same spectra reproduced in Figs. 3d and a, respectively. Signals at m/z 519 and 530 are 63 u lower than signals at m/z 582 and 593, respectively, corresponding to loss of a deuterioacetic acid moiety. Signals at m/z 562 and 623 are the methyl esters formed by methanol addition to the lactonised species at m/z 530 and 591, whilst signals at m/z 609 and 548 are the underdeuterioacetylated species of signals at m/z 654 and 593, respectively.

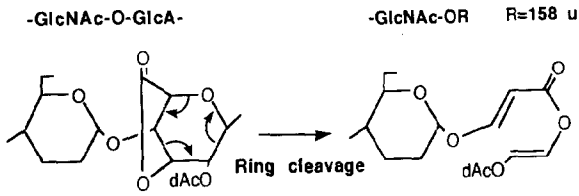
glycosidic oxygen as a free OH group at the reducing end. Thus, the signal at m/z 537 corresponds to monosodiated IdoA–GlcNAc bearing two free OH groups, one at the reducing end and the other as a consequence of sulfite loss. Similarly, the signal at m/z 803 corresponds to IdoA–GlcNAc–GlcA$(OH)_2$. Ions derived from β-cleavage and ring cleavage produce a pair of reducing end fragment ions 28 mass units apart (the ring-cleavage ion is of higher mass, Scheme 2) with the β-cleavage ion having a free OH group at the non-reducing end as a result of the glycosidic cleavage. Additional free OH group(s) are attributed to loss of sulfite(s). Thus,



Scheme 2.

signal pairs at m/z 588/616, 836/864, and 881/909 correspond to monosodiated β-cleavage and ring-cleavage ions of GlcA–aMan$_R$, GlcNAc(OH)–GlcA–aMan$_R$, and GlcNAc–GlcA–aMan$_R$, respectively. Taken together, these ions define the sequence IdoA–GlcNAc–GlcA–aMan$_R$ with the single sulfate group attached to either the GlcNAc residue, giving the pair of signals at m/z 836/864 after loss of sulfite, or the IdoA residue, yielding the pair of signals at m/z 881/909, fully consistent with the sample **1** being a mixture of IdoA(2-OSO$_3$)–GlcNAc–GlcA–aMan$_R$ and IdoA–GlcNAc(6-OSO$_3$)–GlcA–aMan$_R$ as reported previously[11].

Analogous fragment ions were also observed for pyridine-catalysed deuterioacetylated **1** each shifted to 63 u lower, consistent with the lactonised forms being the dominant parent molecular ions. In the negative-ion mode (Fig. 4B), β-cleavage and ring-cleavage ions were observed at m/z 829/857 and 892/920, corresponding to the lactonised and non-lactonised GlcNAc(OSO$_3^-$)–GlcA–aMan$_R$, respectively. These were analogues of those seen in the positive-ion mode except that the sulfate substituent was retained. Non-reducing end fragment ions corresponding to lactonised, monosulfated IdoA–GlcNAc with free hydroxyl and enol groups at the reducing end were present at m/z 593 and 591. The non-lactonised form of the latter was also present at m/z 654. Finally, ions at m/z 750 and 813 (with lactonised and non-lactonised IdoA, respectively) could be rationalised as similar non-reducing end fragment ions resulting from ring cleavage at the lactonised GlcA residue as shown in Scheme 3. The GlcA residue is schemati-



Scheme 3.

