FAST ATOM BOMBARDMENT MASS SPECTROMETRY OF OLIGOSACCHARIDES AND GLYCOPEPTIDES.

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ABSTRACT.

Fast Atom Bombardment (FAB) was first introduced in 1980 and the potential it offered in conjunction with high mass facilities for the analysis of biological molecules was instantly recognised. Although the technique was rapidly exploited for the examination of peptides, its application in the field of carbohydrate analysis was almost totally unexplored at the time the work described in this thesis was begun.

Strategies have been explored which allow FAB-MS to be successfully used in the examination of carbohydrates and glycopeptides: the behaviour of both underivatised and derivatised samples under FAB conditions has been assessed and derivatisation protocols developed in order to obtain structurally useful data from samples.

The development of these techniques has been carried out in parallel with their application to solving the following biological problems:

1) In conjunction with standard carbohydrate techniques the full structures of the carbohydrate portions of Band 3 glycoprotein from adult erythrocytes and of polyfucosyl and sialylated fucosyl lactosaminoglycans from human granulocytes have been unambiguously determined. Enzymic digestion products were sequenced by FAB analysis of peracetyl and permethyl derivatives, while intact glycopeptides were examined after permethylation - characteristic fragmentation occured and so allowed the non-reducing terminal regions of very large molecules to be "mapped".

2) (i) Glycopeptide storage products accumulating in the brains of dogs with fucosidosis have been sequenced by making use of the FAB properties of both underivatised and derivatised samples.

(ii) The mixture of mannosidosis storage products present in crude urine and cell extracts have been profiled using a combined derivatisation and extraction step allowing separation of the derivatised sample from inorganic contaminants. The mixture of derivatives was then characterised by FAB.

3) The structure of haptenic Enterobacteriaceae common antigen (ECA) has been assigned using a combination of approaches including:

(i) FAB of the underivatised sample,

(ii) FAB monitoring of time course reactions,

(iii) FAB of derivatives.

The data proved unambiguously that haptenic ECA is a cyclic molecule and as such is the first naturally occuring cyclic complex oligosaccharide to be described.

For Bobby and Henry.

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CHAPTER 1 : INTRODUCTION.

This thesis describes the development of Fast Atom Bombardment Mass Spectrometry (FAB-MS) as a technique for carbohydrate structure analysis, and presents some examples of its application in solving biological problems.

Carbohydrates are found in a very wide variety of biological systems, either in the free form or as glycoconjugates, and an appreciation of their importance in such systems is growing rapidly. Their distribution includes being found in secretions, in structural material, as cell surface markers and as components of biologically active glycoproteins such as lectins, enzymes, etc., and as such have been assigned roles in immunological, enzymatic and recognition processes.

In order to understand the mechanisms of the systems in which carbohydrates are implicated the structures of the molecules involved must be determined. However, complete structure assignment of carbohydrates is considerably more difficult than that of peptides or polynucleotides, since in addition to obtaining the composition and linear sequence of the residues, it is also necessary to define the anomeric configuration, stereochemistry and linkage of each residue, and to locate any branch points. A wide variety of chemical and spectrometric techniques have been developed for use in such analyses and a combination of these must be employed in order to make a complete structure assignment. Many of these techniques require access to specialist instrumentation and frequently demand a high level of expertise, so that the choice of approach often depends as much on the practical limitations of the laboratory as on the problem under investigation. Routine

structure analysis of carbohydrates, analogous to that of proteins, is possible in very few laboratories since the wide variety of structures found requires that the approach used is dictated by the sample under examination. An initial and very serious drawback of many classical methods which examine intact species is that they are only applicable to relatively small molecules (< 1000 dalton), whereas the majority of biologically important carbohydrates have masses in excess of 1000 dalton. The introduction of FAB-MS, together with the availability of high field magnet technology offered a way round this limitation.

FAB was first introduced in 1981 (1) and its potential as a tool for the analysis of biomolecules was instantly recognised. Its exploitation, particularly in the peptide field, was rapid (2,3,4, 5, 6). In 1981, when the work in this thesis was begun, the application of FAB to problems in the carbohydrate area was still limited. However, preliminary studies of a 3500 molecular weight mono-methyl glucose polymer had indicated that FAB could be valuable for carbohydrate analysis (7). Further, the combined facilities of High Field and FAB-MS, available at the Imperial College laboratory, offered a unique opportunity to develop and apply FAB techniques to very complex carbohydrates.

In order to present the FAB studies in appropriate perspective a short description of some of the more commonly used "classical" techniques is now presented. This discussion is not intended to be comprehensive but merely to provide a background against which to consider the new technique.

1.1 Composition Analysis.

The first step in any structure analysis is to determine the residues comprising the polysaccharide or glycoconjugate. A

monosaccharide mixture is obtained on complete acid hydrolysis which cleaves all the glycosidic linkages; this mixture may then be analysed using one of a variety of detection methods. Early monosaccharide determinations were carried out using colourimetric and paper chromatographic techniques (8) but these have now been superceded by g.l.c. methods (9). Derivatisation is necessary to make the monosaccharides volatile enough for g.l.c. and they are most frequently examined as trimethyl silyl ethers (10) or alditol acetates (11). Methyl ethers were the first volatile derivatives to be used for g.l.c. (12) although they are less easily prepared and less well resolved on g.l.c. than other derivatives (9). Identification of residues is based on the comparison of the g.l.c. behaviour of the unknowns with that of standard derivatives.

1.2 Methylation Analysis.

Methylation analysis may be considered to be a variation on the above approach, in which the intact molecule is permethylated and then degraded : it is to be favoured since data is obtained on linkage position and terminal residues in addition to composition (13). Complete methylation may be achieved using the method of Hakomori (14) and is carried out on intact glycolipids, glycopeptides or oligosaccharide alcohols. The methylated derivative is then degraded using acid hydrolysis to yield partially methylated monosaccharide units. The conditions used for the acid hydrolysis must be varied depending on the data required; hexosyl and amino hexosyl residues may be released using acetolysis (0.5 N sulphuric acid in 95% acetic acid) followed by aqueous acid hydrolysis (15). Under such conditions acid labile neuraminic acids are destroyed, so to release these residues methanolysis is used (16) (0.5 M hydrogen chloride in methanol at 80 ^{o}C for 18 hr.) - neuraminic acids are

obtained as the methyl glycosides. Samples are thus generally divided into two parts - one part is methanolysed and analysed for neuraminic acid while the other is acetolysed and hydrolysed to yield hexose and aminohexose residues. The products of these degradations are identified by g.l.c.- m.s. of the acetates (17) obtained on reduction and acetylation of the neutral and hexosamine residues, and by direct acetylation of neuraminic acids. The combined g.l.c. and MS data allows the methyl substitution pattern of the partially methylated sugars to be assigned, while the g.l.c. retention times allow isomers to be distinguished, e.g. galactose and mannose are retained differently on g.l.c. columns although their mass spectral behaviour is similar. A wide variety of suitable capillary columns is now commercially available on which to separate the products of hydrolysis. Identification of components is based on the EI mass spectral data on the peak, or by comparison with retention times of standard components - g.l.c.- m.s. identification of all the common components of oligosaccharides and glycoconjugates is thoroughly dealt with in the literature, and standard retention times are also well documented (for references see (18)).

Quantitation of the partially methylated derivatives may be calculated from the g.l.c. responses as determined by flame ionisation detection (19, 20).

It is possible to carry out g.l.c.- m.s. analysis on small alkylated samples (4-5 residues) without degradation (21,22) and this can yield important data on the sequence of the residues. If, however, the sample is too large to be sequenced directly by this method methylation analysis can be carried out before and after specific degradation techniques to determine where modifications have taken place.

1.3 Specific Degradations.

After determination of the composition further information on the structure of a polysaccharide may be obtained by partial degradation and examination of the products. Such partial degradation may be achieved using conditions which are known to produce specific cleavages; these methods include both chemical and enzymatic techniques.

1.3.1 Acidic methods. One of the most common methods of specific degradation is acid hydrolysis, e.g. 0.5 M HCl at 80 - 100 ^{o}C for several hours. Under these conditions the most susceptible glycosidic linkages are those formed by furanoses and by deoxy sugars. Within the group of deoxy sugars susceptibility differs, the most labile residues being those in which the deoxy group is vicinal to the glycosidic carbon, e.g. neuraminic acid. Less susceptible are 3,6-dideoxy sugars and finally 6 deoxy residues ; the glycosidic linkage of uronic acids and of 2-amino,2-deoxy sugars is resistant (23). Thus, a large polysaccharide which contains a few labile residues may be partially degraded on acid hydrolysis into smaller fragments which may be further analysed. It is also commonly used to remove fucose and N-acetyl neuraminic acid (Nana) residues from resistant backbones.

Acetolysis provides a complementary technique to aqueous acid hydrolysis since it preferentially cleaves 1→6 linkages which are resistant to acid hydrolysis. Acetolysis involves incubation of the sample with a mixture of glacial acetic acid : acetic anhydride : concentrated sulphuric acid (10 : 10 : 1, v:v:v) and yields peracetylated fragments which are then analysed directly, or after deacetylation. Acetolysis is a convenient means of degrading large branched polymers in which 1→6 branches are present or in which

branches arise from a repeating 1→6 linked backbone, as in the studies of Lee and Ballou on yeast mannans (24).

1.3.2 Release of carbohydrate chains from proteins. Trifluoro-acetolysis has been used to isolate N- and O- glycosidically linked carbohydrate chains from glycoproteins (25) based on the observation that most glycosidic linkages are stable under these conditions,while proteins are degraded by transamidation. The technique involves incubation of the sample with a mixture of trifluoro acetic anhydride and trifluoroacetic acid : the carbohydrate is released as the pertrifluoroacetate in which N-acetyl hexosamine residues are converted to N-trifluoroacetyl hexosamines.

N-glycosidically linked carbohydrates may be removed specifically using anhydrous hydrazine - this reagent causes de-N-acetylation and cleaves all amide bonds - the peptide is thus degraded and the GlcNAc - Asn bond cleaved. Hydrazinolysis may be followed by nitrous acid deamination in the protocol developed by Bayard (as described in (8))- cleavage occurs at $GlcNH_2$ residues with their conversion to 2,5-anhydromannose. The mixture of 2,5-anhydromannose containing oligosaccharides can then be analysed further, e.g. (26).

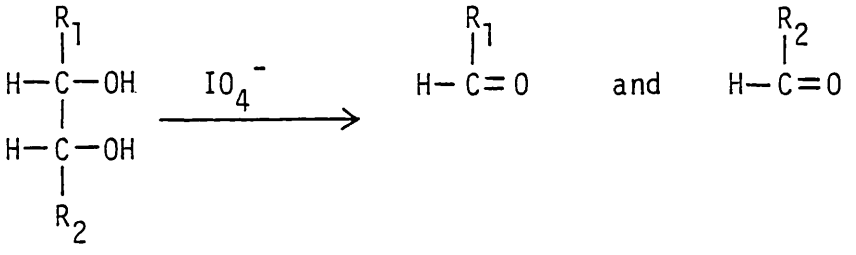
Alternatively hydrazinolysis may be followed by re-N-acetylation and analysis of the intact carbohydrate chain, e.g. (27).

O-linked carbohydrate chains may be specifically removed using alkaline degradation conditions although the released sugar then continues to be degraded by peeling reactions, in which reducing end residues are lost sequentially until an alkaline stable structure is formed (28). It is possible to recover the intact carbohydrate chain if the degradation is performed in the presence of a high concentration of $NaBH_4$ (29), e.g. 1 M $NaBH_4$ in 0.05 N NaOH at 45 ^{o}C. Under these conditions the alkali resistant sugar alcohol is formed

before the peeling reaction can occur.

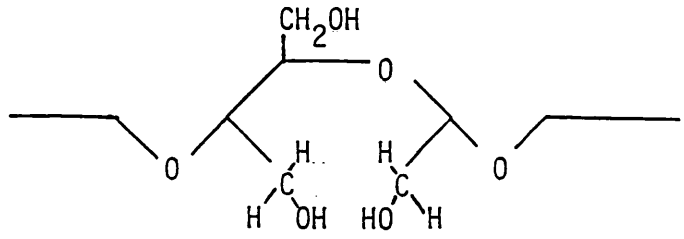
However, in 1981 Rasilio and Renkonen (30) showed that these conditions, which were assumed to specifically release O-linked chains, also caused release of N-linked oligosaccharides in the form of glycopeptides or oligosaccharide alcohols. This GlcNAc - Asn degradation is assumed to be caused by the borohydride included to prevent peeling reactions. The technique is still useful for release of oligosaccharides, although it can no longer be considered to be specific to O-linked chains.

1.3.3 Oxidations. One of the most useful and widely used cleavages is the Smith degradation, introduced in 1959, which cleaves polysaccharide chains by degradation of residues bearing adjacent hydoxyl groups (31). The protocol involves periodate oxidation followed by borohydride reduction and mild acid hydrolysis. The periodate reaction is specific for vicinal hydroxyl groups and may be represented as



The polyhydroxylic nature of carbohydrates renders them perfect substrates for this reaction. Before the introduction of the Smith degradation this oxidation was used on its own in carbohydrate analysis (32); information was provided by the amount of periodate consumed in the reaction, and by analysis of the products formed. The aldehyde groups formed during this oxidation of a sugar residue are in the form of hemiacetals and so the residues remain cyclic.

On borohydride reduction a noncyclic polyalcohol is formed:



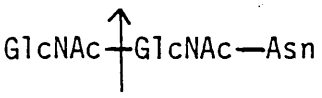
- this is hydrolysed more readily than the glycosidic linkages because of its linear form. The products of acid hydrolysis are thus fragments of the oxidised residues which may be released totally or remain linked as aglycons to other residues, depending on their position in the original residue.

The anomeric configuration of sugar residues may be determined using the chromium trioxide oxidation (33). The protocol is based on the observation that fully acetylated aldopyranose residues where the most stable chair form is produced by an equatorial aglycon (generally the β-form) are oxidised more quickly than the corresponding axial aglycon (34). The technique for anomeric determination involves reduction of the oligosaccharide, acetylation and treatment with chromium trioxide in acetic acid. Sugar analysis of the product identifies the surviving residues which must have thus been α-D linked.

1.3.4 Enzymatic cleavages. In addition to these chemical degradations the use of specific enzymes for structural analysis is becoming more widespread despite the high cost of many of these reagents at present. The enzymes may be considered in three groups - exoglycosidases, endoglycosidases and proteases. The proteases may be used to release carbohydrate chains from glycoproteins by cleavage of peptide bonds

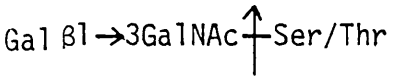
to release glycopeptides. The most widely used example is pronase which is very nonspecific, eventually cleaving most peptide bonds to release glycopeptides with generally only a single amino acid attached. More specific proteases may be used such as trypsin, chymotrypsin, etc. to release glycopeptides with larger peptide moieties, although digestions may require extended incubations since glycosylation may confer resistance to digestion (35).

Endoglycosidases may also be used to release carbohydrate chains from glycoproteins. The products are generally oligosaccharides : glycoaspartamidase cleaves the GlcNAc - Asn bond as long as the Asn residue is not linked to any further amino acids (36), while endo-β-N-acetyl glucosaminidase cleaves as shown:



There are several different forms of this enzyme each with its own specificity for the sugar moiety (37).

The enzyme which releases the carbohydrate chain from O-linked glycoproteins is an endo-α-N-acetyl galactosaminidase which cleaves thus:



(38).

Endo-β-galactosidases cleave the glycosidic bond of β-galactose residues dependent on their specificity for the remainder of the sugar chain.

A wide variety of exoglycosidases is now available - these have been isolated from many different sources. They release a single residue at a time from the nonreducing terminus and include

α- and β- galactosidases, α- and β- mannosidases, β-N-acetyl hexosaminidase, α-N-acetyl galactosidase, α- fucosidase and neuraminidase. A typical strategy for sequencing using specific exoglycosidase enzymes involves labelling of the reducing residue by NaB^3H_4 reduction, followed by chromatography, digestion and rechromatography (39). The running position of the radiolabelled component changes if digestion has resulted in cleavage.

1.4 Examination of Undegraded Species.

In addition to the data obtainable from degradative methods, valuable information can also be derived by analysing the interactions of intact carbohydrates with lectins and antibodies. Both lectins and antibodies are glycoproteins with binding sites on their surfaces which are specific for certain carbohydrate structures, and as such may be used in structural studies.

Lectins were originally found in plants, the most well known being Concanavalin A from the jack bean (40). Almost all lectins contain two or more carbohydrate binding sites and so cause agglutination of red cells which bear the appropriate carbohydrate structure on their surfaces. Very many lectins have now been isolated and their specificities described ; their applications include affinity chromatography, haemagglutination and fluorescence studies (41). Lectins have the advantage of being isolable in gram quantities, while antibodies are inducible and have a wider range of specificities: the availability of hybridoma technology for the production of monoclonal antibodies has ensured the wide use of immunological techniques in the carbohydrate field. Very many monoclonal antibodies are now available from both natural sources, eg the anti-I and anti-i cold agglutinins (42) and from hybridoma cultures, eg(43) and their applications include immunoassays, immune precipitation of cell surface antigens, fluorescence studies and immunostaining

of soluble products or TLC plates (44).

1.5 N.M.R.

Spectroscopic techniques have been used in carbohydrate stuctural studies for many years but recent developments in instrumentation and computer programming have made Nuclear Magnetic Resonance spectroscopy(NMR) particularly useful in this field (45). Both proton and ^{13}C nuclear resonances are useful in carbohydrate work and a variety of data may be obtained depending on the technique employed. The development of magnets with increased field strengths has improved resolution and so enabled the study of molecules with larger molecular weights, while the availability of Fourier Transform has improved sensitivity to the µg level for proton work. High resolution ^{1}H NMR has been used very effectively in structural studies by Vliegenthart and colleagues who have developed the concept of structural reporter groups to allow information to be obtained from the spectra of the complex carbohydrate chains of N-linked glycoproteins (46, 47). Structural reporter groups are protons which resonate at clearly defined positions in the spectrum - their chemical shifts, coupling constants and signal widths contain the information essential for primary structure assignment. These structural reporter groups are

1) anomeric protons - the chemical shift and coupling constant characterise the sugar residue, its configuration and linkage,
2) H-2 and H-3 protons of mannose - these indicate the substitution in branching structures,
3) H-3 of sialic acid - the chemical shift reveals the configuration and linkage and sometimes the position of the residue in the chain,
4) H-5 and methyl protons of fucose - the chemical shifts, with that of the anomeric proton characterise linkage and configuration and define the structural environment especially of the residue

to which the fucose is linked,

5) H-3 and H-4 protons of Gal - in some cases the chemical shifts characterise linkage and configuration of galactose,

6) aminosugar N-acetyl methyl protons - the chemical shifts are very sensitive to even small structural variations.

In addition to these chemical shift and coupling constant data, the line widths of these structural reporter group signals give information about the local mobility of the protons. Apart from the signals for the structural reporter groups, the spectra contain a broad signal in a characteristic position corresponding to nonanomeric sugar skeleton protons, which can not be resolved down to individual protons.

An alternative method for primary structure assignment is that used by Dabrowski and coworkers (48) in which peracetylated derivatives are examined using high resolution proton NMR. This technique allows protons attached to glycosylation sites to be readily recognised since their signals remain unshifted, while protons linked to acetylated carbons undergo a pronounced down field shift.

^{13}C NMR is complementary to proton NMR techniques - it gives a better separation of signals due to the wider range of chemical shifts, although it is less sensitive due to the low natural abundance of the ^{13}C nucleus. The chemical shifts of ^{13}C nuclei are characteristic of their chemical nature and environment, while spin-spin coupling constants yield information about the bond angles between coupled nuclei. ^{13}C NMR spectra may be recorded using broad band proton decoupling, in which case a relatively simple spectrum is obtained containing one signal per atom, or may be obtained as proton coupled spectra - these are more complex

and show the number of protons covalently bound to each carbon atom, and may also contain some splittings or line broadenings due to 2- and 3- bond long range couplings. The assignment of signals in ^{13}C spectra is based on the observation that the chemical shifts produced by the monosaccharide constituents of polysaccharides are similar to those of the free monosaccharides, apart from substituent effects : when a carbon atom is glycosyl substituted its resonance moves downfield, while adjacent carbon atoms generally resonate at slightly higher field than before so allowing sites of glycosylation to be identified. The assignment of resonances in monosaccharides was originally made with reference to a glucose standard and although a complete assignment is rarely possible, especially in a polysaccharide, the anomeric carbon and carbons bearing methylene, amino or substituted amino groups have characteristic resonances and so can be assigned.

In addition to its use in primary structure determination, the advent of high field and computer technologies have made NMR suitable for 3-dimensional studies too (49).The 3-dimensional solution conformation of a molecule is defined by the relative orientation of residues as described by the angles between linkage groups. Information on these orientations may be obtained by examination of

1) inter-residue shielding and deshielding in ^{1}H and ^{13}C spectra,
2) inter-residue ^{1}H-^{13}C and ^{13}C-^{13}C coupling across the glycosidic linkage,
3) inter-residue ^{1}H-^{1}H spin lattice relaxation and nOe phenomena,
4) inter-residue H-bonding.

The data obtainable from such experiments is very complex so that analysis is greatly facilitated by using computer-assisted molecular modelling techniques such as Hard-Sphere Exo-Anomeric

effect calculations (HSEA) (50). HSEA calculations have been used in solution conformation studies on the ABO and Lewis blood group antigens (49) and then extended and applied to the complex linear periodic polysaccharide which carries the Y,O- antigen in Shigella flexneri (51).

A variety of 2-dimensional NMR techniques are now available which are useful in improving the resolution in NMR spectra. This can be useful in sequence analysis and is particularly helpful in interpretation of spectra to yield solution conformation data (52).

1.6 Mass Spectrometry.

The use of EI and CI-MS in combined g.l.c.-m.s. experiments as described above represents only one of many MS applications in the carbohydrate field. A variety of ionisation techniques has now been employed but electron impact is the one which has been most widely used in the structure analysis of carbohydrates - sequence and sometimes linkage may be assigned from the fragment ions formed.

Since carbohydrates are nonvolatile thermally labile compounds EI-MS is generally carried out on their more volatile derivatives, eg methyl ethers, acetates, etc. A very useful analysis of the EI behaviour of carbohydrate derivatives was made by Kochetkov and Chizhov (53) who elucidated the detailed fragmentation and ionisation behaviour of small permethylated carbohydrate molecules. The principles established in that study have since been successfully applied to larger and more complex compounds and EI-MS of permethylated oligosaccharides has played a very important part in structural studies on carbohydrates and glycoconjugates.

The introduction in 1977 of the high field magnet (54, 55), providing a mass range of 3,300 dalton at full sensitivity, made a

huge contribution in the area of biological mass spectrometry in view of the high molecular weights of many bioactive substances, and so had a marked effect in the carbohydrate field.

Some of the most notable EI studies on high mass carbohydrates have been carried out by Karlsson and coworkers (56,57) who have used a variety of techniques, including MS, in the analysis of bioactive glycolipids : EI analysis of the permethylated and the permethylated reduced derivatives allows structural data to be obtained on both the carbohydrate chain and the lipid moiety.

Egge and colleagues have also produced some impressive results using high mass instrumentation, eg during their work on the characterisation of urinary oligosaccharides in human mannosidosis (58) in which samples were examined as the reduced permethylated derivatives; molecular ions were observed above mass 2,000 dalton.

The major disadvantage of EI is the loss of signal intensity at the high mass end of the spectrum, which can result in the loss of the molecular ion, and sometimes some of the higher mass fragment ions. The EI-MS protocol can be improved by additionally examining the underivatised sample by field desorption(FD-MS). FD is a soft ionisation technique in which the sample is ionised from the solid phase producing quasimolecular ions of the $M+H^+$/$M+Na^+$ type;this provides molecular weight data on the sample (59). Although FD is potentially a very useful technique because of its µg level sensitivity and applicability to underivatised samples it has many technical drawbacks which limit its wide use.

In 1981 the new ionisation technique of Fast Atom Bombardment (FAB) was introduced (1) which features many of the advantages of both EI and FD while avoiding many of their problems. In the original studies ionisation was achieved by bombardment of the sample

with a beam of accelerated argon atoms.This beam of neutrals is generated by resonance charge exchange between a beam of accelerated argon ions and neutral argon gas maintained at high pressure in a collision chamber - the resulting beam contains both charged and uncharged species having the same kinetic energy as the original ions ; the charged species are removed by electrostatic deflector plates. It was subsequently shown (60) that the bombarding particles do not need to be neutral and that identical results may be obtained using an ion gun in which no attempt is made to remove ions from the mixture of atoms and ions generated.

Unlike other MS procedures, eg. CI,EI etc., FAB ionisation is carried out from a solution of the sample in a high boiling solvent such as glycerol. The term matrix is used to describe the liquid phase from which the sample is ionised. Although the mechanisms of ionisation and desorption involved in FAB have not been established, sample volatility is not required, merely solubility in the matrix used. Sample ionisation occurs on bombardment of the surface of the matrix with the atom/ion beam. Ionisation in the positive mode occurs by protonation or cationisation to form pseudomolecular ions of the form $M+H^+$, and $M+Na^+$ or $M+K^+$. Analogous ions in the negative mode are $M-H^-$ and $M+Cl^-$. These ions give information about the molecular weight of the sample, while the internal structure is characterised by the fragment ions which may also be formed.

Ionisation and fragmentation may be manipulated by altering the matrix and the solvent in which the sample is loaded. The matrix and solvent must be miscible to allow dispersion of the sample, so that, for example,methanol and aqueous acid solutions are suitable for use with glycerol, although chloroform is not. Use of different matrices can improve the quality of the spectra, eg. thioglycerol,

which is more volatile than glycerol, may be used to improve the intensity of signals, although they are then less persistent. A useful compromise can be arrived at by using a 1:1 (v:v) mixture of glycerol and thioglycerol (61); 1,1,3,3-tetramethyl urea has been found by some workers to be more suitable than glycerol for running glycolipids (62). In addition to variations in the matrix, it is also possible to use additives in order to improve ionisation. This is generally achieved by loading the sample into the matrix in a particular solvent and then adding extra components into the matrix afterwards, eg. aqueous solutions of ammonium (63) or alkali metal salts (64) can improve sensitivity by providing a species which readily cationises to form pseudomolecular ions.

FAB is particularly useful for the analysis of biomolecules which are generally involatile substances with molecular weights in excess of 1000 dalton : since ionisation occurs from solution there is no requirement for sample volatility, and since the technique is essentially a soft ionisation procedure, molecular ions are obtained even from high molecular weight samples.

At the time when this study was first undertaken FAB had been applied to a variety of biological molecules, including peptides (2,65), bleomycins (66), cobalamines (67) and antibiotics and nucleoside phosphates (3), although at this time no papers were published about the application of the technique to carbohydrates or glycoconjugates.

The development of FAB for carbohydrate analysis was carried out in parallel with its application in various structural studies - selected examples of these are presented in the following chapters in order to illustrate the following aspects of the technique:

1) its high mass capability,

2) its sensitivity,

3) its applicability for sequencing,

4) its power to reveal sample heterogeneity and sites of chemical modification.

The examples illustrate the applicability of FAB to several different types of problems including

1) its power to complement and extend classical methods (chapter 2),

2) its use as a sensitive alternative to these methods (chapter 3), and

3) in its application to the study of the Enterobacterial common antigen (chapter 4), its unique ability to solve a structural problem which remained intractable to other techniques.

CHAPTER 2 : CELL SURFACE ANTIGENS.

2.1 Introduction.

Probably the best known examples of cell surface antigens are the ABH blood group determinants carried on red blood cells. Their existence was reported at the turn of the century by Landsteiner (68, 69) who showed that red blood cells could be classified into groups on the basis of antigenic substances on their surfaces. An enormous number of cell surface antigens are now recognised, not just on erythrocytes but on other blood cells and on cells of nervous and somatic tissues as well.

The presence of such highly specific determinants on the surface of cells has aroused much interest and although the particular functions of most of these structures are not understood they are thought to be involved in specific cell-cell interactions. In order to improve our understanding of their biological role, chemical studies have been undertaken on a variety of cell surface antigens and have revealed that in the majority of cases the antigenic structure is borne by the carbohydrate moiety of a glycolipid or glycoprotein.

The major intrinsic glycoprotein of the human erythrocyte membrane is Band 3 glycoprotein which is thought to function as an anion transporter. The molecular weight of the glycoprotein is 90-100,000 dalton (70) of which 7-11,000 dalton is carbohydrate (71). The carbohydrate portion has been shown to carry ABH (72) and Ii (73) antigens and to contain a repeating N-acetyl-lactosaminyl backbone:

$$\cdot(Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3)_n$$

attached to a mannose, N-acetylglucosamine, asparagine core -

this type of structure has been termed lactosaminoglycan.

The structure and the amount of lactosaminoglycan change significantly during differentiation of human erythroid cells, and during the development from foetus to adult (74). The differentiation from immature erythroid cells to the mature form is accompanied by a marked increase in the amount of lactosaminoglycan expressed on the cell surface (75); foetal erythrocytes bear unbranched lactosaminoglycan which has foetal (i) antigenic activity, while adult erythrocytes express the adult (I) antigen carried by branched lactosaminoglycan (76). ABH antigenic specificity is also carried by lactosaminoglycan through substitution of non-reducing terminal galactose residues with antigenic determinants (72).

In addition to its presence in the Band 3 glycoprotein of erythrocytes, the lactosaminoglycan structure has been found in a variety of other cells including Friend erythroleukaemic cells (77), human vascular endothelial cells (78), mouse embryonic cells (79) and human myeloid (granulocyte-monocyte) cells (80).

As well as the changes in the expression of lactosaminoglycan already outlined for red blood cells, studies suggest that similarly significant changes of lactosaminoglycan expression occur during human myeloid cell differentiation (80).

In order to understand the mechanism and the significance of these changes in surface antigen structure and expression a long term programme of investigation was initiated by Dr. Minoru Fukuda (La Jolla Cancer Research Foundation) during which structural studies have been carried out on the surface antigens expressed by erythrocytes and granulocytes at different stages of maturation.

The FAB studies described in this section were carried out

on samples provided by Dr. Fukuda. The objectives of the FAB work were threefold:

a) to establish suitable protocols for analysing complex carbohydrates,

b) to elucidate the fragmentation behaviour of complex carbohydrates and glycopeptides, and

c) to contribute to the structure elucidation programme by providing sequence information which could not be more easily obtained by classical procedures.

The full structure of the carbohydrate moieties present on the Band 3 glycoprotein of adult erythrocytes and the polyfucosyl and sialylated fucosyl lactosaminoglycan of human granulocytes were thus determined by a combination of FAB and classical procedures. The data provided by FAB were essential for the correct interpretation of the results of the classical studies. The FAB results alone will be presented in this thesis since the classical studies were carried out by other workers and are reported, together with the FAB data,in the literature (81, 82, 83).

2.2 Results and Discussion.

During these studies two types of sample were analysed requiring the development of two different analytical strategies. Firstly, specific enzymic digests yielded relatively low molecular weight fragments which were fractionated by gel filtration and analysed by FAB as the underivatised and the acetylated species. During this work a generally applicable procedure (see chapter 3) was developed which allows data to be obtained from samples which contain aqueous soluble contaminants, such as salts. Secondly, high molecular weight glycopeptides and polysaccharides were examined as the permethyl derivatives. These results represent the first observation that very high molecular weight samples can

yield FAB spectra and as such demonstrate a novel application of FAB. The spectra obtained comprise fragment ions derived from the non-reducing end of the molecule and so allow the non-reducing termini of such large molecules to be "mapped".

During this programme of research a large number of samples of both types have been examined allowing an overall view of the FAB behaviour of the different species to be built up. In order to summarise these findings a few specific examples of each type of sample are chosen to illustrate the phenomena observed.

2.2.1 Low molecular weight oligosaccharides. Four samples are discussed here (see table 2.1), three produced from polyfucosyl lactosaminoglycan and the other from sialylated fucosyl lactosaminoglycan, both from human granulocytes. All samples were prepared by Dr. Fukuda by digestion of lactosaminoglycan glycopeptides with endo-β-galactosidase, reduction of the fragments with $NaBH_4$ and isolation of the products by gel filtration.

The hexa/heptasaccharide, tetrasaccharide and trisaccharide fractions were examined underivatised in the positive mode, but no results were obtained. This was interpreted as suppression due to salt contamination, as indicated by the abundant Na^+ adduct ions observed in association with the glycerol cluster ions in all the spectra.

When fraction 30-34 was examined underivatised in positive FAB two weak molecular ions were observed at m/z 839 ($M+H^+$) and 861 ($M+Na^+$) for $Nana,Hex_2HexNAcitol$. Since this molecule carries an overall negative charge the sample was then analysed in the negative mode. Under these conditions three molecular ions were observed, all of the ($M-H^-$) type,at m/z 837 (most abundant), 983 and 1348

TABLE 2.1.
SAMPLES DESCRIBED IN CHAPTER 2.

SAMPLE CODE	SOURCE
Low molecular weight samples;	
Hexa/Heptasaccharide	Polyfucosylated Lactosaminoglycan
Tetrasaccharide	——————" ——————
Trisaccharide	——————" ——————
Fraction 30-34	Sialylated fucosyl lactosaminoglycan
High molecular weight samples;	
Adult Core III	Adult erythrocyte lactosaminoglycan, by limited endo-β-galactosidase digestion.
A-1	Sialylated fucosyl lactosamino-glycan from human granulocytes, by hydrazinolysis of pronase digest.
A-2	
A-3	

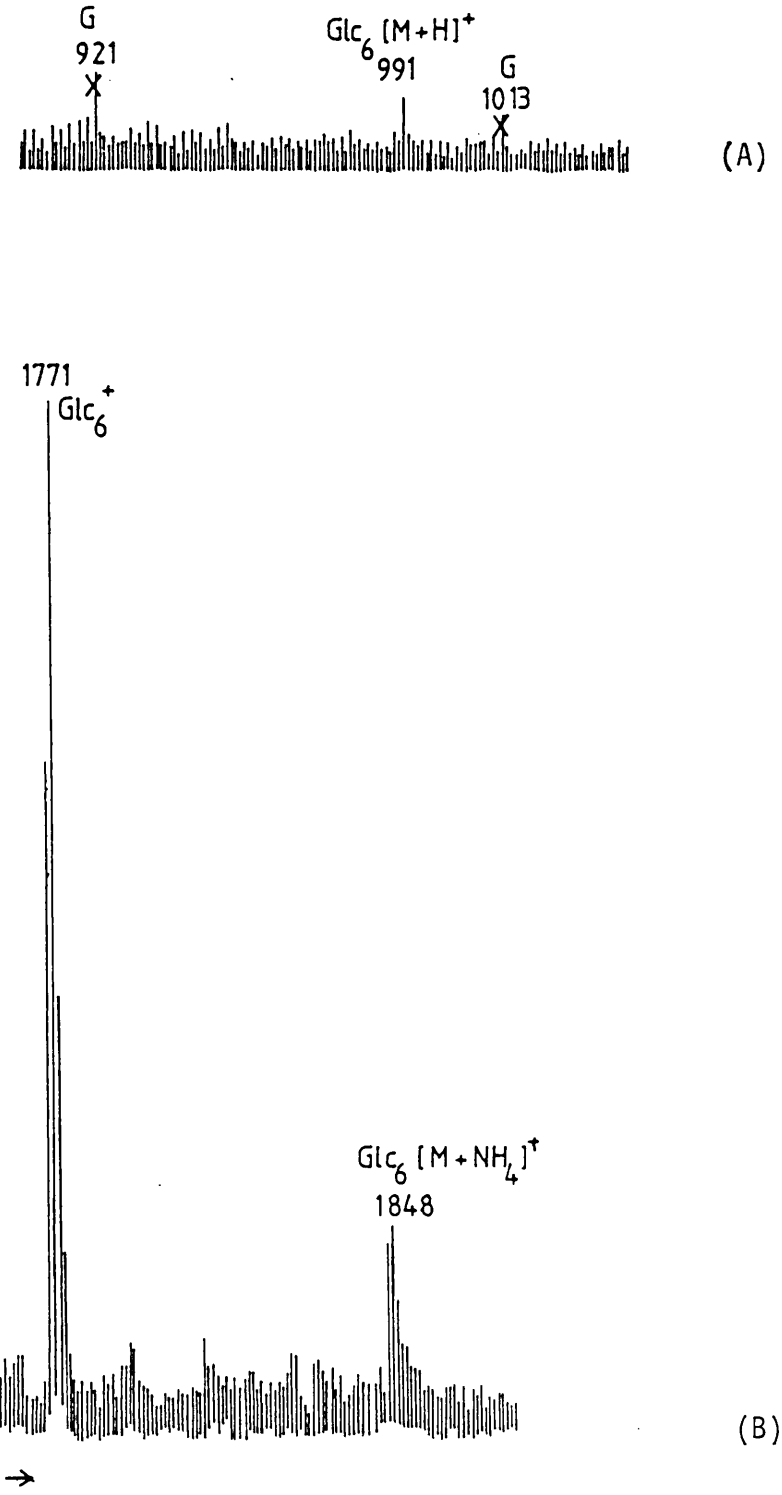


FIG 2.1. Molecular ion region for Glc_5 (A) underivatised, and (B) acetylated. Glycerol cluster ions are marked with a cross and are labelled G. The peak at m/z 1771 represents an A-type ion of composition Glc_6^+ and may be formed from both the Glc_6 and the higher mass components.

corresponding to compositions Nana,Hex_2HexNAcitol, Nana,Fuc,Hex_2 HexNAcitol and Nana,Fuc,$Hex_3HexNAc_2$itol respectively. A derivatisation procedure was devised in which an extraction step could be used to remove the salt. The most satisfactory protocol was found to be acetylation using a modification of the method of Bourne et al (84)(see chapter 5). Acetylation is carried out using trifluoroacetic anhydride and glacial acetic acid in a 10 min room temperature reaction, after which these volatile reagents are removed under a stream of nitrogen. The method has the advantage of being quick and easy to carry out and of proceding in high yield to give a clean product. In contrast, the more standard pyridine/ acetic anhydride procedure, although giving high yield products also gives by-products which were found to suppress FAB ionisation. The per-O-acetyl derivative formed is soluble in organic solvents and so can be readily isolated from salts and other water soluble contaminants by partition in an aqueous/organic system. In addition to these advantages, formation of the peracetyl derivative improves FAB sensitivity 10-50 fold: this was shown by model studies which were carried out on an 8 component mixture of polyglucose oligosaccharides (Glc_4-Glc_{11}). The molecular ion region for the hexameric component obtained on positive FAB analysis of 1/20th of an acetylated 10 μg aliquot (i.e. 0.5 μg) is shown in fig. 2.1. It is compared with the corresponding Glc_6 ($M+H^+$) signal obtainable from 20 μg of the same mixture underivatised.

