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MONOMER SEQUENCE DETERMINATION OF CARBOHYDRATES USING FAST-ATOM BOMBARDMENT MASS SPECTROMETRY OF PERIODATE-OXIDIZED ACETATE ESTER DERIVATIVES*

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ABSTRACT

A derivatization method, adapted from that of Angel *et al.* (ref. 10), for sequencing sugar residues in partially degraded poly- and oligo-saccharides using positive-ion f.a.b.–m.s. is described. Derivative selection provides sequence information by directing fragmentation exclusively to both sides of glycosidic oxygen atoms and, in the case of opened rings, between glycosidic carbon and ring oxygen atoms. Polysaccharides or oligosaccharides are subjected to sequential periodate oxidation, borodeuteride reduction, and acetylation. The derivatized polysaccharides are then subjected to partial degradation, acetylation, and high-performance liquid chromatography (h.p.l.c.) purification. F.a.b.–m.s. data obtained on model compounds, using 3-nitrobenzyl alcohol as matrix for f.a.b.–m.s., demonstrated direction of fragmentation to both sides of the glycosidic oxygen atom in unoxidized residues, and to both sides of the acetal oxygen atoms in oxidized residues. Oligosaccharide linkage and sequence may thus be determined by observing fragmentation from both the reducing and non-reducing ends of the molecule. Two *Salmonella* lipopolysaccharides, derivatized by this procedure, were partially hydrolyzed and then acetylated. Analysis of the h.p.l.c.-purified oligosaccharide derivatives by f.a.b.–m.s. demonstrated the applicability of the technique for sequencing nmol quantities of branched structures.

INTRODUCTION

Early applications of fast-atom-bombardment mass spectrometry (f.a.b.–m.s.)[1] to the analysis of oligosaccharides were associated with technical difficulties regarding sensitivity. Sensitivity can often be increased by derivatization, choice of

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matrix, use of matrix additives, and other factors[1–4]. Permethylation and peracetylation have been utilized extensively in f.a.b.–m.s.[5,6] as well as in chemical ionization (c.i.–m.s.)[7] and electron impact (e.i.–m.s.)[8] mass spectrometry to enhance sensitivity and to obtain partial sequence information. Derivatization alone, however, does not give f.a.b. spectra yielding sequence data when multiple monomers in the oligosaccharide are of the same type, such as multiple hexoses or amino sugars.

Although linkage analysis of derivatives (such as methylated alditol acetates[9]) may be accomplished readily by g.l.c.–m.s., the sequence in which the individual monosaccharides are linked is not always apparent when the oligosaccharide is larger than a trisaccharide. Angel, *et al.*[10] have described a procedure for sequencing oligosaccharides that reintroduced periodate oxidation, followed by reduction with $NaBD_4$ and permethylation of the product. Analysis of the f.a.b. mass spectra of these products provides a means for sequencing, for both order and linkage position, on the basis of characteristic mass additions to the masses of fragment ions arising from the non-reducing terminal sugar.

We have further developed this methodology and adapted it to polysaccharides. Peracetylated rather than permethylated derivatives were used in order in direct fission to the carbon–oxygen bonds of the oligosaccharide backbone. The f.a.b. mass spectra of the peracetylated derivatives give unique information not yielded in the spectra of the permethylated derivatives, in that fission occurs on either side of the glycosidic oxygen atom not only at the reducing terminal, but at internal linkages. Thus, since fragmentation is observed from both the reducing and non-reducing ends of the molecule, redundant sequence information is obtained. Borodeuteride reduction is utilized to differentiate a native primary alcohol function from one introduced by periodate oxidation followed by sodium borodeuteride reduction, and to distinguish 6-linked from 2-linked oxidized reducing terminal residues.

The most common matrices used for obtaining f.a.b. mass spectra of carbohydrates are glycerol and thioglycerol[2]. 3-Nitrobenzyl alcohol has been shown recently to be an effective matrix compound for obtaining high quality f.a.b. mass spectra for a wide variety of compounds[11,12]. We report here the use of 3-nitrobenzyl alcohol as a matrix for f.a.b.–m.s.; it gives spectra for oxidized and peracetylated carbohydrates of greatly enhanced quality compared with the spectra obtained using the more conventional matrices or those of the non-acetylated compounds.

The very simple fragmentation pattern obtained through cleavage predominantly on either side of the glycosidic oxygen and ring oxygen atoms in oxidized ring structures[13] lent itself to the use of computer system, GLYCOSPEC, written in Turbo Pascal (Borland)[14]. The database is currently available to analyze spectra resulting from the periodate-oxidized, borodeuteride-reduced, and peracetylated carbohydrate derivatives, but is readily modified to accomodate other periodate-oxidized derivatives.

F.a.b.–m.s. analysis of purified, partially degraded polysaccharides demonstrates that the combination of this method with linkage analysis yields extensive structural information for both linear and branched oligosaccharides as well as for partially degraded polysaccharides. We demonstrate the utility of this combination of technologies using several model compounds and two *Salmonella* lipopolysaccharides. Preliminary reports of part of this have been published[13,14].

