The enzymic defect and storage products in canine fucosidosis

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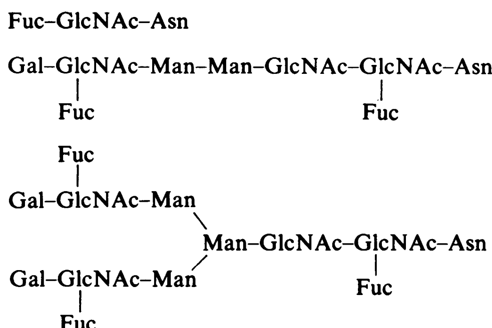
A marked deficiency of α-L-fucosidase and the accumulation of fucose-containing glycoasparagines were found in the brains of two English Springer spaniels suffering from a progressive nervous disorder. Both forms of α-L-fucosidase in normal brain, which are separable by ion-exchange chromatography, are absent from the affected animals. The storage products were characterized by t.l.c., gel filtration, g.l.c. and fast-atom-bombardment mass spectrometry. The postulated structures of the main components are:



The enzymic defect and nature of storage products justify designation of this disorder as canine fucosidosis.

A progressive neuronal storage disease has been described in English Springer spaniels in Australia (Hartley *et al.*, 1982) and the United Kingdom (Littlewood *et al.*, 1983). It is characterized by vacuolation of several types of cells, including neurons and glial cells, throughout the brain. The vacuoles appear empty, suggesting that the stored material is water-soluble. A deficiency of α-L-fucosidase was detected in the leucocytes and fibroblasts of one affected Australian dog (Kelly *et al.*, 1983), suggesting that the disorder was a canine case of the lysosomal storage disease fucosidosis. Human fucosidosis is an autosomal recessive trait in which the deficiency of α-L-fucosidase leads to the accumulation in tissues and excretion in the urine of fucoglycoconjugates (Durand *et al.*, 1982). We have investigated the enzymic defect and the storage products in the brains of two affected dogs to evaluate the disorder as an animal model for human fucosidosis.

Abbreviation used: f.a.b.m.s., fast-atom-bombardment mass spectrometry.

Materials and methods

Tissues

Two English Springer spaniels were diagnosed as suffering from a progressive nervous disorder, characterized by vacuolation of several cell types. Samples of the brain of affected dog 1 (male, 2½ years old) and of the brain and spinal cord of affected dog 2 (male, 3 years old) and brains of two unaffected dogs were removed at post-mortem and

stored at −20°C. For enzyme assays, homogenates (10%, w/v) of the tissues were prepared in distilled water in a Potter–Elvehjem homogenizer and then centrifuged at 18000rev./min (37000*g*) in an MSE High-Speed 18 centrifuge. Enzymic activities were measured in the resultant supernatant. To isolate neutral carbohydrates supernatants were extracted with 5vol. of diethyl ether to remove lipids and passed through a mixed-bed resin (Duolite MB 6113; BDH Chemicals, Poole, Dorset, U.K.), which was eluted with water. The eluate was freeze-dried and redissolved in a smaller volume of water for analysis.

Enzyme assays

Glycosidase activities were assayed as described previously (Burditt *et al.*, 1980), with the appropriate fluorigenic 4-methylumbelliferyl glycoside (Koch–Light Laboratories, Haverhill, Suffolk, U.K.). The pH-dependence of the brain α-L-fucosidase was measured by using the McIlvaine (1921) phosphate/citrate buffer system and a substrate concentration of 0.5mM. In each case 1 unit of activity is defined as a rate of 1 μmol of substrate transformed/min under the conditions specified.

Ion-exchange chromatography

This was carried out as described by Robinson & Thorpe (1973) with a column (2ml capacity) of DEAE-cellulose (Whatman DE-52). Supernatant (1ml) was dialysed against the equilibration buffer before chromatography. Fractions (1.5ml) were collected at 60ml/h with a salt gradient (0–0.25M) in 100ml of the equilibration buffer. The α-L-fucosidase in the fractions was assayed by the standard procedure.