The four lactosaminoglycan samples were acetylated, extracted to remove the salt and analysed by positive FAB. The spectrum obtained from the hexa/heptasaccharide fraction is presented in fig. 2.2. Quasimolecular ions of the $M+H^+$ type give the signals at m/z 2045, 1815, 1527, 1240 and 1010 which correspond to the fully

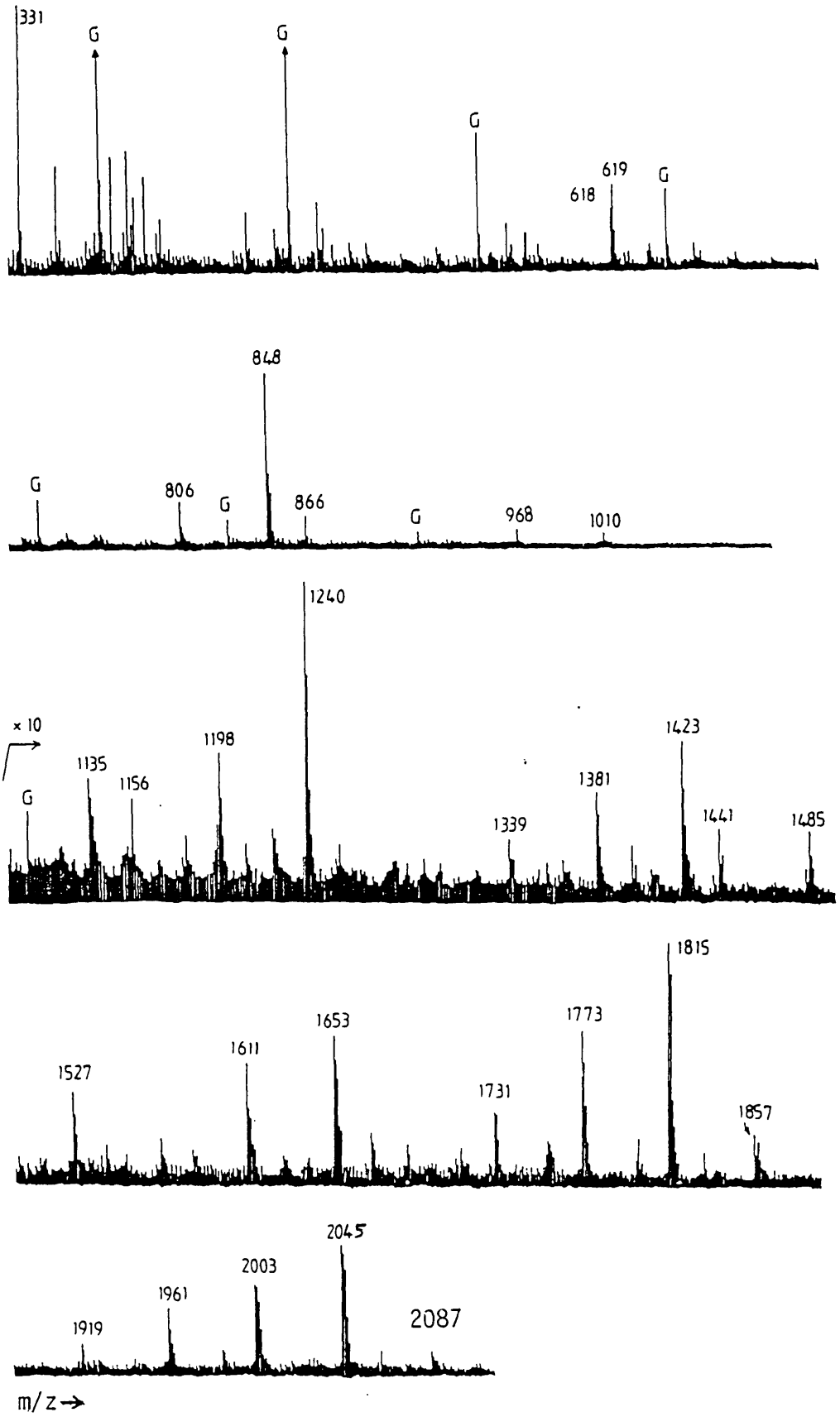
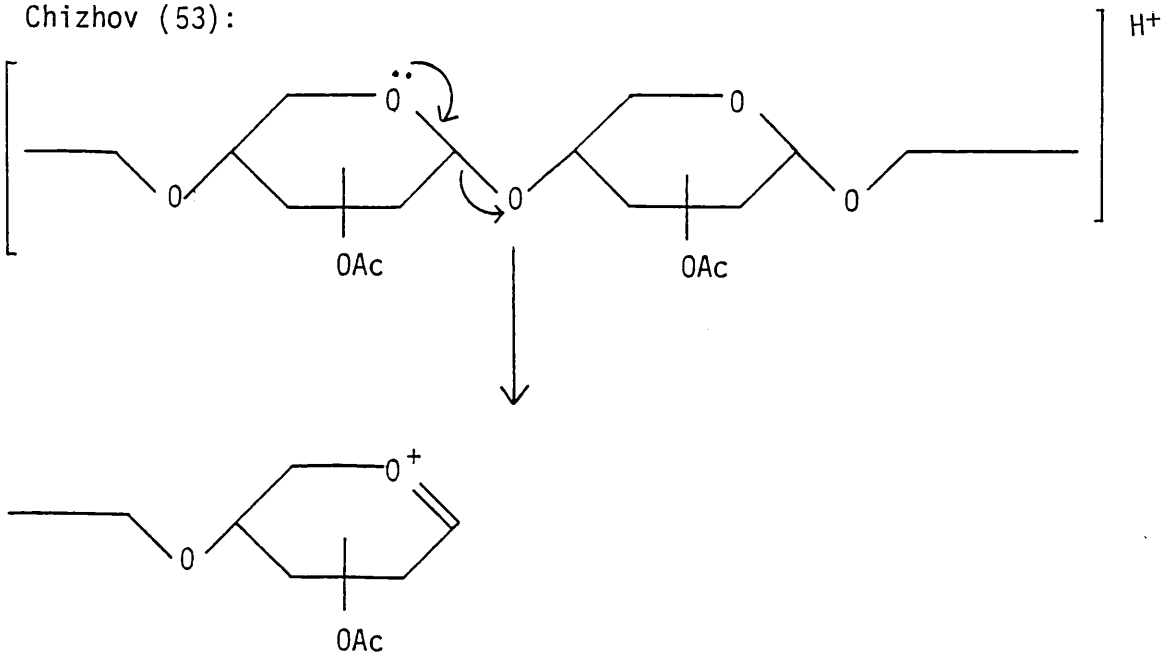


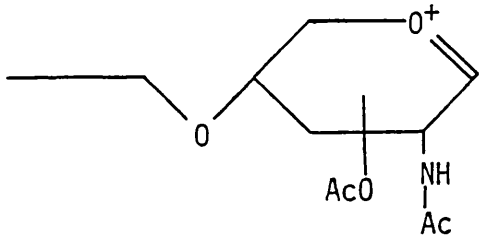
FIG 2.2. Positive FAB spectrum of the per-O-acetylated hexa/hepta-saccharide fraction, acquired at 8 kV accelerating voltage. Peaks originating from the glycerol matrix are marked G.

acetylated species $Fuc_2Hex_3HexNAc_2itol$, $Fuc,Hex_3HexNAc_2itol$, $Fuc,Hex_2HexNAc_2itol$, $Fuc,Hex_2HexNAcitol$ and $Hex_2HexNAcitol$ respectively. Each of these molecular ions is accompanied by a series of 1, 2 or 3 lower mass peaks separated by 42 mass units which represent one or more degrees of underacetylation. Thus the peaks at m/z 2003, 1961 and 1919 represent mono-, di- and tri- underacetylation of the molecular ion at m/z 2045, etc.

It is important to note that in assigning molecular ions in this way compositions only, and not sequence may be inferred. In order to determine the sequence of residues it is necessary to examine the pattern of fragment ions. In this spectrum fragment ions are represented by the peaks at m/z 1653, 1423, 1135, 848 and 618. These fragment ions are typical of the type of fragment ions observed in the spectra of all acetylated samples which have been examined and may be conveniently used to illustrate proposed fragmentation pathways for acetylated species. All fragment ions may be rationalised as arising by glycosidic cleavage with charge retention on the non-reducing terminal portion to yield a species analogous to the A-type fragment ion described by Kochetkov and Chizhov (53):



Additionally it has been observed in this and other work (85) that when amino sugars are present fragmentation preferentially occurs at the aminoglycosidic linkage to yield the ion:



presumably because the presence of the nitrogen atom helps stabilise the positive charge. The specificity of aminoglycosidic cleavage increases as the size of the acetylated molecule increases, presumably because the internal energy imparted during ionisation, when delocalised throughout a large molecule only allows the cleavage of the most susceptible bonds. In smaller molecules nonaminoglycosidic cleavage also occurs but the resultant ions are generally less abundant than the more stable ions shown above. The fragment ions observed in this spectrum may thus be rationalised as A-type ions arising by the favoured aminoglycosidic cleavage. The mass of the ion gives its composition while the known site of cleavage allows the charge bearing residue to be assigned as follows:

m/z 1653 $(Fuc_2Hex_2HexNAc_1)$-$HexNAc^+$

1423 $(Fuc_1Hex_2HexNAc_1)$-$HexNAc^+$

1135 $(Fuc_1Hex_1HexNAc_1)$-$HexNAc^+$

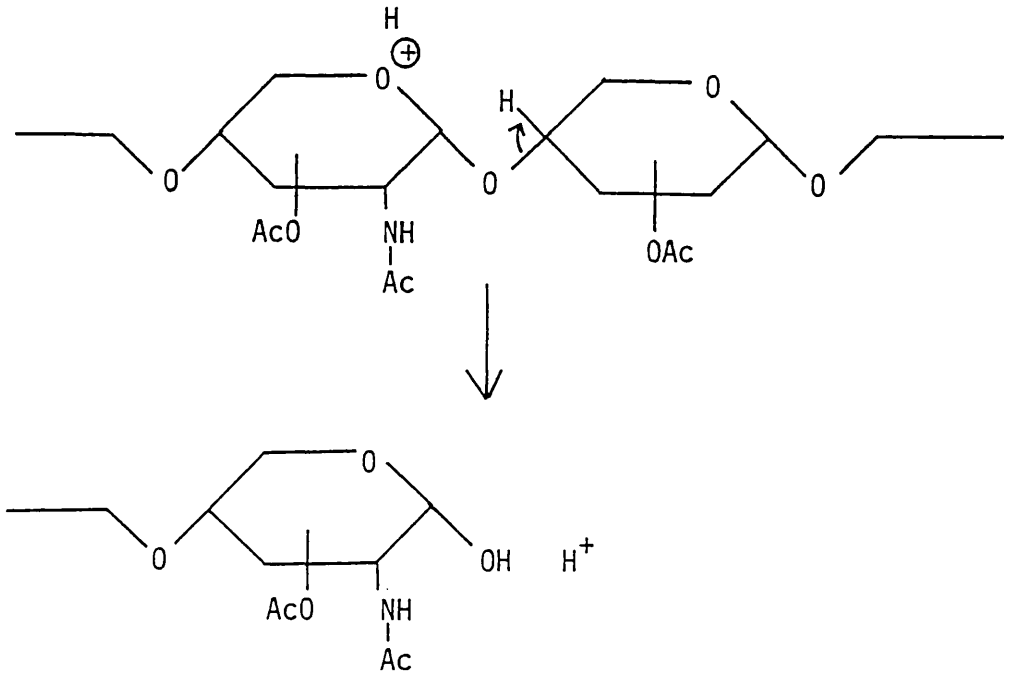
848 (Fuc_1Hex_1)-$HexNAc^+$

618 $Hex_1HexNAc_1^+$

As observed for the molecular ions, the fragment ions are also

accompanied by signals representing one or more degrees of under-acetylation e.g. m/z 1423 and its associated peaks at m/z 1381 and 1339.

The peaks at m/z 1441 and 866, observed as relatively weak signals 18 m.u. above the A-type ions may be explained as arising thus:

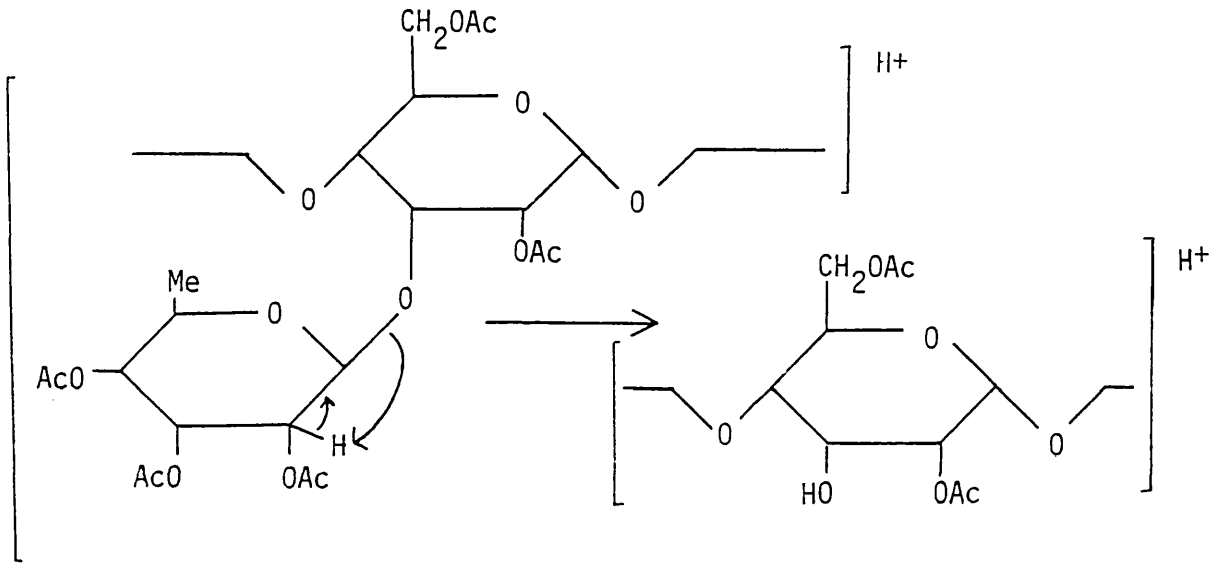


In order to complete the assignment of this spectrum there are two peaks which remain to be discussed. These are not fragment ions but appear 42 m.u. above the m/z 1815 and 2045 molecular ions at m/z 1857 and 2087 respectively. It may be postulated that these arise by overacetylation, the most probable site of which is an N-acetyl hexosamine amino proton.

These experiments give vital information by revealing that the sample is a mixture and in giving the composition of its components. These results alone do not allow deduction of unambiguous sequence information since the origin of each fragment ion can not be

assigned - such information may be obtained from linked scanning experiments, although it is not important for this work.

In contrast,the tetrasaccharide fraction contains one major component and only a very small amount of one minor component. The spectrum is presented in fig. 2.3. The major molecular ion ($M+H^+$) at m/z 1240 is accompanied by signals for one and two degrees of underacetylation at m/z 1198 and 1156 and corresponds to the composition $Fuc_1Hex_2HexNAc_1$itol. The most abundant fragment ion is at m/z 848 (m/z 806 represents one degree of underacetylation) and corresponds to an A-type fragment ion of composition Fuc,Hex, $HexNAc^+$ arising by aminoglycosidic cleavage. The very weak signal at m/z 1010 corresponds to an $M+H^+$ molecular ion with composition $Hex_2HexNAc$itol and represents a very minor contaminant. Although the signal at m/z 968 differs from this molecular ion by 42 m.u. it cannot arise solely from underacetylation since it is so much more abundant than m/z 1010 (cf the relative intensities of m/z 806 and 848, and 1156, 1198 and 1240). It is best rationalised as arising via fragmentation of the major molecular ion at 1240 by a β-cleavage pathway. A plausible mechanism involves glycosidic cleavage and hydrogen transfer:



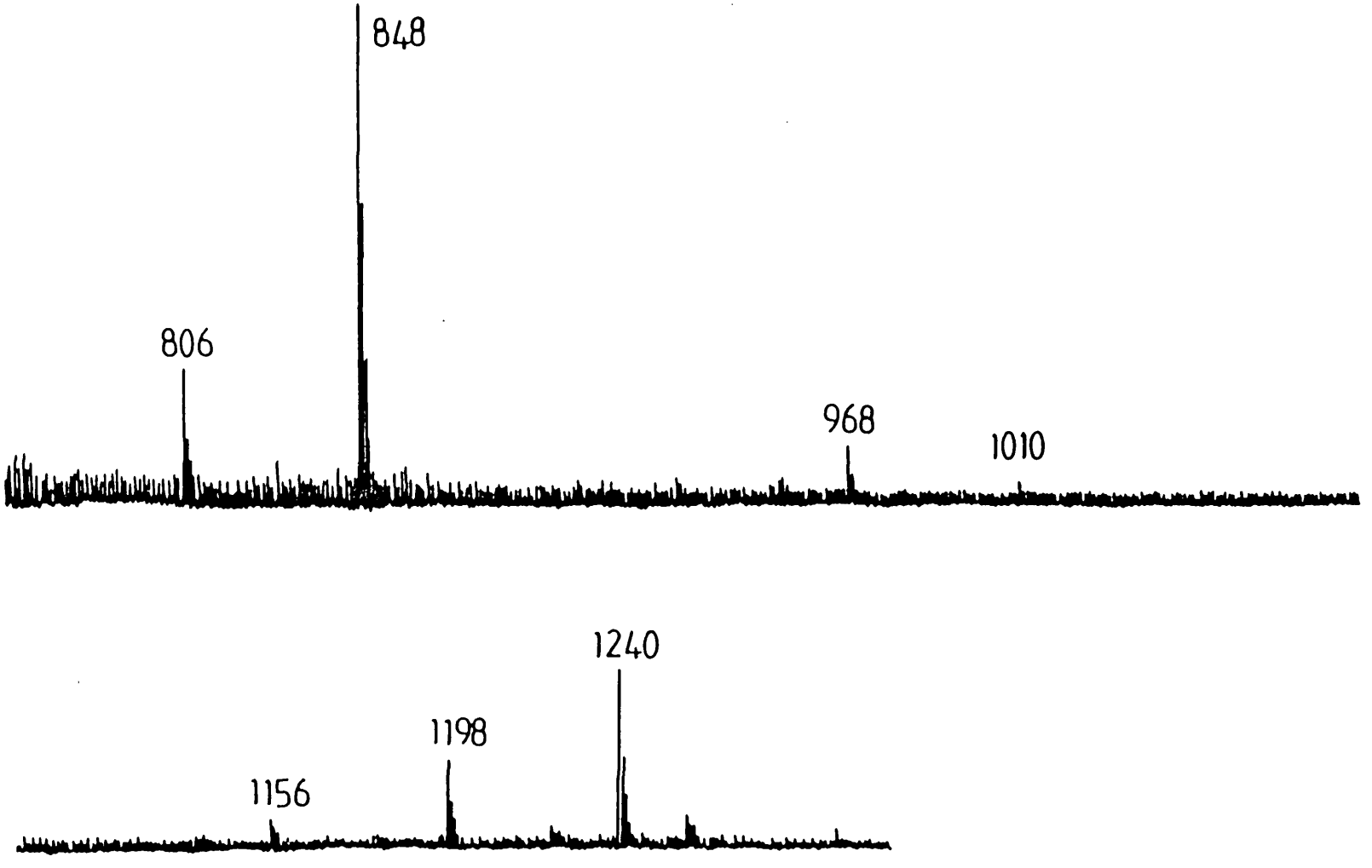
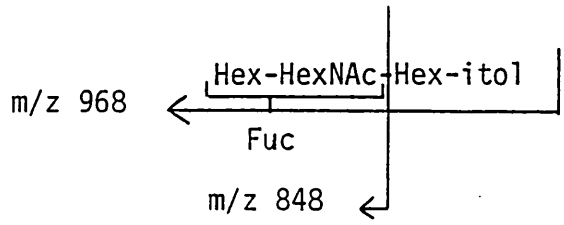


FIG 2.3. Positive FAB spectrumof the per-O-acetylated tetrasaccharide fraction,acquired at 8 kV accelerating voltage.

This type of fragmentation is most frequently observed, as in this case, as the loss of a branched fucose residue. The β-cleavage may occur from a molecular ion,as here, or from an A-type fragment ion (an analogous cleavage has been observed in permethylated samples, see later).

From the data above it is possible to assign the partial structure of the tetrasaccharide as



from which the fragment ions arise as shown. The component giving the minor molecular ion at m/z 1010 is in such low abundance that no fragment ions can be reliably assigned to it. Thus from this experiment it is possible to assign both the partial structure of the major component and identify the presence of a low level contaminant. The presence of the latter had not been identified by the classical studies of Fukuda.

The spectrum obtained from the trisaccharide is shown in fig. 2.4. The most abundant signal is that observed at m/z 1010 which corresponds to the quasimolecular ion ($M+H^+$) for the fully acetylated species $Hex_2HexNAc_1itol$. Associated ions at m/z 968 and 926 correspond to one and two degrees of underacetylation. Two further molecular ions are present in the spectrum; that at m/z 722 corresponds to the fully acetylated disaccharide species HexHexNAcitol while the less abundant signal at m/z 952 (together with its underacetylated counterpart at m/z 911) corresponds to the fucosyl trisaccharide FucHexHexNAcitol. The signal at m/z 618 is assigned to the A-type fragment ion $HexHexNAc^+$ (underacetylated at m/z 576) with its less abundant "plus 18" ion (see above) at

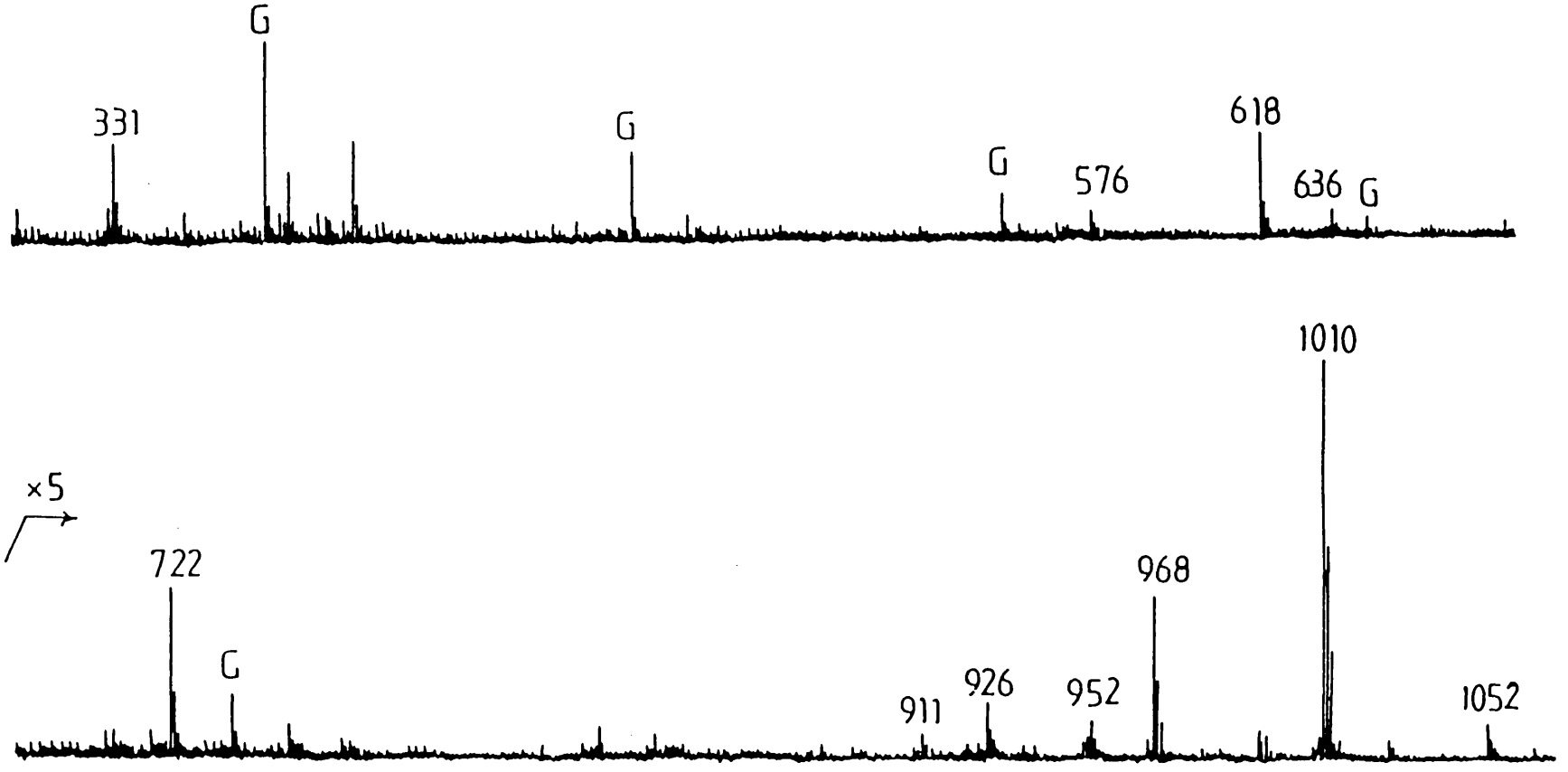


FIG 2.4. Positive FAB spectrum of the per-O-acetylated trisaccharide fraction(acquired at 8 kV). Glycerol cluster ions are marked G.

m/z 636. The signal at m/z 331 corresponds to the A-type ion Hex^+ which arises by the less favoured nonaminoglycosidic cleavage. As mentioned above, such cleavages occur more frequently in smaller molecules such as this one where there are fewer cleavable bonds.

As with the first fraction it is not possible to assign structure from these fragment ions since the sample is a mixture. In these studies it is the data which the FAB provided about the composition of the mixture which was important in the Band 3 study. The FAB data was essential for the correct interpretation of results from methylation analysis and exoglycosidase treatment.

The final sample to be discussed in this section is fraction 30-34 derived from sialylated fucosyl lactosaminoglycan - the spectrum is presented in fig. 2.5. This sample provides a useful contrast with the previous three both because it contains larger components than the other samples and because only molecular ions are seen - no fragmentation occurs. It was demonstrated in these and other experiments that the production of fragment ions from both derivatised and underivatised samples depends on the amount of sample loaded. If the levels are low the successful experiment will produce molecular ions only. More sample is needed if fragmentation data is required. In a study of this type where mixtures are being examined and fragmentation data is not required the amount of sample used can be reduced and molecular ions only are obtained.

$M+H^+$ molecular ions are observed at m/z 2232, 1657 and 1427 for the fully acetylated species $Nana_1Fuc_1Hex_3HexNAc_2itol$, $Nana_1Fuc_1Hex_2HexNAcitol$ and $Nana_1Hex_2HexNAcitol$ as detected in the underivatised spectra. In addition to the signals representing underacetylation associated with each molecular ion a second set

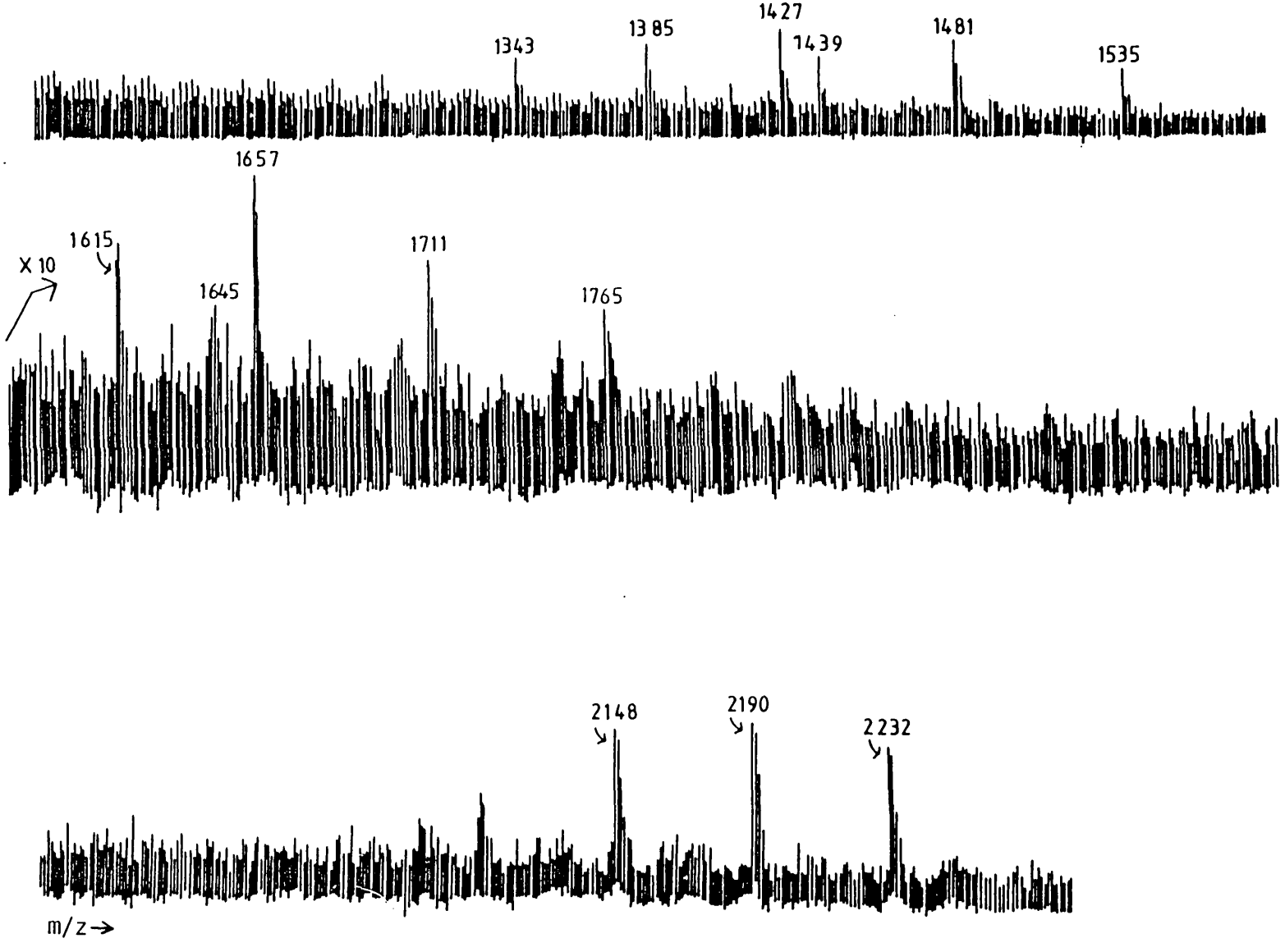
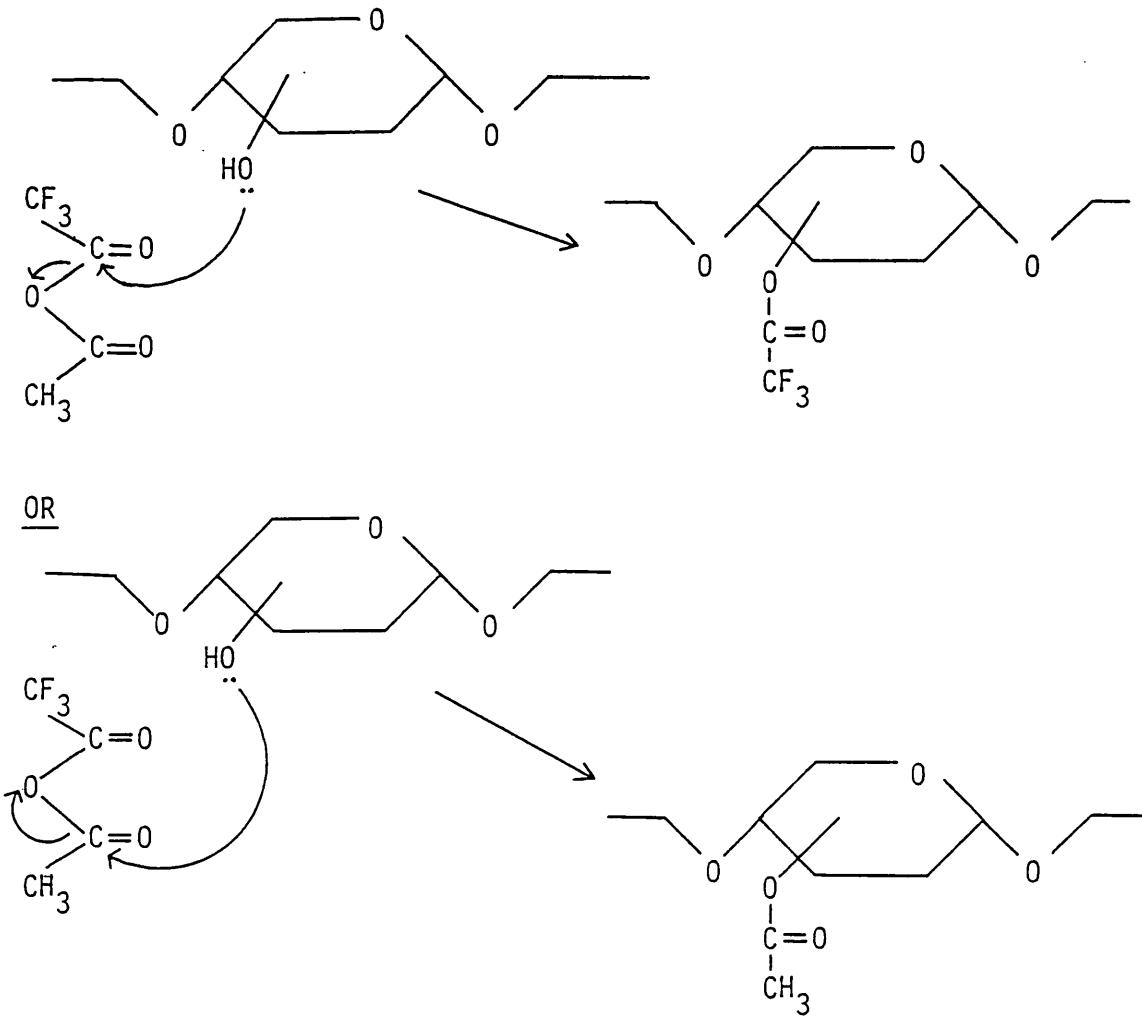


FIG 2.5. Positive FAB spectrum of per-O-acetylated fraction 30-34, acquired at 8 kV.

of peaks associated with the two lower mass molecular ions is observed. The peaks occur 54 m.u. apart on the high mass side of the $M+H^+$ ions and represent the exchange of an acetyl group for a trifluoroacetyl group. The mechanism of the acetylation reaction with TFAA/acetic acid proceeds via the mixed anhydride:



Since the O-trifluoroacetate is more reactive than the O-acetate most TFA groups are lost while the more stable O-acetyl groups are retained. In a small proportion of cases a trifluoroacetyl group is not completely lost and a low abundance ion is seen 54 m.u. above a more intense signal (molecular or fragment ion). The 54 m.u. interval represents the difference between the acetyl group (43 m.u.) and the TFA group (97 m.u.). Trifluoroacetylation may occur simultaneously with underacetylation. Thus the peak at m/z 1439 arises from the component having one degree of underacetylation coupled

with one degree of trifluoroacetylation, while that at m/z 1645 corresponds to three degrees of underacetylation associated with two degrees of trifluoroacetylation.

Although these four samples represent only a small fraction of the samples of this type examined during these studies, they suffice to illustrate the important features of the acetyl derivative which may be summarised as follows:

1) the technique is fast and clean,
2) products are readily isolated,
3) the derivative gives good sensitivity,
4) fragmentation behaviour has been defined e.g.
    i. aminoglycosidic cleavage is favoured,
    ii. fragment ions are usually A-type ions with additional β-cleavage of branching fucose residues.

2.2.2 Large permethylated samples. The second group of samples to be discussed in this chapter comprises the high molecular weight species. These samples were also provided by Dr. Fukuda and were supplied as the permethyl derivatives, (see table 2.1).

These experiments illustrate the novel concept that permethylated samples of very high molecular weight can give spectra when run using an accelerating voltage of 8 kV. The mass range defined by these conditions is 3,300 dalton, but even if the molecular weight of the permethylated derivative is greatly in excess of this it has been shown that spectra may be obtained comprising fragment ions derived from the non-reducing terminus. These fragment ions allow the non-reducing terminus of the antigen molecule to be "mapped".