EXPERIMENTAL

Chemicals and reagents. — Oligosaccharides used as model compounds were obtained from Sigma Chemical Co. (St. Louis, MO 63178). 3-Nitrobenzyl alcohol was obtained from Aldrich Chemical Co. (Milwauke, WI 53233). All chemicals and reagents were of the highest quality available, and all liquid solvents and reagents were glass-distilled prior to use. The *Salmonella* polysaccharide preparations were those previously used in structural work[14,15].

Methods. — Samples (0.5–2.0 mg) of lactose, fucosyllactose, nigeran tetrasaccharide, and lipopolysaccharides from *Salmonella strasbourg* and *Salmonella typhimurium LT2* were dissolved at a concentration of 1.0 mg mL^{-1} in 0.12M $NaIO_4$ and incubated for 48 h at 4° in the dark. Iodate and excess periodate were removed by using Dowex 1-X4 anion-exchange resin (Cl^- form).

The samples were then reduced with $NaBD_4$ (10 mg) for 2 h, followed by decomposition of excess borodeuteride and removal of Na^+ by addition of cation-exchange resin (H^+ form) in excess. Boric acid was then removed by evaporation with MeOH, and water removed by evaporation with EtOH. The samples were then treated with a mixture of pyridine (1 mL) and Ac_2O (2 mL) for 20 min at 100°. Pyridine and Ac_2O were removed by codistillation with toluene. The *Salmonella* lipopolysaccharides alone were then treated with CF_3CO_2H (1 mL, 25°) for 45 sec, followed by addition of Ac_2O (2 mL) and kept at room temperature for 15 min. Toluene was added and the samples were again evaporated.

All samples analyzed in this study were dissolved in $CHCl_3$ and extracted with water. The organic phase was then dried and the samples were redissolved in the developing phase of the reverse-phase h.p.l.c. system. H.p.l.c. employed a developing phase of 65% MeCN and 35% water on a Bio-Rad ODS-10 column (150 × 4 mm) and a Waters Model 440 liquid chromatograph equipped with a differential-refractometer detector. Fractions were collected as detected (10 μg or larger) or based on elution times based on a standard curve using di-, tetra- and hepta-saccharides for calibrations. After evaporation, fractions were dissolved in $CHCl_3$ and aliquots thereof were subjected to f.a.b.–m.s. analyses.

F.a.b. mass spectra were recorded with a VG 70-250HF g.l.c.–m.s. instrument (high-field magnet) equipped with a standard unheated VG f.a.b. ion source and a saddle field gun (Ion-Tech Model B11N) producing a beam of xenon atoms at 8 keV and 1 mA. The spectrometer was adjusted to a resolving power of 2500 and spectra were obtained using an accelerating voltage of 6 keV, which

provides a mass range of about 2600 daltons. Scans were obtained at 10 sec/decade with the various instrumental parameters optimized for positive-ion extraction. 3-Nitrobenzyl alcohol was applied to the stainless-steel stage via glass rod and was spread out as a thin, continuous layer. Compounds were dissolved in $CHCl_3$, at concentration of 1–100 μg μL^{-1}, and 1–1.5 μL (2–200 nmol) of this solution was added via syringe to the center of the stage.

Quantitative determinations of the sensitivity of the methodology were made by recording f.a.b. mass spectra of a serially diluted, derivatized sample which was originally 1 mg of fucosyllactose.

RESULTS AND DISCUSSION

Oligo- and poly-saccharides used had exclusively pyranosyl residues. Presentation of results is simplified by numbering carbon atoms in oxidized residues as in the original unoxidized rings. Pyranose ring-oxygen atoms are referred to as such, whether or not oxidation has opened the ring.

Successful application of this methodology may require additional information regarding the mode of attachment of the individual residues, as by methylation linkage-analysis[9], or from literature information on related compounds.

Periodate oxidation of non-reducing terminal hexoses and 6-deoxyhexoses, and internal hexoses substituted at O-6, results in loss of C-3 as formic acid. Periodate oxidation of internal hexoses substituted at O-2 or O-4 produces oxidatively opened rings without loss of carbon atoms. Internal 3-substituted hexoses, with or without additional linkages, are not oxidized. Non-reducing terminal 3,6-dideoxyhexoses and branched hexoses substituted at O-2 and O-4 are not oxidized.