Protein determination

Protein was measured by the Folin method (Lowry *et al.*, 1951), with bovine serum albumin as standard.

Analysis of neutral oligosaccharides

(1) *Determination of total carbohydrate*. The total carbohydrate content of tissue extracts was determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956). Standard curves for fucose and mannose were used over the range 0–100μg.

(2) *T.l.c., gel-filtration chromatography and g.l.c.* These were carried out as described previously (Abraham *et al.*, 1983).

(3) *Analysis of oligosaccharides by f.a.b.m.s.* Carbohydrate-containing material was isolated from de-ionized tissue extracts by preparative gel filtration for analysis by f.a.b.m.s.

(*a*) *N*-Acetylation. The sample was dissolved in water (100μl) to which was added acetic anhydride/[$^2H_6$]acetic anhydride/methanol (1:1:8, by vol.) (500μl). The reaction mixture was left at room temperature for 30min and the reagents were removed under vacuum.

(*b*) Permethylation. Permethylation was carried out by using a double-permethylation modification of the Hakomori procedure (Hakomori, 1964). A sodium methylsulphinylmethide base was prepared by heating NaH in dimethyl sulphoxide (50μg/μl) at 90°C for 20min, followed by cooling and centrifugation. The *N*-acetylated sample was dissolved in dry dimethyl sulphoxide (100μl) in a stoppered tube, and 300μl of the base was added. After 75s methyl iodide (50μl) was added and the reaction was allowed to proceed at room temperature for 10min. Additional base (500μl) was then added, followed by methyl iodide (500μl), and the reaction was continued for a further 20min at room temperature. The reaction was terminated by the addition of water (2ml), and the products were isolated by using a Sep-Pak procedure based on work of Waeghe *et al.* (1983). A C_{18} reverse-phase Sep-Pak was pre-washed with the following solvents sequentially: water (5ml), acetonitrile (5ml), ethanol (5ml) and water (5ml). The quenched reaction mixture was loaded on to the Sep-Pak, which was then washed with water (1.2ml) to elute salts. The Sep-Pak was then eluted with 15% (v/v) acetonitrile in water (0.6ml), 50% (v/v) acetonitrile in water (0.6ml), 75% (v/v) acetonitrile in water (0.6ml), acetonitrile (0.6ml) and ethanol (0.6ml). Each elution was collected separately and dried down under vacuum. The *N*-acetylpermethyl derivative was usually recovered in the 50%-acetonitrile wash.

(*c*) Peracetylation. Peracetyl derivatives were prepared by a modification of the procedure of Bourne *et al.* (1949). The sample was dissolved in trifluoroacetic anhydride/acetic acid (2:1, v/v) (200μl) in a stoppered tube and left at room temperature for 10min. Reagents were removed under a stream of N_2, and the product was dissolved in chloroform (1ml) and washed with water (3 × 2ml) to remove any salt contamination. The chloroform was removed by evaporation under a stream of N_2.

(*d*) F.a.b.m.s. F.a.b.m.s. spectra were obtained by using a VG Analytical High Field ZAB 1F mass spectrometer operated in the positive mode at 8kV and 7kV accelerating voltages giving mass ranges of 3300 and 3800 respectively. The atom gun was operated at 10kV 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 300s for a complete coverage of the mass range defined by the accelerating voltage

used. Samples were dissolved in aq. 5% (v/v) acetic acid or methanol (unmodified and modified samples respectively) and loaded into a glycerol/monothioglycerol mixture (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.

Results

Enzymic defect

(*a*) *Glycosidase activities in brains of normal and affected dogs.* The glycosidase activities in extracts of a normal dog brain and of the brains of two affected dogs were measured with a series of synthetic fluorigenic glycoside substrates (Table 1). There was a marked deficiency of α-L-fucosidase in the brains and the spinal cord of the affected dogs. The activities of seven other lysosomal glycosidases were elevated 2.5–25-fold in the affected dogs, whereas that of β-D-glucosidase was unaltered. The secondary hyperactivity of other lysosomal enzymes is characteristic of lysosomal storage diseases. It is concluded that the affected animals are cases of canine fucosidosis, resulting from a deficiency of α-L-fucosidase.