Permethylated samples behave in a similar way to acetylated

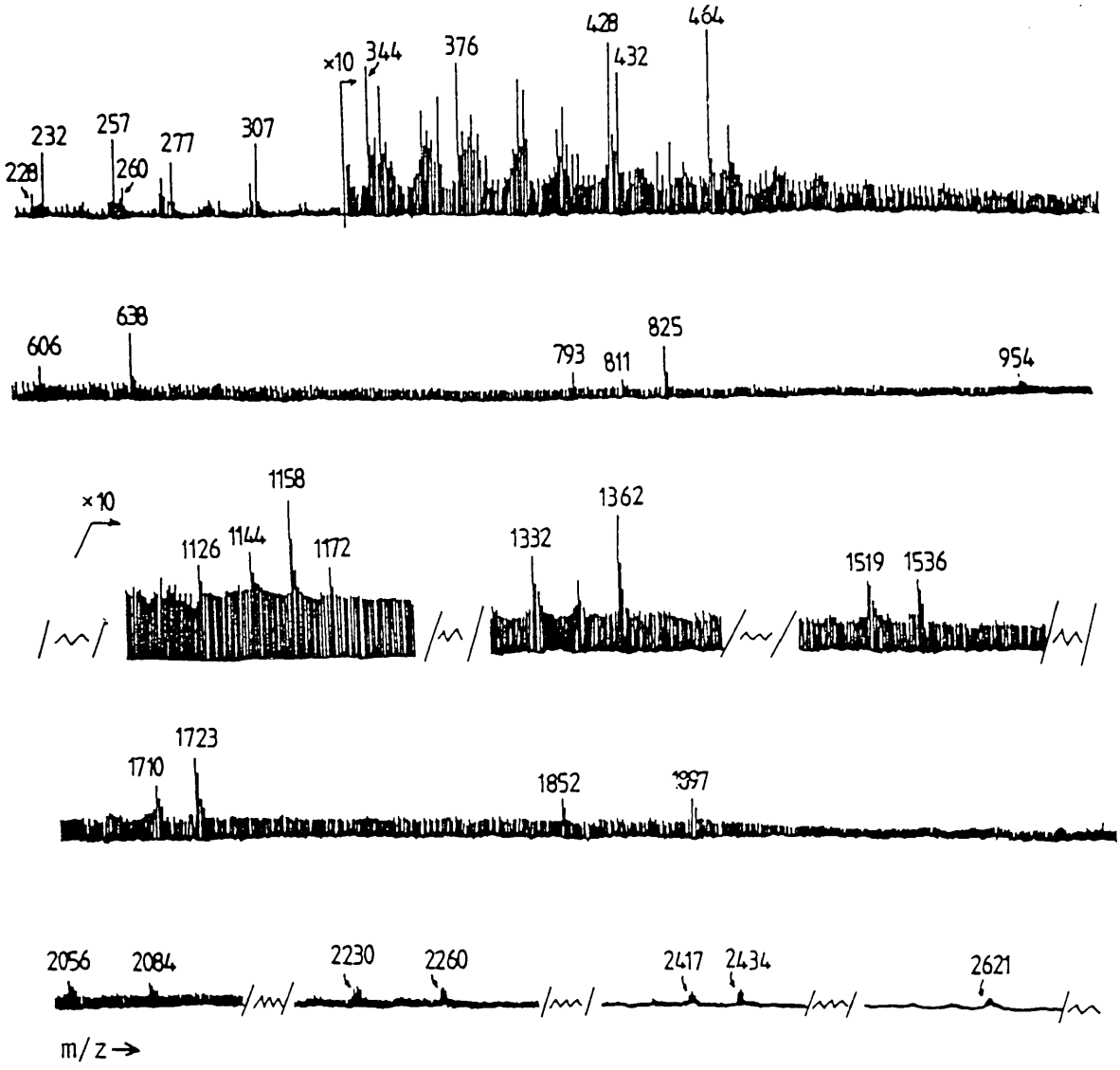


FIG 2.6. Positive FAB spectrum of permethylated adult Core III, (8 kV.)

TABLE 2.2.

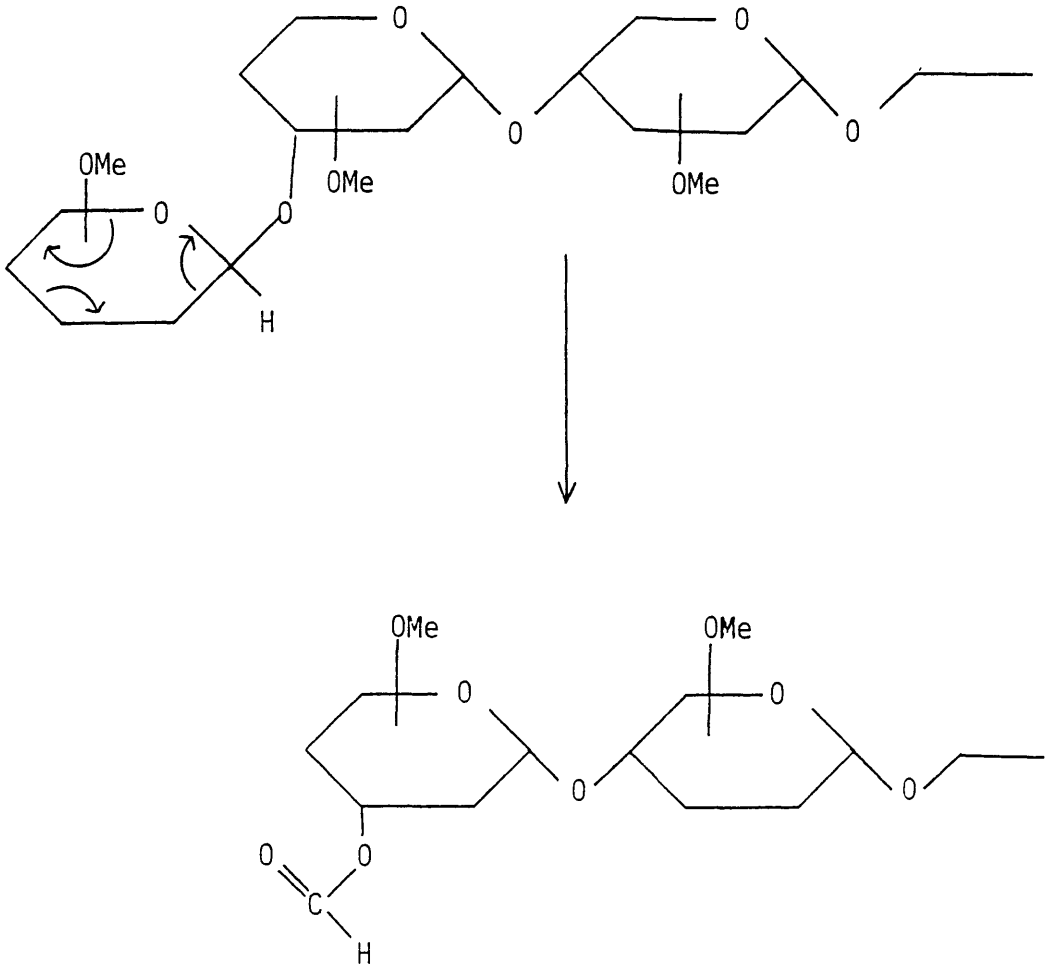
ASSIGNMENT OF PEAKS OBSERVED IN THE POSITIVE FAB SPECTRUM OF PERMETHYLATED ADULT CORE III (SEE FIG. 2.6.).

M/Z	ASSIGNMENT
2621	$NanaHex_5HexNAc_5^+$
2608	$Fuc_2Hex_5HexNAc_5^+$
2434	$FucHex_5HexNAc_5^+$
2417	$NanaHex_4HexNAc_5^+$
2260	$Hex_5HexNAc_5^+$
2230	$FucHex_4HexNAc_5^+$
2084	$Nana_2Hex_3HexNAc_3^+$
2056	$Hex_4HexNAc_5^+$
1897	$NanaFucHex_3HexNAc_3^+$
1852	$Hex_3HexNAc_5^+$
1723	$NanaHex_3HexNAc_3^+$
1710	$Fuc_2Hex_3HexNAc_3^+$
1536	$FucHex_3HexNAc_3^+$
1519	$NanaHex_2HexNAc_3^+$
1362	$Hex_3HexNAc_3^+$
1348	β-cleavage of Fuc from 1536
1332	$FucHex_2HexNAc_3^+$
1172	Ring cleavage of Fuc in 1332
1158	$Hex_2HexNAc_3^+$
1144	β-cleavage of Fuc from 1332
954	$HexHexNAc_3^+$
825	$NanaHexHexNAc^+$
811	undermethylation of 825
793	loss of methanol from 825
638	$FucHexHexNAc^+$
606	loss of methanol from 638
464	$HexHexNAc^+$
432	loss of methanol from 464
376	$Nana^+$
344	loss of methanol from 376
260	$HexNAc^+$
228	loss of methanol from 260.

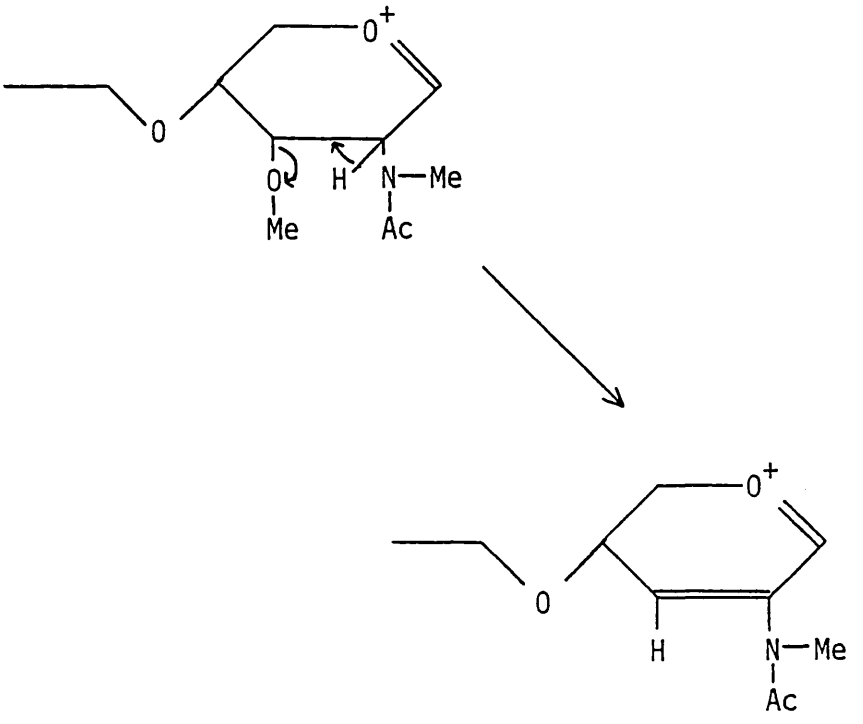
ones under positive FAB conditions, fragmenting at the glycosidic bond to give the A-type fragment ion described by Kochetkov and Chizhov (53). When amino sugar residues are present in the molecule aminoglycosidic fragmentation occurs almost exclusively, while nonaminoglycosidic cleavage is rarely, if ever, observed - this is in contrast with the behaviour of acetylated samples where such cleavages may occur in low abundance. Such aminoglycosidic cleavages give rise to the fragment ions observed when analysing such high molecular weight samples.

Although permethylation is less convenient to perform than acetylation (samples must be extensively purified after derivatisation) it gives a similar improvement in sensitivity and has the advantage of introducing a relatively low molecular weight group at each substitution - the mass increase on substituting a methyl group for a proton is 14 m.u. as compared with 42 m.u. for a similar substitution of an acetyl moiety. When dealing with high molecular weight samples, as in this study, the specificity of the fragmentation and the mass considerations make the permethyl derivative the one of choice.

The positive FAB spectrum of permethylated adult core III is presented in fig. 2.6 and a summary of peak assignments is given in table 2.2. Since the parent molecule is very large, no molecular ions are detected; all peaks represent A-type fragment ions which arise by aminoglycosidic cleavage; m/z 1348 and 1144 have undergone additional β-cleavage of fucose - the latter is too intense to be due to undermethylation of 1158. The signal at 1172 may be rationalised as arising from the ion at 1332 by ring cleavage of the fucose residue :



Undermethylation arises in an analogous way to underacetylation - by the failure to exchange a proton for a methyl group, resulting in a mass interval of 14 m.u. Although the probability of undermethylation arising increases as the size of the molecule, and therefore the number of sites for methylation increases, the degree of undermethylation in this sample is so low that only the lower mass ions are intense enough for their undermethylated ions to be seen. A second feature of the lower mass ions which provides useful information is the peak denoting the loss of methanol, observed as a loss of 32 m.u. This may be rationalised as occuring by β-elimination of methanol from the A-type ion to form the stable species shown below:



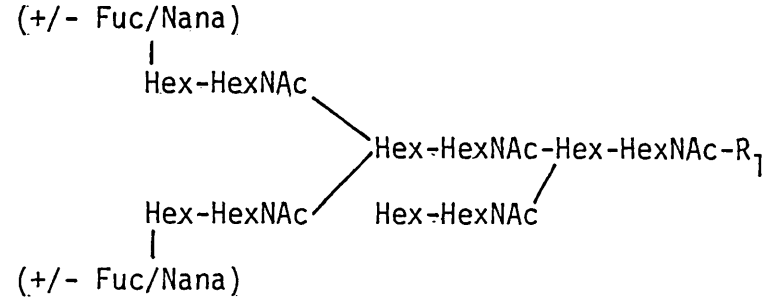
In this spectrum the ability of the peak at m/z 638 (Fuc,Hex, $HexNAc^+$) to lose methanol is significant; in order to eliminate methanol and form the structure above, position 3 of the hexosamine residue must carry a methoxyl group and therefore since fucose is known to be linked to hexosamines only at the 3 position the fucose residue must be linked to the terminal hexose.

Analysis of the pattern of fragment ions allows information to be obtained about the branching structure of the parent molecule. Two series of fragment ions are observed in approximately equal abundance:

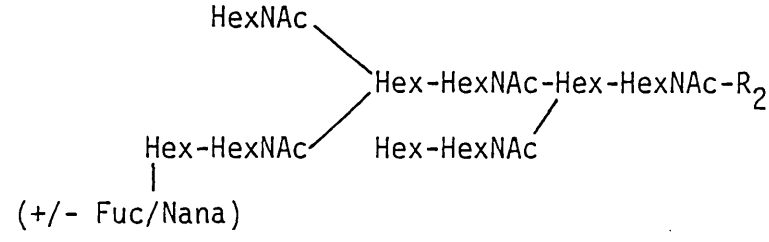
1) those of general structure $Hex_xHexNAc_x^+$ and their fucosylated and sialylated derivatives,
2) those of general structure $Hex_yHexNAc_{y+1}^+$ and their fucosylated and sialylated derivatives.

It is significant that no $HexNAc_2$ or $HexNAc_4$ containing fragments are observed, but that $HexNAc_1$, $HexNAc_3$ and $HexNAc_5$ ions are abundant. Since fragmentation is known to occur at aminoglycosidic bonds the only way such a pattern may be obtained from a molecule containing the lactosamine backbone is from the following parent structures:

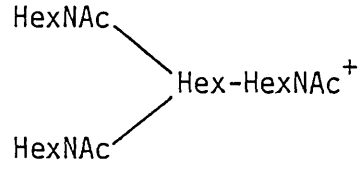
a)



b)



All fragment ions observed may be derived from one or other of these structures by a single aminoglycosidic cleavage with the exception of the peak at m/z 954. This corresponds to an ion of composition $Hex_1HexNAc_3^+$ and represents the branching structure



This ion could arise from parent structure b) if in a proportion of these structures the non-reducing terminal hexose is absent.

These data suggest that the core III glycopeptide contains at least two branches, one terminated in structure a) and the other in structure b). The final structure for core III (see fig. 2.7)

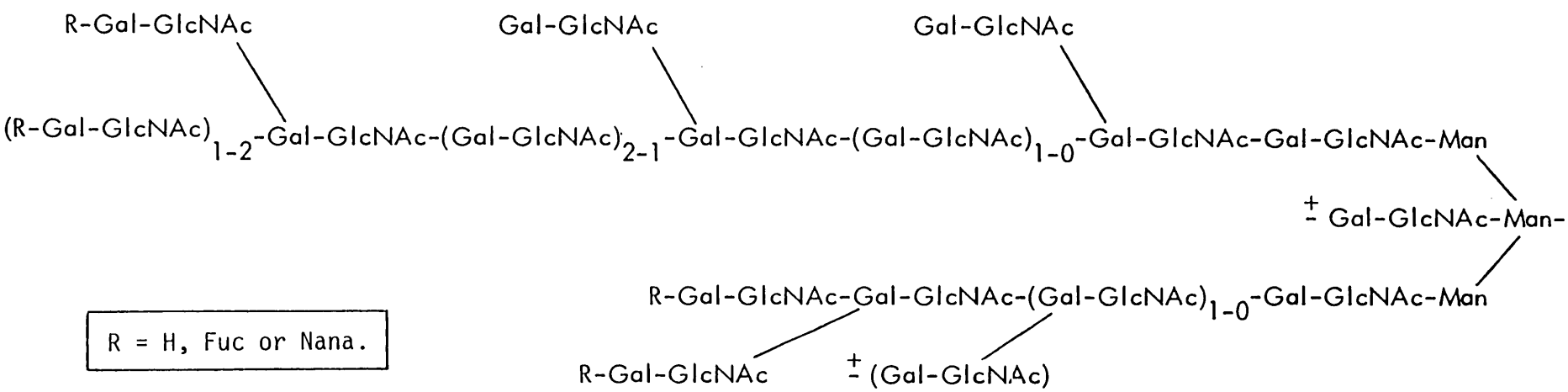


FIG 2.7. Structure of branched lactosaminoglycan from Band 3 of adult human erythrocytes.

was obtained by combining these FAB data with the results of classical studies which are published in full in the literature (81).

The remaining high mass permethylated samples were all obtained from sialylated fucosyl lactosaminoglycan from human granulocytes and represent three fractions from the same preparation eluted from an ion-exchange column. These samples provide a useful contrast with the previous Band 3 glycopeptide since the structural studies showed that the side chains are unbranched and the position and extent of sialylation and fucosylation are different.

The positive FAB spectra of samples A-1, A-2 and A-3 are presented in fig. 2.8 A, B and C and the structures of the fragment ions observed in these spectra are summarised in table 2.3.

A-1 contains three types of side chain as indicated by the following series of fragment ions:

1) unsubstituted;

$HexHexNAc^+$ (464), $Hex_2HexNAc_2^+$ (913), $Hex_3HexNAc_3^+$ (1362).

2) fucosylated;

i. monofucosylated;

$FucHexHexNAc^+$ (638), $FucHex_2HexNAc_2^+$ (1087), $FucHex_3HexNAc_3^+$ (1536),

ii. difucosylated;

$FucHexHexNAc^+$ (638), $Fuc_2Hex_2HexNAc_2^+$ (1261), $Fuc_2Hex_3HexNAc_3^+$ (1710).

3) Sialylated;

$Nana^+$ (376), $NanaHexHexNAc^+$ (825), $NanaHex_2HexNAc_1^+$ (1029), $NanaHex_2HexNAc_2^+$ (1274), $NanaHex_3HexNAc_3^+$ (1723).

In addition to allowing the identification of these three side chains the spectrum provides information on the position of

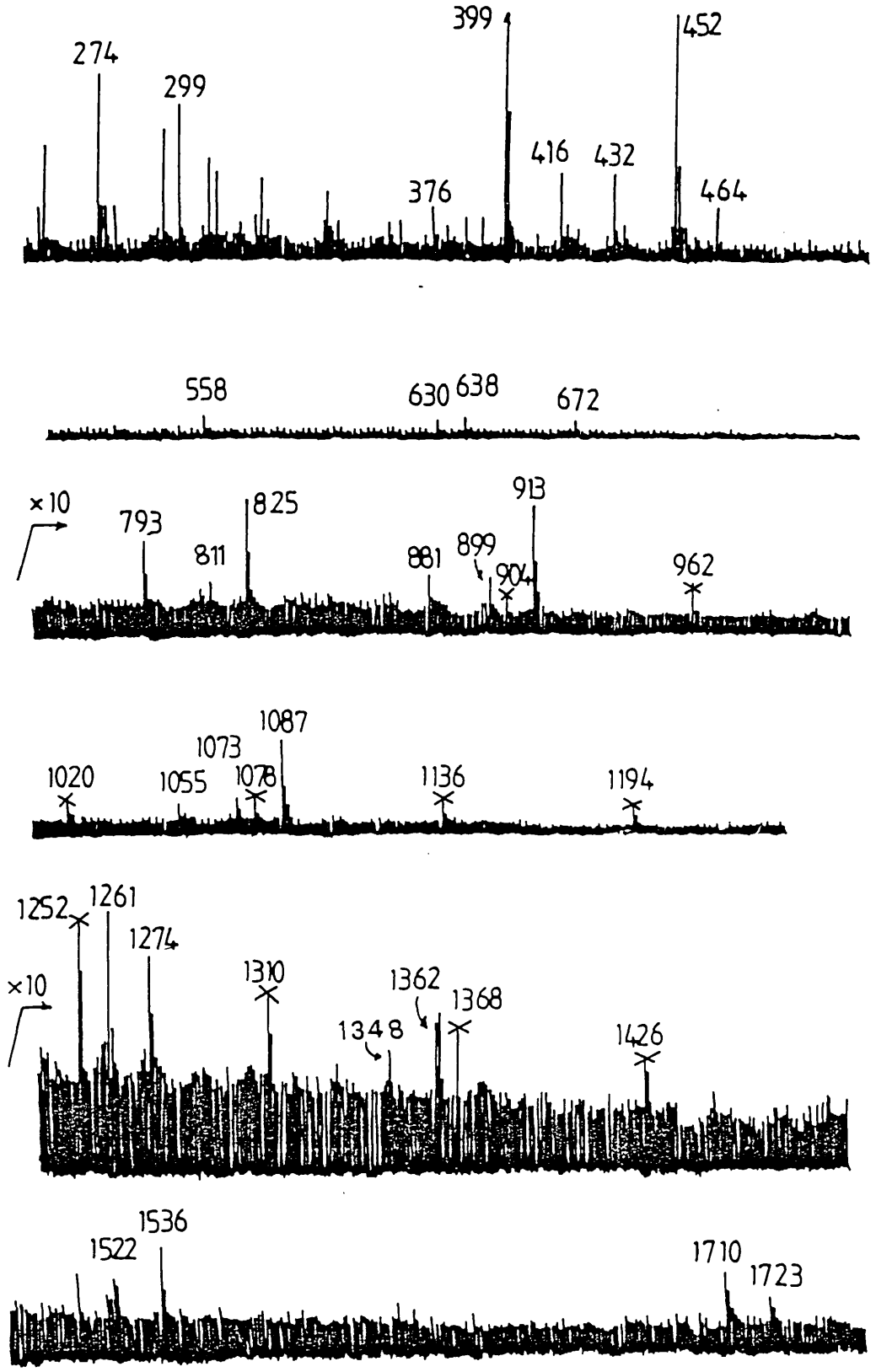


FIG 2.8 A. Positive FAB spectrum of permethylated A-1, acquired at 8 kV. The peaks marked with a cross are separated from each other by 58 m.u. and represent impurities in the sample.

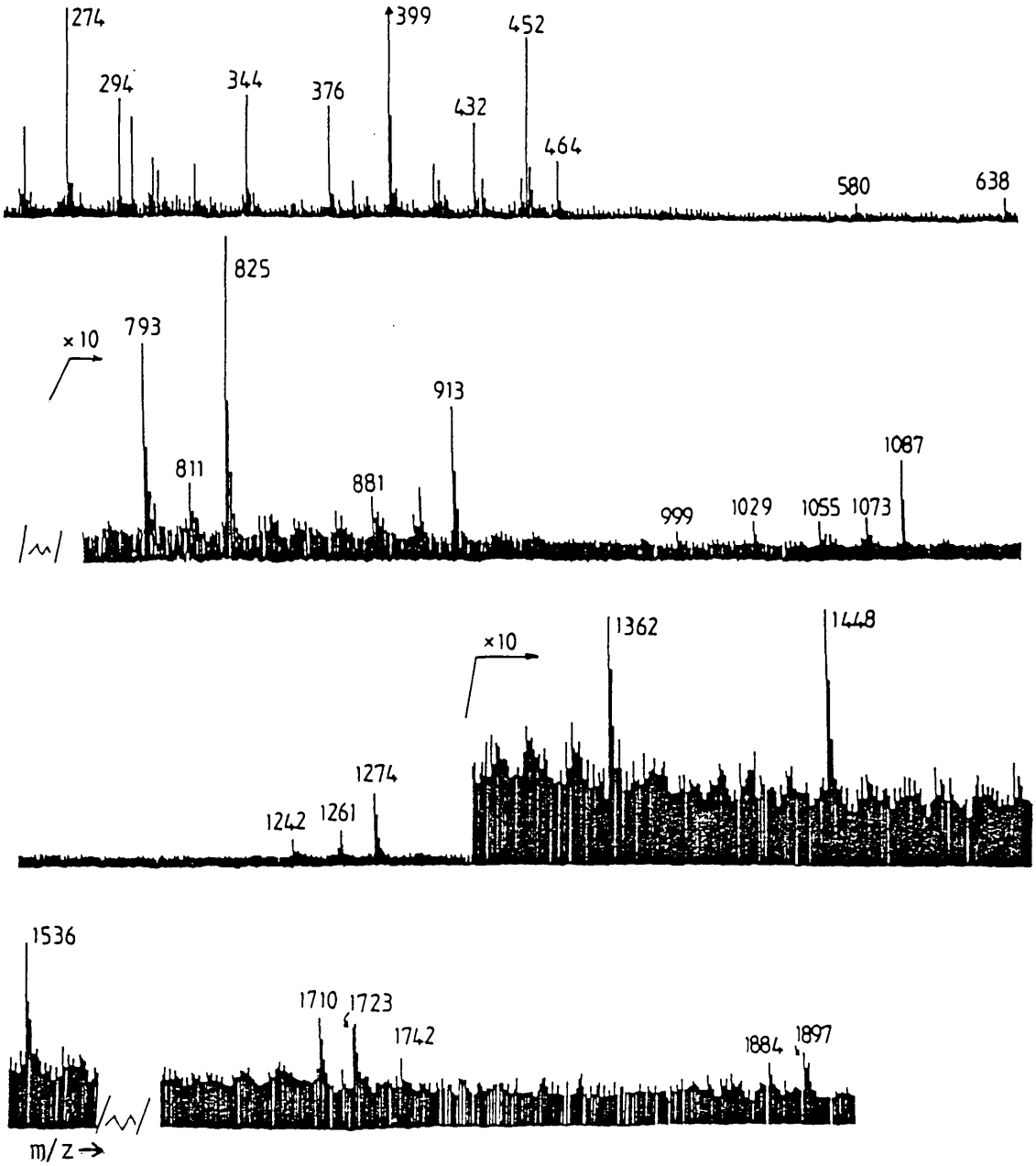


FIG 2.8 B. Positive FAB spectrum of A-2, acquired at 8 kV.

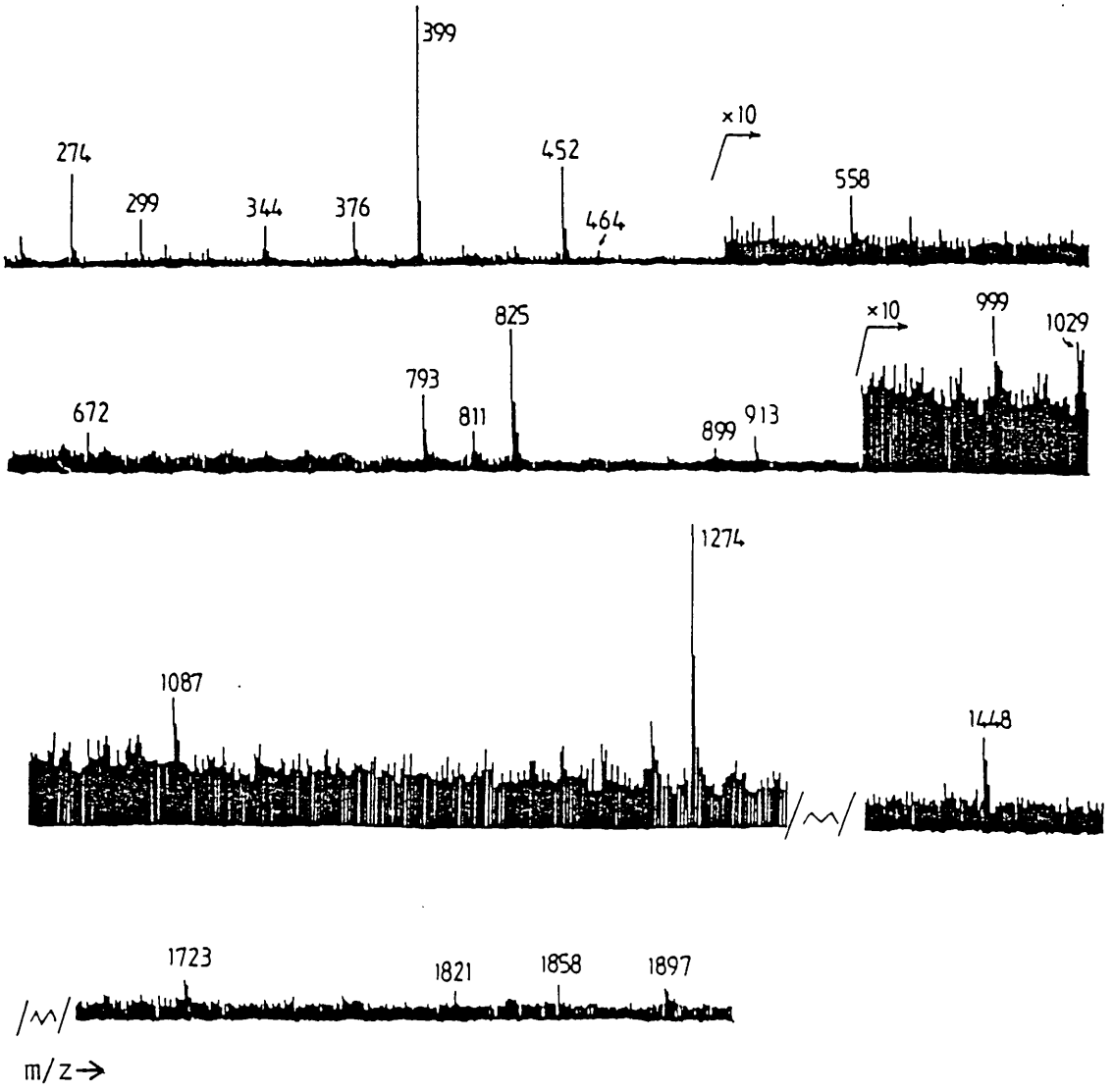


FIG 2.8 C. Positive FAB spectrum of permethylated A-3,acquired at 8 kV.

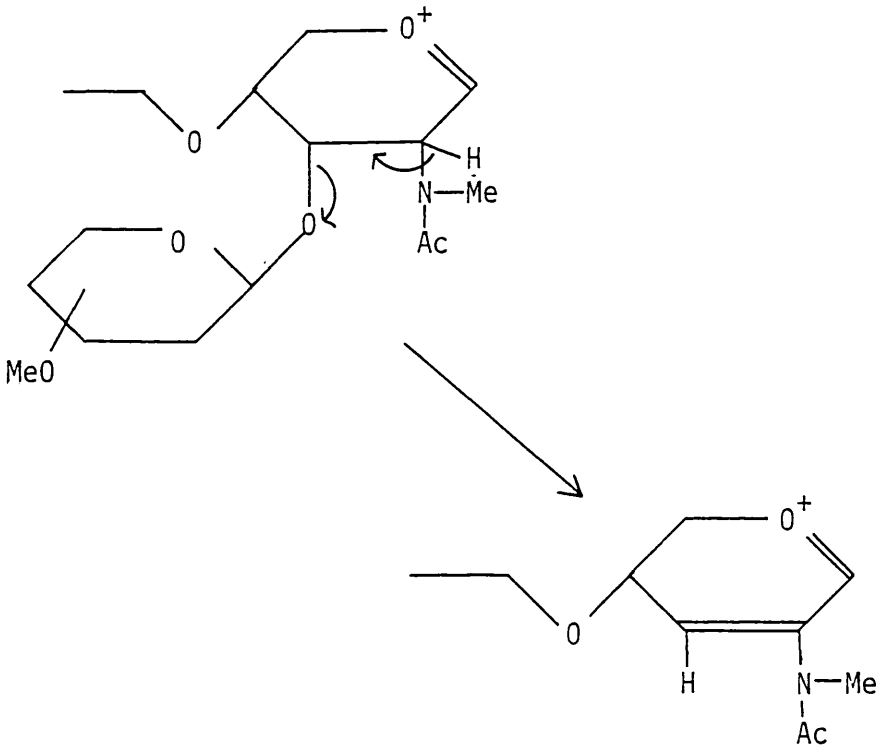
TABLE 2.3.

SUMMARY OF STRUCTURE ASSIGNMENTS FOR THE FRAGMENT IONS OBTAINED FROM PERMETHYLATED SAMPLES A-1, A-2 AND A-3 FROM SIALYLATED FUCOSYL LACTOSAMINOGLYCAN FROM HUMAN GRANULOCYTES.

M/Z	ASSIGNMENT	PRESENCE IN SAMPLE:		
		A-1	A-2	A-3
1897	$NanaFucHex_3HexNAc_3^+$	-	+	+
1884	$Fuc_3Hex_3HexNAc_3^+$	-	+	-
1723	$NanaHex_3HexNAc_3^+$	+	+	+
1710	$Fuc_2Hex_3HexNAc_3^+$	+	+	-
1536	$FucHex_3HexNAc_3^+$	+	+	-
1522	β-cleavage of Fuc from 1710	+	-	-
1448	$NanaFucHex_2HexNAc_2^+$	-	+	+
1362	$Hex_3HexNAc_3^+$	+	+	-
1348	β-cleavage of Fuc from 1536	+	-	-
1274	$NanaHex_2HexNAc_2^+$	+	+	+
1261	$Fuc_2Hex_2HexNAc_2^+$	+	+	-
1242	loss of methanol from 1274	-	+	-
1087	$FucHex_2HexNAc_2^+$	+	+	+
1073	β-cleavage of Fuc from 1261	+	+	-
1055	loss of methanol from 1087	+	+	-
1029	$NanaHex_2HexNAc^+$	-	+	+
999	$NanaFucHexHexNAc^+$	-	+	+
913	$Hex_2HexNAc_2^+$	+	+	+
899	β-cleavage of Fuc from 1087	+	+	+
881	loss of methanol from 913	+	+	+
825	$NanaHexHexNAc^+$	+	+	+
811	undermethylation of 825	+	+	+
793	loss of methanol from 825	+	+	+
638	$FucHexHexNAc^+$	+	+	-
464	$HexHexNAc^+$	+	+	+
432	β-cleavage of Fuc from 638	+	+	+
376	$Nana^+$	+	+	+

The peaks at m/z 399 and 452 represent impurities and are marked on the spectrum with a cross.

fucosylation. The signals at m/z 913 and 825 ($Hex_2HexNAc_2^+$ and $NanaHexHexNAc^+$) readily lose methanol indicating that position 3 of the hexosamine at which cleavage has occured is occupied by a methoxyl group. In contrast, the signal at m/z 638 for $FucHexHexNAc^+$ does not lose methanol (no peak at m/z 606). This indicates that fucose is linked to position 3 of the hexosamine residue in both the mono- and di- fucosylated forms, since the $FucHexHexNAc^+$ ion may derive from either parent. The peak at m/z 432 represents the product of β-elimination of fucose from position 3 in an analogous way to the elimination of methanol, to form an ion of the same structure:



The peak at m/z 432 could also be obtained by loss of methanol from $HexHexNAc^+$ at m/z 464. However, an examination of the peak intensities reveals that m/z 432 is more abundant than m/z 464 whereas all other peaks arising by loss of methanol are less

intense than their parent ions (e.g. 913 and 881). m/z 432 cannot, therefore, be explained solely as loss of methanol from m/z 464 and so can be assigned as the product of β-elimination of fucose from m/z 638.

Sample A-2 contains the same signals as sample A-1 but additionally contains a sialylated fucosylated chain which gives rise to the fragments:

$NanaFucHexHexNAc^+$ (999), $NanaFucHex_2HexNAc_2^+$ (1448), $NanaFucHex_3HexNAc_3^+$ (1897).

One further peak is observed in this spectrum which is not present in A-1 - it is the weak signal at m/z 1029 which can be rationalised as the product of a rare hexosyl cleavage:

$$Nana\text{-}Hex\text{-}HexNAc\text{-}Hex^+$$

or alternatively could represent a low abundance hexosyl side branch:

$$\begin{array}{l} Nana\text{-}Hex\text{-}HexNAc^+ \\ \quad\quad\quad\quad\quad\quad | \\ \quad\quad\quad\quad\quad\quad Hex \end{array}$$

The proportion of sialylated chains is higher in sample A-2 than in A-1 e.g. in A-1 m/z 825 and 913 are of approximately equal intensities, while in A-2 m/z 825 is almost twice as abundant as 913, also cf m/z 1274 and 1261 etc. It must be pointed out that although it is valid to compare the ratio of intensities of peaks between several spectra in this way, a direct comparison of different peaks within a spectrum cannot be made since the stability and bond lability of the different species will not be the same e.g. a sialylated ion will have a different internal energy compared with a fucosylated ion.

