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FAST-ATOM-BOMBARDMENT MASS-SPECTROMETRIC STRATEGIES FOR SEQUENCING SULPHATED OLIGOSACCHARIDES*

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ABSTRACT

A strategy is presented for the structural analysis of sulphated oligosaccharides. The oligosaccharides are permethylated to leave sulphate groups intact, and the products examined by negative f.a.b.-m.s. The fragmentation observed from such compounds in the negative mode is described for the first time. The sulphates are then chemically replaced by acetyl groups, so producing a derivative that is examined in the positive mode. This procedure yields sequence data and defines the residues on which the sulphates were originally located. The strategy is illustrated using glycosaminoglycan fragments.

INTRODUCTION

Glycosaminoglycans are highly charged anionic polysaccharides usually found in the connective tissues of most animal species. There are seven major types of glycosaminoglycans which vary in their distribution and have similar, yet distinct structural characteristics. A variety of biological functions has been described for the different polymers and their fragments and the number is increasing rapidly to include roles in the control of cell growth and differentiation[1,2], the modulation of angiogenesis[3,4], the control of proliferation in vascular muscle cells[5], and modulatory effects at various points in the coagulation and clot-forming pathways[6–8]. Despite the increasing interest being shown in the glycosaminoglycans, it remains a difficult task to reliably obtain detailed structural information on these molecules.

One of the most important developments in this area has been the introduc-

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tion of fast-atom-bombardment mass spectrometry (f.a.b.-m.s.). Reinhold and co-workers[9] were the first to show that fragments of chondroitin sulphate and heparin can be successfully analysed by f.a.b.-m.s. despite the difficulties associated with the presence of multiple anionic functional groups. It was observed that the majority of the ion current is carried by molecular weight-related ions together with fragment ions formed by consecutive eliminations of sulphite moieties. Sequence-containing ions are weak or absent. Similar results have been reported for other polysulphated oligosaccharides[10–12]. These studies of native sulphated oligosaccharides have highlighted a number of problems which potentially limit the effectiveness of f.a.b.-m.s. for characterising small quantities of polysulphated oligosaccharides derived from biological matrices: (*a*) the polydisperse nature of the molecular ion region, due to the presence of several species differing in their counter-cation content, results in inherently low sensitivity; (*b*) a fragment ion formed by loss of sulphite is indistinguishable from the molecular ion of the corresponding oligosaccharide having one less sulphate moiety; hence, f.a.b.-m.s. of native samples cannot define the compositions of mixtures differing in sulphate content; (*c*) the major fragmentation pathways, *i.e.*, losses of sulphite, are structurally uninformative and sequence information is minimal; (*d*) salt buffers are frequently used during the purification of polysulphated oligosaccharides and it is often difficult to desalt native samples to a level which permits high sensitivity f.a.b.-m.s.

Poor sensitivity, salt contamination, and insufficient or ambiguous fragmentation are problems not solely confined to the f.a.b.-m.s. analysis of polysulphated oligosaccharides, although the presence of several charged groups, such as sulphates, does exacerbate the situation. Problems such as these have been resolved for many other classes of carbohydrates by converting the native samples into their peracetylated or permethylated derivatives which, on examination by f.a.b.-m.s., yield molecular weight and sequence data at high sensitivity[13]. We now show that the same derivatisation protocols, with minor changes in strategy, are applicable to polysulphated oligosaccharides, and that sequence information as well as the location of the sulphated residues can be reliably obtained. Further, we propose a general derivatisation/f.a.b.-m.s. strategy for the sensitive characterisation of such molecules. The method may also be applied to samples with a relatively "high salt" content.

EXPERIMENTAL

Materials. — Unless otherwise stated, all reagents were obtained from Sigma Chemical Co. Sep-Pak cartridges were purchased from Waters Ltd. Sulphated oligosaccharides were a tetrasaccharide fraction derived from heparan sulphate (HS-4) previously described[14] as HS-4 fraction III, and a hexasaccharide-rich fraction derived from dermatan sulphate (DS-6) of pig mucosal origin following digestion[15] with chondroitinase ABC.