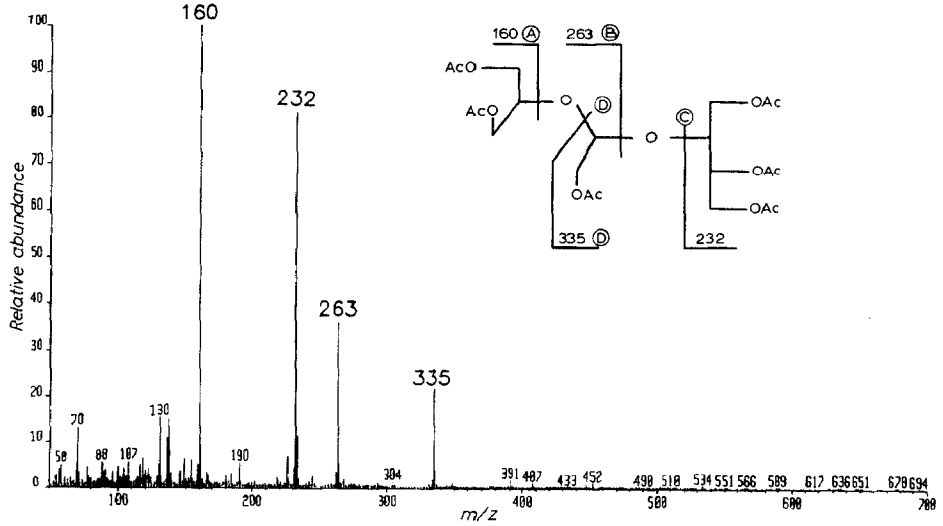

Fig. 1. F.a.b. mass spectrum of derivatized lactose.

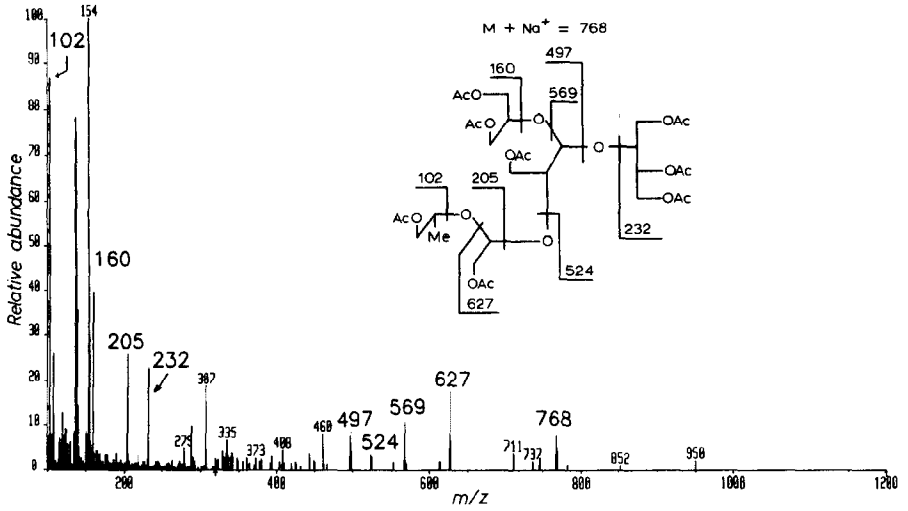

Fig. 2. F.a.b. mass spectrum of derivatized fucosyllactose.

Initial analysis of the f.a.b. mass spectra revealed simple, readily interpretable cleavage-patterns. The f.a.b. mass spectrum of the compound derived from lactose (Fig. 1 illustrates the four simple and predominant types of cleavage observed (for simplicity, deuterons introduced by borodeuteride reduction following periodate oxidation are not shown).

Fragmentation of the molecule with charge retained on the non-reducing end resulted in ions with *m/z* values of 160 and 263. The *m/z* 160 ion arises by cleavage between C-5 and the ring oxygen of the original non-reducing terminal residue. This type of cleavage is hereafter designated as type A. The ion having *m/z* 263 results from cleavage between the glycosidic carbon and oxygen. This type of cleavage is hereafter designated as type B.

Fragmentation of the molecule with charge retention on the reducing end resulted in ions having *m/z* 232 and 335 (Fig. 1). The ion having *m/z* 232 is attributable to cleavage between the reducing alditol and the glycosidic oxygen. This type of cleavage is hereafter designated as type C. The ion having *m/z* 335 is attributable to cleavage between the glycosidic carbon and ring oxygen. This type of cleavage is hereafter designated as type D. A peak at *m/z* 534 of low abundance is attributable to MNa^+ (not shown).

Fucosyllactose, having non-reducing terminal L-fucose, 2-linked galactose and 4-linked glucose residues, was used as a quantitative standard. Approximately 2 nM (1 μg) of starting material, subjected to the derivatization procedure, gave the spectrum shown in Fig. 2. This spectrum demonstrates clearly that the compound can be sequenced readily in a manner similar to that described for lactose (Fig. 1). At this level of sample the matrix signals *m/z* 136, 154, 289, 307, and 460 are visible but the signal/noise ratio for the sample is still high. Type A fragments having *m/z* 102 and 160 demonstrate the oxidized ring structures of the non-

reducing terminal fucose and the 2-linked galactosyl residues, respectively. The presence of *m/z* 205 indicates a non-reducing terminal fucosyl residue. The fragment ion *m/z* 205 demonstrates the presence of a non-reducing terminal 6-deoxyhexosyl residue and *m/z* 497 suggests a sequence of either Fuc-(1→2)-Gal or Fuc-(1→4)-Gal. The type D fragment *m/z* 569 eliminates the alternative of O-4 substitution. From the type C fragment (*m/z* 232) and type B fragment (*m/z* 497) it follows that the 4-linked glucose is the reducing end. The type D fragment (*m/z* 627) corroborates the sequential conclusions. The presence of MNa^+ = 768 provides information of the mass of the intact molecule. These results indicate the potential of this methodology for sequence information on oligosaccharides in the low nanomole range.