Sample A-3 contains the unsubstituted sialylated and sialylated fucosyl side chains present in A-1 and A-2 but contains only one

fragment ion indicating the presence of a monofucosylated unsialylated side chain; m/z 1087 for $FucHex_2HexNAc_2^+$. The absence of a peak at m/z 638 for $FucHexHexNAc^+$ and the presence of m/z 464 for $HexHexNAc^+$ suggest that the fucose is not linked to the first but to the second hexosamine residue:

$$\begin{array}{l} \text{Hex-HexNAc-Hex-HexNAc}^+ \\ \qquad\qquad\quad | \\ \qquad\qquad\quad \text{Fuc} \end{array}$$

The peak at m/z 881 corresponds to the expected elimination of a fucose residue linked in this way. However, as pointed out above, this type of peak may also arise by loss of methanol from an unfucosylated parent,in this case from m/z 913 ($Hex_2HexNAc_2^+$). The relative intensities of 881 and 913 are such that 881 may be explained solely as loss of methanol from 913 (cf 376 and its "minus methanol" peak at 344) so that it is not possible to use the presence of 881 in assigning the structure of the monofucosylated side chain.

A similar anomaly arises on fucosyl β-cleavage - the resultant ion has the same mass as the monoundermethylated unfucosylated species and again assessment must be made of the relative intensities of other undermethylated and parent species of similar mass before assigning a β-cleavage product, e.g. in the spectrum of A-1 m/z 1522 could arise by undermethylation of 1536. However, no other peaks of similar mass show any undermethylation so that 1522 must correspond to fucosyl β-cleavage from m/z 1710 ($Fuc_2Hex_3HexNAc_3^+$).

The unsubstituted side chain in A-3 is presumably shorter than in A-1 and A-2 since a $Hex_3HexNAc_3^+$ fragment is not produced.

A consideration of relative peak intensities shows that A-3 is more highly sialylated than A-2: the sialylated peaks at m/z 825 and 999 are more intense with respect to nonsialylated m/z 913 in

TABLE 2.4.

SUMMARY OF STRUCTURES PRESENT IN SAMPLES A-1, A-2 AND A-3 FROM SIALYLATED FUCOSYL LACTOSAMINOGLYCAN FROM HUMAN GRANULOCYTES.

A-1:

Hex-HexNAc-Hex-HexNAc-Hex-HexNAc

Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

Nana-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc

A-2:

As for A-1, but additionally

Nana-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

Nana-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

Nana-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

A-3:

Hex-HexNAc-Hex-HexNAc

Nana-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc

Nana-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

Nana-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

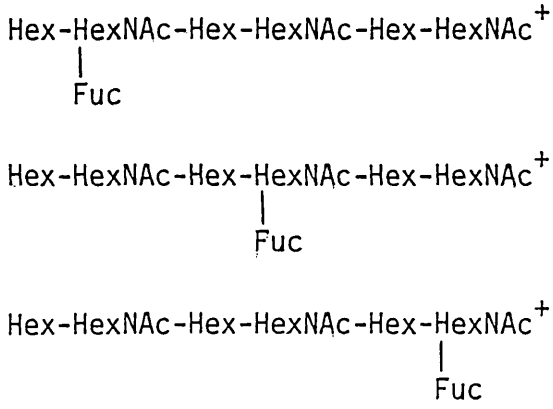
Nana-Hex-HexNAc-Hex-HexNAc-Hex-HexNAc
|
Fuc

Hex-HexNAc-Hex-HexNAc
|
Fuc

A-3 than in A-2.

A summary of the structures present in each sample is given in table 2.4.

Because the samples contain both substituted and unsubstituted chains it is not possible to assign the position of substitution in all structures, e.g. an ion with composition $FucHex_3HexNAc_3^+$ could have three structures:



The presence of the m/z 638 fragment shows that some of the fucosylated side chains have terminal fucose, but does not specify that all do. Similarly m/z 464 may arise from either of the second two structures, but can also come from the unsubstituted branches. Again, due to the mixture of substituted and unsubstituted side chains it is not possible to prove or disprove the second two structures on the basis of whether they eliminate fucose - elimination of fucose from these structures cannot be distinguished from elimination of methanol from unsubstituted species. All possible structures have thus been drawn except that

1) all fucose residues have been drawn in 1→3 linkage to hexosamine. Since 638 never eliminates methanol it may be deduced that all terminal fucose residues are 1→3 linked to hexosamine. This specifies the presence of a Fuc α 1→3 HexNAc transferase which in the simplest case may be assumed to mediate all fucosylation

reactions, so that internal fucosylations have also been represented in 1→3 linkage to hexosamine,

2) all Nana residues are considered to be hexose linked as in NanaFucHexHexNAc$^+$ (m/z 999) and are assumed to be terminal.

Analysis of these samples illustrates the essential features of the FAB behaviour of permethylated samples, which may be summarised as:

1) the derivative gives good sensitivity,
2) the mass increase on per ıethylation is smaller than on acetylation,
3) very large (>10,000 dalton) molecules will fragment to give low molecular weight fragment ions which allow elucidation of branching patterns, sites of substitution, etc.,
4) fragmentation behaviour has been defined, e.g.
   i. aminoglycosidic cleavage is favoured,
   ii. fragment ions are usually A-type with additional β-cleavage of branched fucose residues,
   iii. Hex/HexNAc substitution of fucose can be assigned by examination of the products of β-elimination from fucosylated parent ions.

2.3 Conclusion.

In addition to the important part these results played in the structure elucidation studies mentioned, much useful information was gained about the FAB behaviour of different derivatives and structures. Many obseıvations made during these studies provided useful background experience which has allowed the acetylation and permethylation protocols to be confidently applied to the samples in the following chapters.

CHAPTER 3 : LYSOSOMAL STORAGE DISEASES.

3.1 Introduction.

The lysosomal storage diseases are a group of hereditary diseases arising from the deficiency of the lysosomal exoglycosidases involved in the catabolism of glycoconjugates. The enzymic deficiency results in incomplete catabolism and leads to the accumulation of oligosaccharides, glycopeptides and glycolipids in the tissues and urine of patients. The storage of these products causes distortion of the tissues and gives rise to the clinical symptoms which characterise this group of diseases; marked facial dismorphia, multiple skeletal abnormalities and severe mental retardation.

The enzymic deficiency and associated storage products have been identified for several such diseases and interest has focussed on finding suitable models for the study of the human condition.

This chapter describes the use of FAB techniques in the structure analysis of storage products in two of the lysosomal storage diseases; fucosidosis and mannosidosis.

3.2 Fucosidosis.

3.2.1 Introduction. Fucosidosis was first reported in humans by Durand, who described an autosomal recessive trait characterised by the deposition of lipid like material in the tissues (86). The disease was later demonstrated to arise from a deficiency of lysosomal α-L-fucosidase (87) and the consequent accumulation of storage products. In 1971 Dawson and Spranger reported the isolation and characterisation of a fucose containing glycolipid accumulated in the liver of patients (88), and studies have since been carried out on the storage products in a variety of different organs, including the brain (89, 90). In addition, the structures of

urinary oligosaccharides and glycopeptides have also been published (91,92,39).

More recently, studies on English Springer spaniels have revealed cases of a progressive neuronal storage disease (93,94) which is characterised by vacuolation of neurones and glial cells in the brain. A deficiency of α-L-fucosidase was detected in the leucocytes of one dog (95) suggesting that the disease was a case of canine fucosidosis.

This section describes the use of derivatisation procedures in conjunction with FAB-MS to identify the storage products accumulating in the brains of affected dogs.

3.2.2 Results and Discussion. The glycopeptide samples (II,IIb and IV) analysed in this section were provided by Dr. Bryan Winchester and colleagues(Queen Elizabeth College, London) and were obtained as the three major peaks after bio-gel P4 fractionation of brain extracts from two affected dogs. The details of the extractions and associated studies performed by these workers have been published (96).

FRACTION III

The most detailed analysis was carried out on this fraction since it was available in the greatest quantity.

Positive FAB of the underivatised sample yielded a spectrum containing only one very intense molecular ion cluster at m/z 1520 ($M+H^+$) which corresponds to a glycopeptide(s) of composition $Fuc_2Hex_3HexNAc_3$ Asn.

Treatment of III with a 1:1 (v:v) mixture of $(CH_3CO)_2CO$: $(C^2H_3CO)_2CO$ in methanol (a reagent which rapidly acetylates α-amino groups of peptides) caused the molecular ion signal to shift to a 1:1 doublet at m/z 1562 and 1565. This indicates incorporation of

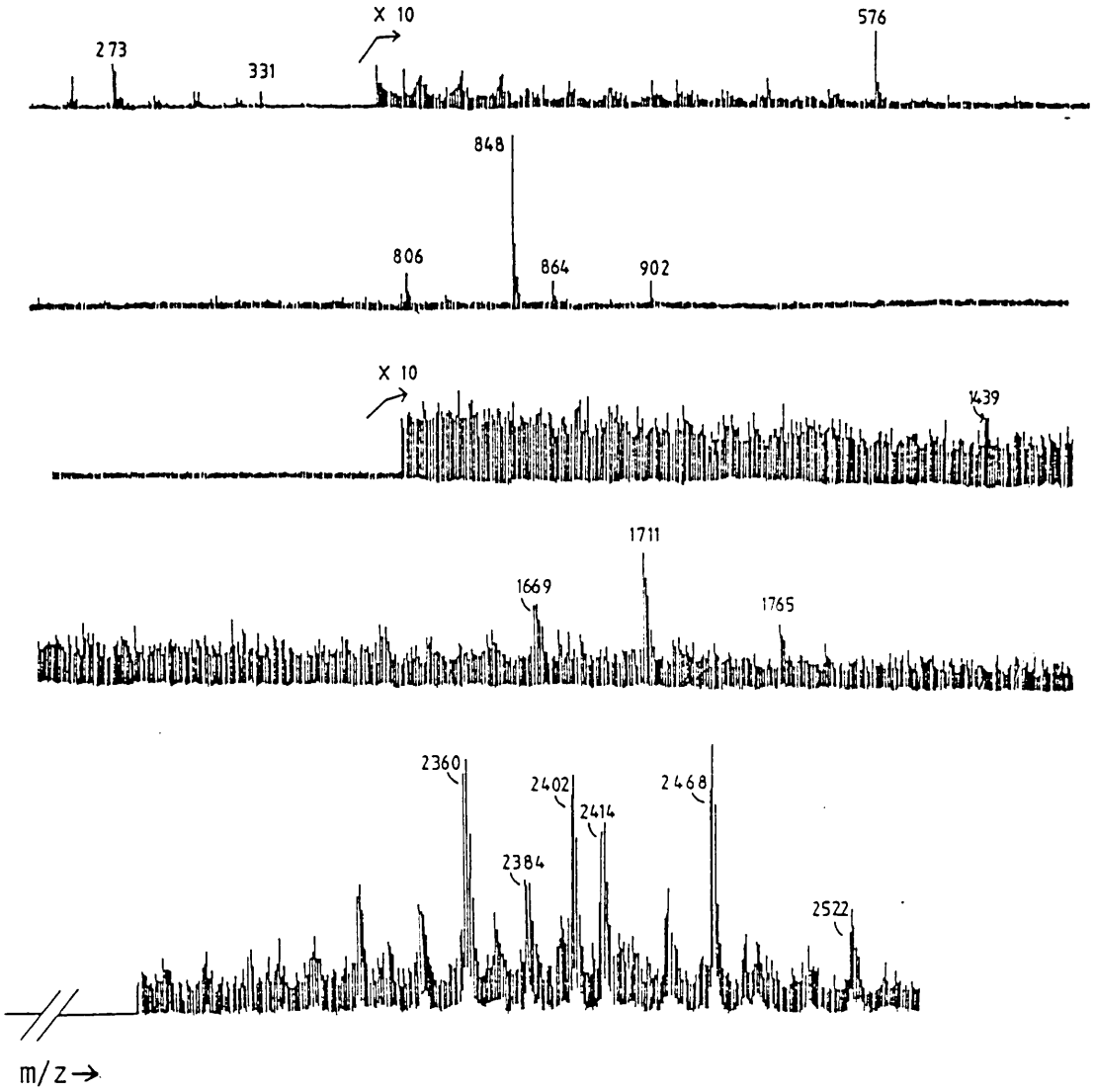


FIG 3.1. Positive FAB spectrum of peracetyl fraction III from canine fucosidosis brain (8 kV).

one acetyl group and provides further evidence for the glycopeptide nature of III. Amino acid analysis was used to confirm the presence of Asx.

The internal structure of III was determined by FAB analysis of per-O-acetyl and per-O-methyl derivatives. The positive FAB spectrum of the per-O-acetylated sample is presented in Fig 3.1. The signals at m/z 2360 and 2402 are molecular ions corresponding to the fully O-acetylated glycopeptide ($M+H^+$ - m/z 2360) and the overacetylated glycopeptide ($M+H^+$;2402). This signal is much more intense than those observed (p. 38) for overacetylated carbohydrates and the additional acetyl is probably on the Asn. A-type sequence ions are observed at m/z 1711, 1439 (very weak), 848, 576, 331 and 273 corresponding to $FucHex_3HexNAc_2^+$, β-cleavage of fucose from 1711, $FucHexHexNAc^+$, β-cleavage of fucose from 848, Hex^+ and Fuc^+ respectively.

Permethylation of the 1:1 ($H:^2H$) N-acetyl derivative of III was then carried out and the positive ion FAB spectrum recorded (Fig 3.2). When glycopeptides are examined it is necessary to protect the α-amino group by acetylation in order to prevent quaternisation during methylation, since such a product would be charged (97).

Molecular ion signals were observed as 1:1 doublets at m/z 1926/1929 and 1940/1943 corresponding to the fully methylated species (1926/1929) and to the overmethylated molecule (1940/1943) in which C-methylation has presumably occured. The molecular ions are the only signals containing the 1:1 label, indicating that fragment ions are derived from the carbohydrate part of the molecule. Signals attributable to A-type sequence ions were observed at m/z 1710, 1291, 1103, 842, 810, 654, 638, 622, 450, 432, 413 and 391. The most abundant of these ions were those at m/z 1710, 1291 and 638 which are assumed therefore to arise via the favoured hexosamine

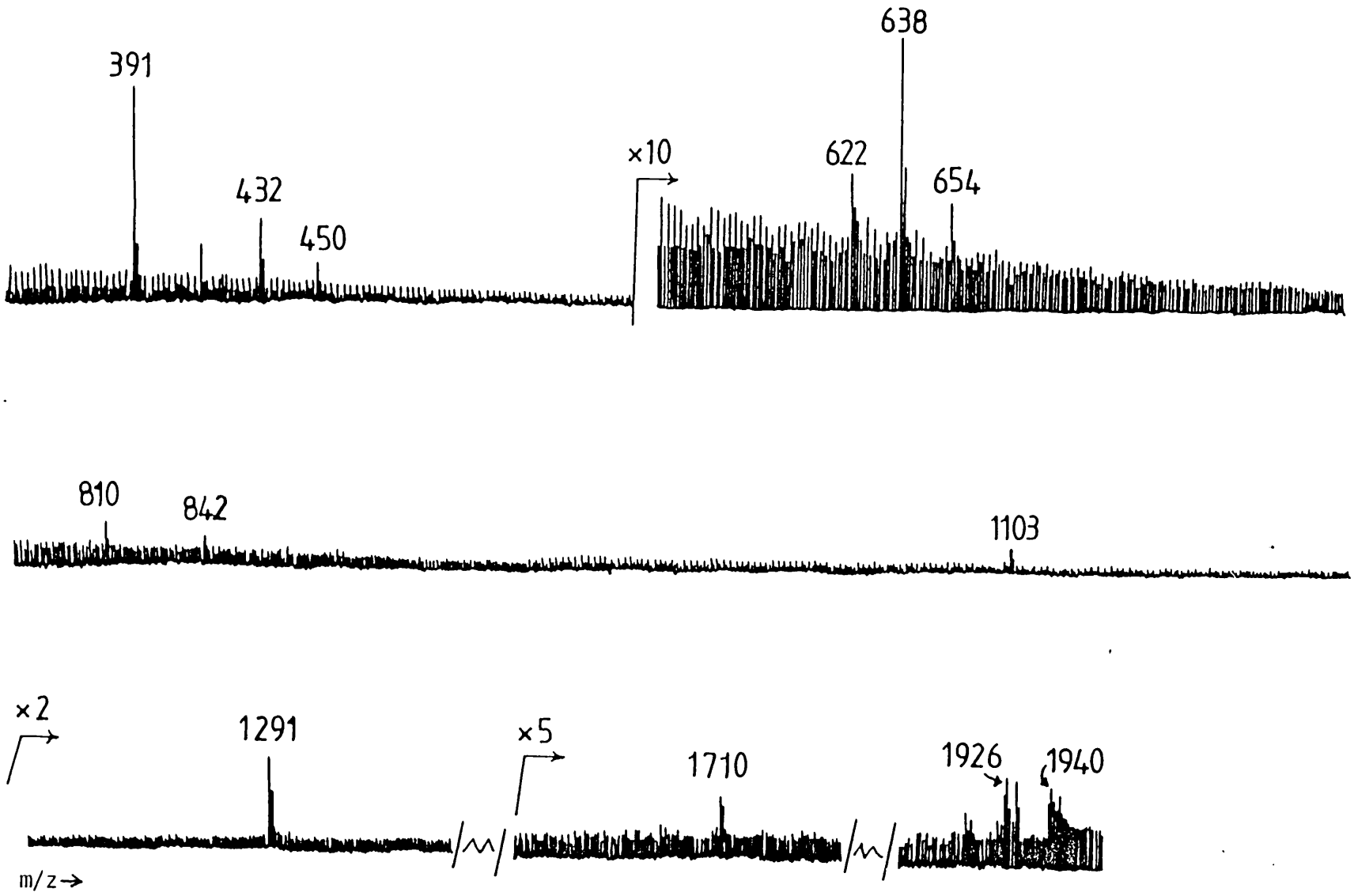
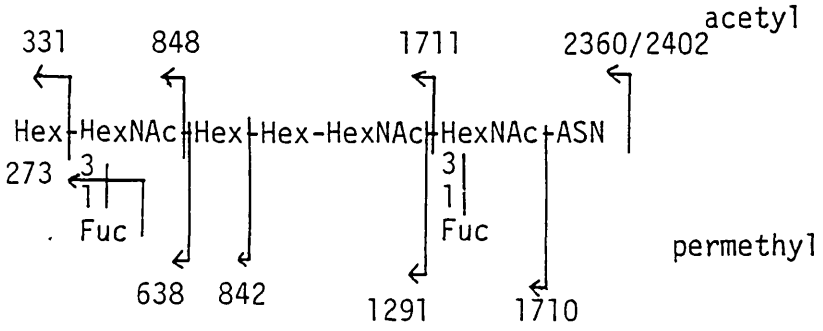


FIG 3.2. Positive FAB spectrum of 1:1 N-acetyl permethyl fraction III.

cleavage. These ions can be assigned the compositions $Fuc_2Hex_3HexNAc_3^+$, $FucHex_3HexNAc_2^+$ and $FucHexHexNAc^+$ respectively. The less abundant ions at m/z 1103 and 450 are formed on β-cleavage of fucose from $FucHex_3HexNAc_2^+$ (m/z 1291) and $FucHexHexNAc^+$ (m/z 638) respectively. The very weak signal at m/z 842 corresponds to an A-type ion of composition $FucHex_2HexNAc^+$ and is presumably the product of a hexosyl cleavage. This signal is accompanied by associated ions at m/z 810, which is produced by loss of methanol from 842, at m/z 654, which is the product of fucosyl β-cleavage from 842, and at m/z 622 corresponding to the loss of methanol from the β-cleavage ion at m/z 654. These data are consistent with the structure



from which the major ions arise as shown.

Linkage of the external fucose residue.

Two pieces of evidence support the structure drawn above in which the external fucose is linked 1→3 to hexosamine rather than to the terminal hexose.

1) The Hex^+ ion (m/z 331) in the spectrum of the acetylated sample can only arise if the non-reducing terminal hexose residue is unsubstituted. Since the fucose is not linked to the non-reducing terminal hexose it must be linked to the adjacent hexosamine in order to give the $FucHexHexNAc^+$ ions observed in both the acetyl and permethyl spectra.

2) As has already been described in chapter 2, if the 3 position

of hexosamine in the permethyl derivative is unsubstituted, the m/z 638 ion readily loses methanol to yield a signal at m/z 606. When fucose is substituted at position 3 of hexosamine an analogous signal is observed at m/z 432 arising from elimination of fucose. The spectrum of the permethyl derivative contains an abundant ion at m/z 432 and no m/z 606 peak was observed, thus allowing the above structure to be assigned to the major, or probably the sole component of fraction III.

Fraction IV.

Positive FAB of fraction IV contained pseudomolecular ions at m/z 482 ($M+H^+$), 504 ($M+Na^+$) and 526 ($M-H^+ + 2Na^+$). At higher mass a second cluster of ions was observed at m/z 985 ($2M+Na^+$), 1007 ($2M-H^+ + 2Na^+$) and 1029 ($2M-2H^+ + 3Na^+$). These signals correspond to molecular ions for the monomer and the dimer of a component with composition FucHexNAcASN. These results indicate that a large amount of salt is present in the preparation which seems to promote formation of the cationised dimeric species.

The remainder of the sample was per-O-acetylated and FAB spectra of the derivative were obtained in the positive mode. The largest peak in the spectrum was at m/z 734 corresponding to the $M+H^+$ molecular ion for fully acetylated FucHexNAcASN. One other major signal was observed at m/z 717 which probably derives from contaminants in the sample.

It is worth noting that although aminohexosyl cleavages are favoured no fragment ion was observed for $FucHexNAc^+$. This is consistent with the observation made during the course of this work that very small molecules generally do not fragment in the same way as their larger counterparts. Amino acid analysis was carried out

on the remainder of the acetyl sample and confirmed the presence of Asx. The only reasonable structure which may be deduced from such results is

Fuc-HexNAc-ASN

since known biosynthetic pathways exclude the possibility of HexNAc-Fuc-ASN.

Fraction II.

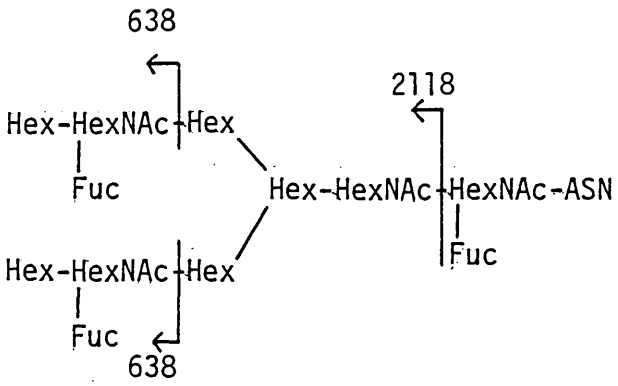
FAB of the underivatised sample produced a spectrum containing three molecular ion signals, so indicating that the sample contained a mixture of components. The major signal was observed at m/z 2193 ($M+H^+$) corresponding to a composition of $Fuc_3Hex_5HexNAc_4ASN$, with a less abundant molecular ion at m/z 2031 ($M+H^+$) corresponding to one hexose unit less ($Fuc_3Hex_4HexNAc_4ASN$). A weak molecular ion was observed at m/z 1520 indicating some contamination of fraction II with the component observed in fraction III. In order to determine the structure(s) corresponding to these compositions the N-acetyl permethyl derivative of fraction II was analysed. The spectrum contained only two major A-type fragment ions. These were observed at m/z 638 and 2118 and correspond to $FucHexHexNAc^+$ and $Fuc_2Hex_3HexNAc_3^+$. A minor fragment ion was observed at m/z 432 but no signal was present at m/z 606. These data indicated that the non-reducing fucosyl residues are linked to hexosamine and not hexose residues (see discussion of fraction III). The absence of signals at m/z 1087 and 1261 precludes the presence of structures which can give rise to the ions

$$\text{Hex-HexNAc-Hex-HexNAc}^+ \quad (\text{Fuc})$$

and

$$\text{Hex-HexNAc-Hex-HexNAc}^+ \quad (\text{Fuc}, \text{Fuc}).$$

The only structure which can accomodate all these data is shown below, in which the fragment ions arise as indicated:

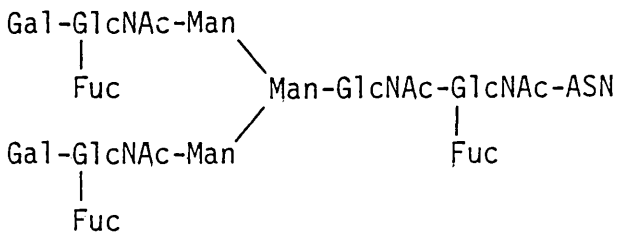


The less abundant molecular ion (m/z 2031 in the underivatised spectrum) corresponding to one fewer hexosyl residue is presumably derived from the related structure which lacks a non-reducing terminal hexose. No fragment ions were observed which could be rationalised as cleavage products from this component, illustrating that, as seen in chapter 2, not all species in a mixture fragment to the same extent.

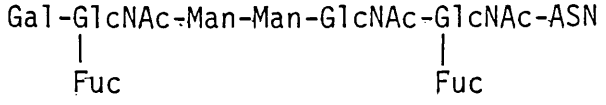
These results demonstrate the way in which the choice of suitable derivatives for FAB analysis can be used to provide unambiguous structure information.

3.2.3 Conclusion. The structures of the three major storage products isolated from the brains of two dogs displaying symptoms of fucosidosis were determined by FAB analysis of acetyl and permethyl derivatives. Based upon established biosynthetic pathways and the sugar analysis data obtained by the other workers in the course of this study (96) the structures are postulated to be:

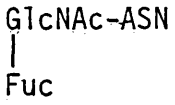
II



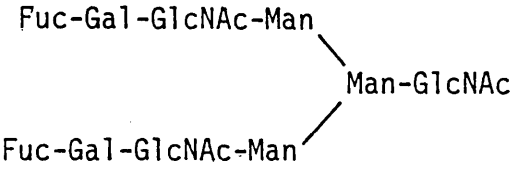
III



IV



It is interesting to compare these structures with those determined for the storage products in the brains of humans with fucosidosis (90): the major components were found to be the oligosaccharides



and Fuc-GlcNAc

The glycolipid Fuc-Gal-GlcNAc-Gal-Glc-Ceramide was a minor component. There are important differences between these and the canine products:

1) the canine products are glycoasparagines, while the major human products are free oligosaccharides, which suggests greater or different glucosaminidase activity in the human system,
2) fucose is galactose linked in the human but is linked to hexosamine in the dog which indicates the presence of different fucosyl transferase activities,
3) no hexasaccharide is reported in the human preparation, suggesting the absence of the endo-mannosidase activity which exists in the dog to produce the structure seen in fraction III.

These discrepancies reflect differences in the anabolic and catabolic pathways of glycoprotein metabolism in dogs and humans and therefore indicate that caution should be exercised in drawing parallels between the animal and the human conditions.

3.3 Mannosidosis.

3.3.1 Introduction. α-mannosidosis is a hereditary disease

characterised by the deficiency of lysosomal acidic α-mannosidase. It was first described in humans (98) but since then cases of feline (99) and bovine (100) α mannosidosis have also been reported. The oligosaccharides accumulated in the tissues and urine of patients have been thoroughly investigated with over 20 structures published eg. (101-104). In contrast, the storage products in the animal disorders are not well characterised, although the structure of a single urinary oligosaccharide (105) and the composition of two storage oligosaccharides (from brain (106) and lymph nodes (107)) have been reported for the bovine disease. Tentative structures have been assigned for the remaining bovine and feline oligosaccharides on the basis of their chromatographic behaviour (108).

In addition to these examples of hereditary mannosidosis, the incidence of an induced mannosidosis was reported in animals after prolonged ingestion of swainsonine (109). This 1,2,8-trihydroxylated indolizidine alkaloid is found in plants of the genus Swainsona (110), in certain species of locoweed (111) and in the fungus Rhizoctonia leguminicola (112) and has been shown to be a potent inhibitor of lysosomal α-mannosidase (113). Swainsonine intoxication therefore produces a neurological disorder which is the phenocopy of the genetically induced disease, and so provides a convenient model system for comparison with the naturally occuring disorder.

This section describes the use of a combination of derivatisation techniques which allows FAB-MS to be used in the rapid screening of crude biological extracts for oligosaccharide storage products. Examples are given for products isolated from urine and from cell lysates and both the naturally occuring and the swainsonine induced conditions are examined.

3.3.2 Results and Discussion. The samples analysed in this section

TABLE 3.1.

MANNOSIDOSIS URINE SAMPLES EXAMINED IN CHAPTER 3.

ORIGIN	SAMPLE
Feline	Crude whole urine
Bovine	Crude whole urine
	Main component D]
	Main component E] isolated by preparative T.L.C.
	Main component G]
Ovine	Crude whole urine

were provided by Dr. Bryan Winchester and colleagues (Queen Elizabeth College, London). The urinary samples were obtained as the neutral fraction after ion exchange chromatography of lyophilised urine. The cell extracts were prepared by similar chromatography of the supernatant after cell lysis.

Urinary Samples.

Samples of urinary oligosaccharides were analysed from feline and bovine hereditary mannosidosis and from sheep swainsonine induced mannosidosis. Details of the samples are given in table 3.1. The feline and bovine samples were analysed underivatised using positive FAB. The spectrum obtained from underivatised feline whole urine is shown in Fig 3.3. The majority of the signals may be assigned as cluster ions formed between the glycerol matrix and the urea which contaminates the crude urine extract eg. m/z 737 and 829 are $glycerol_7$ and $glycerol_8$ polymers, m/z 797 and 889 are $glycerol_7$ and $glycerol_8$ polymers each combined with one urea molecule, and m/z 765 and 857 are $glycerol_6$ and $glycerol_7$ polymers each combined with two urea molecules. Only the weak signal at m/z 911 derives from an oligosaccharide and corresponds to the molecular ion $M+H^+$ for $Hex_3HexNAc_2$. The remaining underivatised samples failed to give any meaningful data in similar experiments, with all significant signals in the spectra being attributable to urea and glycerol cluster ions. Such results are not unexpected since crude extracts of this type may be expected to contain large amounts of urea and other contaminants which suppress FAB ionisation of the oligosaccharides. It was found that a sufficiently clean sample for direct FAB analysis could be prepared by per-O-acetylation. The per-O-acetyl oligosaccharides formed are soluble in organic solvents and so may be separated from the aqueous soluble contaminants by partition in an aqueous/chloroform

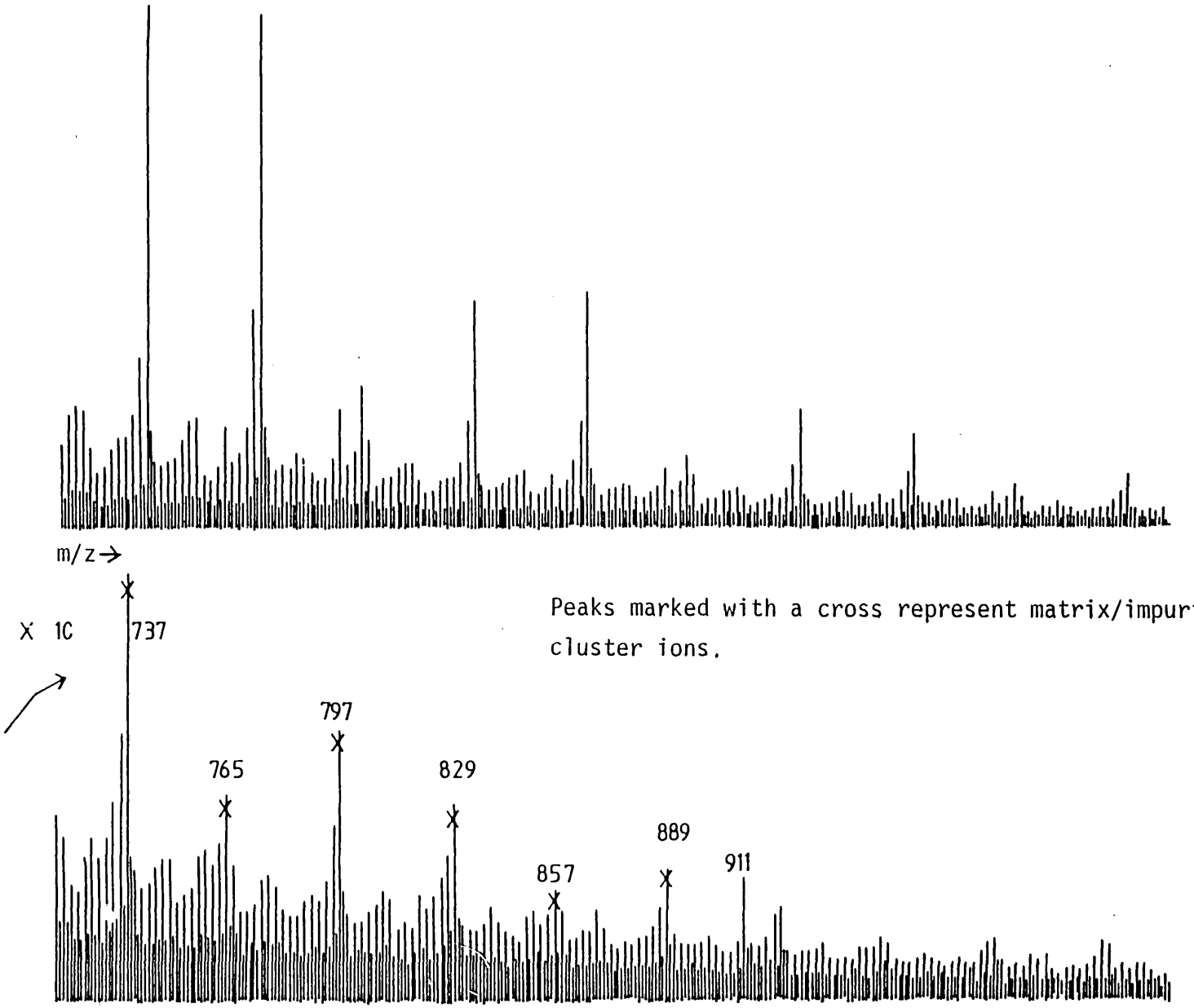


FIG 3.3. Positive FAB spectrum of underivatised Feline mannosidosis urinary oligosaccharides (8kV)

system. Per-O-acetylation was carried out on all samples and the chloroform soluble products analysed by FAB. Results were obtained in all cases except that of bovine whole urine. The spectrum obtained from per-O-acetylated feline whole urine is presented in Fig 3.4. $M+H^+$ molecular ions were observed at m/z 1211, 1499 and 1787 which correspond to acetylated oligosaccharides with a free reducing centre (designated $M—OH/H^+$) having compositions of $Hex_2HexNAc_2$, $Hex_3HexNAc_2$ and $Hex_4HexNAc_2$ respectively, the Hex_3 component being the most abundant. However, in addition to these signals another set of molecular ions was observed one mass unit above the first set. These signals were more intense than would be expected from the ^{13}C contribution at these masses, and correspond in composition to the replacement of a HexNAc with a Hex residue (acetylated residue mass for Hex = 288, HexNAc = 287) so that the peaks at m/z 1212, 1500 and 1788 are assigned to the series of acetylated oligosaccharides containing a single HexNAc and 3,4 and 5 hexose residues respectively. All the remaining intense signals in the spectrum may be assigned as follows:

1) A-type fragment ions. These are of low abundance in this sample although the peaks at m/z 1481/1482 may be assigned as A-type fragment ions of composition $Hex_3HexNAc_2^+$ and $Hex_4HexNAc^+$, with a further ion at m/z 1194 for $Hex_3HexNAc^+$.

2) "minus 42" ions arise due to underacetylation eg. at m/z 1457/1458, 1415/1416, 1373/1374, etc.

3) "plus 54" ions arise by substitution of an acetyl group with a trifluoroacetyl group (see chapter 2) eg. at m/z 1553/1554, and 1841/1842.