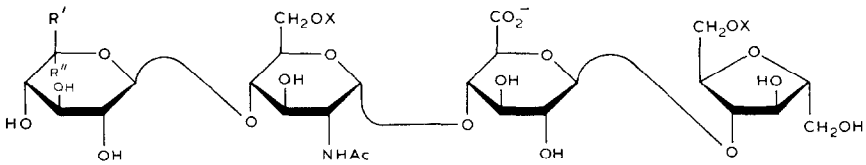
Peracetylation. — This was performed as previously described[16], using a mixture of trifluoroacetic anhydride and acetic acid (2:1, v/v).

Permethylation. — A modified Hakomori[17] procedure was used. Samples were dissolved in 100 μL of dried, distilled dimethyl sulphoxide and 100 μL of methylsulphinylmethanide base (freshly prepared by mixing 0.1 g of sodium hydride with 400 μL of methyl sulphoxide and heating for 10–15 min at 100°) were added. After 5 min at room temperature, 100 μL of methyl iodide were added and the reaction was allowed to continue for a further 20 min. The sequential additions of base and methyl iodide were repeated using 400 μL of each reagent and the same reaction times. The reaction was quenched by the addition of 0.5 mL of distilled water and excess of methyl iodide was removed by bubbling a stream of oxygen-free nitrogen through the mixture, prior to drying under vacuum. The dried residue was dissolved in 200 μL of 1:1 methanol–water and applied to a Sep-Pak cartridge. Equilibration and elution of the samples were performed as previously described[18]. In some experiments, the reaction mixture was loaded directly onto the Sep-Pak after quenching and removal of methyl iodide. Both procedures gave identical results.

The recently described sodium hydroxide procedure[19] for permethylation was assessed for applicability to polysulphated samples. Samples were dissolved in dimethyl sulphoxide (100 μL), NaOH (dried, powdered, 0.5 mg) was added, followed by methyl iodide (0.5 mL), and the mixture was shaken for 10 min. The reaction was quenched with water and carefully neutralised with 0.1M HCl. Methyl iodide was removed under a stream of nitrogen and the product mixture was then purified on Sep-Pak as described for the Hakomori procedure.

Fast-atom-bombardment mass spectrometry. — F.a.b.-mass spectra were obtained as previously described[18], using a VG Analytical ZAB-HF mass spectrometer fitted with an M-Scan f.a.b. gun. Each sample was run using a glycerol–1-thioglycerol (1:1) matrix containing 0.5 μL of 0.1M HCl, except for experiments designed to optimise matrix conditions.

Reduction and permethylation. — Oligosaccharides were treated with sodium borohydride as previously described[20]. At the end of the reaction, the sample was dried down five times from acetic acid in methanol (10% v/v) and then five times from methanol to remove borates, and then permethylated as described above.



1 $R' = H, R'' = CO_2^-$ for IdoA ; $R' = CO_2^-, R'' = H$ for GlcA
$X = SO_3^-$

RESULTS AND DISCUSSION

Optimisation of matrix conditions using a heparan sulphate oligosaccharide (HS-4). — The negative f.a.b.-mass spectrum of HS-4[14] (**1**) acquired after loading a mildly acidic solution (5–10 μg of HS-4 in 1 μL of aqueous 5% acetic acid) into a matrix composed of glycerol and 1-thioglycerol is shown in Fig. 1a. The spectrum contains a very weak signal for the deprotonated molecular ion of the species which contains no counter cations (*m/z* 878). The major molecular ion current is distributed between *m/z* 900, 922, and 944, corresponding to the mono-, di-, and tri-sodium salts, respectively. Loss of sulphite affords the fragment ions at *m/z* 798, 820, and 842. The polydisperse nature of the molecular ion region is considerably improved after addition of 0.1M HCl to the matrix. Fig. 1b shows the spectrum of the same sample of HS-4 acquired after removal of the probe from the source, addition of 1 μL of 0.1M HCl, and re-insertion of the probe. Since a significant proportion of the sample ionised in the first experiment, the signals in the second spectrum (Fig. 1b) are somewhat less intense than in the first (Fig. 1a). The data