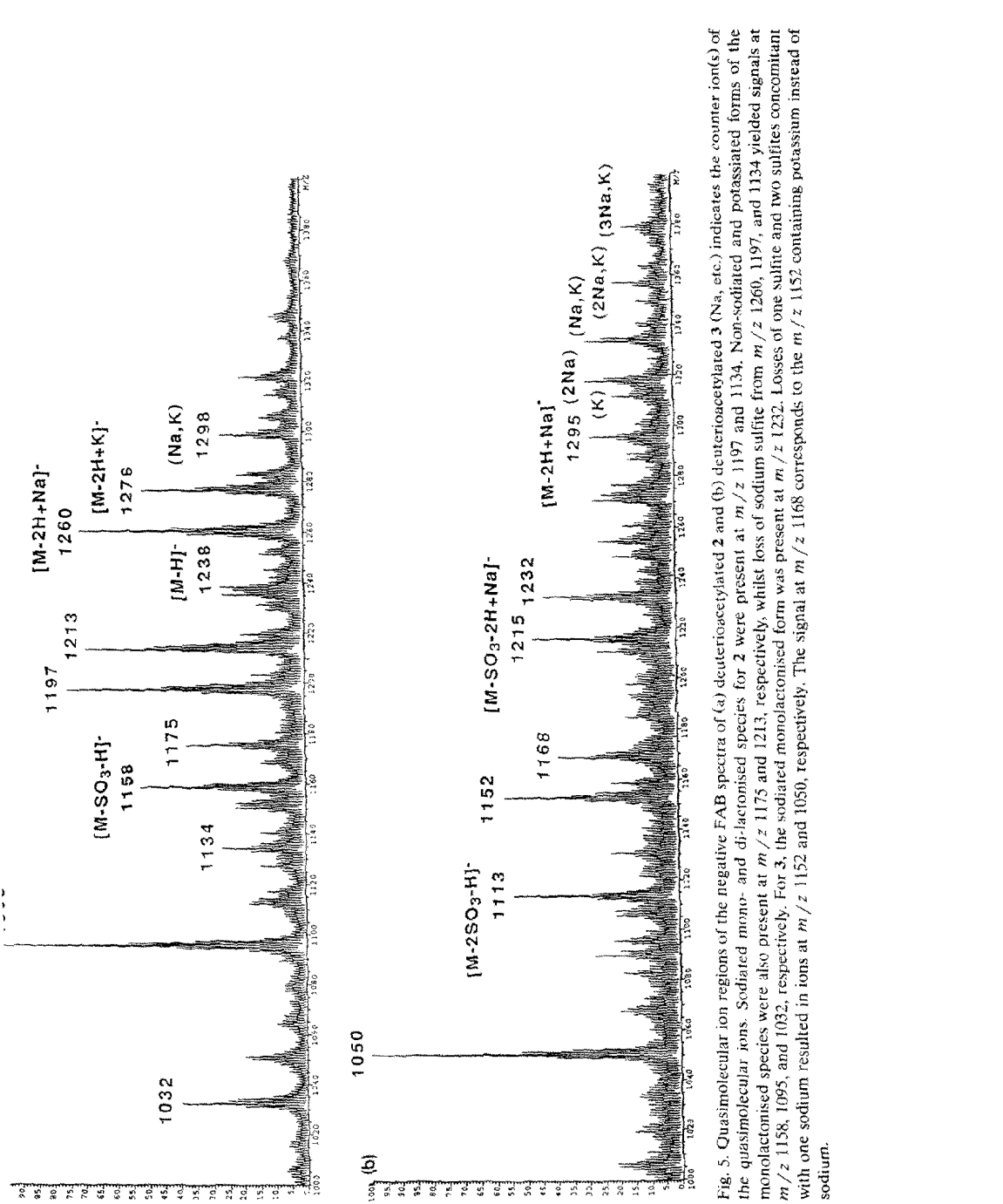


Fig. 5. Quasimolecular ion regions of the negative FAB spectra of (a) deuterioacetylated **2** and (b) deuterioacetylated **3** (Na, etc.) indicates the counter ion(s) of the quasimolecular ions. Sodiated mono- and di-lactonised species for **2** were present at m/z 1197 and 1134. Non-sodiated and potassiated forms of the monolactonised species were also present at m/z 1175 and 1213, respectively, whilst loss of sodium sulfite from m/z 1260, 1197, and 1134 yielded signals at m/z 1158, 1095, and 1032, respectively. For **3**, the sodiated monolactonised form was present at m/z 1232. Losses of one sulfite and two sulfites concomitant with one sodium resulted in ions at m/z 1152 and 1050, respectively. The signal at m/z 1168 corresponds to the m/z 1152 containing potassium instead of sodium.

cally represented as a five-membered lactone involving the carboxylic group and the OH at C-3, but alternative lactones are possible.

In summary, data on the monosulfated heparin tetrasaccharide showed that acetylation can be effected without any desulfation, using either pyridine or 1-methylimidazole as the base catalyst. The reaction is simple and, provided the sample to be derivatised is relatively free of salt or other impurities, the acetylated products are sufficiently clean to be analysed directly after drying down to give good quality FABMS spectra in both positive- and negative-ion modes at the 1-μg loading level. The pyridine-catalysis conditions, which involved heating at 80°C for 2 h, appeared to promote lactonisation of the uronic acids more readily than the milder 1-methylimidazole-catalysed conditions (1 h, room temperature). Thus, although the non-lactonised molecular ion was observed, the lactonised forms are the dominant species in the spectra of pyridine-catalysed derivative. Finally, in the negative-ion mode, the molecular ions were the dominant signals whilst, in the positive-ion mode, fragment ions resulting from loss of the sulfite moiety and hence the negative charge, are usually more intense than the molecular ions. Some sequence-related ions were also present.