It must be noted that when examining a mixture A-type fragment ions do not allow unambiguous structure assignment since it is not

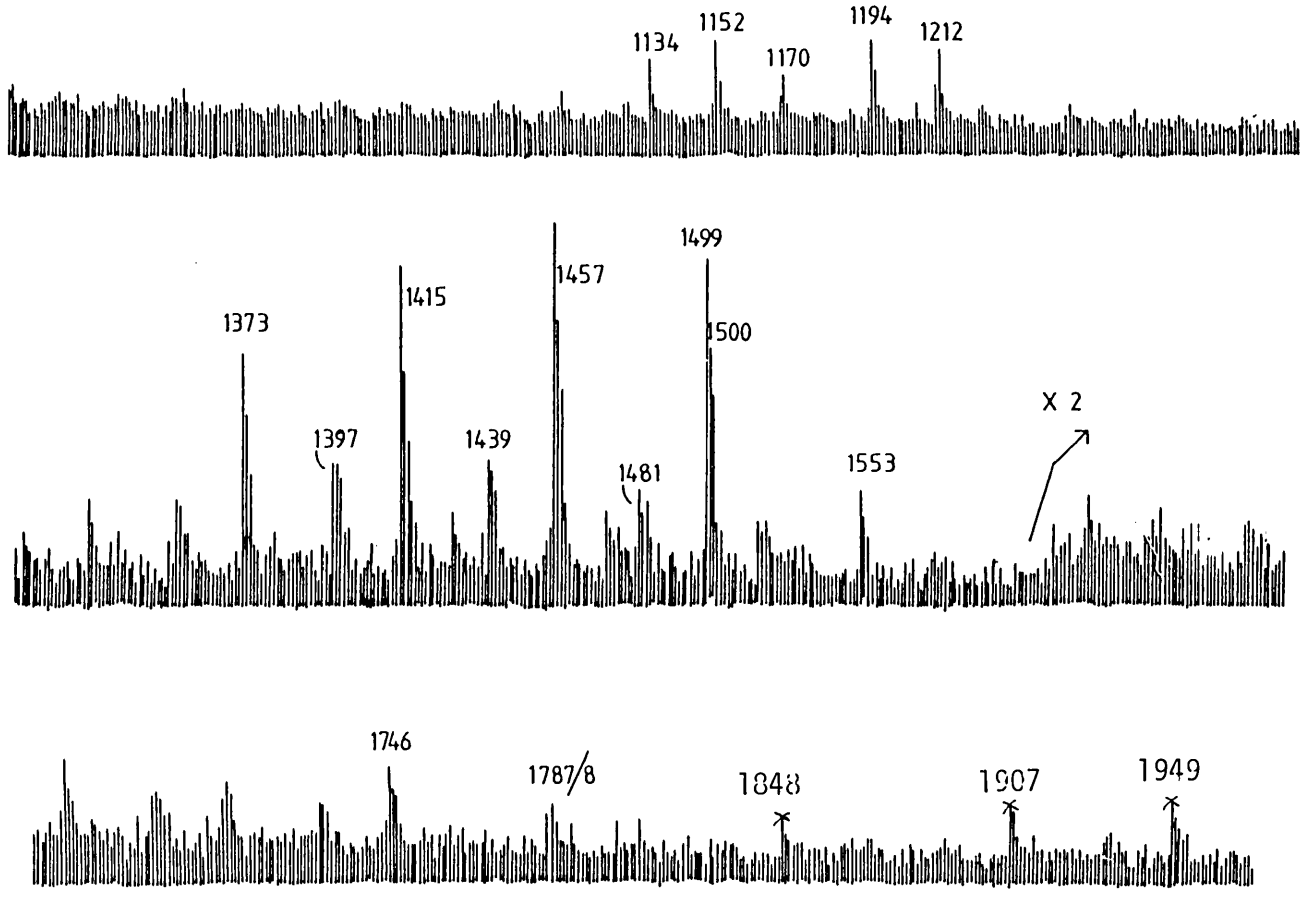


FIG 3.4. Positive FAB spectrum of peracetyl Feline mannosidosis urinary oligosaccharides (8kV). Peaks marked x assumed to be due to impurities since they are only observed in some scans.

TABLE 3.2.

COMPOSITION OF OLIGOSACCHARIDES IDENTIFIED IN MANNOSIDOSIS URINE EXTRACTS BY FAB-MS OF PER-O-ACETYL DERIVATIVES.

FELINE	$Hex_{2-4}HexNAc_2$	$Hex_3HexNAc_2$	most abundant
	$Hex_{3-5}HexNAc_1$	$Hex_4HexNAc$	
BOVINE	$Hex_{2-5}HexNAc_2$		
	$Hex_{3-6}HexNAc$		
SHEEP	$Hex_{2-5}HexNAc_2$		
	$Hex_{2-6}HexNAc$		

TABLE 3.3.

SUMMARY OF COMPOSITION ASSIGNMENTS FOR THE MOLECULAR IONS OBSERVED FROM ACETYLATED BOVINE MAIN COMPONENTS D, E AND G.

MAIN COMPONENT D.

m/z 1211	$M+H^+$ for —OH	$Hex_2HexNAc_2$

MAIN COMPONENT E.

m/z 1211	$M+H^+$ for —OH	$Hex_2HexNAc_2$
1212	$M+H^+$ for —OH	$Hex_3HexNAc$
1499	$M+H^+$ for —OH	$Hex_3HexNAc_2$
1500	$M+H^+$ for —OH	$Hex_4HexNAc$

MAIN COMPONENT G.

m/z 1787	$M+H^+$ for —OH	$Hex_4HexNAc_2$
1788	$M+H^+$ for —OH	$Hex_5HexNAc$
2075	$M+H^+$ for —OH	$Hex_5HexNAc_2$
2076	$M+H^+$ for —OH	$Hex_6HexNAc$

possible to determine the parent molecule(s) from which they arise. However, by examination of the molecular ions alone it can be seen that feline whole urine contains the components given in Table 3.2. These structures include the molecules deduced from the chromatographic studies (108) i.e. $Man_5GlcNAc$ and $Man_3GlcNAc_2$ or $Man_1GlcNAc_3$.

Several attempts were made to obtain FAB data on the bovine whole urine sample after per-O-acetylation although all failed. All spectra showed signs of being suppressed despite exhaustive extraction, suggesting that the bovine urine contained a higher proportion of urea and salts in relation to oligosaccharides than the feline, or that a different contaminant was present which was not susceptible to removal by chloroform/aqueous partition.

In contrast, the three bovine main components D,E and G produced good data on FAB analysis of the per-O-acetyl derivatives. The molecular ions observed and the compositions assigned are given in Table 3.3. A-type fragment ions were observed in each spectrum corresponding to the aminoglycosidic cleavages observed in the feline samples. It is interesting to note that although these three fractions were isolated after TLC separation, main components E and G are shown by FAB analysis to be complex mixtures and not single components as expected.

The spectrum obtained on FAB analysis of per-O-acetylated urine from swainsonine intoxicated sheep is shown in Fig 3.5. Pairs of molecular ions, all of comparable abundance were observed at m/z 2075/2076, 1787/1788, 1499/1500, 1211/1212 and finally a single peak at 924. These correspond to components with compositions $Hex_5HexNAc_2$ and $Hex_6HexNAc$, $Hex_4HexNAc_2$ and $Hex_5HexNAc$, $Hex_3HexNAc_2$ and $Hex_4HexNAc$, $Hex_2HexNAc_2$ and $Hex_3HexNAc$ and $Hex_2HexNAc$ respectively. In addition to these signals which arise from the species generally produced using the TFAA/glacial acetic acid procedure i.e. molecules unacetylated at the reducing

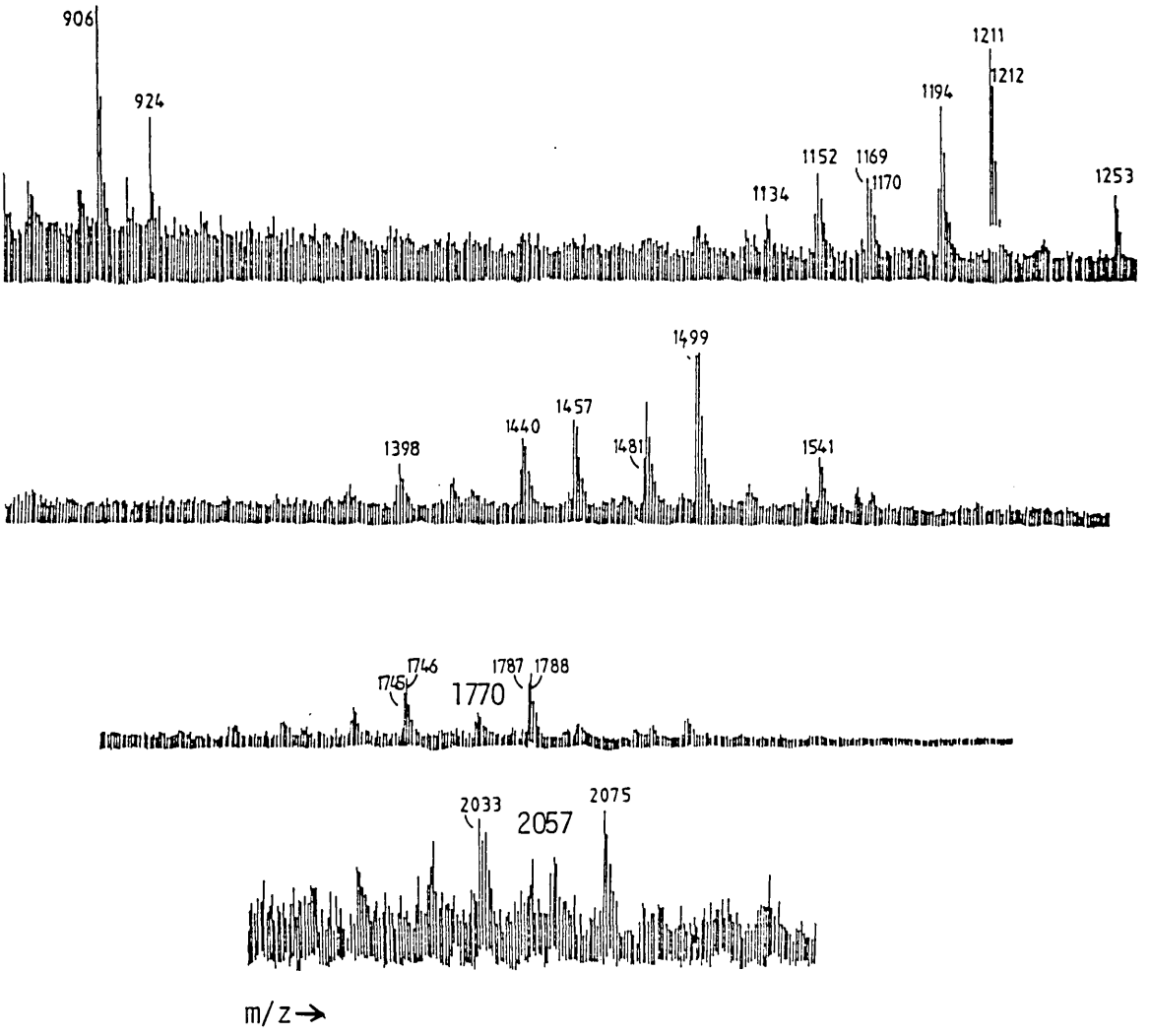


FIG 3.5. Positive FAB spectrum of peracetylated urinary oligosaccharides from swainsonine intoxicated sheep (8 kV).

centre, a second set of molecular ions ($M+H^+$) were observed in this spectrum 42 m.u. higher than the first. These correspond to the fully acetylated derivatives and are particularly pronounced for the $Hex_2HexNAc_2$, $Hex_3HexNAc$, $Hex_3HexNAc_2$ and $Hex_4HexNAc$ components. A-type fragment ions are present at m/z 906 ($Hex_2HexNAc^+$), 907 (Hex_3^+), 1193 ($Hex_2HexNAc_2^+$), 1194 ($Hex_3HexNAc^+$), 1195 (Hex_4^+), 1481 ($Hex_3HexNAc_2^+$), 1482 ($Hex_4HexNAc^+$), 1483 (Hex_5^+), 1769 ($Hex_4HexNAc_2^+$), 1770 ($Hex_5HexNAc^+$), 1771 (Hex_6^+), 2057 ($Hex_5HexNAc_2^+$) and 2058 ($Hex_6HexNAc^+$).

In addition, a series of signals separated by 42 m.u. is observed to the low mass side of each cluster, arising from incompletely acetylated components. The oligosaccharides present in this sample are summarised in Table 3.2.

Since this work was carried out two reports have been published on the structure elucidation of components isolated from the urine of swainsonine intoxicated sheep (114,115). One study (114) reported the presence of $HexNAc_2$ components containing between 2 and 5 hexose residues, while the other study (115) also described the $Hex_4HexNAc_2$ and $Hex_5HexNAc_2$ species. The acetylation work described here identified these four components but also detected the three additional $HexNAc_1$ species.

The technique of acetylation and FAB analysis of crude samples is fast and convenient and so has important potential for the rapid screening of large numbers of crude extracts for oligosaccharide content. The protocol does have the disadvantage that, although components in a complex mixture can be identified, little or no unambiguous structure information about the components may be obtained from this experiment. Frequently, the components are present in such low amounts that no fragmentation occurs and molecular ions alone are generated. However, even in the cases where fragmentation does occur, the information such fragments afford is limited in a

mixture since it is not possible to identify which molecule produced which fragment. In order to obtain structure information by FAB, fractionation of the mixture would be necessary. Purified components could then be structured by analysis of suitable derivatives.

The coincidence of the ^{13}C-isotope peaks of the $HexNAc_2$-containing oligosaccharides with the signals arising from components containing Hex instead of HexNAc can give rise to problems if the higher mass component is of low abundance, or when dealing with derivatives higher in mass than about 1600 m.u. In such cases it becomes difficult to decide whether the higher molecular weight species are present.

In order to resolve this problem it was necessary to use a derivative which produces a mass separation of more than 1 m.u. between derivatised hexose and hexosamine residues. Two different approaches will be described here, the first involving a permethylation protocol.

Reverse permethylation.

The permethylated residue masses of hexose (204) and hexosamine (245) are sufficiently different to allow HexNAc and $HexNAc_2$ compounds to be readily distinguished. In addition the FAB behaviour of permethylated oligosaccharides was already known so that permethylation suggested itself as a suitable solution. Permethylation was attempted several times on feline whole urine and on bovine main component E but it was not possible to carry out the derivatisation procedure on the crude extracts and obtain a permethylated sample which yielded a FAB spectrum. These difficulties were probably due to the crude nature of the sample. To overcome these purity problems permethylation was carried out on an acetylated sample which had already been cleaned up during the extraction procedure. The permethylation protocol used was exactly the same as for an underivatised sample but was carried out on what remained after FAB analysis of the acetylated feline

whole urine. The positive FAB spectrum obtained is shown in Fig 3.6. The molecular ion ($M+H^+$) peaks at m/z 945 ($Hex_2HexNAc_2$), 1149 ($Hex_3HexNAc_2$) and 1353 ($Hex_4HexNAc_2$), and m/z 904 ($Hex_3HexNAc$), 1108 ($Hex_4HexNAc$) and 1312 ($Hex_5HexNAc$) confirm the components observed in the acetylation experiment. In addition, further molecular ions were present at m/z 1557,1761, 1516 and 1720 for $Hex_5HexNAc_2$, $Hex_6HexNAc_2$, $Hex_6HexNAc$ and $Hex_7HexNAc$ respectively.

The strategy of acetylation followed by permethylation has a variety of factors which make it useful for problems of this type:
1) the sample has already been purified to a certain extent during the extraction procedure necessary for the acetylated sample,
2) acetylated samples do not keep well, especially after being dissolved in methanol for FAB and dried down again, due to the instability of O-acetyl groups, particularly in methanol. This is not a problem here since the acetyl groups are required to be removed by the DMSO base during the permethylation reaction. Thus old acetyl samples can be used in this procedure so conserving material.

The disadvantage of using this reverse permethylation method to distinguish $HexNAc_1$ and $HexNAc_2$ components is that the permethylation protocol is much less convenient than that for acetylation. Thus the other method that has been used is analysis of deuteroacetylated derivatives.

d-Acetylation.

The separation between residue masses of deuteroacetyl hexose (297) and hexosamine (293) is 4 m.u.

d-Acetylation was carried out on bovine main components D, E and G using the same conditions as previously but substituting deuteroacetic acid for glacial acetic acid. FAB analysis of d-acetyl main component D produced a spectrum containing a single molecular

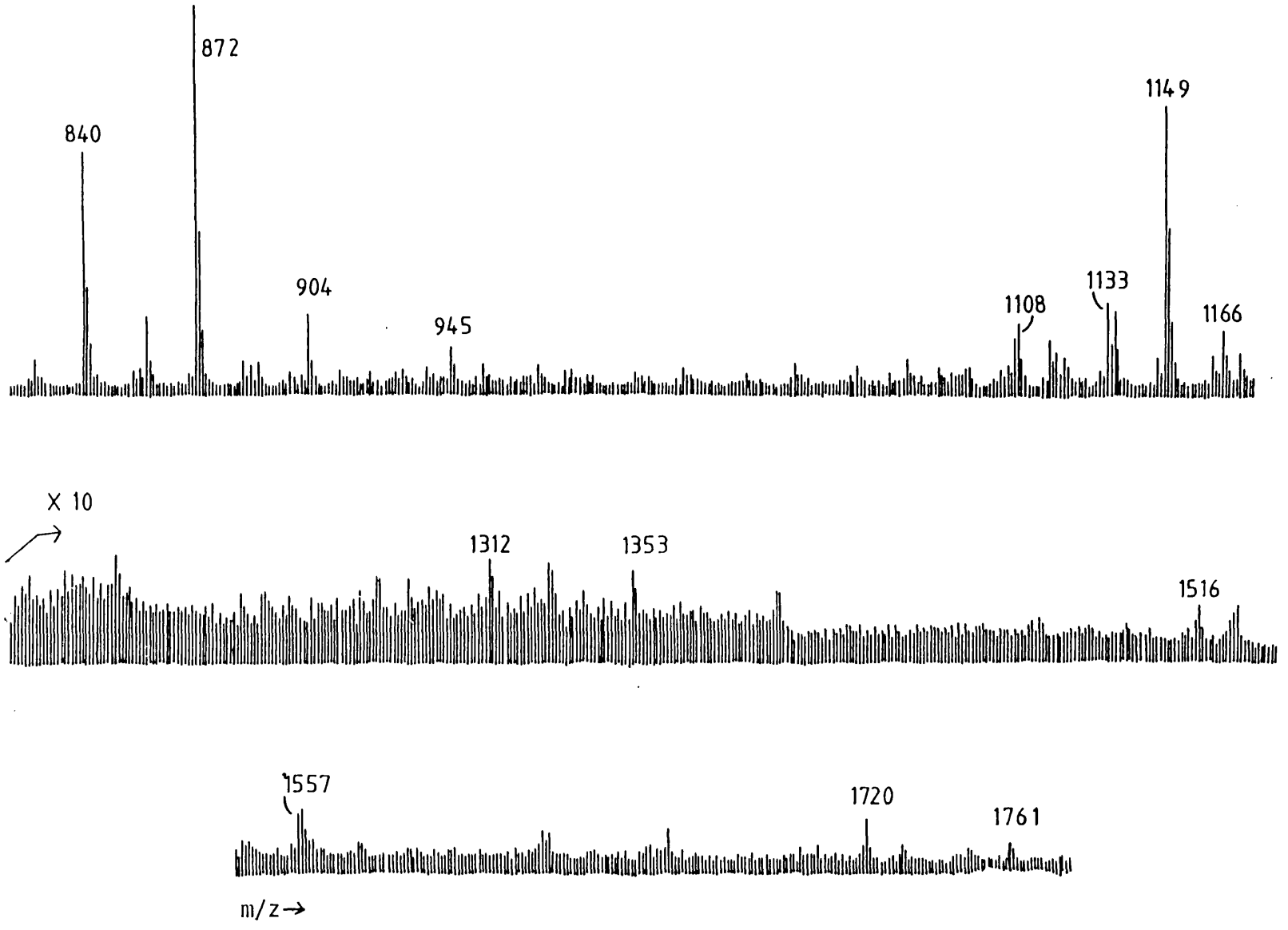


FIG 3.6. Positive FAB spectrum of reverse permethylated Feline mannosidosis urinary oligosaccharides (8 kV). m/z 872 corresponds to an A-type ion of composition $Hex_3HexNAc^+$, and 840 to loss of MeOH.

ion cluster with peaks at m/z 1244 for $Hex_2HexNAc_2$ with a free reducing terminus and m/z 1289 for the same species fully d-acetylated. Associated A-type fragment ions were observed at m/z 1226 for $Hex_2HexNAc_2^+$ and 933 for $Hex_2HexNAc^+$. No peaks were observed 4 m.u. above either molecular ion which confirms the conclusion reached on examination of the original acetyl spectrum, that $Hex_3HexNAc$ is not present in detectable quantities.

The spectrum obtained on FAB analysis of d-acetylated bovine main component E is presented in Fig 3.7. $M+H^+$ molecular ions corresponding to deuteroacetylated species with underivatised reducing centres are represented by the signals at m/z 1244 ($Hex_2HexNAc_2$), 1541 ($Hex_3HexNAc_2$) and 1545 ($Hex_4HexNAc$) while fully d-acetylated $M+H^+$ molecular ions for the $Hex_2HexNAc_2$ and the $Hex_3HexNAc_2$ species only are seen at m/z 1289 and 1586 respectively. The signals at m/z 1226, 1230, 1523 and 1527 correspond to A-type fragment ions of composition $Hex_2HexNAc_2^+$, $Hex_3HexNAc^+$, $Hex_3HexNAc_2^+$ and $Hex_4HexNAc^+$ respectively. Remaining signals may be assigned as arising by under-acetylation or TFA substitution of these major ions. It must be noted that although the original acetylation experiment on this fraction suggested that a component of composition $Hex_3HexNAc$ may be present, no signal for such a component (m/z 1248) is observed in the deuteroacetylated spectrum.

FAB analysis of d-acetyl bovine main component G yielded two molecular ions for species with free reducing centres: m/z 1838 for $Hex_4HexNAc_2$ and 2135 for $Hex_5HexNAc_2$. Peaks were not observed for the $Hex_5HexNAc$ or $Hex_6HexNAc$ components (m/z 1842, 2139) demonstrating that the need for a means of checking the interpretation of the acetyl experiment is increased when higher mass components are being examined.

The major advantage of using the d-acetylation method over reverse

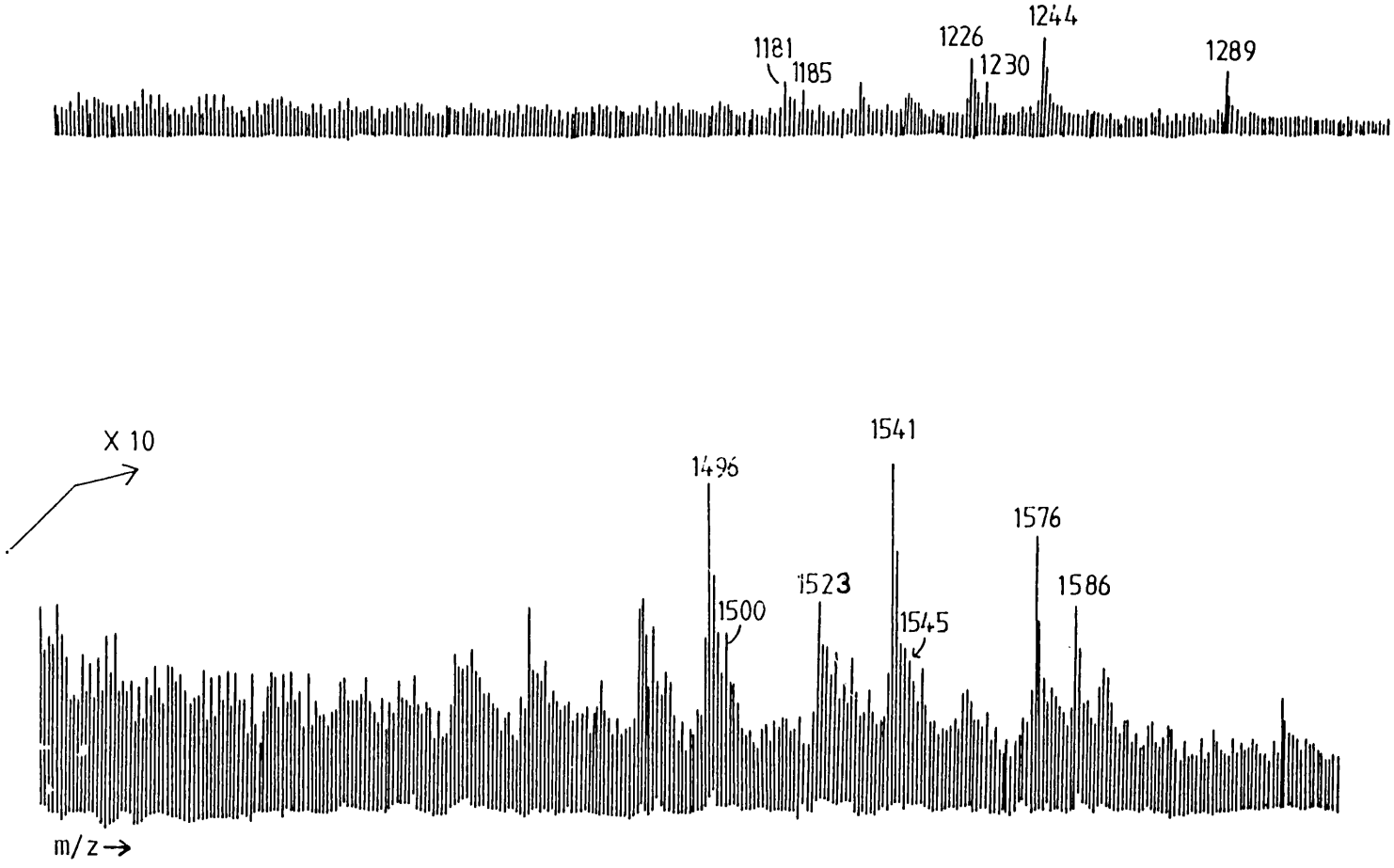


FIG 3.7. Positive FAB spectrum of perdeuteroacetyl main component E from bovine mannosidosis urine (8 kV).

permethylation is the speed and convenience of the procedure. In addition, deuteroacetylation is probably cheaper, despite the costliness of deuterated reagents, since only small quantities are used and Sep-Paks, used to isolate the permethylated products, are expensive. Reverse permethylation is to be favoured when high molecular weight samples are to be examined since, although both derivatives give comparable sensitivity the mass increase on permethylation is not as great. Additionally, reverse permethylation is to be favoured if acetylation data suggests that the higher mass component is present in low abundance since permethylation removes its signal totally from the molecular ion region of the lower mass species and so makes it easier to decide whether or not significant amounts are present.

Cell Samples.

Four cell extracts from human fibroblasts were examined from:

a) normal fibroblasts,

b) normal fibroblasts incubated with swainsonine,

c) mannosidosis fibroblasts,

d) mannosidosis fibroblasts incubated with swainsonine.

All samples were per-O-acetylated and extracted by partition between chloroform and water. The chloroform soluble products were examined using positive FAB.

Analysis of sample a) produced a spectrum containing glycerol and thioglycerol cluster ions only, demonstrating that nothing was present to suppress the ionisation of the matrix.

Sample b) gave a FAB spectrum containing molecular ions at m/z 1212, 1500 (very weak), 1788 and 2076 corresponding to the following species with free reducing centres: $Hex_3HexNAc$, $Hex_4HexNAc$, $Hex_5HexNAc$ and $Hex_6HexNAc$ respectively. A-type fragment ions were observed at m/z 1194 ($Hex_3HexNAc^+$), 1195 (Hex_4^+), 1770 ($Hex_5HexNAc^+$) and 2058

($Hex_6HexNAc^+$)In contrast to the urinary samples analysed above there was no evidence for the presence of any $HexNAc_2$ components in this cell lysate. Additionally it is interesting to note that the $Hex_4HexNAc$ component is very weak by comparison with the intense peaks for $Hex_3HexNAc$ and $Hex_5HexNAc$.

The FAB spectrum obtained from acetylated sample c) is shown in Fig. 3.8. The peaks at m/z 924, 1212, 1500, 1788 and 2076 represent molecular ions for free reducing end species of composition $Hex_2HexNAc$, $Hex_3HexNAc$, $Hex_4HexNAc$, $Hex_5HexNAc$ and $Hex_6HexNAc$. Again there is no evidence for any $HexNAc_2$ components. However, a second series of molecular ions is present in this spectrum corresponding to hexose polymers without any hexosamine. In contrast to the hexosamine containing molecular ions these polymeric hexose species are observed as the fully acetylated species cationised with an ammonium ion (designated $-OAc/NH_4^+$). This difference may be presumed to arise due to the absence of the readily protonatable amide group in the hexosamine residue. Molecular ions of the hexose series are observed at m/z 984, 1272,1560, 1848 and 2136 for Hex_3-Hex_7. Additionally low abundance molecular ions of the $-OH/NH_4^+$ and $-OH/H^+$ types are presentfor each species in this series.

A-type fragment ions are represented by the peaks at 619 (Hex_2^+), 906($Hex_2HexNAc^+$), 907 (Hex_3^+), 1194 ($Hex_3HexNAc^+$), 1195 (Hex_4^+), 1482 ($Hex_4HexNAc^+$), 1483 (Hex_5^+) , 1771 (Hex_6^+) and 2059 (Hex_7^+).

Acetylated sample d) produced a very weak spectrum containing only two very weak molecular ions at m/z 1212 and 1213 for $Hex_3HexNAc$ and Hex_4-OH/H^+.

Reverse permethylation was carried out on samples b), c) and d) in order to examine more closely the difference between the genetic and induced mannosidosis states, and to attempt to improve the data available

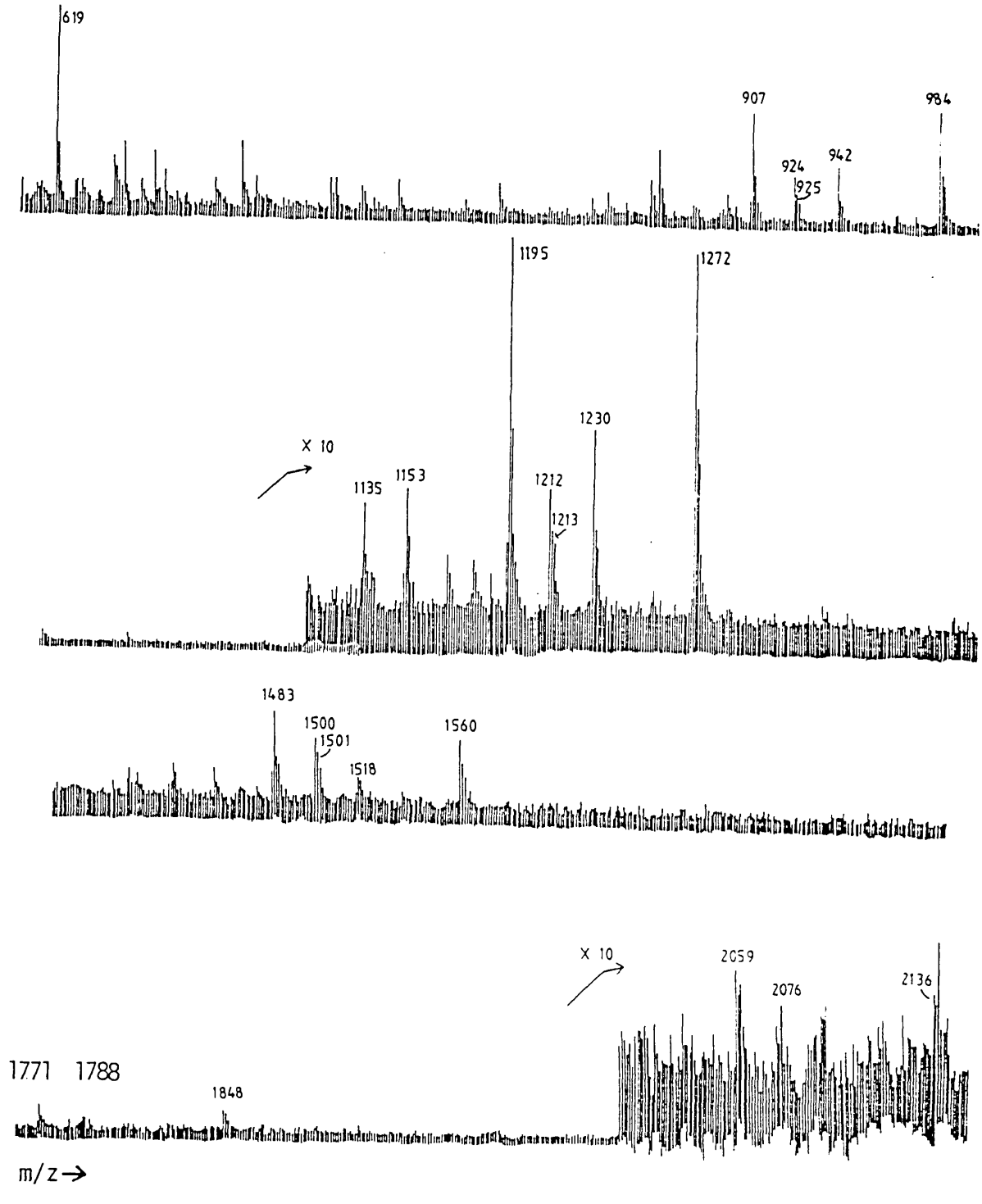


FIG 3.8. Positive FAB spectrum of peracetyl oligosaccharides from mannosidosis cells (8 kV).

on sample d).

Reverse permethylation of sample b) gave $M+H^+$ molecular ions at m/z 904 for $Hex_3HexNAc$ (also m/z 921 for $M+NH_4^+$), a very weak m/z 1108 for $Hex_4HexNAc$, m/z 1312 for $Hex_5HexNAc$ (also m/z 1329 for $M+NH_4^+$), m/z 1516 for $Hex_6HexNAc$, m/z 1720 for $Hex_7HexNAc$ and m/z 1924 for $Hex_8HexNAc$. These signals confirm the assignments made from the acetylated experiments and allow the additional Hex_7 and Hex_8 components to be identified. The low intensity of the $Hex_4HexNAc$ molecular ion seen in the acetyl spectrum is reproduced on reverse permethylation, demonstrating that this component is genuinely present in low abundance and that the poor signals do not arise due to sample handling problems during the initial experiment.

FAB of the reverse permethylated sample c) gave a spectrum with molecular ions at m/z 659 and 676 ($M+H^+$ and $M+NH_4^+$; Hex_3), 700 ($M+H^+$; $Hex_2HexNAc$), 863 and 880 ($M+H^+$ and $M+NH_4^+$; Hex_4), 904 and 921 ($M+H^+$ and $M+NH_4^+$; $Hex_3HexNAc$), 1084 ($M+NH_4^+$; Hex_5), 1108 and 1125 ($M+H^+$ and $M+NH_4^+$; $Hex_4HexNAc$), 1288 ($M+NH_4^+$; Hex_6), 1312 ($M+H^+$; $Hex_5HexNAc$), 1492 ($M+NH_4^+$; Hex_7) and 1516 ($M+H^+$; $Hex_6HexNAc$). The pattern of intensities displayed by these ions is of interest, particularly for comparison with that noted for the acetylated sample. The $M+H^+$ ion is always the major or only ion observed for the hexosamine containing species, while the predominant molecular ion representing the hexose polymers is of the $M+NH_4^+$ type. This may be explained, as for the acetyl derivative, because the nitrogen atom in the amino sugar is sufficiently basic to protonate readily and acquire charge in this way. When this nitrogen is absent, as in the hexose polymers, the molecule, having no group sufficiently basic to protonate easily can acquire charge by association with an ammonium ion, present as contamination in the thioglycerol matrix.

FAB analysis of reverse permethylated sample d) yielded only two weak signals representing $M+H^+$ molecular ions at m/z 1312 and 1516 for $Hex_5HexNAc$ and $Hex_6HexNAc$ respectively. The data derived from both the acetylation and the reverse permethylation experiments on sample d) are disappointing since in both cases only weak signals were obtained and although in each case the signals observed were assignable to compounds of expected compositions, the results are not consistent and a much more intense spectrum is expected from a successful experiment. The poor data may be due to one or more of the following:

1) the presence of some contaminant which prevents successful derivatisation and/or ionisation,
2) low yield of carbohydrate extraction from this batch of cells,
3) absence of significant amounts of storage products in the mass range examined.

In summary, these results show that normal fibroblasts, incubated with swainsonine accumulate a single series of oligosaccharides containing one HexNAc residue, while fibroblasts from a mannosidosis patient accumulate these and also a series of polyhexose oligosaccharides.

The results demonstrate that swainsonine administration causes the accumulation of a different set of storage products from those due to genetic mannosidosis and therefore the assumption that swainsonine intoxication produces a perfect phenocopy of genetic mannosidosis requires reinvestigation.

3.3.3 Conclusion. The combination of acetylation, extraction and reverse permethylation techniques, coupled with FAB analysis of the products formed has been found suitable for the rapid analysis of crude urine and cell lysate samples for oligosaccharide storage products.

CHAPTER 4 : ENTEROBACTERIAL COMMON ANTIGEN.

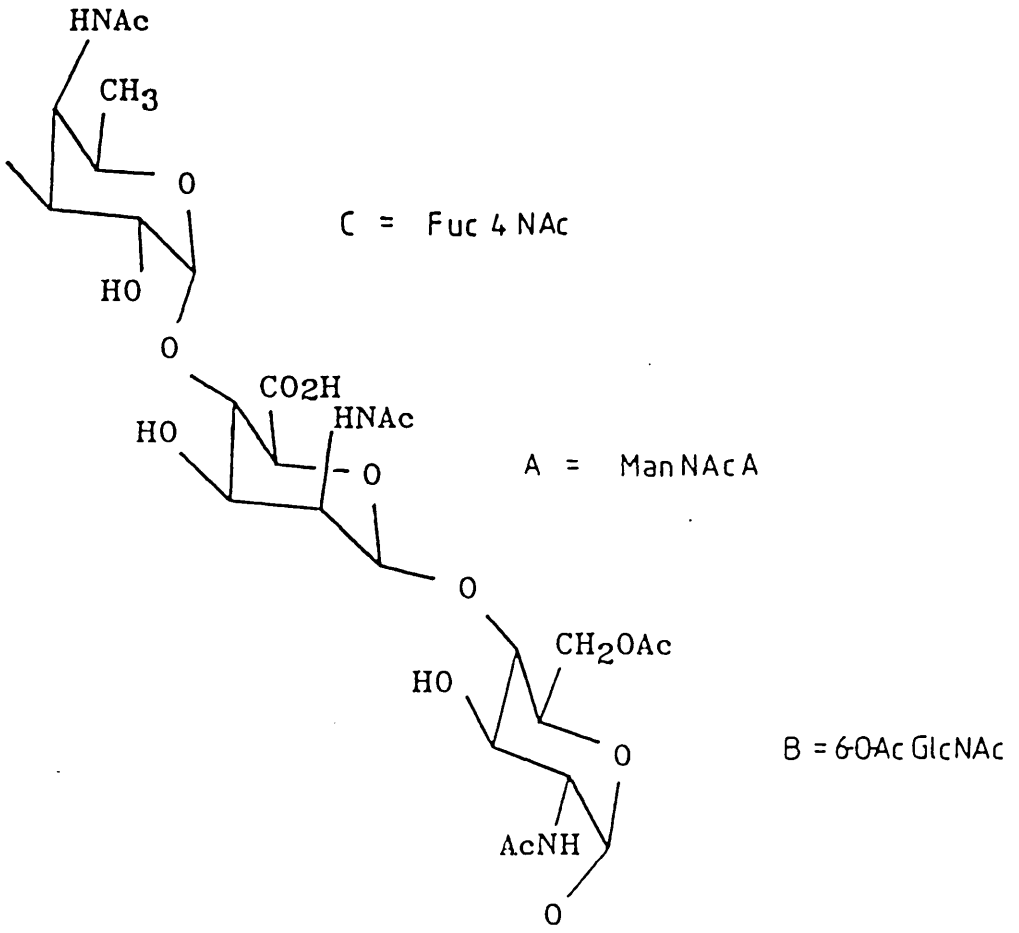
4.1 Introduction.