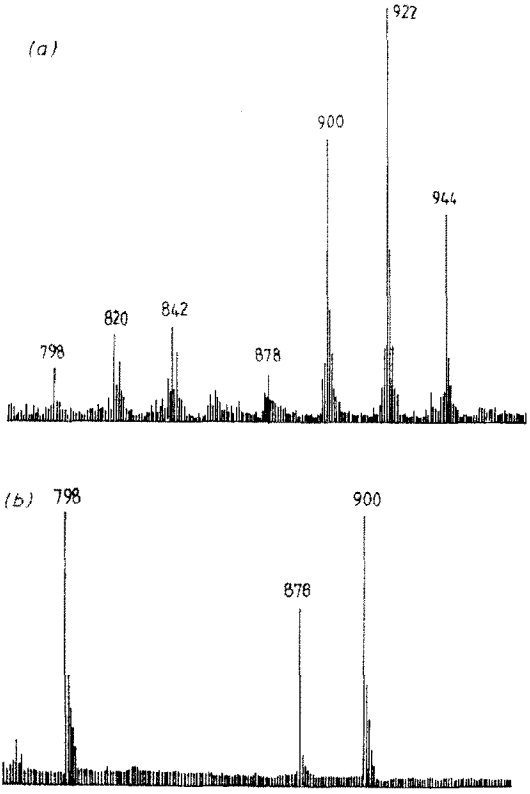


Fig. 1. Negative-ion-mode f.a.b.-mass spectra (acquired at −8 kV) of underivatised HS-4, recorded (*a*) in a matrix of glycerol–1-thioglycerol (1:1, v/v), and (*b*) after removal of the probe, addition of 1 μL of 0.1M HCl, and re-insertion of the probe without any further addition of sample.

are, nevertheless, of high quality and, importantly, the ion current is now distributed among only three ions, namely, the deprotonated molecular ion (*m/z* 878), its mono-sodium salt (*m/z* 900), and the fragment ion at *m/z*798. We have observed similar behaviour with other polysulphated oligosaccharides, namely, the increased abundance of the true (M − H)*, a decrease in the polydispersity, and, usually, an increased signal-to-noise ratio for molecular ions and fragment ions after dosing with 0.1M HCl. In the time scale of the experiment, desulphation was not observed.

It has been reported[21] that crown ethers improve the quality of negative f.a.b.-mass spectra of some biopolymers, by acting as proton acceptors. The efficacy of crown ethers for sulphated compounds was therefore investigated, using HS-4 as a model compound. The f.a.b. spectrum of HS-4 obtained using the matrix recommended by Isobe and co-workers[21], namely, 15-crown5–glycerol (1:10), contained only background signals. After addition of 0.1M HCl, the spectrum shown in Fig. 2a was obtained. The spectrum is similar to Fig. 1b but considerably less intense. However, a spectrum comparable in quality to Fig. 1b was obtained after removal of the probe from the source, addition of monothioglycerol, and re-insertion of the probe (Fig. 2b). Thus, it appears that the crown ether does not improve spectral quality, and that monothioglycerol and dilute acid are the key factors in obtaining good data.

It is possible that crown ethers may help in obtaining f.a.b. data from slightly salty samples by scavenging sodium, but we have had no success using the crown ether for samples fairly heavily contaminated with salt after reduction. These reduced samples, however, gave excellent data after the derivatisation procedures described below.

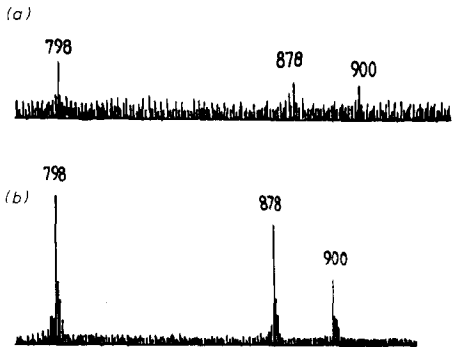


Fig. 2. Negative-ion-mode f.a.b.-mass spectra (acquired at −8 kV) of underivatised HS-4, recorded (*a*) in a matrix of 15-Crown5–glycerol (1:10) after addition of 1 μL of 0.1M HCl, and (*b*) after removal of the probe, addition of 1 μL of 1-thioglycerol, and re-insertion of the probe without any further addition of sample.