Smith degradation of carbohydrates yields mixtures of oligosaccharides separable only with difficulty and with loss of material. The simultaneous f.a.b.–m.s. analysis of nigeran tetrasaccharide and a related trisaccharide illustrates this problem and the ability of this methodology to resolve mixtures of oligosaccharides. The f.a.b.–m.s. analysis gave the data shown in Fig. 3. The *m/z* fragments at 1186 and 822 are attributable to the sodiated molecular ions of the modified nigeran tetrasaccharide and the related trisaccharide. The oligosaccharide is composed of glucose residues substituted at O-3 and O-4. Sequence data thus does not need to consider 2- and 6-substituted alternatives. As discussed later, however, the methodology when applied to a homopolysaccharide derivative does yield the sequence defined as order and linkage. The fragments 160, 263, 551, 740 and 843 constitute types A, B, B, A, and B fragmentation from the non-reducing end, respectively. *M/z* 160 and 263 arise from the non-reducing terminal residue. The type B fragment having *m/z* 551 demonstrates that the non-reducing terminal residue (*m/z* 263) is linked to O-3 of the adjacent sugar residue. *M/z* 555 would be the expected fragment, had this linkage been at O-2 or O-4 and the O-6 substitution would have appeared as *m/z* 483. The fragment *m/z* 843 (type B) indicates an oxidized 2- or 4-linked residue added to the disaccharide derivative signaled by *m/z* 551. The type A fragment (*m/z* 740) is 551 plus 189 and demonstrates that the 3-linked residue is linked to the 4-position of an oxidized ring structure, thus eliminating the O-2 alternative. From these data, it follows that the sequence from the non-reducing end is Glc-(1→3)-Glc-(1→4)-Glc-X, where X is an oxidized reducing terminus. Hexoses at the reducing end are oxidized to yield type C fragments which are unique to the type linkage. Hexose substituted at O-2, O-3, O-4, and O-6 would yield *m/z* 161, 304, 232, and 160, respectively. (Had borohydride been used in place of borodeuteride, reducing-terminal 2-linked and 6-linked hexoses would have both yielded type C fragments of *m/z* 159). Fig. 3 shows both *m/z* 304 and 232, and demonstrates the presence of both 3-substituted and 4-substituted reducing hexoses. The other type C fragment from the reducing end results from the addition, to the reducing end, of 288 for a 3-linked residue. The spectrum reveals the presence of *m/z* 520 and 596 which suggests, respectively, the presence of a 3-linked residue penultimate to the 4-linked reducing terminal (*m/z*

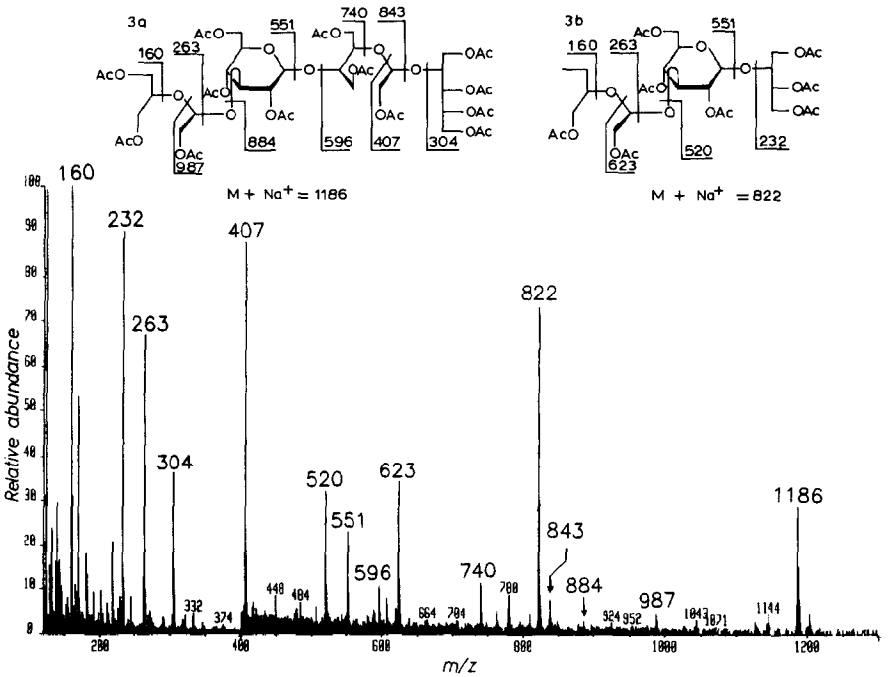

Fig. 3. F.a.b. mass spectrum of derivatives of nigeran tetrasaccharide and a related trisaccharide resulting from Smith degradation.