The Enterobacterial Common Antigen (ECA) is a bacterial antigen common to all the family Enterobacteriaceae. It is distinct from the classical surface antigens of that family, denoted O, K and H, which have been and continue to be extensively studied and which have provided the basis for the taxonomy of this family.

ECA was first described in 1962 by Kunin et al (116) and since then much research has been devoted to the study of its chemical nature and also its distribution and biological properties.

ECA is known to exist in two separate forms; in wild type bacteria ECA exists only in the nonimmunogenic or haptenic form, while in the rough or R mutants, in addition to the haptenic form, a proportion of the ECA is immunogenic, being linked to the lipopolysaccharide of the bacterial envelope (117).

Until recently, despite continued chemical studies, very little useful information was available about the structure of ECA. However, in 1978 it was shown (118,119) that the main constituents of ECA were 2-acetamido-2-deoxy-D-glucose (GlcNAc) and 2-acetamido-2-deoxy-mannuronic acid (ManNAcU). This was followed in 1983 by the demonstration that the additional sugar residue 4-acetamido-4,6-dideoxy-D-galactose (Fuc-4-NAc) is present in ECA and that with the other two residues it forms the trisaccharide repeating unit shown over (120). No further information was available until the experiments described in this chapter allowed the complete structure of haptenic ECA to be assigned.



ECA trisaccharide repeating unit

The samples examined in this chapter were provided by Prof. Bengt Lindberg (University of Stockholm, Sweden) and the FAB results described constitute part of a collaborative study which also included NMR and classical chemical experiments and which is presented in full elsewhere (121).

4.2 Results and Discussion.

The ECA preparation provided was thought to contain the trisaccharide repeating structure and also small amounts of phosphate, fatty acids and amino acids. It was not known whether these were covalently bound to ECA or were merely contaminants, or whether any further structures were included which had not been detected.

The original ECA sample was examined without derivatisation below mass 3000 dalton in both the positive and negative modes. The positive spectrum contained signals at m/z 2513, 2471 and 2429 at high mass and then m/z 837, 650, 608, 463, 246 and 218 at low mass. The high mass signals are each separated by 42 m.u. and it was postulated that they represented different degrees of acetylation, presumably on hydroxyl groups. m/z 2429 corresponds to the calculated $M+H^+$ quasimolecular ion for the trisaccharide repeated four times but without the mass increment to account for the terminal H— and —OH groups i.e. $((ABC)_4 + H)^+$; m/z 2471 and 2513 then correspond to the mono- and di- acetylated forms of this. The spectrum contained no evidence for the components containing the terminal H— and —OH groups 18 m.u. above any of these molecular ion signals. The negative FAB spectrum contained only three signals : m/z 2511, 2469 and 2427 which represent the $M-H^-$ molecular ions for the same components so corroborating the positive mode data. The low mass fragment ions in the positive spectrum had no counterparts in negative. They correspond to A-type ions (see chapter 2) with the following compositions : $Ac-(A_1B_1C_2)^+$ (m/z 837); $Ac-(A_1B_1C_1)^+$ (m/z 650); $(A_1B_1C_1)^+$ (m/z 608); $Ac-(A_1B_1)^+$ (m/z 463); $Ac-B_1^+$ (m/z 246) and A_1^+ (m/z 218).

These data provide a variety of useful information:

1) none of the ions correspond in mass to components containing phosphate, fatty acid or amino acid residues,

2) the molecular ions may be explained in one of the following ways

   a. the molecules are cyclic and so do not have the H— and —OH groups which terminate linear molecules:

   cyclic —A-B-C-A-B-C-A-B-C-A-B-C-

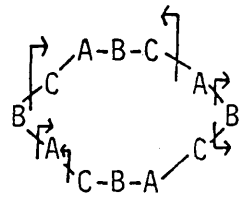
   VS linear H-A-B-C-A-B-C-A-B-C-A-B-C-OH

b. the molecules are linear and so bear the terminal H— and —OH group but contain a site of dehydration either within a single residue or between two residues:

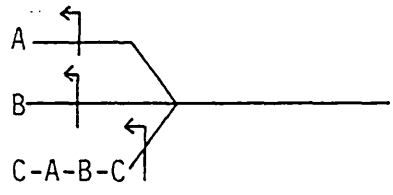
eg. H-A-B-C-A-B-C-A-B-C-A-B-C-OH
↑
dehydro

3) the A-type ions Ac-$(A_1B_1C_2)^+$, A^+ and Ac-B^+ could arise

a. by random double cleavage and proton transfer (i.e.β-cleavage at the non-reducing terminus and oxonium ion formation at the reducing terminus) from a cyclic molecule eg.



b. from a branched linear molecule with at least three different non-reducing termini eg.



or c. from a mixture of linear molecules in which some species terminate in A, some in B and some in C,

4) the presence of Ac-B^+ and A^+ suggest that B contains the additional acetyl groups.

In order to confirm that the three different molecular ions arise due to varying degrees of O-acetylation a sample of ECA was provided which had been subjected to de-O-acetylating conditions. The sample was analysed in positive and negative FAB and the spectra are shown in Figs. 4.1 and 4.2 respectively.

Considering first the molecular ion regions:

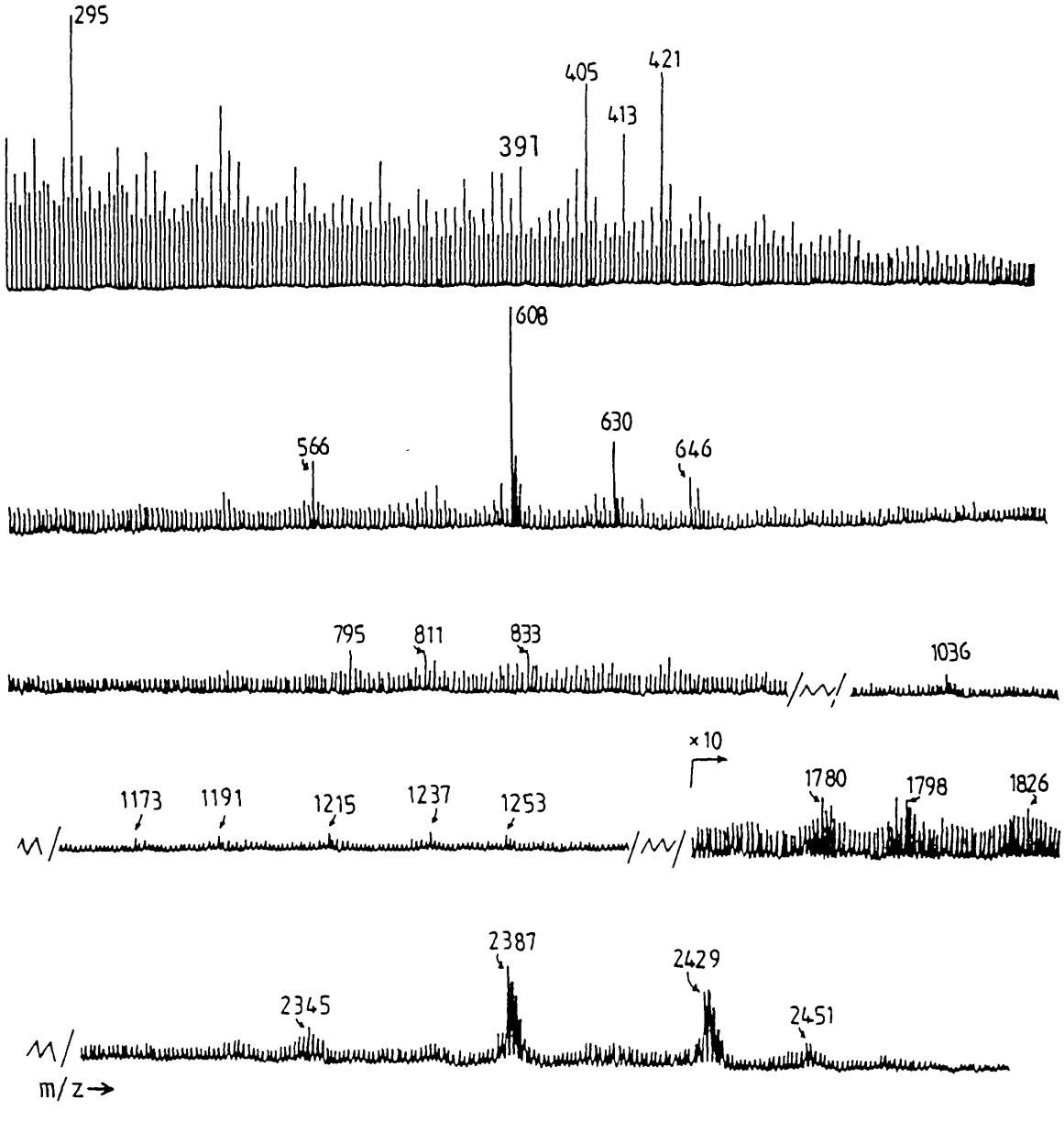


FIG 4.1. Positive FAB spectrum of de-O-acetylated ECA (8 kV).

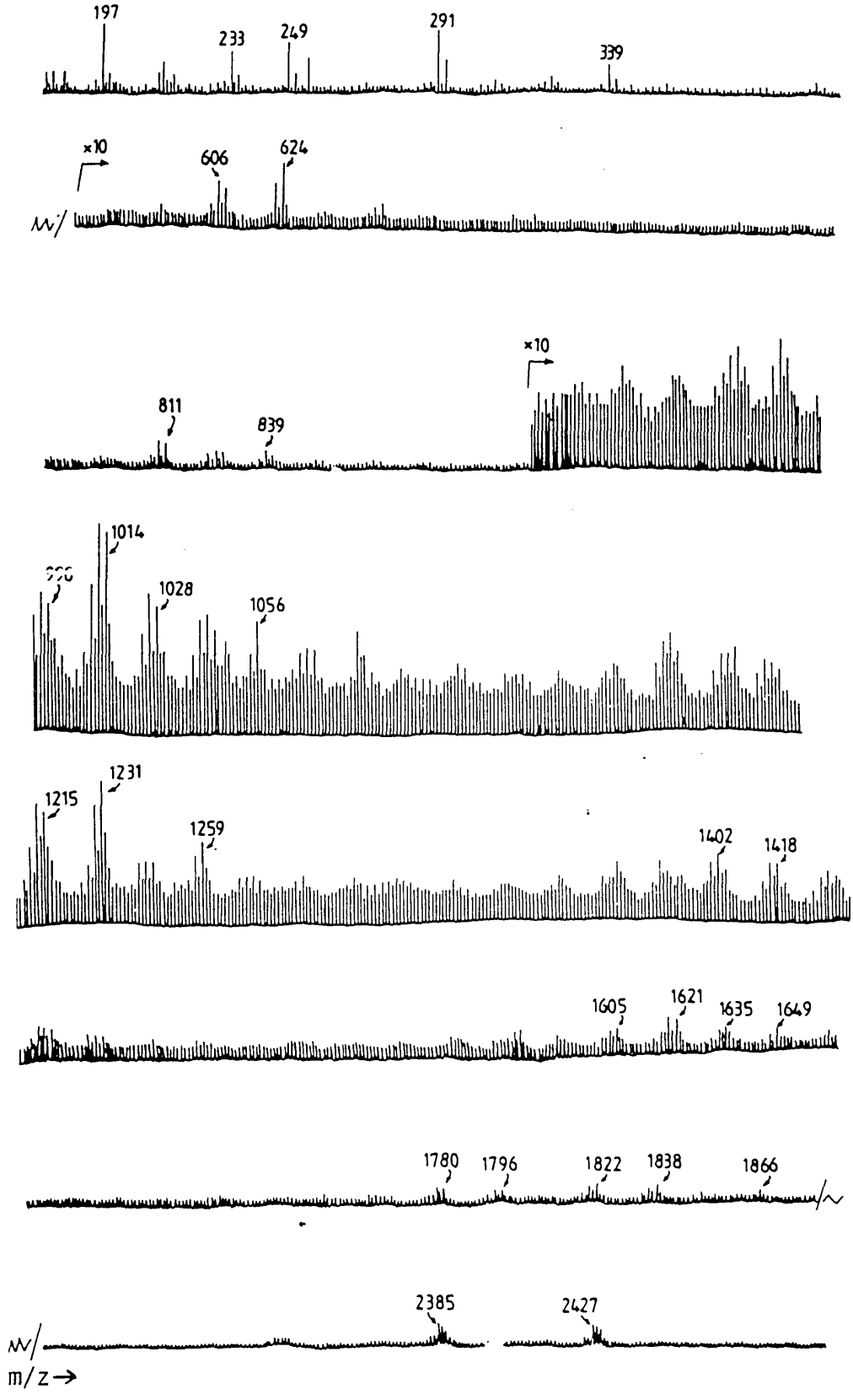


FIG 4.2. Negative FAB spectrum of de-O-acetylated ECA (8 kV).

the positive spectrum contains $M+H^+$ molecular ions at m/z 2345, 2387 and 2429 with the corresponding $M-H^-$ ions at m/z 2343, 2385 and 2427 in the negative spectrum. These again differ by 42 m.u., the highest mass signal in each case representing the cyclic/dehydrated $(ABC)_4$ species with no O-acetylation, while the peaks 42 and 84 m.u. down from these suggest de-N-acetylation - this could arise as a side reaction during the de-O-acetylation, or alternatively may already exist in the native molecule and be "masked" by the O-acetylation.

The remainder of the spectra are very complex although it is possible to assign some of the peaks (see tables 4.1 and 4.2). The negative spectrum is particularly difficult to interpret : the most intense "fragment" ion in each cluster is usually that of general structure $H\text{-}A_x\text{-}B_y\text{-}C_z\text{-}OH$ which is negatively charged due to deprotonation, probably of the carboxylic acid moiety. Such a structure could arise from a linear dehydrated molecule by a β-cleavage type reaction with charge retention on the non-dehydrated portion of the molecule, or by a reaction analogous to hydrolysis from a cyclic molecule

eg.

Alternatively, these signals could represent molecular ions formed in low abundance by hydrolytic cleavage during the de-O-acetylation procedure, although this seems unlikely since equivalent "molecular ions" are not observed in the positive mode. These ions are each accompanied by three further signals 2, 16 and 18 mass units down from them. Plausible structures for such masses may be represented as:

2 m.u. down:

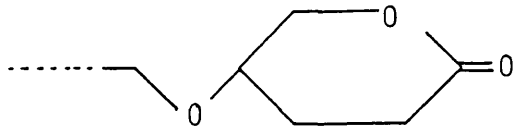


TABLE 4.1.

PEAK ASSIGNMENTS FOR POSITIVE FAB SPECTRUM OF DE-O-ACETYL ECA.

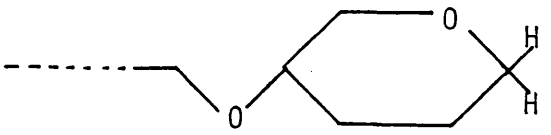
M/Z	ASSIGNMENT
391	$H\text{-}B_1C_1^+$
405	$H\text{-}A_1C_1^+$
421	$H\text{-}A_1B_1^+$
566	deacetyl $H\text{-}A_1B_1C_1^+$
608	$H\text{-}A_1B_1C_1^+$
630	$H\text{-}A_1B_1C_1$ $/Na^+$
646	$H\text{-}A_1B_1C_1$ $/K^+$
795	$H\text{-}A_1B_1C_2^+$
811	$H\text{-}A_1B_2C_1^+$
833	$H\text{-}A_1B_2C_1$ $/Na^+$
1036	$H\text{-}A_1B_2C_2$ $/K^+$
1173	deacetyl $H\text{-}(ABC)_2^+$
1191	————
1215	$H\text{-}(ABC)_2^+$
1237	$H\text{-}(ABC)_2$ $/Na^+$
1253	$H\text{-}(ABC)_2$ $/K^+$
1780	deacetyl $H\text{-}(ABC)_3^+$
1796	————
1798	————
2345	dideacetyl $H\text{-}(ABC)_4^+$

TABLE 4.2.

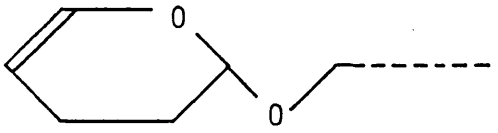
PEAK ASSIGNMENTS FOR NEGATIVE FAB SPECTRUM OF DE-O-ACETYL ECA.

M/Z	ASSIGNMENT
624	$(H\text{-}ABC\text{-}OH - H^+)^-$
811	$(H\text{-}ABC_2\text{-}OH - H^+)^-$
839	"plus 28" ion for 811
1014	$(H\text{-}AB_2C_2\text{-}OH - H^+)^-$
1028	$(H\text{-}A_2BC_2\text{-}OH - H^+)^-$
1231	$(H\text{-}A_2B_2C_2\text{-}OH - H^+)^-$
1259	"plus 28" ion for 1231
1418	$(H\text{-}A_2B_2C_3\text{-}OH - H^+)^-$
1621	$(H\text{-}A_2B_3C_3\text{-}OH - H^+)^-$
1635	$(H\text{-}A_3B_2C_3\text{-}OH - H^+)^-$
1649	"plus 28" ion for 1621
1796	deacetyl $(H\text{-}A_3B_3C_3\text{-}OH - H^+)^-$
1838	$(H\text{-}A_3B_3C_3\text{-}OH - H^+)^-$
1866	"plus 28" ion for 1838

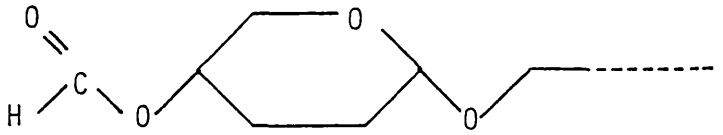
16 m.u. down:



18 m.u. down:



Additionally, some of the major fragment ions are accompanied by "plus 28" ions which may be rationalised as deriving from cleavage, not at the glycosidic linkage but across the adjacent sugar ring (as described in chapter 2) to yield an ion of the type:



In the positive spectrum fragment ions may be assigned as A-type ions in which the carboxylic acid frequently occurs as the sodium or potassium salt. From the composition of the assignable fragment ions in both the positive and negative spectra no unambiguous sequence data can be obtained: fragments of composition $A_2B_1C_2$ and $A_1B_2C_2$ still suggest that the parent species is either cyclic (fragments generated by double cleavage at random points in the ring) or linear containing a mixture of species or non-reducing termini but does not distinguish between these possibilities.

A further attempt was made to obtain a homogeneously acetylated preparation - the de-O-acetylated sample was derivatised using an N-acetylation protocol (see chapter 5) in which a 1:1 mixture of H:^{2}H acetic anhydride was used. This was designed to allow easy identification of incorporated acetyl groups. The positive and negative spectra obtained from this derivative are presented in

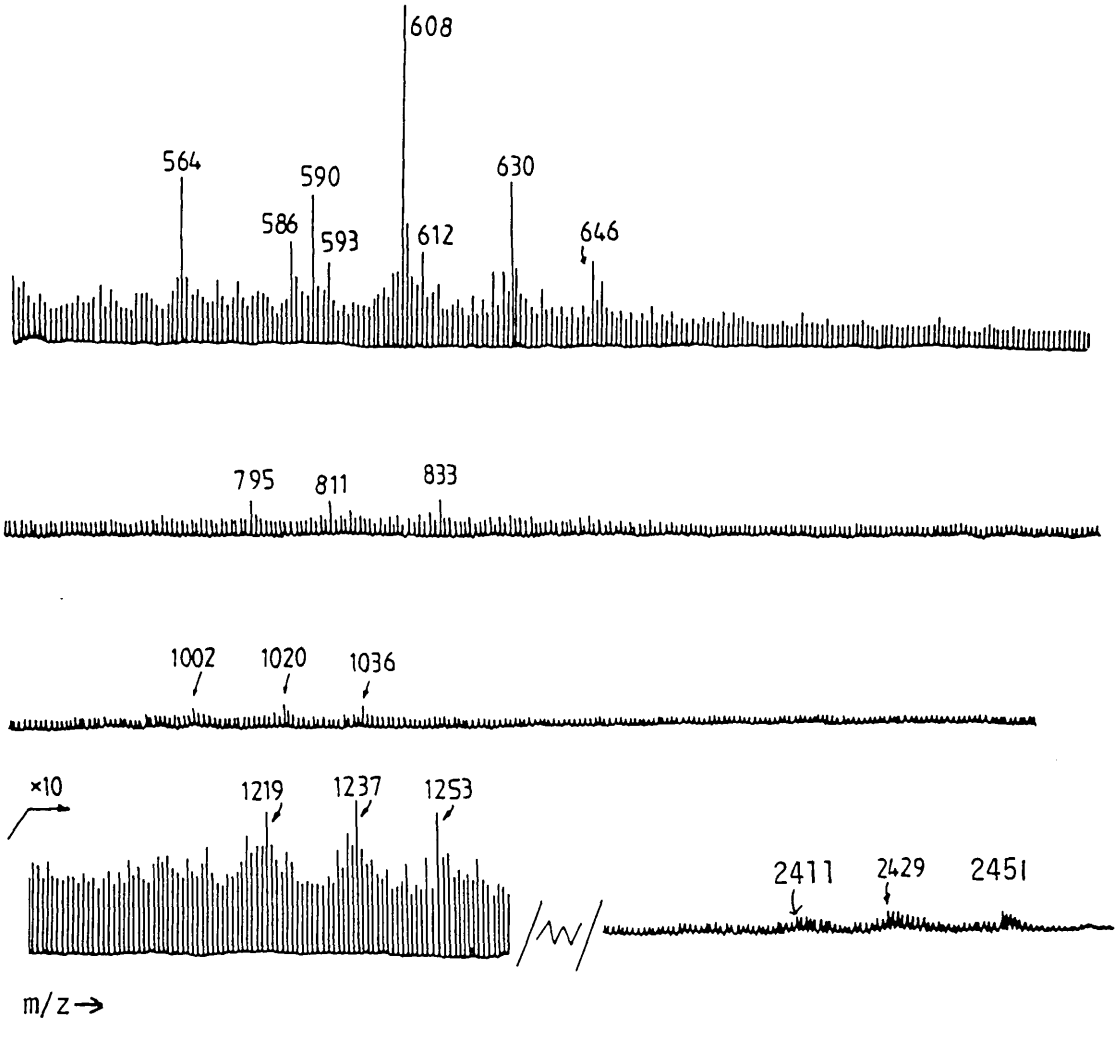


FIG 4.3. Positive FAB spectrum of de-O-acetylated ECA treated with Ac_2O:d_6-Ac_2O(1:1)/MeOH. (8 kV).

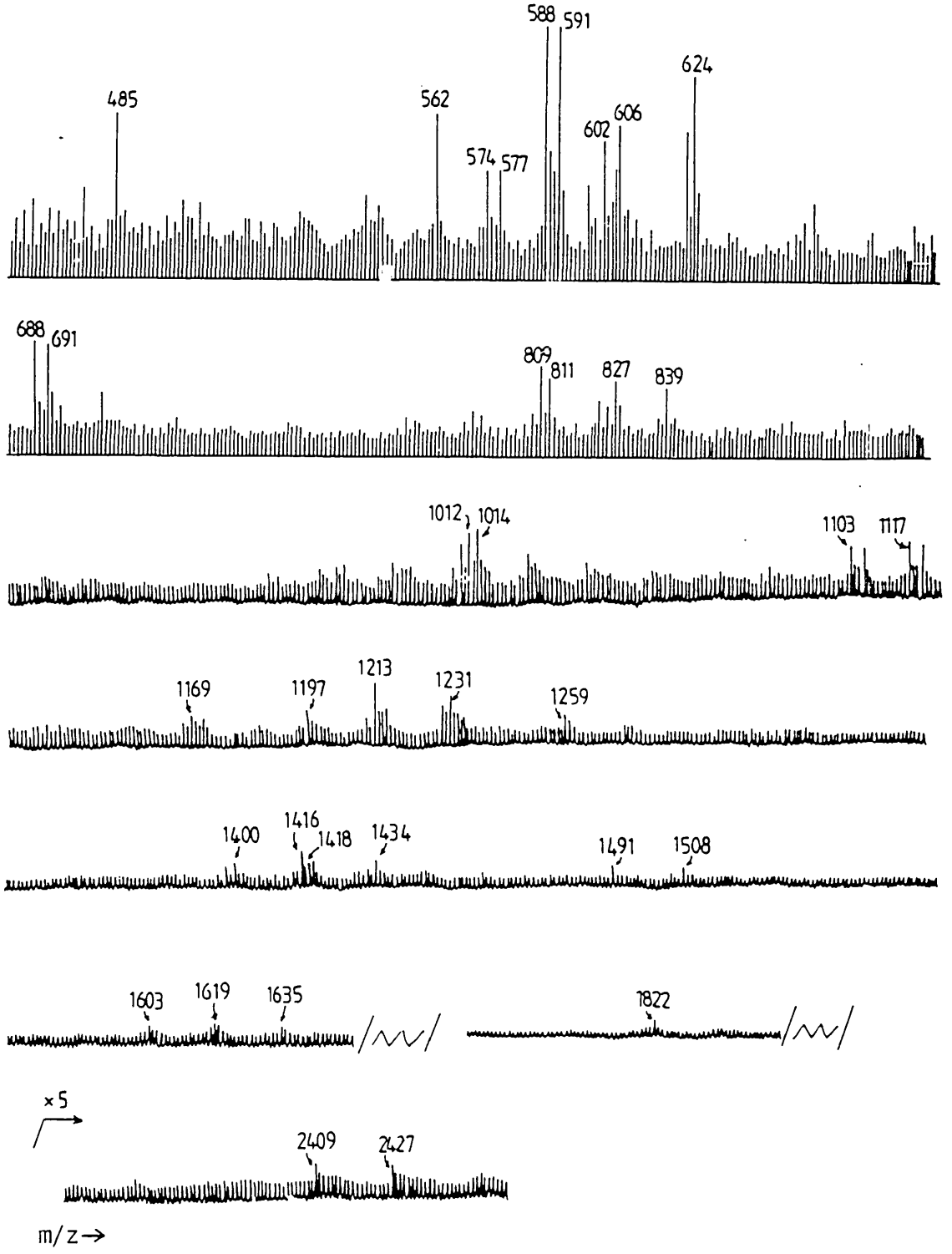


FIG 4.4. Negative FAB spectrum of de-O-acetylated ECA treated with Ac_2O:d_6-Ac_2O(1:1)/MeOH. (8 kV).

in Figs. 4.3 and 4.4 respectively.

Considering the positive spectrum (Fig. 4.3) the presence of a single acetylated species is indicated by the pseudomolecular ions at m/z 2429 ($M+H^+$) and 2451 ($M+Na^+$) for the fully N-, de-O-acetylated $(ABC)_4$ species without "ends". The molecular ion cluster does not display the simple 1:1 isotopic pattern expected on incorporation of the 1:1 H:^{2}H acetyl group. This may be due to a variety of reasons:

1) the signal is at sufficiently high mass for the peak due to the natural abundance of ^{13}C to be more intense than the nominal mass peak, so distorting the shape of the signal,
2) the de-O-acetylated species contained peaks for zero, one and two degrees of de-N-acetylation, so that the new molecular ion after re-N-acetylation may be expected to comprise components containing zero, one and two labelled acetyl groups which will again distort the peak shape by superimposing three recognisable patterns i.e. the normal molecular ion, the 1:1 labelled molecular ion arising by single acetyl incorporation, and the 1:2:1 labelled triplet from double incorporation,
3) the intensity of the molecular ion region is poor so that any anomalies in peak shape or isotope patterns are difficult to distinguish.

The remainder of the spectrum is interpreted in Table 4.3, from which a few important features should be noted:

1) many of the signals are accompanied by peaks corresponding to a loss of 18 m.u. - this may be explained as loss of water, although why the N-acetylation reaction should cause dehydration is unclear,
2) there is only one possible pair of peaks which could represent the incorporation of a 1:1 label i.e. m/z 590/593 , although

TABLE 4.3.

PEAK ASSIGNMENTS FOR POSITIVE FAB SPECTRUM OF 1:1 H:^{2}H N-ACETYL DE-O-ACETYL ECA.

M/Z	ASSIGNMENT
564	unassigned
590	H-ABC^{+} minus 18
593	could form a triplet with 590
608	H-ABC^{+}
612	H-ABC /Na^{+} minus 18
630	H-ABC /Na^{+}
646	H-ABC /K^{+}
795	H-ABC$_2^{+}$
811	H-AB$_2$C^{+}
833	H-AB$_2$C /Na^{+}
1002	H-AB$_2$C$_2$ /Na^{+} minus 18
1020	H-AB$_2$C$_2$ /Na^{+}
1036	H-AB$_2$C$_2$ /K^{+}
1219	H-(ABC)$_2$ /Na^{+} minus 18
1237	H-(ABC)$_2$ /Na^{+}
1253	H-(ABC)$_2$ /K^{+}
2411	M+H^{+} molecular ion minus 18

the isotopic pattern is distorted presumably by some additional component in the m/z 590 peak. This assignment is not supported by a similar pattern in the 608/611 signals from which the former pair have been postulated to arise by dehydration,

3) the appearance of the peak at m/z 564 on N-acetylation cannot readily be explained.

An examination of the negative spectrum (Fig. 4.4) supports the conclusion reached from the positive results that full N-acetylation has been achieved - the single $M-H^-$ molecular ion at m/z 2427 corroborates those seen in the positive spectrum, while a similar signal 18 m.u. down confirms the"dehydrated"species. The remainder of the spectrum contains many of the signals observed in the negative spectrum of de-O-acetylated ECA. Additional signals are observed at m/z 485, 562 (analogous to the unassigned 564 in positive), 602, 704, 827 (for$(H-A_1B_2C_1-OH - H)^-$), 1169,1197, 1434 (for $(H-A_2B_2C_2-OH-H)^-$), 1491 and 1508 and also the following pairs of 1:1 doublets: m/z 574/577, 588/591 (confirming the tentative assignment of 590/593 in positive as a labelled doublet), 688/691, 1103/1106 and 1117/1120. It is disturbing that the majority of these signals were apparently produced during the derivatisation reaction and yet cannot be assigned. Some indication of their origin may be obtained from the observation that in two cases pairs of doublets are separated by 14 m.u., i.e. 574/577 and 588/591; and 1103/1106 and 1117/1120. Since methanol was used in the acetylation reaction such characteristic mass intervals could derive from some degradative pathway involving methanol incorporation, methylation or methyl esterification. In order to test this hypothesis a second acetylation was carried out in 1:1 $H:^2H$ methanol using 100%^1H-acetic anhydride (see chapter 5). The positive and negative FAB spectra obtained from these derivatives

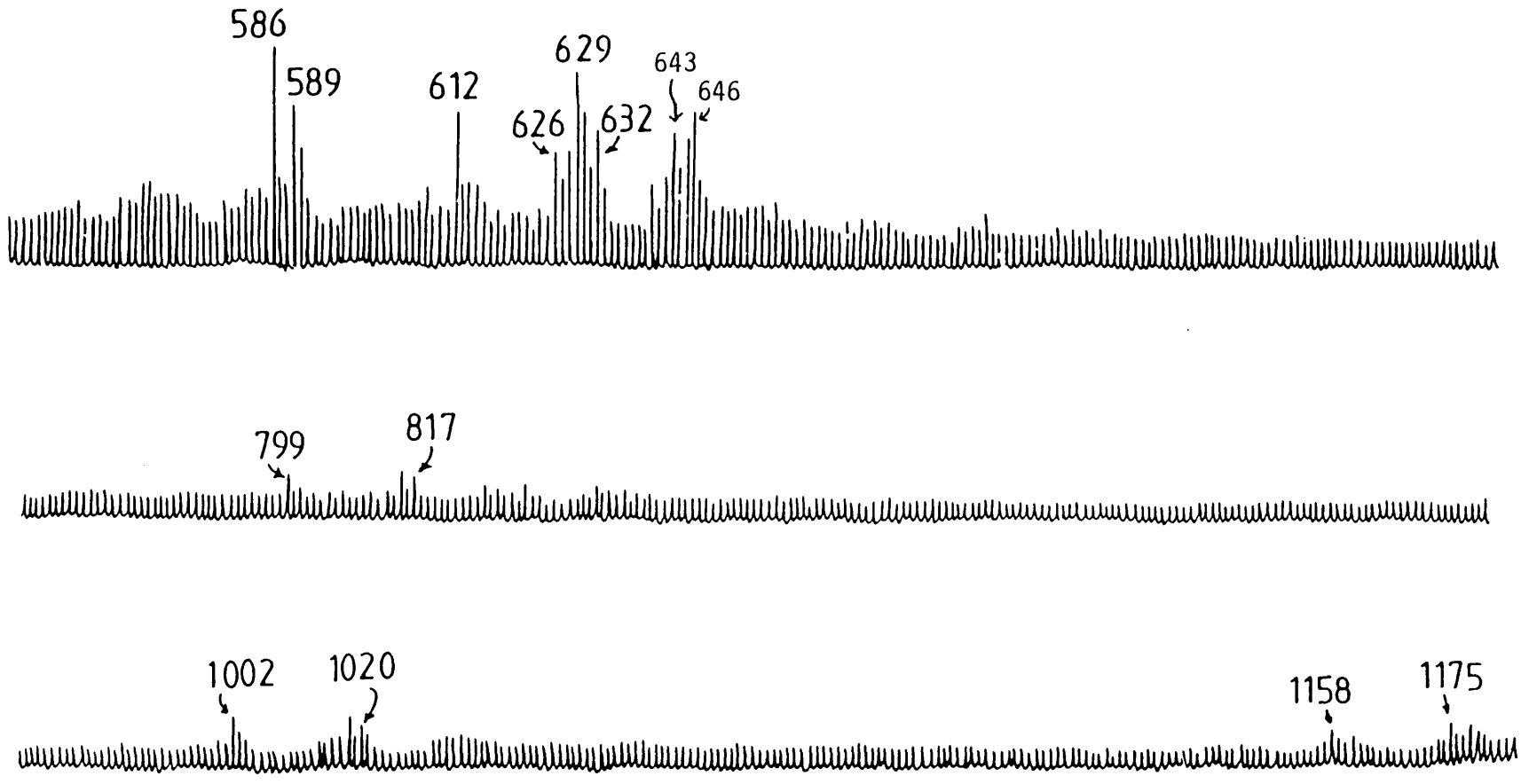


FIG 4.5. Positive FAB spectrum of de-O-acetylated ECA treated with Ac_2O/MeOH:d_4-MeOH(1;1). (8 kV).