*M is defined as the molecular weight of the neutral species in which all of the sulphate and carboxylic acid groups are protonated.

Peracetylation. — The peracetylated sample gave excellent positive f.a.b. data (Fig. 3a), as anticipated from previous experience with other classes of oligosaccharides[18]. Acetylation of HS-4 under acidic conditions resulted in a fully acetylated, desulphated product, giving an (M + H) molecular ion at *m/z* 1140. Less-intense ions at *m/z* 1182 and 1224 were also observed for the mixed anhydride, formed on reaction between one or both of the carboxyl groups of the uronic acid residues and the reagent. In addition to these abundant molecular ions, observable at the 0.1–0.5-μg level, sequence-affording A-type fragment ions[22] are present at *m/z* 590 ($HexAHexNAc^+$) and 850 ($HexAHexNAcHexA^+$). Abundant (M − H) molecular ions were observed in the negative-ion f.a.b. spectrum for the fully acetylated species but not, as expected, for the mixed anhydride (data not shown). Although peracetylation results in chemical loss of the sulphate moieties, it is recommended as a useful first step in characterising an unknown sample since it (*a*) requires considerably less material than is needed for other analyses, (*b*) will yield data however salty the sample may be, (*c*) involves very simple experimental manipulation, and (*d*) provides valuable information on the size heterogeneity of the oligosaccharide backbone and the residue sequence of the components. It is very helpful in subsequent experiments to know the degrees of polymerisation of the components in the sample. Furthermore, as shown later, the removal of sulphates by a simple derivatisation procedure can be exploited in a strategy for ascertaining their location in the sequence.

Permethylation. — Permethylation has been shown to facilitate the f.a.b. analyses of carbohydrates and glycoconjugates by improving sensitivity and directing fragmentation to A-type cleavages[18,23,24]. In order to establish whether permethylated derivatives would facilitate the f.a.b.-m.s. of polysulphated carbohydrates, it was necessary to establish that full methylation could be achieved without loss of the sulphate groups (other workers have suggested that sulphate groups may be lost during Hakomori permethylation[12]) and that the resulting derivatives afforded useful fragmentation patterns. These criteria were examined using HS-4, which, after Hakomori permethylation, gave a product that was eluted from the Sep-Pak with acetonitrile–water (1:1) and afforded the negative f.a.b.-mass spectrum shown in Fig. 3b. Signals corresponding to the expected disulphated, fully methylated molecule are present at *m/z* 1032 and 1054 [(M − H) and (M + Na − 2H), respectively]. Mass-spectrometric cleavage of sulphite gives the signal at *m/z* 952, which is accompanied by a signal 32 m.u. lower at *m/z* 920, due to elimination of methanol. In contrast to the spectra obtained from native samples, the low mass end of the spectrum contains major signals arising from glycosidic cleavages. The signal at *m/z* 574 is consistent with cleavage on the reducing side of the glycosidic oxygen, between the sulphated GlcNAc and the GlcA, accompanied by a methyl transfer. Methyl transfers have been previously observed in chemical ionisation spectra of permethylated oligosaccharides[25]. Evidence that the ion with *m/z* 574 is indeed produced by methyl transfer was obtained after per(trideuteriomethylation) of HS-4. The corresponding fragment ion shifts to *m/z* 595 (see Fig. 3c), indicating