When our study was extended to include di- and tri-sulfated heparin tetrasaccharides, FABMS analyses in the positive-ion mode became less tractable. This was partly due to the molecular ion current being spread amongst various sodium- and potassium-cationised adducts, hence lowering the sensitivity afforded. In addition, complex fragmentations which resulted in loss of negative charges were highly favoured, yielding clusters of signals that were not readily interpretable (data not shown). In contrast, in the negative-ion mode, good quality spectra were obtained from di- and tri-sulfated tetrasaccharides. Data from the 1-methylimidazole-catalysed deuterioacetylated disulfated **2** are presented in Fig. 5a. An abundant quasimolecular ion $[M - 2H + Na]^-$ is present at m/z 1260 together with related cationised species. Other major signals are attributed to lactonised components and to losses of sodium sulfite (see figure legend). Similarly, deuterioacetylated trisulfated **3** (Fig. 5b) afforded clusters of quasimolecular ions varying in cation content, together with fragment ions resulting from cleavage of one or two sodium sulfite moieties. Sequence-related fragment ions were of low abundance in the spectra of acetylated **2** and **3**. Experiments to enhance fragmentation by collision-induced dissociation are in progress. These experiments are practicable at the low microgramme level because of the high abundance of the quasimolecular ions.

CONCLUSION

Heparin oligosaccharides produced from deaminative cleavage are well suited for FABMS analyses. The m/z values of the molecular ions obtained from native material and permethyl and peracetyl derivatives in the negative-ion mode provide data which define the size and the heterogeneity of the sample as well as the

degree of sulphation. Since deaminative cleavage occurs at each *N*-sulfated glucosamine site, resulting in the reducing end $aMan_R$ after reduction, additional glucosamine residues in the oligosaccharide fragments isolated will be *N*-acetylated. In the positive mode, this ensures a favoured mass-spectrometric cleavage site[23] for both the permethyl and peracetyl derivatives, giving abundant non-reducing end sequence ions at each HexA–HexNAc repeat. Important structural data are provided by the β-eliminative degradation products produced during the permethylation reaction. They yield characteristic pairs of ions which unambiguously define the number of sulfate substituents on each disaccharide repeat. Additional sequence information is provided by the fragment ions, mostly arising from β-cleavages, and from the molecular ions of the products of methanolysis. Further, we have shown that base catalysed peracetylation procedures are compatible with sulfated samples and lead to little or no desulfation. These derivatives afford high quality spectra.

The present study constitutes the first rigorous FABMS study of permethylated and peracetylated heparin derivatives. The methodology used in this work is designed to maximise the amount of structurally useful data present in the mass spectra. In some structural studies, it may not be necessary to carry out derivatisation procedures, notably when large quantities of very clean sample permit the detection of ring- and glycosidic-cleavage ions from underivatised samples. However, in our experience, most biological applications demand the better quality data afforded by derivatives. Further, with judicious choice of strategy, a range of data can be obtained from a single sample for example, by implementing the following series of steps: (*i*) deuterioacetylation and positive and negative FABMS analyses of a portion of the products (typically 10% for each analysis); (*ii*) permethylation of the remaining derivatised sample, followed by negative and positive FABMS of aliquots of the permethyl derivative; (*iii*) methanolysis/positive FABMS of the remainder of the permethylated derivative. In related studies in our laboratory (Khoo, Dell, Morris, unpublished work with Sugahara), we have shown that it is, indeed, feasible to carry out sequential reactions of this type starting with a few micrograms of material.

The derivatisation strategies reported in this paper are applicable to the analysis of a wide range of sulfated oligosaccharides. We expect that the FAB protocols described by us, as well as by others in the field[12–15,21], coupled with advances in purifications and mass analysis technology, will permit structural characterisation of biologically important sulfated carbohydrates which can be isolated in only trace amounts from biological matrices.

ACKNOWLEDGMENTS

The Imperial College work is supported by a Medical Research Council Programme Grant and Wellcome Trust Grant 030826/1.27 to HRM and AD. KHK is a recipient of a Wellcome Prize Studentship. MM and UL are supported by Grant

2309 from the Swedish Medical Research Association and by Italfarmaco S.P.A., Milan, Italy.