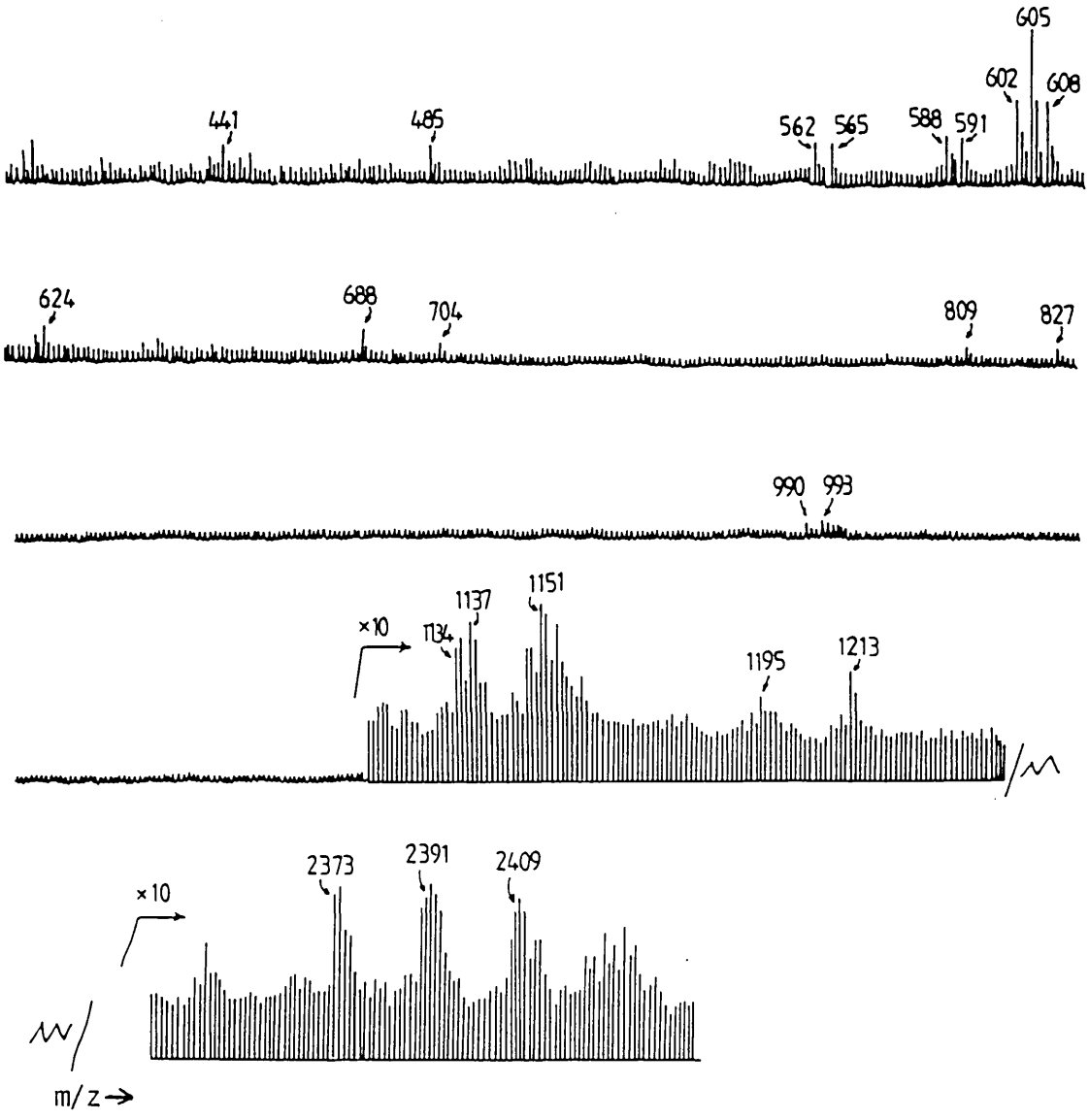


FIG 4.6. Negative FAB spectrum of de-O-acetylated ECA treated with Ac_2O/MeOH:d_4-MeOH(1:1). (8 kV).

are presented in figs 4.5 and 4.6 respectively.

The positive spectrum shows three clusters of ions which contain the 1:1 label separated by three mass units and so demonstrate incorporation of a methyl group from the methanol: 586/589 and 643/646 have the characteristic 1:1 pattern due to incorporation of a single group, while the 626/629/632 cluster has the 1:2:1 pattern indicating a species containing two labelled groups. The signals at 612, 1002 and 1020 were observed in the previous experiment, while 817 with its "minus 18" peak at m/z 799 may be assigned as the sodium salt of $A_1B_1C_2^+$; m/z 1158 and 1175 are unassigned.

The negative spectrum corroborates the positive with a 1:1 doublet at 588/591. A comparison with the negative spectrum of the previous acetylation shows that this 588 peak contains both an acetyl group and a methyl group. Further comparisons show that the peak at 562 contains one methyl group (appearing as a 1:1 doublet at m/z 562/565 in fig. 4.6) and that the peak at m/z 602 contains two methyl groups (it appears as a 1:2:1 triplet at m/z 602/605/608). Further doublets are observed at m/z 990/993 and 1134/1137, and a triplet at m/z 1148/1151/1154, none of which correspond to peaks seen in the previous experiment. Dehydration seems particularly marked in this experiment - the molecular ion is absent and instead a series of three "minus 18" ions is present at m/z 2409, 2391 and 2373.

Several conclusions can be drawn following a consideration of these two experiments:

1) the molecular ion region contains information on the fate of the entire molecule - conditions which specifically acetylate primary amino groups give a single uniformly acetylated product as

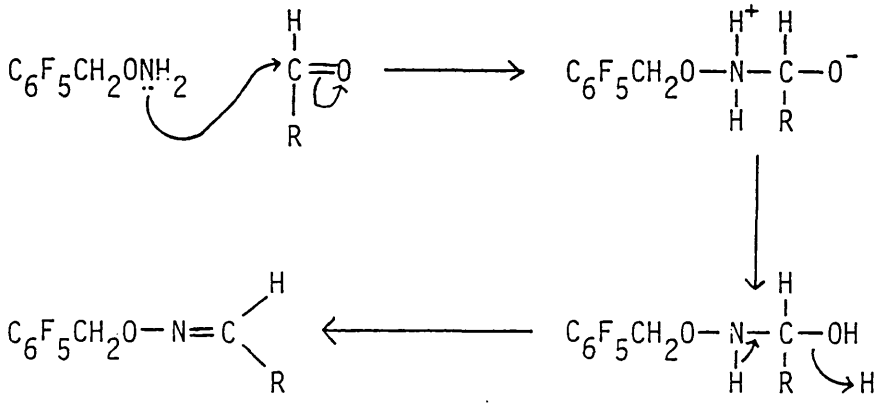
shown by both positive and negative spectra, so confirming the hypothesis that the heterogeneity revealed by the de-O-acetylation reaction is due to free amino groups. In addition, loss of the elements of water from the entire molecule during the reaction is indicated by the "minus 18" peaks in association with the molecular ions,

2) the remainder of the spectrum provides data on the rest of the molecule - in addition to the fragment ions which come from the intact molecule during the FAB experiment, and which correspond to those in the native and de-O-acetyl spectra, a second set of low mass ions is observed here. Some of these have been shown to incorporate N-acetyl groups, methyl groups or both, some contain neither but appear in both of the labelling experiments, while some are only observed in one of the labelling experiments. Most of these peaks cannot be assigned a feasible structure, although they must arise due to cleavage of the parent molecule in the reaction mixture, and it appears that this cleavage is to some extent random since the signals are not all reproducible between experiments.

Despite being unable to assign these signals the experiments are useful in both confirming the presence of free amino groups and in illustrating the hazards of performing derivatisation reactions on samples of unknown structure. The degradation of ECA under such mild conditions as those used for N-acetylation indicates that the structure is labile and so provides a warning about the choice of further derivatisation protocols, and suggests the use of mild conditions in degradative procedures.

Having resolved the origin of the molecular heterogeneity in the ECA preparation it was necessary to determine the structural basis for the molecular weight observed i.e. 2428. The two

alternative structures proposed were the cyclic and the linear dehydrated forms; a cyclic molecule should contain no reducing terminus, while a linear dehydrated molecule could do (unless the site of dehydration includes the reducing centre). Thus the approach used was to attempt to demonstrate the presence of a reducing terminus by derivative formation - if a reducing group could be proven the cyclic model could be discarded. The pentafluorobenzyl oxime derivative was chosen since it reacts with the carbonyl group of the reducing residue:



increasing the residue mass of the reducing terminus by 195 m.u. so that any fragment ions deriving from this end of the molecule are shifted by this mass increment (63). The PFB oxime derivative also has the property of improving the sensitivity of samples in negative FAB since it stabilises negative charge (122).

The positive and negative spectra obtained from the PFB oxime of de-O-acetyl ECA are shown in Fig. 4.7 and 4.8. The positive spectrum contains signals for two sets of molecular ions : those at m/z 2429 and 2387 represent the underivatised species and its de-O-acetylated form, while the signals at m/z 2624 and 2582 represent their PFB derivatives. The fragment ions are those already seen in the positive spectrum of de-O-acetyl ECA, none contain the PFB group. It is to be expected that species without the PFB group will ionise preferentially

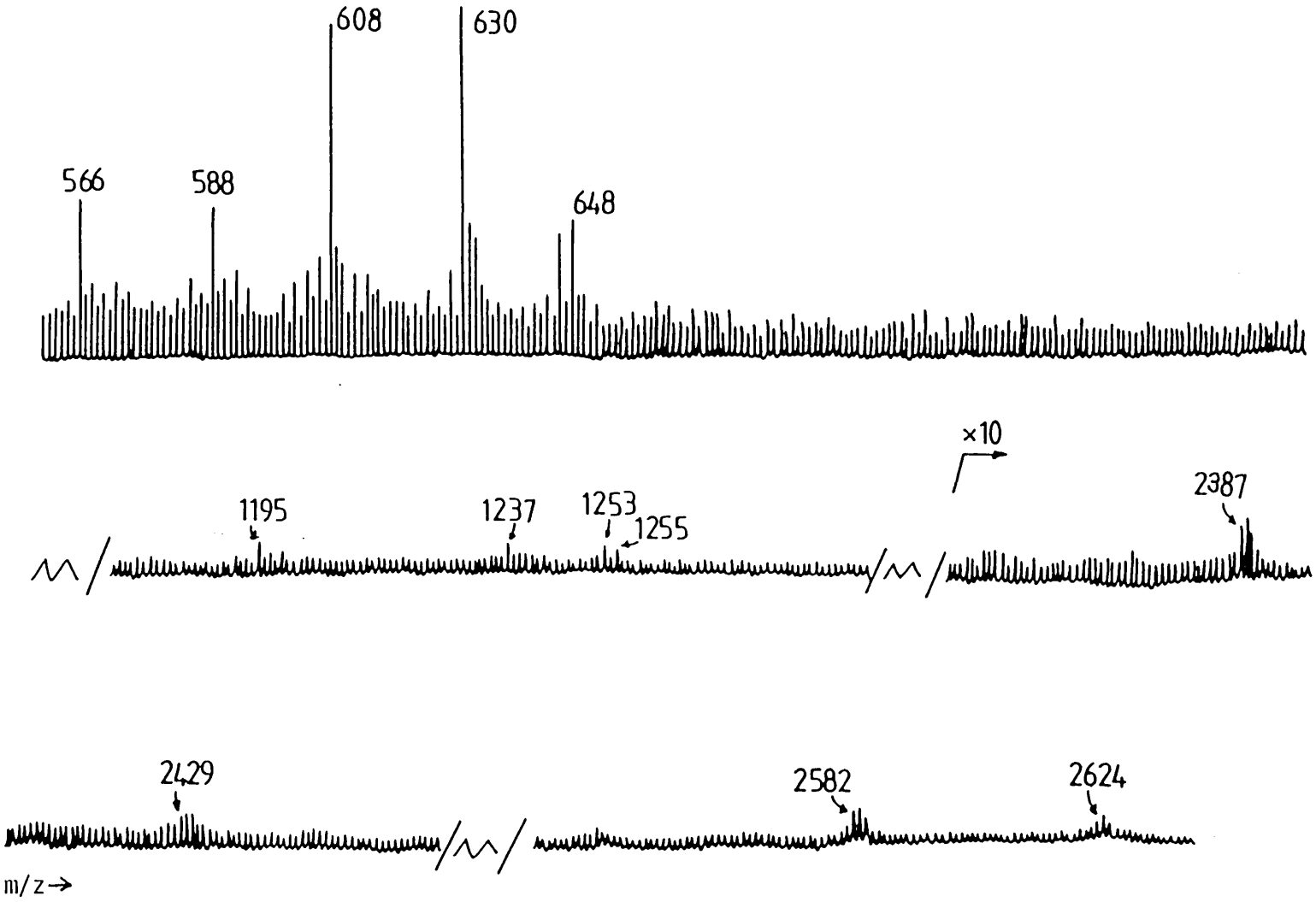


FIG 4.7. Positive FAB spectrum of PFB derivative of de-O-acetylated ECA (8 kV).

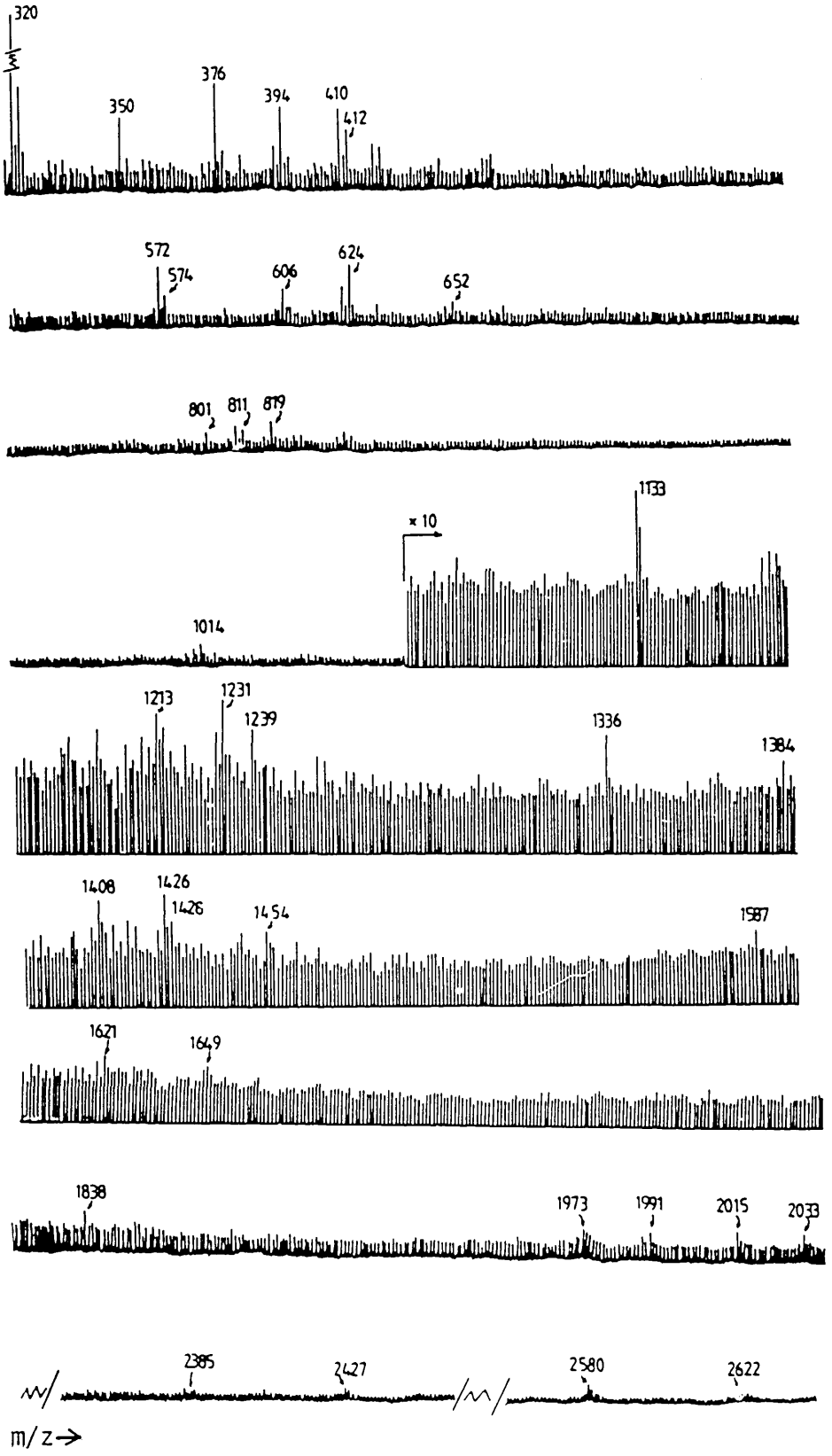


FIG 4.8. Negative FAB spectrum of PFB derivative of de-O-acetylated ECA (8 kV).

in positive while those containing the group will ionise better in negative. In the negative spectrum the molecular ions at m/z 2427, 2385, 2622 and 2580 corroborate those seen in the positive spectrum. Some of the fragment ions correspond to those seen in the negative spectrum of de-O-acetyl ECA and several of these are accompanied by their PFB containing counterparts: eg. m/z 801 from 606; 819 from 624; 1426 from 1231 and 2033 from 1838. Two further signals also correspond to species containing the 195 m.u. increment - m/z 1239 represents $(A_2B_2C\text{-}O\text{-}PFB\text{-}H)^-$ and interestingly m/z 1621 represents the incorporation of a second PFB group in m/z 1426 and so corresponds to $(H(ABC)_2\text{-}OH/PFB_2\text{-}H)^-$. The signals at m/z 1133, 1336, 1384 and 1587 do not represent the simple incorporation of one or more PFB groups and are unassigned.

Unfortunately the results of this experiment are ambiguous: at first examination the incorporation of a PFB group into both the molecular and fragment ions suggests the presence of a free reducing terminus in the molecule. However, two observations indicate that this may not be the only explanation for the data:

1) the sensitivity of the reaction. The PFB derivative of samples containing free reducing termini can normally be produced using as little as 5 µg of native material to yield a fully derivatised product (122). However, when 100 µg of ECA were used no results were obtained at all, so that the reaction was scaled up and ½ mg of starting material used to produce the derivative from which these spectra were obtained. Even under such conditions, in the negative mode where PFB containing species may be expected to ionise particularly well, the molecular ion for the derivatised molecule is only slightly more intense than that for the native species. This suggests that the PFB derivative is only a minor component of the ECA preparation even

after prolonged reaction times.

2) the ability to incorporate a second PFB group (m/z 1621). Since galacturonic acid polymers were found to incorporate two PFB groups (122) it was proposed that the second site for derivatisation could be the carbonyl of a carboxylic acid moiety. In order to test this proposal, model studies were carried out using N-acetyl neuraminic acid (Nana). Reaction of Nana with pentafluorobenzyl hydroxylamine hydrochloride under the same conditions used for the ECA sample produced a derivative containing two PFB groups. This yielded molecular ions at m/z 700 (positive) and m/z 698 (negative). Then this derivative was subjected to a 1 hr. methyl esterification reaction using 1:1 H:^{2}H methanolic hydrogen chloride and the product analysed by FAB. No signals were observed for the di-PFB oxime or the methyl ester of the di-PFB species. Instead doublets were observed for the methyl ester of mono-PFB-Nana at m/z519/522 in positive ($M+H^{+}$) and at m/z 553/556 in negative ($M+Cl^{-}$). These results suggest that the second PFB oxime is formed on the carbonyl of the carboxylic acid.

Returning to the PFB derivative of ECA, it is quite possible that the oxime is formed on the acidic carbonyl group of residue A since more than one group is incorporated and since the yield of the reaction is much poorer than might be expected from a reducing end reaction.

These data still do not allow the cyclic and the linear dehydrated models to be distinguished so that a new set of ECA preparations was supplied and further structural studies undertaken.

A native ECA preparation was supplied which had been separated into three fractions by chromatography on silica gel. This preparation had been chemically analysed for end groups with inconclusive results. The three fractions were examined in negative FAB: the spectra showed

that the fractions differed in the degrees of acetylation but also, and more importantly revealed on examination up to mass 3300 dalton the presence of high mass compounds containing five trisaccharide repeating units. These components had been overlooked in the earlier ECA preparations which were only analysed below mass 3000. The earlier samples were then re-examined up to mass 3300 and were also found to contain the five trisaccharide repeat species. The observation of these high mass components in the earlier samples after completion of that section of the study does not invalidate any of the conclusions made from that work.

Fraction 1 was only partially soluble so that the data obtained was poor with $M\text{-}H^-$ molecular ions at m/z 3182 and 3224 for the sodium salts of $(ABC)_5$ with 3 and 4 acetyl groups - no signals were observed for the $(ABC)_4$ species.

Fraction 2 was the main fraction and gave molecular ions $(M\text{-}H)^-$ at m/z3224, 3182, 3140, 2533 and2491 for the sodium salts of $(ABC)_5$ with 4,3 and 2 acetyl groups, and $(ABC)_4$ with 2 and 1 acetyls.

Fraction 3 gave $M\text{-}H^-$ molecular ions at m/z 3118, 3076, 2491 and 2449 for $(ABC)_5$ with 2 and 1 acetyls and the sodium salts of $(ABC)_4$ with one and zero acetyls respectively.

The higher molecular weight components revealed by these experiments appear to be analogous to the components already identified - both have masses corresponding to multiple trisaccharide repeats but without the 18 m.u. to account for the terminal H— and —OH groups.

In order to distinguish between the cyclic and the linear dehydrated models for these molecules the products of reactions specific for both reducing and non-reducing termini were examined - a cyclic molecule has no termini while a linear dehydrated species

will have at least one unmodified terminal residue.

$NaBD_4$ reduced ECA was supplied and examined by FAB. Under the reaction conditions used a free reducing terminal residue should be reduced to the corresponding alcohol, with incorporation of 4 m.u. in the form of two deuterium atoms. FAB showed that the only effect of the reaction was to cause de-O-acetylation of the molecules - there was no evidence for any reduction having occured; molecular ions were observed for $(ABC)_4$ and $(ABC)_4$ minus an acetyl (m/z 2429 and 2387 in positive, 2427 and 2385 in negative) and for $(ABC)_5$ and $(ABC)_5$ minus one and two acetyl groups (m/z 3036, 2994 and 2952 in positive, 3034, 2992 and 2950 in negative). From these results no reducing terminus could be detected. To probe the putative non-reducing end of the molecule the periodate oxidised borodeuteride reduced product was then examined. Examination of the structure of the trisaccharide reveals that only a nonreducing terminal residue would have vicinal hydroxyl groups and so be susceptible to oxidation by periodate. FAB showed that the sample was heavily contaminated with salt and so the signals were rather weak. In the negative mode $M-H^-$ molecular ions were observed at m/z 3056 (sodium salt of $(ABC)_5$), 2449 (sodium salt of $(ABC)_4$), 2427 ($(ABC)_4$), 2407 (sodium salt of $(ABC)_4$ minus one acetyl) and 2385 ($(ABC)_4$ minus one acetyl), so that again the only effect of the reaction was to cause de-O-acetylation, with no evidence for any oxidation having occured.

This lack of reactivity towards both reducing and non-reducing end reagents suggests that ECA is a cyclic molecule. Further evidence for the cyclic nature of ECA was obtained from a variety of hydrolysis experiments. Mild acid hydrolysis using aqueous hydrochloric acid was performed on the product of the borodeuteride reduction - this sample was chosen as it represents a less complex mixture of components

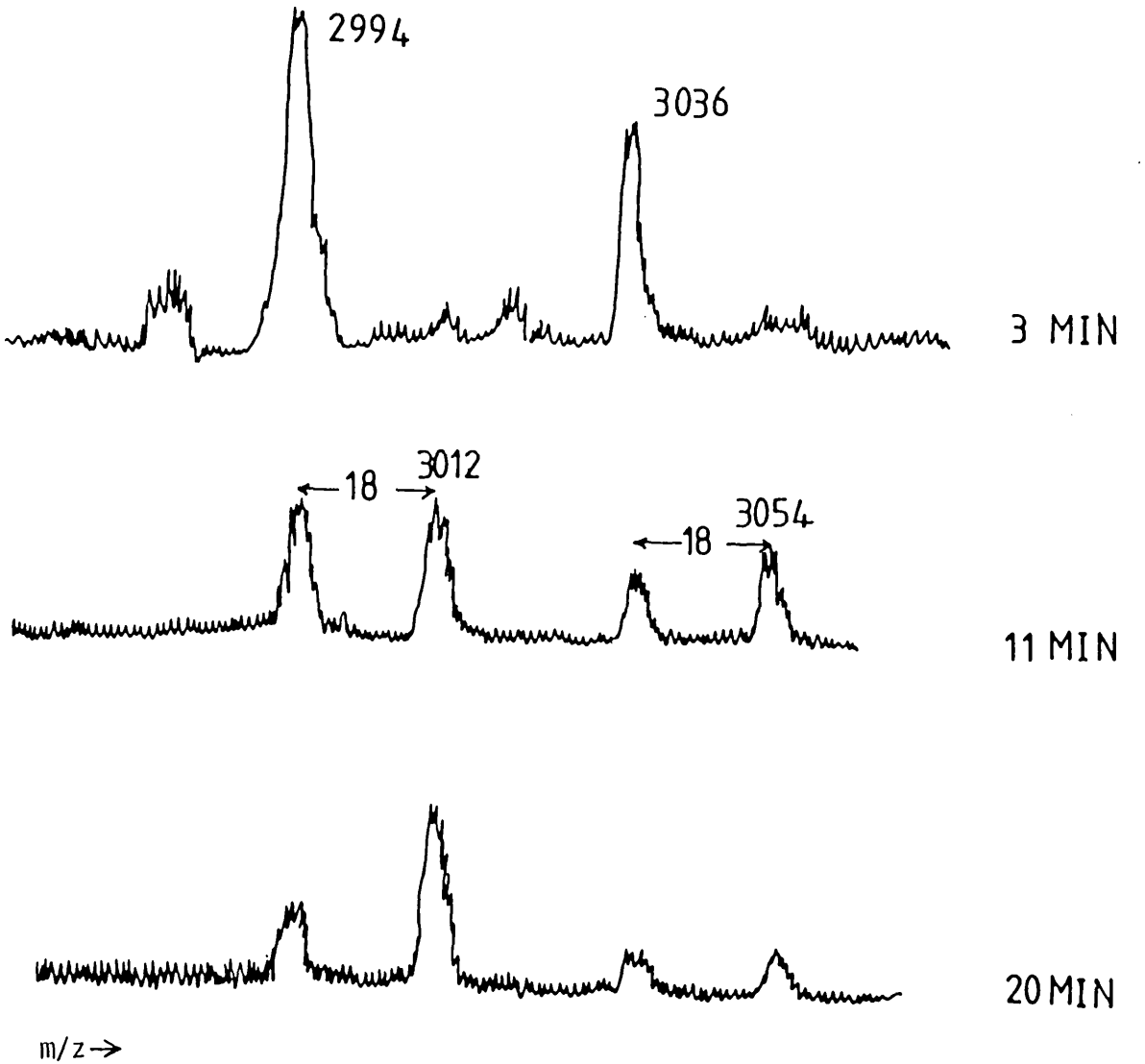
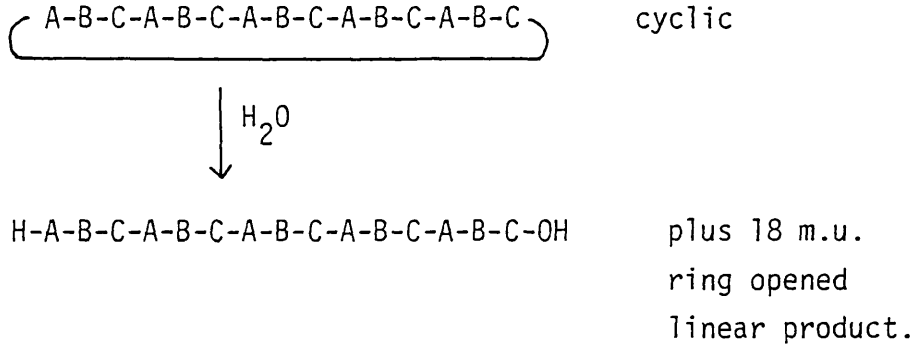


FIG 4.9. Time course hydrolysis (6M HCl) of ECA; scans of the $(ABC)_5$ molecular ion region.

than the untreated fractions, and it was available in greater quantities. The sample was incubated with aqueous hydrochloric acid (see chapter 5) and the progress of the reaction monitored by FAB analysis of aliquots removed at frequent time intervals. Scans were recorded in the 3200-2900 dalton mass range so that modifications to the molecular ions for the $(ABC)_5$ and $(ABC)_5$ mono-de-acetyl species could be observed. Single hydrolytic cleavage of a cyclic molecule may be expected to yield a linear species with incorporation of the elements of water. Scans of the $(ABC)_5$ molecular ion region for the 3, 11 and 20 min. aliquots are shown in Fig 4.9. The 3 min. sample gave intense molecular ions for the cyclic species $(ABC)_5$ (m/z 3036) and de-acetyl $(ABC)_5$ (m/z 2994) only; no signals were observed 18 m.u. above these for linear derivatives. After 11 minutes the peaks for the cyclic species were still present but each was accompanied by signals of approximately the same intensity 18 m.u. above it (m/z 3054 and 3012) corresponding to the ring opened forms:



The 20 min. aliquot contained signals for both the linear and cyclic species although the ring opened form was now the more abundant. The 33 min. aliquot gave no signals above 3000 dalton, indicating that as the reaction proceeds the larger linear species are degraded. A full spectrum was obtained for this aliquot and is shown in Fig. 4.10. The signals observed correspond to $M+H^+$ molecular ions for linear fragments composed of multiples of trisaccharide repeating units

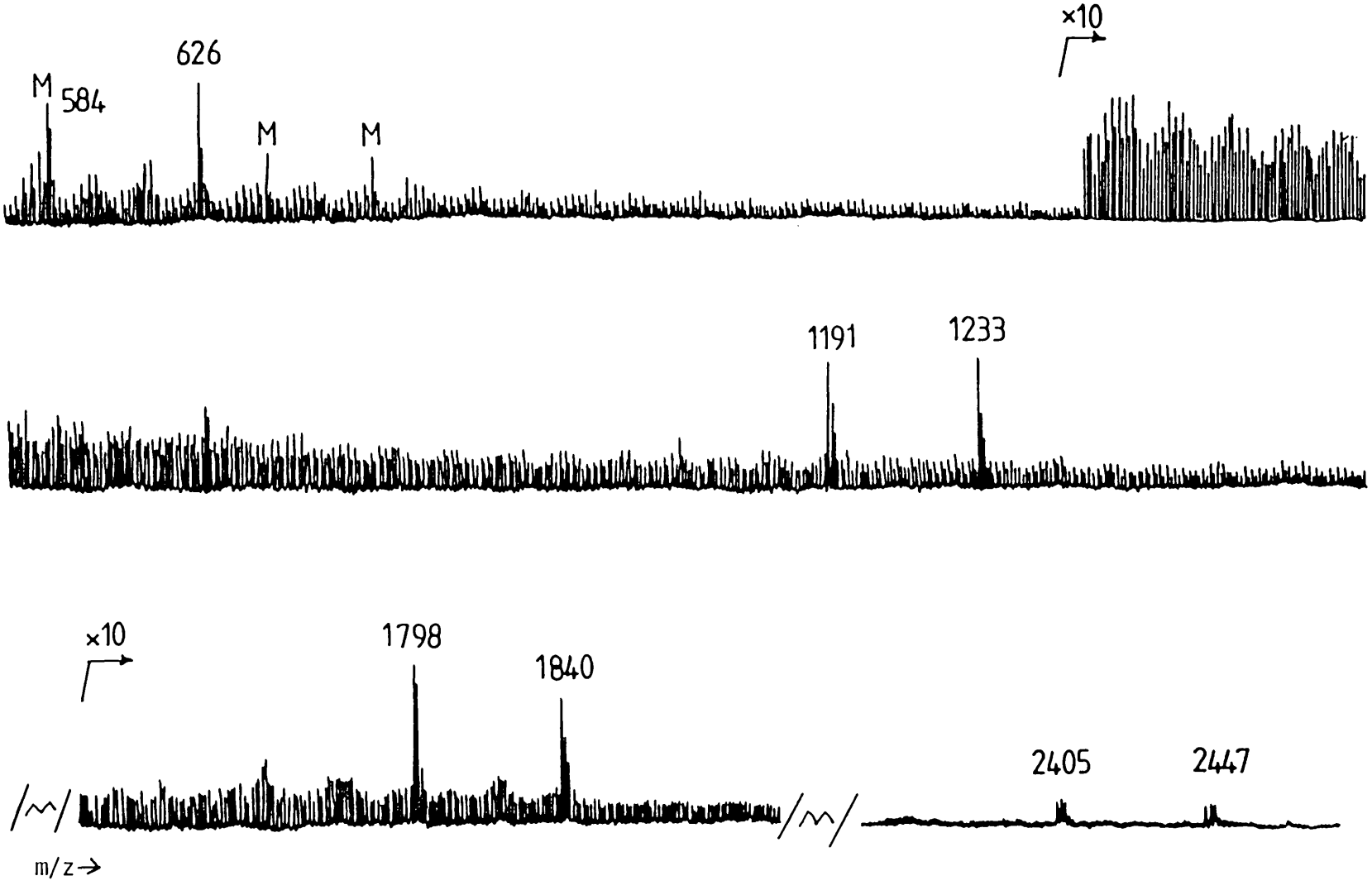


FIG 4.10. Positive FAB spectrum of 33 min. aliquot from 6M HCl hydrolysis of ECA (8 kV).

Signals arising from the matrix are marked with an M.

and showing varying extents of de-N-acetylation. m/z 2447 corresponds to a linear fragment containing four trisaccharide repeating units, with m/z 2405 representing the de-N-acetylated form. Similarly the signals at m/z 1840/1798, 1233/1191 and 626/584 arise from the analogous fragments containing 3, 2 and 1 trisaccharide repeats respectively. It is interesting to note that under these conditions one site seems to be more susceptible to hydrolytic cleavage than the others, so that cleavage always occurs between repeating units, demonstrating that the arrangement of repeating units in the molecules is homogeneous.

These results indicate that hydrolysis proceeds via an initial single cleavage which opens up the ring, and that this is followed by further cleavages which degrade the large linear products into smaller fragments.

In contrast, the products of hydrolysis with acetic acid do not only represent the products of cleavage between repeating units, but indicate a less specific process. The product of a 10 hr. acetic acid hydrolysis (1% aqueous at 100 ^{o}C) of ECA was supplied by Prof. Lindberg and examined using positive FAB.No signals were observed for either the cyclic or linear $(ABC)_5$ components indicating that quite extensive hydrolysis had occured. The lower mass signals represent molecular ions for hydrolytic fragments of the general type $(ABC)_x$ as before, or $(ABC)_xBC$ which indicates that two of the three linkages in ECA are susceptible to cleavage under these conditions. A full interpretation of the signals is given in table 4.4.

A comparison of these two hydrolytic experiments illustrates the advantage of using FAB to follow the progress of a reaction - being able to monitor the extent of hydrolysis directly from the reaction mixture provides much more information than can be obtained

TABLE 4.4.

PEAK ASSIGNMENTS FOR POSITIVE FAB SPECTRUM OF ECA AFTER 10 HR ACETIC ACID HYDROLYSIS.

M/Z	ASSIGNMENT
2447	$H\text{-}(ABC)_4\text{-}OH + H^+$
2429	cyclic $(ABC)_4 + H^+$
2405	de-N-acetyl $H\text{-}(ABC)_4\text{-}OH + H^+$
2387	de-N-acetyl cyclic $(ABC)_4 + H^+$
2252	$H\text{-}(ABC)_3BC\text{-}OH + Na^+$
2230	$H\text{-}(ABC)_3BC\text{-}OH + H^+$
2188	de-N-acetyl $H\text{-}(ABC)_3BC\text{-}OH + H^+$
1840	$H\text{-}(ABC)_3\text{-}OH + H^+$
1798	de-N-acetyl $H\text{-}(ABC)_3\text{-}OH + H^+$
1645	$H\text{-}(ABC)_2BC\text{-}OH + Na^+$
1623	$H\text{-}(ABC)_2BC\text{-}OH + H^+$
1581	de-N-acetyl $H\text{-}(ABC)_2BC\text{-}OH + H^+$
1255	$H\text{-}(ABC)_2\text{-}OH + Na^+$
1233	$H\text{-}(ABC)_2\text{-}OH + H^+$
1191	de-N-acetyl $H\text{-}(ABC)_2\text{-}OH + H^+$
1038	$H\text{-ABCBC-OH} + Na^+$
1016	$H\text{-ABCBC-OH} + H^+$
974	de-N-acetyl $H\text{-ABCBC-OH} + H^+$
648	$H\text{-ABC-OH} + Na^+$
626	$H\text{-ABC-OH} + H^+$
608	$ABC + H^+$

if a reaction is terminated at an arbitrary point and the products analysed later.

A sample in which a portion of this acetic acid hydrolysate had been treated with $NaBD_4$ was also supplied by Prof. Lindberg. Examination of this in positive FAB produced only a partial spectrum due to salt contamination, although it was possible to determine that incomplete reduction of each linear fragment had occured. The ability of hydrolysed ECA to react with $NaBD_4$ demonstrates that reducing termini have been generated during the hydrolytic reaction. However, since the hydrolysis was so extensive and the $(ABC)_5$ species degraded it is not possible to demonstrate that hydrolysis causes a single cleavage which opens the ring and generates a reducing terminus - $H\text{-}(ABC)_4\text{-}OH$ could arise by this route from cyclic $(ABC)_4$, but could equally well be generated by more extensive degradation of $(ABC)_5$.

In order to demonstrate that on hydrolytic ring cleavage a reducing terminus is generated a time course methanolysis reaction was carried out. The conditions used for methyl esterification (i.e. treatment with methanolic hydrogen chloride at room temperature) generally convert carboxylic acids to their methyl esters, and methyl glycosides are formed on reducing termini. The borodeuteride treated ECA preparation (chosen for the reasons given above) was reacted with methanolic hydrogen chloride at room temperature for 30 min. After 20 min. an aliquot was removed and analysed by FAB-MS. The positive spectrum showed that complete methyl esterification of each molecular species had occured, $(ABC)_4$ incorporating four methyl groups and $(ABC)_5$ incorporating five, as indicated by the molecular ions at m/z 2485 and 3106 respectively. These results indicate that methyl esterification is complete, with incorporation of one methyl group for each carboxylic acid moiety (present in residue A), but

that at this stage no hydrolytic ring opening has occured. To promote hydrolysis the reaction mixture was then incubated at 37 ^{o}C and 1 µl aliquots were removed after 10, 20, 30, 40, 60 and 90 min. for positive FAB analysis of the molecular ion regions. These scans allowed the progress of the reaction to be monitored. Gradually the intensity of the signals for the cyclic species decreased, while a new peak appeared 32 m.u. above each cyclic molecular ion corresponding to the methyl glycoside of the linear hydrolytic cleavage product:

$$\underbrace{(ABC)_5} \longrightarrow \quad H\text{-}(ABC)_5\text{-}OMe \quad \text{plus 32 m.u.}$$

Examination of the molecular ion region after 90 min. showed that ring opening was complete and so a full scan was recorded - the spectrum obtained is presented in Fig. 4.11. The spectrum indicates that extensive hydrolysis has occured and that fragments ranging in size from a single trisaccharide repeat upwards have been generated - a full interpretation of the spectrum is given in table 4.5. An examination of the relative intensities of the signals indicates that methanolytic cleavage preferentially occurs between repeating units, although fragments originating from cleavage within repeating units are also present. The results of this methanolysis reaction are complementary to those from acid hydrolysis and support the cyclic model- the incorporation of 32 m.u. is consistent with hydrolysis of a cyclic molecule to generate a reducing terminus which is converted to the methyl glycoside.