TABLE I

GLYCOSPEC PRINTOUT[a] OF ITS SOLUTIONS OF THE F.A.B.-M.S. DATA SHOWN IN FIG. 3

Analysis from reducing end: Hex-(1→3)-Hex-(1→4)-Hex; Hex-(1→3)-Hex-(1→4)-Hex-(1→3)-Hex

Analysis from non-reducing end: Hex-(1→3)-Hex-(1→4)-Hex-(1→3)-Hexı Hex-(1→3)-Hex-(1→4)-Hex

Proposed peak attributions

m/z *Symbolic representation*[b]	*Corresponding fragment of intact structure*
232 (R4LHEX)	→4)-Glc
304 (R3LHEX)	→3)-Glc
263 (NRHEX)	Glc-(1→
520 (R4LHEX=232)+(3LHEX=288)	→3)-Glc-(1→4)-Glc
551 (NRHEX=263)+(3LHEX=288)	Glc-(1→3)-Glc-(1→
596 (R3LHEX=304)+(4LHEX=292)	→4)-Glc-(1→3)-Glc
623 (520)+(NRHEX E 103)	-Glc[C,1-2]-(1→3)-Glc-(1→4)-Glc
740 (551)+(4LHEX E 189)	Glc-(1→3)-Glc-(1→4)-Glc[C,3-6]
822 (740)+(OAc)+Na^+	Glc-(1→3)-Glc-(1→4)-Glc + Na^+
843 (551)+(4LHEX=292)	Glc-(1→3)-Glc-(1→4)-Glc-(1-
884 (596)+(3LHEX=288)	→3)-Glc-(1→4)-Glc-(1→3)-Glc
987 (884)+(NRHEX =103)	→Glc[C,1-2]-(1→3)-Glc-(1→4)-Glc-(1→3)-Glc
1186 (843)+(R3LHEX=304)+Na^+	Glc-(1→3)-Glc-(1→4)-Glc-(1→3)-Glc + Na^+

[a]The data base also contained linkage-analysis data that demonstrated the presence of 3- and 4-substituted glucose residues. [b]

520), and a 4-linked residue penultimate to the 3-linked reducing terminal (*m/z* 596). *M/z* 884 is derived from 596 + 288, demonstrating that a 3-linked residue is linked to the 4-linked penultimate residue from the reducing end. Type D fragments, formed by the addition of *m/z* 103 and indicative of C-1 and C-2 of a residue oxidized at C-3 and beyond, are signaled by *m/z* 623, and 987. *M/z* 623, along with MNa^+ 822 and other sequential ions, demonstrates that a non-reducing terminal residue is linked to O-3 of the residue (1→4)-linked to the reducing end, thereby revealing the presence of a trisaccharide. The tetrasaccharide arises from *m/z* 884 plus 103 and demonstrates that an oxidized residue is linked also to the 3-linked derivative, which was (1→4)-linked to the residue (1→3)-linked to the reducing end.

The A, B, C, and D type fragmentations shown in Fig. 3 can be derived only from a mixture of a tri-, and tetra-saccharide and shows the resolving power of this sequence method, which gives structural information from both the reducing and non-reducing ends of the oligosaccharide derivatives. Thus, a degraded sample or a limited mixture of structures may be resolved without complete purification of each component. The redundancy of the overlapping information obtained strengthens confidence in low-abundance, higher-mass ions such as *m/z* 884. Note that ions formed on each side of any of the ring or glycosidic oxygen atoms are present in the spectrum. The sum of the *m/z* values of each of these pairs of complementary fragment-ions plus sodium plus oxygen gives the expected MNa^+ ion for the tri-, or tetra-saccharide at *m/z* 822 or 1186, respectively, as shown in Fig. 3.

The simple fragmentation patterns from both the reducing and non-reducing end, described in Figs. 1–3, invited the use of computer system GLYCOSPEC, designed to deduce possible structural solutions to a given database. The investigator creates the database from known sugar- and linkage-analysis data, to reduce the possible linkage combination to manageable numbers. Peaks relevant or irrelevant from the f.a.b.–m.s. analysis are then added to the data base and GLYCOSPEC is asked to produce solutions. Given the linkage analysis and significant and insignificant f.a.b.–m.s. peaks shown in Fig. 3, GLYCOSPEC produces the solutions shown in Table I.

F.a.b.–m.s. analysis of mixtures of linear oligosaccharides is here demonstrated to be possible. Application of the methodology to the analysis of mixtures of branched and linear oligosaccharides is the ultimate test of its usefulness.

To ascertain the applicability of the methodology to complex branched polysaccharide structures, we subjected *Salmonella* polysaccharides of known structure to sequential periodate oxidation, borodeuteride reduction, partial acid hydrolysis, and acetylation. The oligosaccharide derivatives were fractionated by h.p.l.c. into tri- through penta-saccharide, and octa- through deca-saccharide fractions. The *S. typhimurium* LT2 lipopolysaccharide contained, from sugar and linkage analysis, equimolar amounts of non-reducing terminal abequosyl- (3,6-dideoxy-D-*xylo*-hexose = Abe), 4-linked L-rhamnosyl, 3-linked D-galactosyl and, 2,3-disubstituted