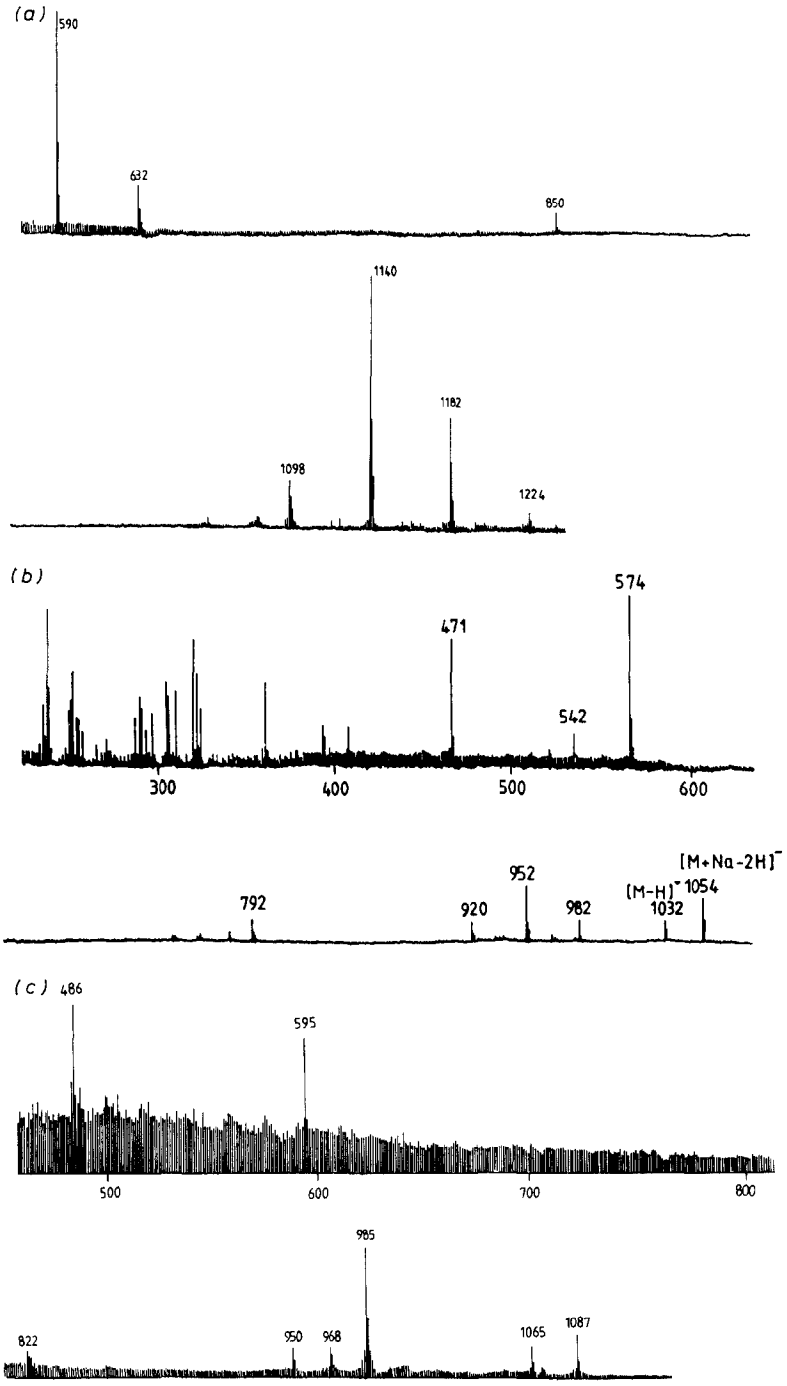
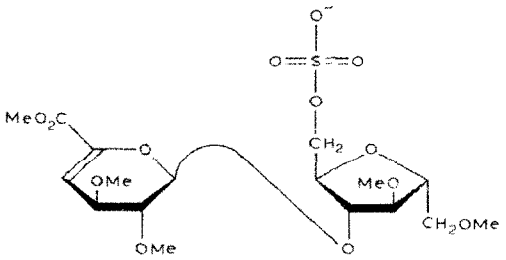


Fig. 3. (*a*) Positive-ion-mode f.a.b.-mass spectrum (acquired at +8 kV) of peracetylated HS-4, recorded in a matrix of glycerol–1-thioglycerol (1:1, v/v). (*b*) Negative-ion-mode f.a.b.-mass spectrum (acquired at −8 kV) of permethylated HS-4, recorded in a matrix of glycerol–1-thioglycerol (1:1, v/v). (*c*) Negative-ion-mode f.a.b.-mass spectrum (acquired at −8 kV) of trideuteriomethylated HS-4, recorded in a matrix of glycerol–1-thioglycerol (1:1, v/v)

the presence of seven methyl groups in the *m/z* 574 ion, which is the exact number expected for the fragment ion resulting from glycosidic cleavage with methyl transfer. The presence of one sulphate group in the non-reducing disaccharide is confirmed by the mass value of the *m/z* 574 fragment ion. A releated sequence ion is present at *m/z* 792, corresponding to the non-reducing disaccharide plus an additional GlcA residue, thus confirming that the second sulphate group is on the anhydromannitol residue. An additional major fragment ion occurs in the low mass region of the spectrum at *m/z* 471, shifting to *m/z* 486 after per(trideuteriomethylation). The mass value and the presence of five methyl groups are consistent with structure **2**, *i.e.*, the product of elimination of the first two residues of the tetrasaccharide.