The final proof that haptenic ECA is a cyclic molecule was provided by examination of the perdeuteromethyl derivative of the $NaBD_4$ treated ECA. The perdeuteromethylated derivative was prepared to obviate the ambiguities arising from undermethylation; the

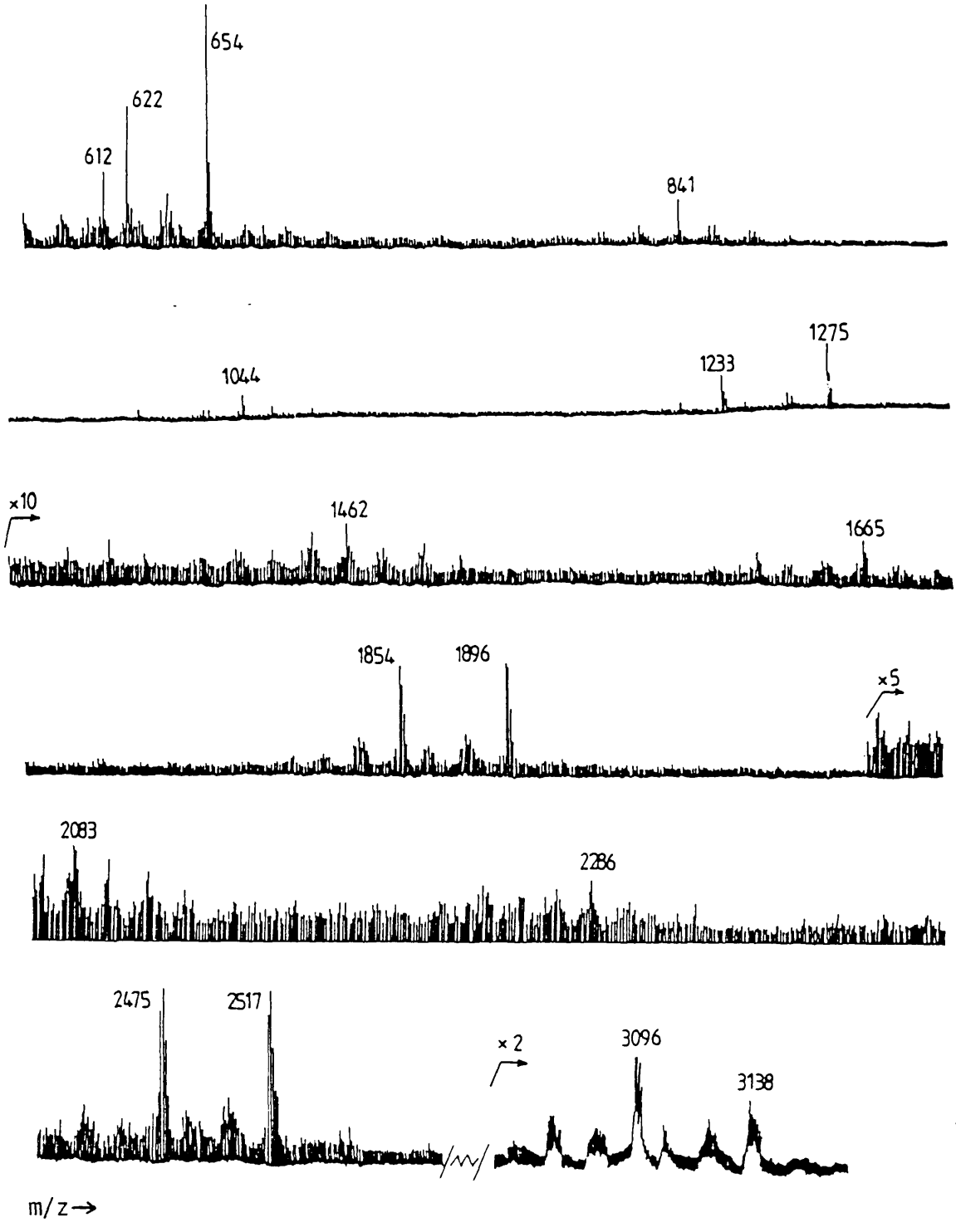


FIG 4.11. Positive FAB spectrum of 90 min. aliquot from methanolysis of ECA (8 kV).

TABLE 4.5.

PEAK ASSIGNMENTS FOR POSITIVE FAB SPECTRUM OF 90 MIN ALIQUOT FROM METHANOLYSIS OF DE-O-ACETYL ECA.

M/Z	ASSIGNMENT
3138	H-$(MeABC)_5$-OMe $+H^+$
3096	de-O-acetyl H-$(MeABC)_5$-OMe $+H^+$
2517	H-$(MeABC)_4$-OMe $+H^+$
2475	de-O-acetyl H-$(MeABC)_4$-OMe $+H^+$
2286	H-$(MeABC)_3$BC-OMe $+H^+$
2083	H-$(MeABC)_3$C-OMe $+H^+$
1896	H-$(MeABC)_3$-OMe $+H^+$
1854	de-O-acetyl H-$(MeABC)_3$-OMe $+H^+$
1665	H-$(MeABC)_2$BC-OMe $+H^+$
1462	H-$(MeABC)_2$C-OMe $+H^+$
1275	H-$(MeABC)_2$-OMe $+H^+$
1233	de-O-acetyl H-$(MeABC)_2$-OMe $+H^+$
1044	H-(MeABC)BC-OMe $+H^+$
841	H-(MeABC)C-OMe $+H^+$
654	H-(MeABC)-OMe $+H^+$
622	H-(MeABC)-OMe $+H^+$ minus methanol
612	de-O-acetyl H-(MeABC)-OMe $+H^+$

difference in residue mass between permethylated A and B is 14 m.u. and so may not be distinguished from one degree of undermethylation; the mass difference between perdeuteromethyl A (residue mass 268) and B (residue mass 254) is still 14 m.u. but the interval observed on underdeuteromethylation is increased to 17 m.u. allowing undermethylation to be correctly identified.

The perdeuteromethylated derivative of ECA was examined in the positive mode, and the molecular ion region investigated in three separate experiments. Accelerating voltages of 7 kV, 6 kV a.d 5 kV were used so that scans were obtained up to the 5300 mass range. The derivative gave very good FAB data with well resolved signals up to mass 3800 - two major molecular ion clusters were observed, one for $(ABC)_5$ and one for $(ABC)_4$. Additionally the 6 kV scan revealed the presence of a very small amount of a higher mass component for $(ABC)_6$ - the sensitivity enhancement obtained on permethylation allowed this species to be identified when it was not observed in the spectra of the underivatised samples. The 7 kV scan is presented in Fig. 4.12. Each cluster contains four major signals all separated by 17 m.u. indicating extensive undermethylation. The highest mass peak in each cluster represents the $M+H^+$ molecular ion for the fully methylated species; m/z 3716 for cyclic permethylated $(ABC)_5$ and m/z 2973 for cyclic permethylated $(ABC)_4$. The mass of the molecular ions allows linear and cyclic molecules to be distinguished: the mass of a fully deuteromethylated cyclic molecule differs from its similarly derivatised linear counterpart by the mass of the "ends" i.e. 18 m.u. plus the mass of the two deuteromethyl groups which will be incorporated at the ends i.e. a total of 52 m.u.

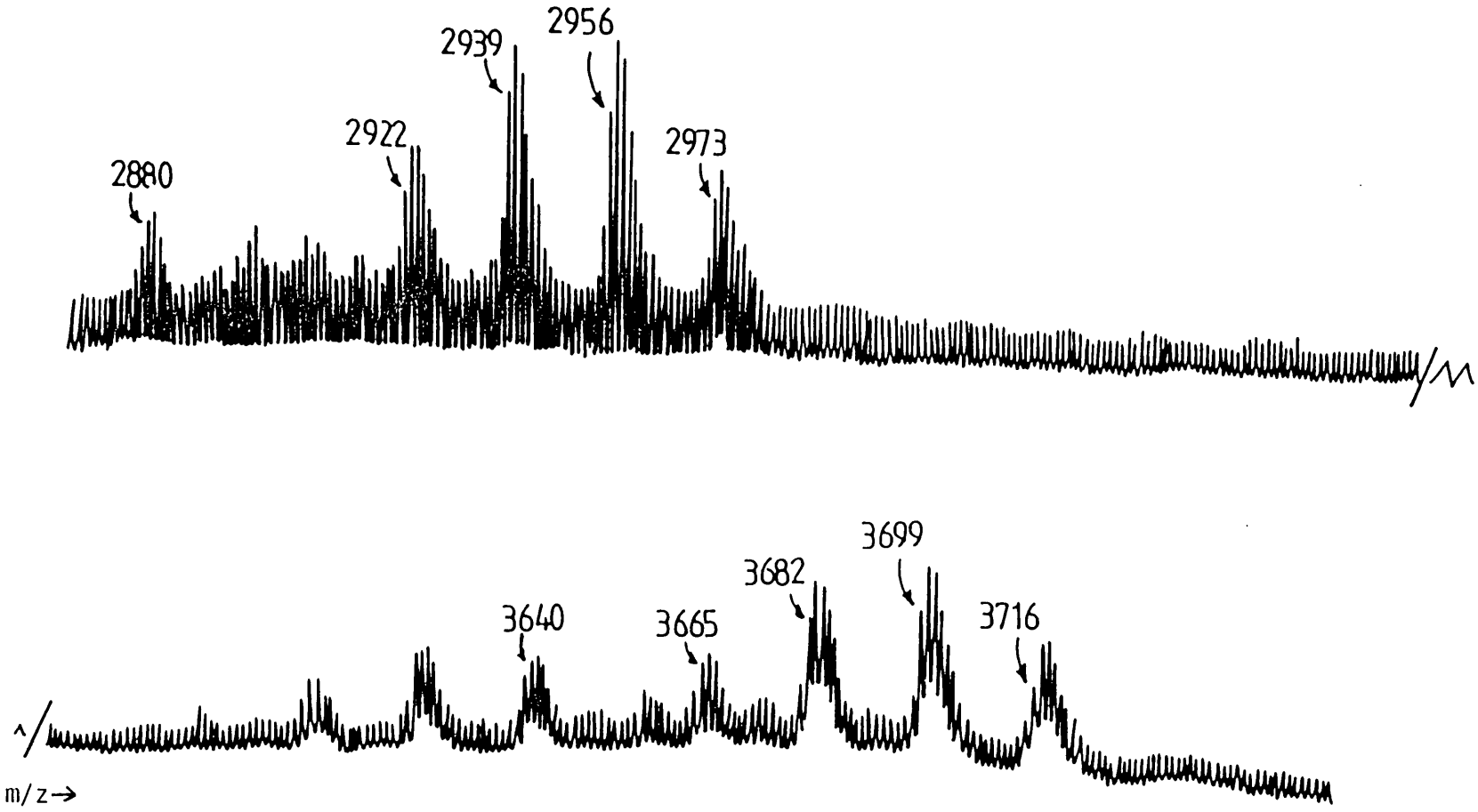
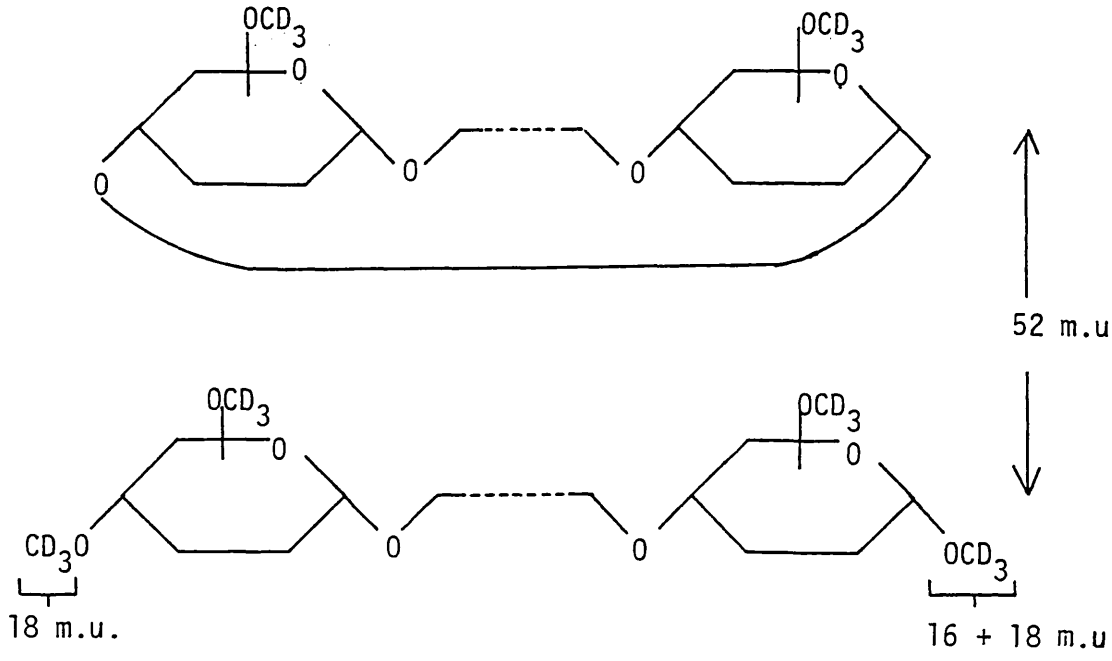


FIG 4.12. Positive FAB of the molecular ion regions of deuteropermethyl ECA (7 kV).



The molecular ions at m/z 3716 and 2973 are consistent with cyclic species since they correspond in mass to a whole number of derivatised trisaccharide repeats, (743 m.u.) without the 52 m.u. increment required by a linear molecule i.e. $(5 \times 743) + H^+ = 3716$

and $(4 \times 743) + H^+ = 2973$

Also there is no evidence for any signals 52 m.u. above these molecular ions so the possibility that the preparation contains a mixture of cyclic and linear species may be excluded.

In addition to the molecular ions a complex spectrum of lower mass fragment ions was obtained - the spectrum acquired at 8 kV accelerating voltage is presented in Fig 4.13. The generation of fragment ions from a cyclic molecule requires that two cleavages occur liberating charged linear fragments. All fragment ions observed here may be rationalised as the A-type ions already described for permethylated samples, in which charge resides on the reducing terminal residue in the form of an oxonium ion. The generation of the non-reducing terminus is more complex, but the ions observed may be postulated to arise from one of three cleavages:

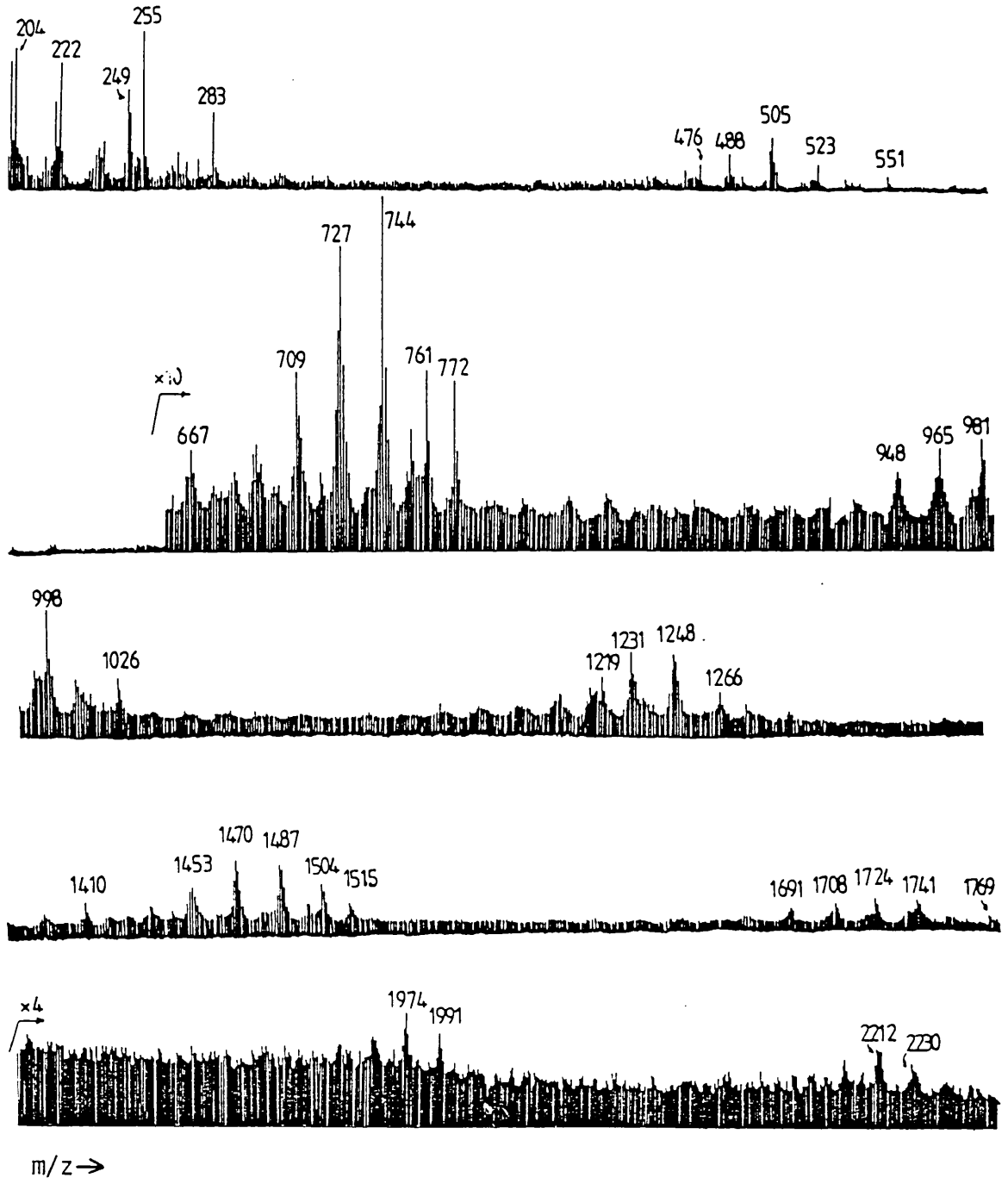
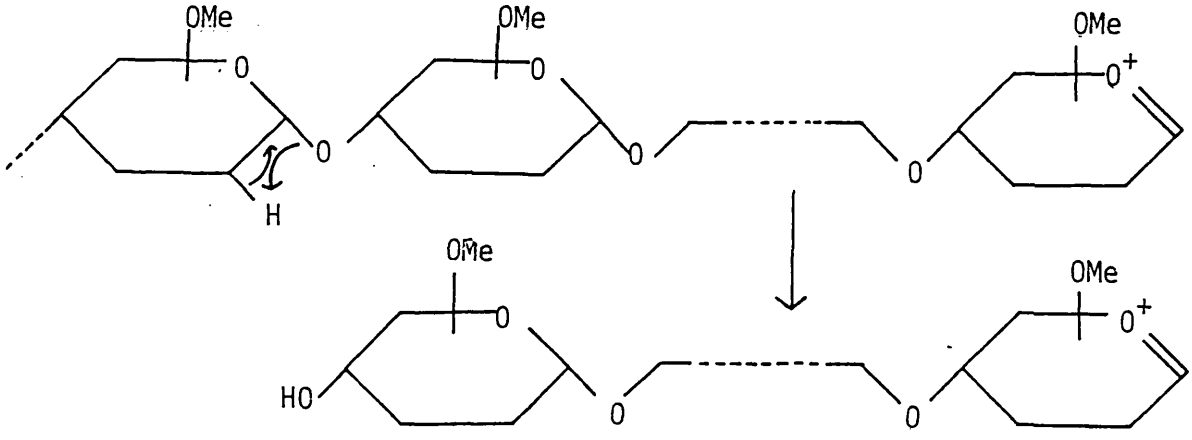


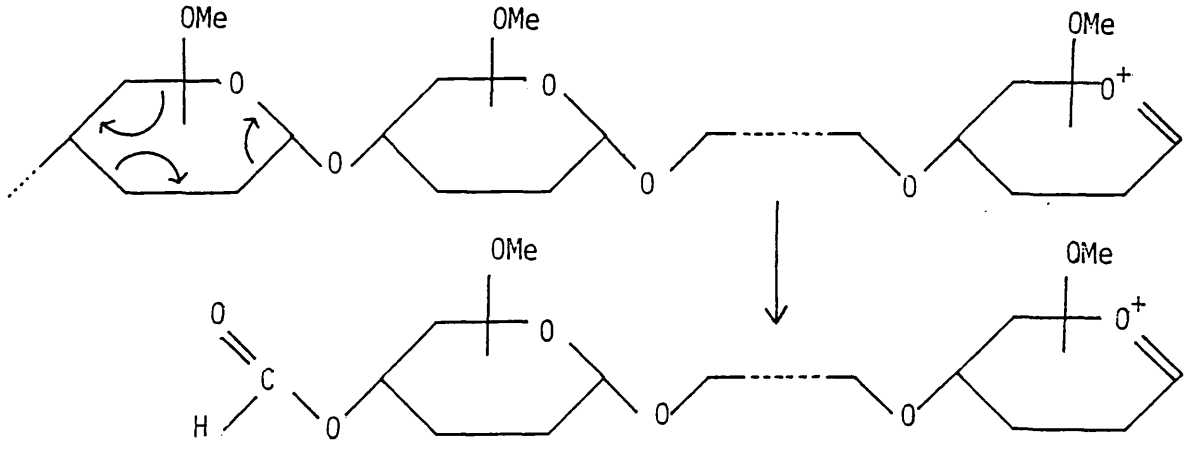
FIG 4.13. Positive FAB spectrum of deuteropermethyl ECA (8 kV).

1) β-cleavage (already described - see chapters 2 and 3)



The new non-reducing end residue carries one free OH group.

2) Sugar ring cleavage (see chapter 2)



The new non-reducing end carries a formyl group - this ion is seen 28 m.u. above the β-cleavage product.

3) Glycosidic elimination

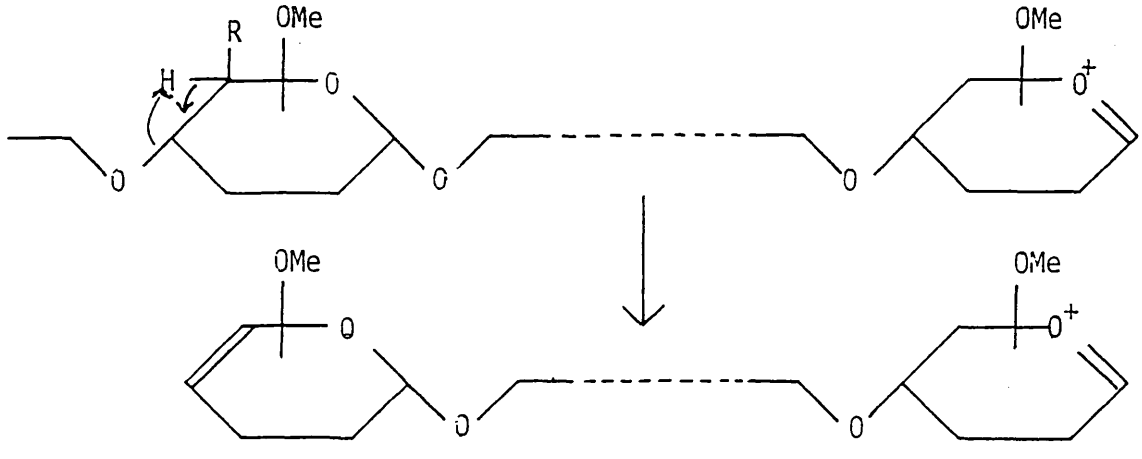


TABLE 4.6.

MAJOR FRAGMENT IONS OBSERVED IN THE SPECTRUM OF DEUTEROPERMETHYL ECA.

(A dash indicates that the ion of the composition given in the first column is not present in the spectrum).

COMPOSITION	TYPE I	TYPE II	TYPE III
A	269	—	—
B	255	283	237
C	222	250	204
AB	523	551	505
BC	476	504	—
AC	—	—	—
ABC	744	772	726
A_2BC	—	—	994
AB_2C	998	1026	980
ABC_2	965	993	947
A_2B_2C	1266	1294	1248
AB_2C_2	1219	1247	—
A_2BC_2	—	—	—
$A_2B_2C_2$	1487	1515	1469
$A_3B_2C_2$	—	—	—
$A_2B_3C_2$	1741	1769	—
$A_2B_2C_3$	1708	—	1690
$A_3B_3C_2$	—	—	1991
$A_2B_3C_3$	—	1990	—
$A_3B_2C_3$	—	—	1958
$A_3B_3C_3$	2230	—	2212

The new non-reducing end residue is "dehydrated", and is seen 18 m.u. below the β-cleavage product.

Table 4.6 presents a summary of the cleavage ions observed in the spectrum of deuteropermethylated ECA - all ions in the spectrum may be rationalised as one of these structures or its undermethylated counterpart.

4.3 Conclusion.

The only rational explanation of the results discussed here is that haptenic ECA is a mixture of cyclic polysaccharides containing 4, 5 and a small amount of 6 trisaccharide repeating units. The results of the NMR and chemical experiments which were carried out in parallel with the FAB studies are consistent with this conclusion, although the FAB results were essential for proof of the cyclic structure.

Until this study there were only two examples of cyclic saccharides known - the Shardinger dextrins (α-,β- and γ-cyclodextrin) and the β-1,2-glucans, elaborated by species of Rhizobium and Agrobacterium, which have recently been independently demonstrated by two groups (123,124) to be cyclic. The structure of the $(ABC)_5$ component is shown in Fig. 4.14. ECA represents the first example of a naturally occuring cyclic complex polysaccharide.

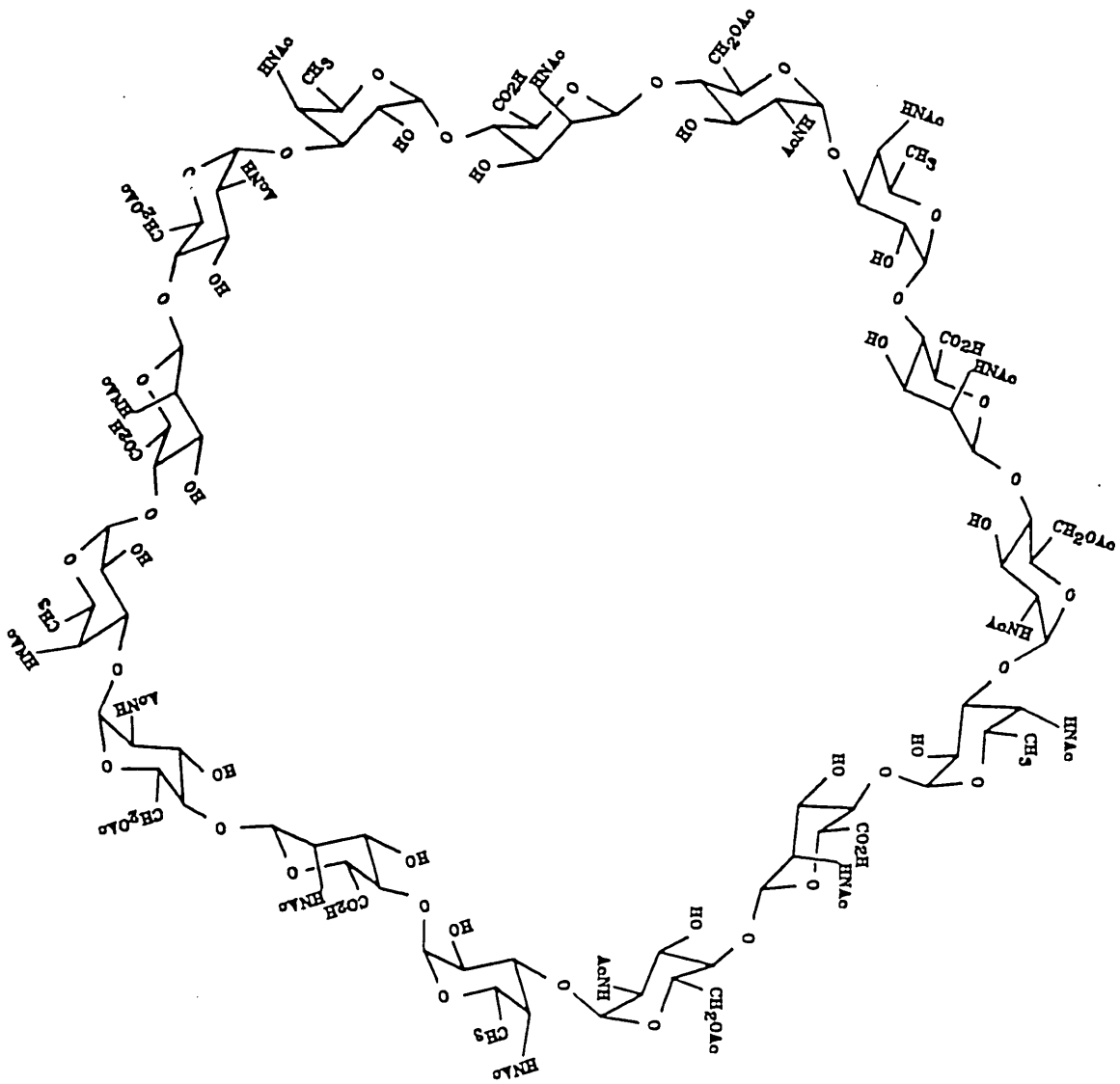


FIG 4.14. The cyclic structure of haptenic Enterobacterial Common Antigen.

CHAPTER 5 : METHODS.

5.1 Fast Atom Bombardment Mass Spectrometry.

FAB mass spectra were obtained using a VG Analytical High Field ZAB 1F mass spectrometer operated in the positive and negative modes at accelerating voltages of 8 kV, 7 kV, 6 kV and 5 kV which give mass ranges of 3,300, 3,800, 4,400 and 5,280 respectively. The atom gun was operated at 10 kV and xenon was used as the bombarding gas. Spectra were recorded on u.v. sensitive oscillographic paper and were counted manually. The instrument was scanned in a mass controlled mode and linear scans were performed at a scan rate of 300 sec for complete coverage of the mass range defined by the accelerating voltage used. Samples were dissolved in 5% aqueous acetic acid or methanol (for underivatised and derivatised samples respectively) and loaded into a mixture of glycerol and monothioglycerol (1:1, v:v, 1 µl) on the stainless steel target. Between 1 and 10 µg of sample was loaded depending on the quantity available.

5.2 Per-O-acetylation.

Per-O-acetylation was carried out using a modification of the method of Bourne et al (84). The sample (25- 0.5 µg, depending on the amount of sample available) was dissolved in a mixture of trifluoroacetic anhydride and glacial acetic acid (2:1, v:v, 200 µl) in a stoppered tube and incubated at room temperature for 10 min. The reagents were then removed under a stream of nitrogen. The dry product was dissolved in chloroform (1 ml) and washed with water (3 x 2 ml) to remove any aqueous soluble contamination. The chloroform was removed by evaporation under a stream of nitrogen.

5.3 N-acetylation.

1:1 N-acetylation was carried out on both the fucosidosis glycopeptide (chapter 3) and on de-O-acetyl ECA (chapter 4). The sample (approximately 30 µg) was dissolved in water (100 µl) and to this was added a mixture of acetic anhydride:$^{2}H_{6}$-acetic anhydride:methanol(1:1:8, v:v:v, 500 µl). The reaction mixture was incubated at room temperature for 30 min. and the reagents then removed under vacuum.

A modifi ation of this reaction was also performed on de-O-acetyl ECA in which the 1:1 label was included in the methanol instead of the acetic anhydride: the sample, dissolved in water as above, was mixed with a mixture of acetic anhydride: methanol:$^{2}H_{4}$-methanol (1:2:2, v:v:v, 500 µl) and incubated at room temperature for 30 min as before, when the reagents were removed under vacuum.

5.4 Permethylation.

Permethylation was carried out on N-acetylated samples (fucosidosis glycopeptide, chapter 3) or on per-O-acetylated samples (mannosidosis oligosaccharides, chapter 3). The procedure used was a double permethylation modification of the method of Hakomori (14). A dimsyl sodium base was prepared by heating sodium hydride in dimethyl sulphoxide (50 µg/µl) at 90 ^{o}C for 20 min. followed by cooling and centrifugation (20 min. at 3,000 RPM). The sample was dissolved in dry dimethyl sulphoxide (100µl) in a stoppered tube and 300 µl of the base was added. After 75 sec. methyl iodide (50 µl) was added and the reaction was allowed to proceed at room temperature for 10 min. Additional base (500 µl) was then added, followed by methyl iodide (500 µl) and the

reaction continued for a further 20 min. at room temperature. The reaction was terminated by the addition of water (2 ml) and the products isolated by extraction into chloroform ; chloroform (1 ml) was added to the quenched reaction mixture and the aqueous layer discarded. The chloroform layer was then washed with water (3 x 2 ml) and dried down under a stream of nitrogen. Further purification of the products was achieved using a Sep-Pak procedure based on that of Waeghe et al (125). A C_{18}-reverse phase Sep-Pak was pre-washed using the following sequence of solvents : water (5 ml), acetonitrile (5 ml), ethanol (5 ml) and water (5 ml). The dry products of the extraction were dissolved in methanol (200 µl) and loaded onto the Sep-Pak, which was then washed with water (1.2 ml) to elute salts. The sample was eluted from the Sep-Pak using 15% aqueous acetonitrile (0.6 ml), 50% aqueous acetonitrile (0.6 ml),75% aqueous acetonitrile (0.6 ml), acetonitrile (0.6 ml) and ethanol (0.6 ml). Each of these fractions was collected separately and dried down under vacuum. The permethylated product was generally recovered in the 50% acetonitrile wash.

5.5 Formation of the pentafluorobenzyl oxime derivative.

Pentafluorobenzyl oxime derivatives of both de-O-acetyl ECA and N-acetyl neuraminic acid were prepared (chapter 4). The first attempt to form this derivative of ECA was made using 100 µg of sample which was mixed with solid pentafluorobenzyl hydroxylamine hydrochloride (5.65 mg) and then mixed with anhydrous pyridine (200 µl) in a stoppered tube. The reaction was carried out at 100 ^{o}C for 2 hr. and was terminated by removal of the pyridine under a stream of nitrogen. This derivative failed to give FAB data so that the procedure was repeated using more sample : ½ mg

of sample and 25 mg of solid reagent were used and mixed with 200 µl of pyridine. The reaction conditions used were otherwise unchanged.

The PFB derivative of Nana was obtained on reaction of 200 µg of Nana with 1.7 mg of solid reagent in 200 µl of pyridine under the conditions given above.

5.6 Methyl esterification of pentafluorobenzyl Nana.

The pentafluorobenzyl oxime derivative of N-acetyl neuraminic acid was subjected to methyl esterification by mixing the solid derivative (200 µg) with methanolic ($^{1}H:^{2}H$, 1:1,v:v) hydrogen chloride (200 µl, 1M) in a stoppered tube. This was incubated at room temperature for 1 hr. when the reaction was terminated by removal of the reagents under vacuum.

5.7 Acid hydrolysis of de-O-acetyl ECA.

De-O-acetyl ECA (100 µg) was mixed with hydrochloric acid (20 µl, 6M) in a stoppered tube and incubated at 60 ^{o}C. At intervals aliquots (1 µl) were removed and analysed by FAB-MS in the positive mode - aliquots were added directly into the matrix and run without further manipulation.

5.8 Methanolysis of de-O-acetyl ECA.

De-O-acetyl ECA (150 µg) was mixed with methanolic hydrogen chloride (20 µl, 1M) in a stoppered tube and incubated at room temperature for 30 min. After 20 min. an aliquot (1 µl) was removed, loaded directly into the matrix and analysed by FAB in the positive mode. The remaining mixture was then incubated at 37 ^{o}C and further aliquots (1 µl) removed at intervals and analysed in the same way.

CHAPTER 6 : CONCLUSION.

The aim when this work was begun three years ago was to develop the new technique of FAB-MS for the analysis of carbohydrates and then to apply it in structural studies of genuine biological samples. The results presented in this thesis illustrate some of the ways in which FAB can be exploited to solve structural problems, and demonstrate the two particularly strong points of the technique:

1) it is versatile and can be used to obtain a variety of types of information depending on the sample handling protocols employed,
2) it complements and enhances many of the classical methods already available.

Several carbohydrate applications of FAB have recently been reported in the literature (7,85,122,124,126,127,128,129,130,131) although it is interesting that other workers use FAB merely as an alternative ionisation technique to obtain data which could equally well, if not more sensitively (131) be provided by EI. In contrast, few studies have exploited the unique ability of FAB to give data in situations where other methods fail eg. in the examination of crude mixtures, impure samples, unusual structures or heterogeneous preparations.

This work has clearly demonstrated the potential of FAB which has been successfully applied to a number of previously intractable biological problems. Continued investigations to improve the range of derivatisation and handling procedures will further extend the contribution of FAB in the carbohydrate and glycoconjugate field.

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