(*b*) *pH-dependence of α-L-fucosidase in normal and affected dog brain.* To investigate whether α-L-fucosidase assayed at other pH values was also deficient, the pH-dependence of the α-L-fucosidase in normal and affected dog brain was investigated. The activity–pH curve for α-L-fucosidase in normal dog brain has a pH optimum of 6.5 and a shoulder at pH 5.5 (Fig. 1), suggesting the presence of more than one enzymic component. Only very low activity at pH 6.5 and 5.5 was detected in the affected dog brain. Thus, if two forms of α-L-fucosidase are present in normal dog brain, they are both deficient in fucosidosis.

(*c*) *Ion-exchange chromatography on DEAE-cellulose of α-L-fucosidase in normal and affected dog brain.* To ascertain whether canine α-L-fucosidase occurs in multiple forms, an extract of the normal dog brain was analysed by ion-exchange chromatography on DEAE-cellulose (Fig. 2). Two forms of α-L-fucosidase were separated. The bound and unbound forms were called α-L-fucosidase I and II respectively, by analogy with human α-L-fucosidase (Robinson & Thorpe, 1973). Although the α-L-fucosidase I had a slightly higher ratio of activity at pH 6.5 to that at pH 5.5, the activity–pH profiles for the two separated forms were very similar. This indicated that the shape of the activity–pH profile for the whole normal brain extract was not due to

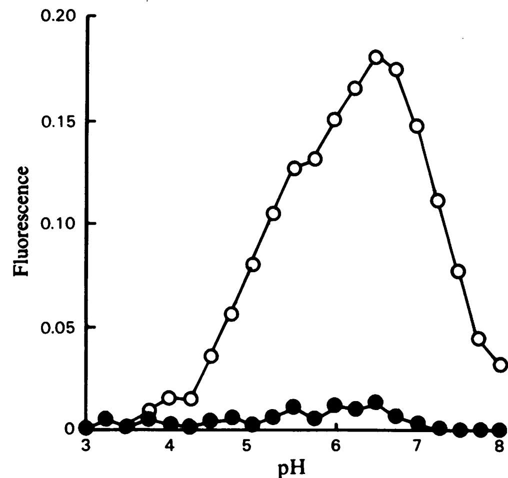


Fig. 1. *pH-dependence of α-L-fucosidase in normal and affected dog brain*
For experimental details see the text. ○, Control; ●, affected dog 2.

Table 1. *Glycosidase activities in dog tissues*
All activities were measured, as described by Burditt *et al.* (1980), with the appropriate fluorigenic 4-methylumbelliferyl glycoside (0.5 mM) in phosphate/citrate buffer, pH 4.0, unless otherwise stated.

	Activity (munits/mg of protein)				
	Normal brain		Affected brain		Spinal cord
Enzyme	Puppy	Adult	Dog 1	Dog 2	Dog 2
β-D-Mannosidase	0.05	0.10	0.52	0.54	0.25
α-D-Mannosidase	1.44	1.12	28.80	32.40	22.30
α-D-Glucosidase	0.14	0.20	0.62	1.32	0.48
β-D-Glucosidase	0.08	0.08	0.08	0.13	0.30
β-D-Glucosidase (pH 5.5)	0.18	0.05	0.05	0.05	0.42
α-D-Galactosidase	0.16	0.13	0.97	2.28	1.27
β-D-Galactosidase	1.88	1.70	4.23	6.43	2.62
α-L-Fucosidase (pH 5.5)	0.53	0.50	0.01	0.03	0.06
β-D-*N*-Acetylhexosaminidase	20.69	18.31	139.2	197.1	149.6
β-D-Glucuronidase	0.41	0.17	4.26	9.94	4.11