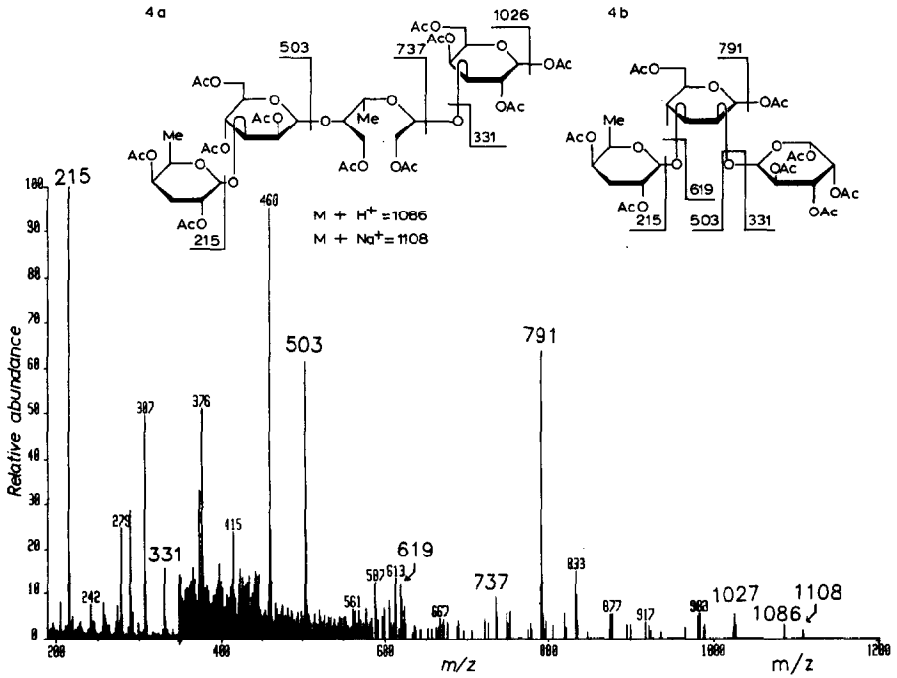

Fig. 4. F.a.b. mass spectrum of derivatives of LPS from *Salmonella typhimurium*.

D-mannosyl residues. *M/z* for a non-reducing abequosyl type B fragment is 215. Type B or C fragmentation of the other three residues, when internal, results in adjacent type B or C fragment-ions plus 234, 288, and 245, respectively. As terminals, these are 43 mass units larger. The fragment having *m/z* 331, resulting from type B, or C cleavage, is attributable to an unoxidized, non-reducing or reducing terminal (Figs. 4a or 4b, respectively). Because of the hydrolysis step in the procedure, an originally internal 3-linked hexosyl residue could, in fact, be present at either end of an isolated oligosaccharide derivative as *m/z* 331, and originally branched residues may appear as chain residues (adjacent type B or fragment plus *m/z* 288) in the final derivative. A reducing-terminal rhamnosyl residue would appear as *m/z* 174. Evaluation of alternative acylation reagents after Smith degradation is currently ongoing.

The spectrum derived from the partially degraded and purified *S. typhimurium* LT2 lipopolysaccharide is shown in Fig. 4. Table II lists the possible solutions according to GLYCOSPEC to the *m/z* values in ascending order from the non-reducing end. The database contained the linkage and f.a.b.–m.s. data. The non-reducing ends are Abe (NR36DDH) and non-reducing hydrolyzed 3-linked hexoses (NRH3LHEX), resulting from Smith degradation of the oxidized polysaccharide. Fragmentation from the non-reducing end yields the linear structure 4A and the branched structure 4B. Based on the fragmentation principles displayed in the spectra so far, fragments of approximately equal size and intensity from either

TABLE II

GLYCOSPEC SOLUTIONS TO F.A.B.-M.S. DATA, GIVEN A DATA BASE THAT CONTAINED ALL EXPECTED LINKAGES FROM THE LINKAGE ANALYSIS AND ANTICIPATED RESULTS FROM THE PARTIAL ACID HYDROLYSIS STEP INVOLVED IN THE SAMPLE PREPARATION

m/z *Symbolic representation*[a]	*Corresponding fragment of intact structure*
Structure 4A	
215 (NR36DDHEX=215)	Abe-(1→
503 (215)+(3LHEX=288)	Abe-(1→3)-Man-(1→
737 (503)+(4L6DHEX=234)	Abe-(1→3)-Man-(1→4)-Rha-(1→
1026 (737)+(3LHEX=288)	Abe-(1→3)-Man-(1→4)-Rha-(1→3)-Gal-(1→
1086 (1026)+(OAc)+1 = M+H$^+$	Abe-(1→3)-Man-(1→4)-Rha-(1→3)-Gal-OAc + H$^+$
Structure 4B	
215 (NR36DDHEX=215)	Abe-(1→
331 (NRH3LHEX=331)	Gal-(1→
791 (331)+[NR36DDH B 215]+(B23LHEX=245)	Gal-(1→2)-[Abe-(1→3)]-Man