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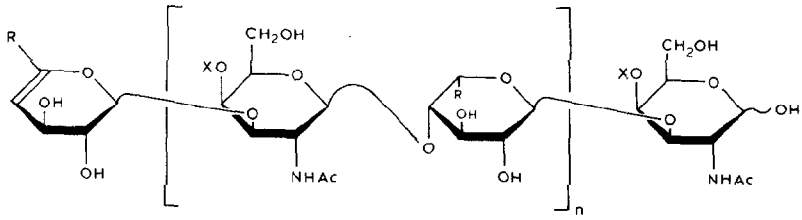
The permethylated sample of HS-4 was examined in the positive-ion mode in order to check for the presence of permethylated, desulphated components which may be expected to ionise preferentially in the positive mode. No evidence for such species was obtained. Having established that the sulphate groups are not lost during permethylation and that sequence-containing ions are produced from permethylated, sulphated molecules in the negative-ion mode, we applied the method to a more challenging sample. A sample of dermatan sulphate oligosaccharides (DS-6, of the general structure **3**), which was too salty to yield f.a.b. data when native, was permethylated and subjected to f.a.b.-m.s. The spectrum shown in Fig. 4 was obtained. Molecular ions are present at *m/z* 1644 (M + 2Na − 3H) for a hexasaccharide, and at *m/z* 2195 (M + 3Na − 4H) for an octasaccharide. Both species show strong signals corresponding to the mass-spectrometric cleavage of one and two sodium sulphite moieties from the molecular ions. In addition, intense sequence-containing fragment ions are present. These are formed by glycosidic cleavages accompanied by methyl transfer and allow sequencing from the non-reducing ends of the molecules. These ions also permit the identification of all sulphated residues, with a single ambiguity at the non-reducing end due to lack of cleavage at the first residue. The fragment ion compositions are given in Table I.

Recently, an alternative to the Hakomori permethylation procedure has been proposed for carbohydrates. This involves the use of sodium hydroxide as the base and is a simpler, cleaner reaction, which is claimed to produce more fully methylated samples[19]. Its applicability to sulphated samples was therefore investigated.



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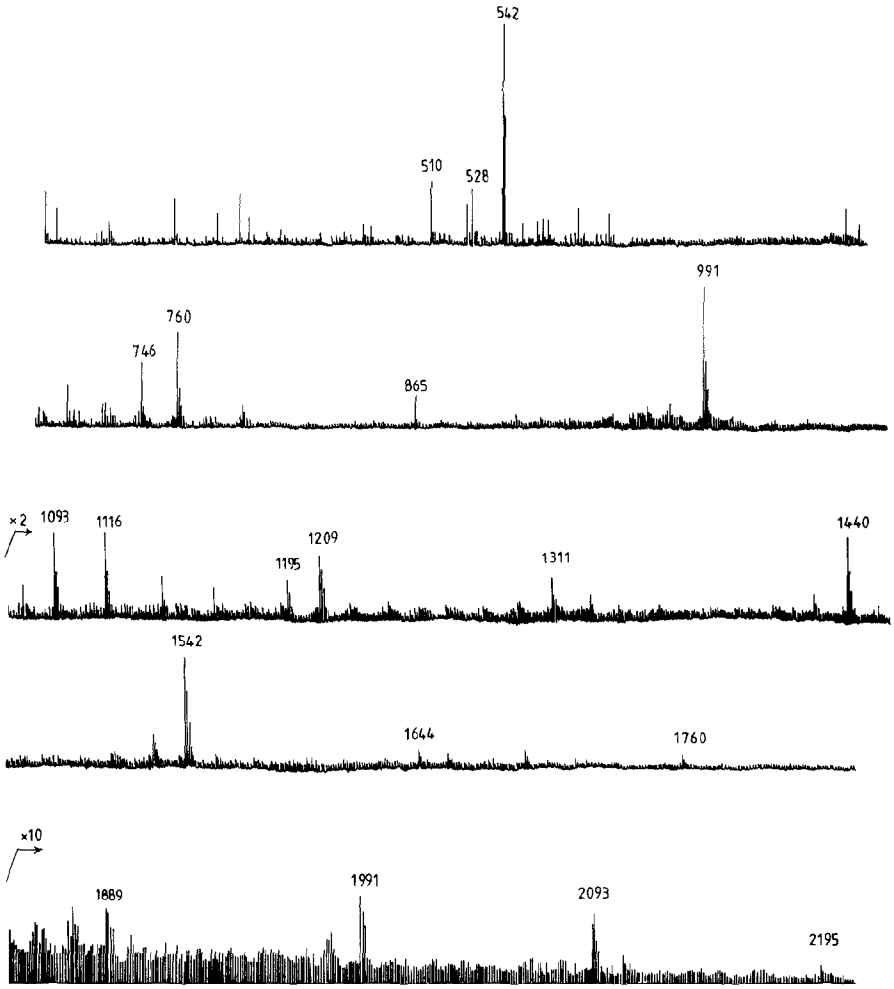