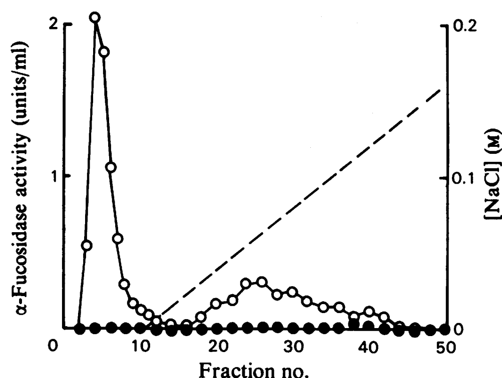


Fig. 2. *Ion-exchange chromatography on DEAE-cellulose of α-L-fucosidase in normal and affected dog brain*
Supernatants (1 ml) from normal and affected dog were applied to a column. Fractions (1.5 ml) were collected at 60 ml/h and assayed for α-L-fucosidase. For full experimental details see the text. ○, Control; ●, affected dog 1; ----, NaCl gradient.

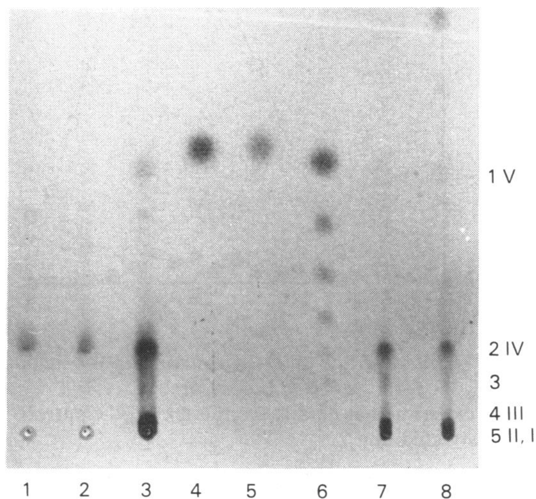


Fig. 3. *T.l.c. of neutral oligosaccharides in brain and spinal cord of affected dogs*
T.l.c. of extracts of tissues was carried out on silica gel with propan-1-ol/water (8 : 3, v/v) as the solvent. Oligosaccharides were detected with orcinol/ H_2SO_4. Track 1, normal puppy brain; track 2, normal adult dog brain; track 3, brain, affected dog 1; track 4, mannose; track 5, fucose; track 6, dextran hydrolysate; tracks 7 and 8, brain and spinal cord respectively of affected dog 2. I–V indicate mobilities of material in peaks I–V separated by gel filtration (Fig. 4).

two forms with different pH optima. α-L-Fucosidase forms I and II were almost completely absent from the brain of an affected dog, suggesting that the two forms are genetically and structurally related. Human α-L-fucosidase can be separated into two forms that differ in their charge, size and pH-dependence at lower pH values (Robinson & Thorpe, 1973). Both forms are absent in human fucosidosis (Alhadeff *et al.*, 1974).

Characterization of storage products

(*a*) *Carbohydrate content of normal and affected tissues.* The carbohydrate content of crude aqueous extracts (10%, w/v) of the control brains and of affected brains and spinal cord was measured by using the phenol/H_2SO_4 method. The affected brains contained 17.0 and 13.8 mg of carbohydrate/g wet wt., expressed as equivalent weight of fucose as determined from a standard curve for fucose, compared with 2.9 and 2.3 mg/g wet wt. in the controls. This indicated that the deficiency of α-L-fucosidase leads to accumulation of carbohydrate.

(*b*) *T.l.c. of oligosaccharides in extracts of normal and affected brains.* The neutral water-soluble oligosaccharides extracted from the brains of the normal and affected dogs were analysed by t.l.c. (Fig. 3). Large amounts of oligosaccharides were detected in the affected tissues, but not in the controls. Three main components, 2, 4 and 5 in order of increasing size, were present in the affected tissues. Component 2 had a similar mobility to that of a pentasaccharide in the dextran hydrolysate, whereas component 4 had a very low mobility and component 5 remained at the origin. Minor components, 1 and 3, had mobilities similar to those of a monosaccharide and a heptasaccharide.