end of the molecule should be found. The major fragment (*m/z* 503) proposed by GLYCOSPEC and the minor fragment (*m/z* 619) would arise from the acetylated trisaccharide Gal-(1→2)-[Abe-(1→3)]-Man having *m/z* 850 by cleavage at the Gal and Abe linkage, respectively. The proposed Abe-(1→3)-Man- structure having *m/z* 503 is not absolutely conclusive with respect to linkage, as the partial hydrolysis could have removed the 2-linked galactosyl residue. Reversal of the two linkages would give the same result. Two arguments may be offered for the proposed linkage assessment. Firstly, the abequosidic linkage is so much more acid-labile than the galactosidic that it is likely that the disaccharide fragment arises from the reducing end of the polysaccharide. Secondly, if the abequosidic linkage in the fragment signaled by *m/z* 503 had been to O-2 of mannose, a fragment having *m/z* 443, arising from loss of acetic acid, would be expected[10] (see later). *M/z* 619 is attributable to the corresponding fragment where the abequosidic residue has been hydrolyzed. That *m/z* 619 is only a minor component in the spectrum suggests that the fragment mainly derives from the non-reducing end of a linear oligosaccharide rather than from the reducing end of the branched trisaccharide.

This result illustrates that GLYCOSPEC, given linkage information and f.a.b.–m.s. peaks, can propose solutions and thereby provide unbiased sets of possibilities for consideration. In the absence of linkage and f.a.b.–m.s. data

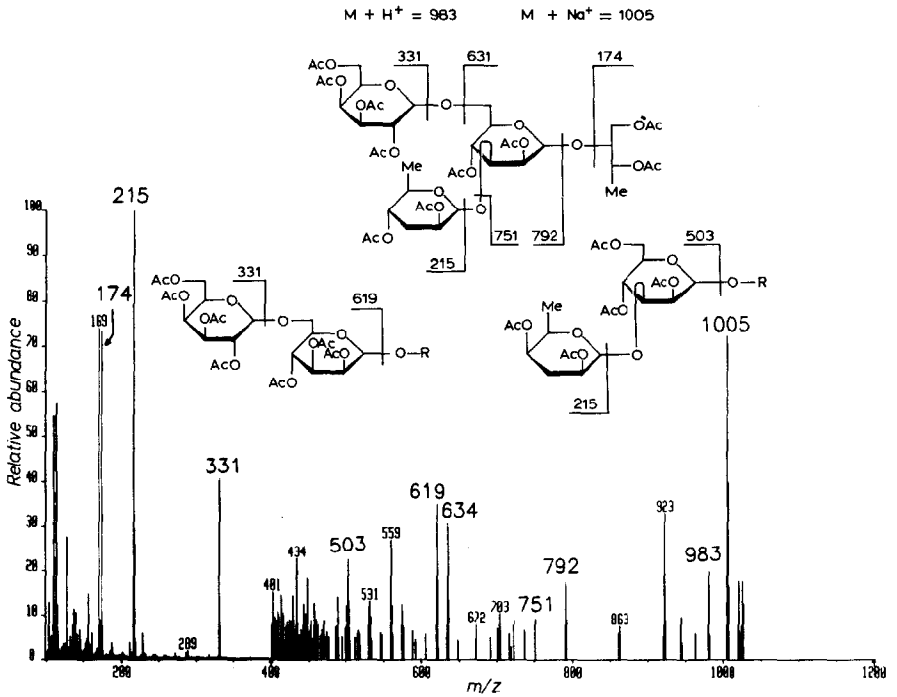

Fig. 5. F.a.b. mass spectrum of an early h.p.l.c. fraction of derivatives of LPS from *Salmonella strasbourg*.