Fig. 4. Negative-ion-mode f.a.b.-mass spectrum (acquired at −7 kV) of permethylated DS-6, recorded in a matrix of glycerol–1-thioglycerol (1:1, v/v).

TABLE I

COMPOSITIONS OF FRAGMENT IONS OBSERVED IN THE NEGATIVE-ION-MODE F.A.B.-MASS SPECTRUM (SEE FIG. 4) OF PERMETHYLATED DS-6

m/z	*Composition*
1311	$\Delta HexA_3HexNAc_2(SO_3^-)_2(Na^+)_1$–OMe
1209	loss of Na sulphite from 1311
1195	one methyl group less than 1209
1093	$\Delta HexA_2HexNAc_2(SO_3^-)_2(Na^+)_1$–OMe
991	loss of Na sulphite from 1093
760	$\Delta HexA_2HexNAc_1(SO_3^-)_1$–OMe
746	one methyl group less than 760
542	$\Delta HexA_1HexNAc_1(SO_3^-)_1$–OMe
528	one methyl group less than 542
510	elimination of methanol from 542

using HS-4 as a model. We have successfully applied this method in the methylation of non-sulphated oligosaccharides and have produced clean, salt-free products after the introduction of a chloroform extraction step prior to the Sep-Pak fractionation. This extraction step is not applicable when examining sulphated samples, so that HS-4 samples which had been permethylated using sodium hydroxide were still extremely salty, even after Sep-Pak purification, and f.a.b. spectra could not be obtained. The Hakomori procedure gives good data and is clearly superior for this type of sample.

Finally, the applicability of the Hakomori procedure to samples that had been reacted with sodium borohydride and still contained considerable quantities of salt was investigated. HS-4 was subjected to normal reducing conditions[20] followed by minimal clean-up (see Experimental). Attempts to obtain f.a.b. spectra without further purification were unsuccessful, even with the use of a crown ether in the matrix. However, after Hakomori permethylation and Sep-Pak fractionation, an excellent negative f.a.b. spectrum was obtained. The data were identical to those shown in Fig. 2b, showing no loss in sensitivity due to the original salt contamination and no loss of sulphate during the reduction conditions normally used for oligosaccharides[20] (other workers, using very vigorous conditions, have reported possible loss of sulphates[12]). HS-4 is not a reducing oligosaccharide and therefore is not altered by sodium borohydride.