REFERENCES

1 L. Kjellén and U. Lindahl, *Annu. Rev. Biochem.*, 60 (1991) 443–475.
2 D.A. Lane, in D.A. Lane and U. Lindahl (Eds.), *Heparin; Chemical and Biological Properties, Clinical Applications*, Arnold, London, 1989, pp 363–391.
3 R.L. Jackson, S.J. Busch, and A.D. Cardin, *Physiol. Rev.*, 71 (1991) 481–539.
4 U. Lindahl, L. Thunberg, G. Bäckström, J. Riesenfeld, K. Nordling, and I. Bjork, *J. Biol. Chem.*, 259 (1984) 12368–12376.
5 B. Casu, in D.A. Lane and U. Lindahl (Eds.), *Heparin; Chemical and Biological Properties, Clinical Applications*, Arnold, London, 1989, pp 25–49.
6 R.J. Lindhardt, H. Wang and S.A. Ampofo, in D.A. Lane, I. Bjork, and U. Lindahl (Eds.) *Heparin and Related Polysaccharides*, Plenum, New York, 1992, in press.
7 J.E. Shively and H.E. Conrad, *Biochemistry*, 15 (1976) 3932–3942.
8 I. Jacobson and L. Rodén, *Fed. Proc.*, 39 (1980) 1636–1640.
9 L. Thunberg, G. Bäckström, and U. Lindahl, *Carbohydr. Res.*, 100 (1982) 393–410.
10 M.J. Bienkowski and H.E. Conrad, *J. Biol. Chem.*, 260 (1985) 356–365.
11 M. Kusche, G. Torri, B. Casu, and U. Lindahl, *J. Biol. Chem.*, 265 (1990) 7292–7300.
12 S.A. Carr and V.N. Reinhold, *J. Carbohydr. Chem.*, 3 (1984) 381–401.
13 V.N. Reinhold, S.A. Carr, B.N. Green, M. Petitou, J. Choay, and P. Sinaÿ, *Carbohydr. Res.*, 161 (1987) 305–313.
14 L.M. Mallis, H.M. Wang, D. Loganathan, and R.J. Linhardt, *Anal. Chem.*, 61 (1989) 1453–1458.
15 R.J. Linhardt, H.M. Wang, D. Loganathan, D.J. Lamb, and L.M. Mallis, *Carbohydr. Res.*, 225 (1992) 137–145.
16 A. Dell, M.E. Rogers, J.E. Thomas-Oates, T.N. Huckerby, P.N. Sanderson, and I.A. Nieduszynski, *Carbohydr. Res.*, 179 (1988) 7–19.
17 A. Dell, H.R. Morris, F. Greer, J.M. Redfern, M.E. Rogers, G. Weisshaar, J. Hiyama, and A.G.C. Renwick, *Carbohydr. Res.*, 209 (1991) 33–50.
18 H.R. Morris, M.R. Thompson, D.T. Osuga, A.I. Ahmed, S.M. Chan, J. Vandenheede, and R. Feeney, *J. Biol. Chem.*, 253 (1978) 5155–5161.
19 A. Dell, *Methods Enzymol.*, 193 (1990) 647–660.
20 E. Hunt and H.R. Morris, *Biochem. J.*, 135 (1973) 333–343.
21 P. Scudder, P.W. Tang, E.F. Hounsell, A.M. Lawson, H. Mehmet, and T. Feizi, *Eur. J. Biochem.*, 157 (1986) 365–373.
22 J. Kiss, *Adv. Carbohydr. Chem.*, 29 (1974) 229–303.
23 A. Dell, *Adv. Carbohydr. Chem. Biochem.*, 45 (1987) 19–72.
24 A. Dell, N.H. Carman, P.R. Tiller, and J.E. Thomas-Oates, *Biomed. Environ. Mass Spectrom.*, 16 (1988) 19–24.
25 J.R. Woodward, D. Craik, A. Dell, K.-H. Khoo, S.L.A. Munro, A.E. Clarke, and A. Bacic, *Glycobiology*, 2 (1992) 241–250.
26 A. Dell, R.A. McDowell, and M.E. Rogers, in J.J. Harding and M.C.C. Crabbe (Eds.), *Post-Translational Modifications of Proteins*, CRC Press, Boca Raton, FL, 1991, pp 185–216.
27 A.L. Chan, PhD Thesis, University of London, 1991.