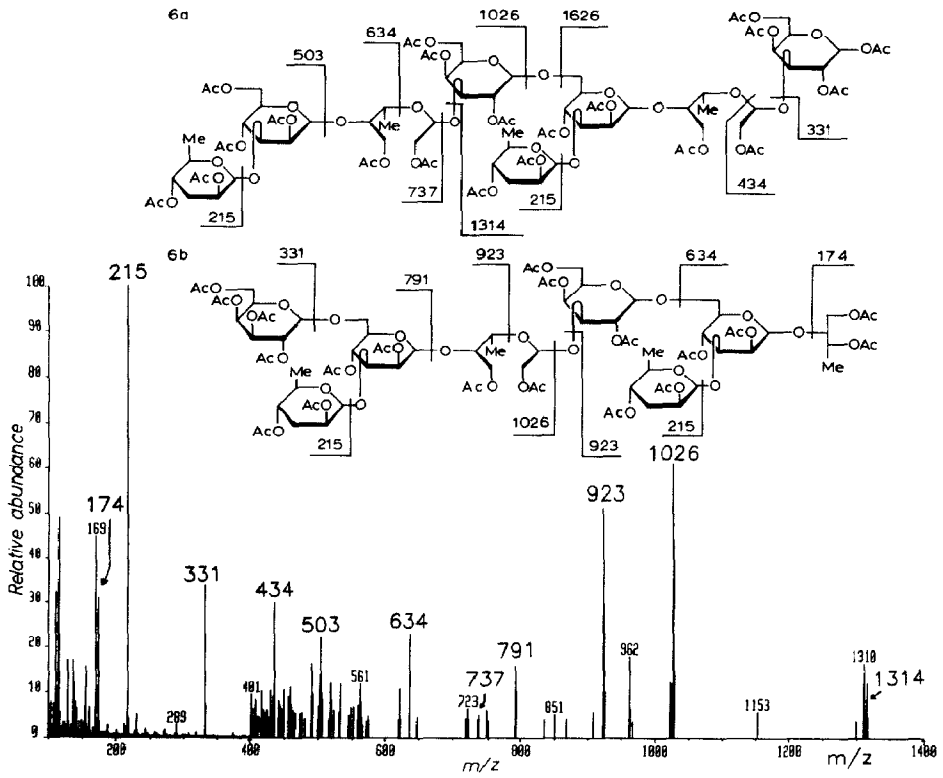

Fig. 6. F.a.b. mass spectrum of a late h.p.l.c. fraction of derivatives of LPS from *Salmonella strasbourg*.

information, there would be 4! × 8 × 8 × 6 × 4 = 36,864 solutions to a Man, Gal, Rha, Abe tetrasaccharide.

The spectra obtained for two h.p.l.c. fractions of partially degraded derivatives of *Salmonella strasbourg* lipopolysaccharide are shown in Figs. 5 and 6. The polysaccharide differs from the previous one only in that the mannose residue is substituted at O-3 and O-6 and by replacement of abequose by tyvelose (3,6-di-deoxy-D-*arabino*-hexose = Tyv) as the non-reducing terminal. Rhamnose is 4-linked and galactose is substituted at O-3. Fig. 5 shows results from an early h.p.l.c. fraction containing one major and two minor components. The fragmentation pattern is that expected from the major structure in Fig. 6. The type B fragments (*m/z* 215 and 331) demonstrate the presence of the non-reducing terminal tyvelosidic and hydrolyzed 3-linked galactosidic residues, the presence of which is corroborated by the type C fragments *m/z* 634 and 751, respectively. Assessment of the site of attachment of the two residues to the branched mannosidic residue is possible. The 6-linkage of the formerly 3-linked galactose residue to mannose could be assigned because of the existence of an oligosaccharide derivative, constituting a hydrolysis product from which the dideoxyhexose is missing. The primary type B fragment, *m/z* 619, is observed. Loss of 60 (acetic acid) beta to the charge, produces

the secondary fragment *m/z* 559, indicating that the C-2–C-3 bond was intact, and O-3 was acetylated. Thus, O-3 had been substituted by the dideoxyhexose. As may be seen, the hydrolysis product signaled by the fragment having *m/z* 503 did not lose acetic acid, because of the 3-linkage. Other cleavage patterns are as observed in other Figs., in addition to type C cleavage at the 6-linkage of the branch hexose.

Fig. 6 shows the mass spectrum of an h.p.l.c. fraction of the hydrolyzate that corresponds to an octa- to deca-saccharide. The type B and A fragments suggest the presence of both linear (6a) and branched (6b) non-reducing ends. These could be combined with the two primary reducing ends, indicated by the type C and D fragments, and depicted in Fig. 6, giving a total of four possible major structures. The spectrum clearly demonstrates that the fragmentation pattern obtained, on account of the redundancy of overlapping fragments, allows for the safe assignment of sequence for hexasaccharides and for reasonable assessment of larger structures. Furthermore, the repetitive nature of the polysaccharide is readily established.

The combined methodologies of linkage analysis and f.a.b.–m.s. of periodate-oxidized, borodeuteride-reduced, acetylated oligosaccharides and subsequently, partially hydrolyzed, and reacetylated polysaccharides, coupled to a computer-based system that uses an appropriate database from data obtained from these methodologies, offers a powerful tool for structural assessment of most biologically and pathophysiologically significant carbohydrate components. The structurally significant epitope recognized by a protein would rarely be larger than a hexasaccharide[17].

The quantitative analysis showed that nmol quantities of oligosaccharides may be sequenced for linkage and order. Polysaccharides may require somewhat larger amounts of material, because of the need for partial hydrolysis to generate oligomers for sequencing and resolution by h.p.l.c. with sufficient care in derivatization and purification, 100 μg of polysaccharide is adequate for complete sequence-analysis. Care must be taken not to over-hydrolyze to the extent of total loss of more-labile monomers.

Spectra obtained in our early studies using a glycerol matrix were of poor quality and sensitivity; no useful spectra were obtained at high mass[12]. However, sensitivity was improved by at least an order of magnitude upon using 3-nitrobenzyl alcohol matrix.

All of the spectra were obtained with a relatively small double-focusing instrument with manual sample-introduction. Use of the methodology reported here, in combination with a more-modern high transmission instrument, perhaps with a diode array detector, should yield greatly improved results on smaller sample sizes than those achieved in the current work. Further improvements may be achieved by utilization of dynamic f.a.b. for the direct transfer from the h.p.l.c. output to the ion source.

M.s.–m.s. of these derivatives may help to resolve the problem of iso-substituted hexoses within the same oligosaccharide.

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