Combination of permethylation and peracetylation. — Although an improvement in sensitivity was achieved for the sulphated oligosaccharides after permethylation, and sequence data could be obtained, the intrinsically poor sensitivity of negative ion f.a.b.-m.s., and the less predictable fragmentation pathways observed in the negative ion mode, compared with the positive, may present difficulties in analysis. It was reasoned that a suitable strategy for high-sensitivity f.a.b. analysis in the positive ion mode could be simply devised by first permethylating and then substituting each sulphate residue with an acetyl group using the

$(CF_3CO)_2O$/glacial acetic acid protocol described in the Experimental. The partially acetylated, permethylated molecule would then be amenable to positive f.a.b.-m.s. and would be expected[24] to cleave to yield sequence ions whose masses would define the original location of the sulphate groups, now marked by acetyl moieties. The strategy was assessed using HS-4. Fig. 5 shows the positive f.a.b. spectrum of HS-4 obtained after sequential permethylation and acetylation. A strong (M + H) ion is present at *m/z* 958, corresponding to the expected composition of HS-4 after permethylation and retention of sulphates, followed by replacement of both sulphates with acetates. The major fragment ion at *m/z* 506 is formed by A-type cleavage at the GlcNAc residue, fragmentation behaviour which is that expected for permethylated derivatives of molecules containing amino sugar residues[23]. Further, these permethylated, acetylated derivatives are also suitable for classical methylation analysis procedures using g.l.c.–m.s.[20].

Proposed general strategy for f.a.b.-m.s. analysis of sulphated oligosaccharides. — Sulphated oligosaccharides can be successfully permethylated with retention of the sulphate groups and the resulting derivatives yield sequence ions in the negative ion mode. The sulphate groups can be readily removed from the permethylated molecules and replaced with acetyl groups. The resulting products can then be subjected to all the chemical/f.a.b.-m.s. strategies that have been devised for other classes of carbohydrates[18,24,26]. We now propose the following strategy for analysing sulphated oligosaccharides. (*1*) Part (10%) of the native sample is removed for f.a.b. analysis in the negative ion mode. If data are obtained, they give information on the molecular weight of the components, an indication of the complexity of a mixture, and define the maximum number of sulphate groups on each component. (*2*) A further part (10%) is then subjected to peracetylation, especially if step (*1*) failed to give data. This step should always give data at high sensitivity and they define the composition of the oligosaccharides present and their residue sequences. (*3*) The remainder of the native sample is then permethylated by the Hakomori method and 10% of the products are analysed by negative f.a.b.-m.s. The spectra gained from this analysis are often complex, but should allow compos-

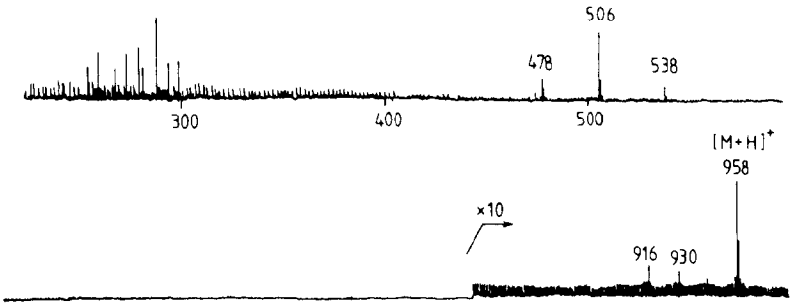

Fig. 5. Positive-ion-mode f.a.b.-mass spectrum (acquired at +8 kV) of sequentially permethylated, acetylated HS-4, recorded in a matrix of glycerol–1-thioglycerol (1:1, v/v).

itions, sequences, and sulphated residues to be assigned. (*4*) The remaining 90% of the permethylated sample is acetylated and 10% of the products are examined in the positive mode. The spectra allow composition and sequence to be defined unambiguously, the sites of sulphation being marked by acetyl moieties. If only a limited amount of sample is available, the sensitivity of the analysis may be greatly enhanced if the f.a.b. analyses in steps (*1*) and (*2*) are omitted and the entire sample is permethylated, followed by acetylation, and examination by f.a.b.-m.s. only after the final acetylation step. (*5*) If sequence or sulphation-site data remain incomplete or ambiguous at this stage, we recommend that the remainder of the sample be used for the time-course methanolysis procedure previously reported[26]. To remove any possible ambiguities arising from their hydrolysis, the acetyl groups may be chemically replaced prior to methanolysis by carrying out a Hakomori methylation on the partially methylated, partially acetylated product, using trideuteriomethyl iodide so that acetyl groups are replaced with trideuteriomethyl groups.

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