(*c*) *Gel filtration on Bio-Gel P4.* Several carbohydrate components were resolved when an extract of the affected brain was fractionated by preparative gel filtration on Bio-Gel P4 (Fig. 4). The polymeric material in peak I, which remained at the origin in t.l.c., was not investigated any further. Peak II could be resolved into two peaks by analytical gel filtration. The material in peak II also remained at the origin in t.l.c. The carbohydrates in peaks III, IV and V were found to correspond to components 4, 2 and 1 observed by t.l.c. (Fig. 3). The material in the fractions corresponding to peaks II, III and IV was pooled as indicated in Fig. 4, and purified further by rechromatography on the Bio-Gel P4 column before analysis by g.l.c., amino acid composition and f.a.b.m.s.

(*d*) *G.l.c.* Derivatives of fractions II, III and IV (10–100 nmol of carbohydrate) were formed and analysed by g.l.c. Fraction III contained fucose, *N*-acetylglucosamine, mannose and galactose in the proportions 1.15 : 1.7 : 1.0 : 1.4. The same sugars were found in fraction II, but the proportions varied from one experiment to another, reflecting

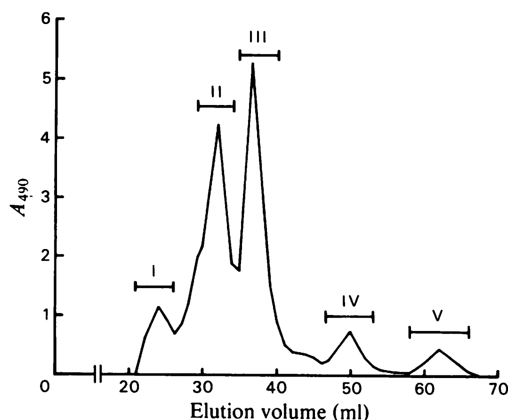


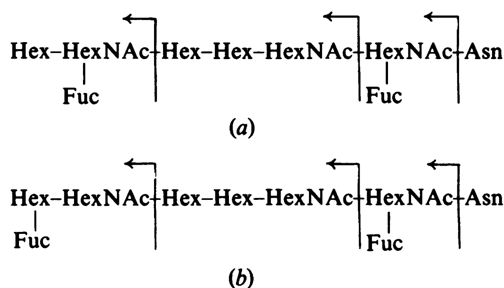
Fig. 4. *Gel-filtration chromatography on Bio-Gel P4 of neutral oligosaccharides in brain of affected dog 1*
Extract of brain (1.5ml) containing approx. 1.5mg of oligosaccharide was fractionated on a column (1cm × 100cm) of Bio-Gel P4, which was eluted with water (8ml/h). Carbohydrates were detected by using phenol/H_2SO_4. The fractions (1ml) pooled for purification of the oligosaccharide are shown (⊢—⊣).

the heterogeneity of this fraction. In contrast, only fucose and *N*-acetylglucosamine were detected in fraction IV, in a ratio of 1:0.75.

(*e*) *Identification of the storage products by f.a.b.m.s.* The most detailed structural analysis was performed on fraction III, since this was available in the greatest quantity. F.a.b.m.s. of fraction III afforded an excellent positive-ion spectrum containing only one molecular-ion cluster at *m/z* 1520 $(M+H)^+$ (see Fig. 5*a*). This signal shifted to a 1:1 doublet at *m/z* 1562 and 1565 after the sample was acetylated with acetic anhydride/[$^2H_6$]acetic anhydride (1:1, v/v) in methanol, a reagent that rapidly acetylates amino groups in peptides. These data indicated that fraction III contained one major component of M_r 1519 having one free amino group and thus was likely to be a glycopeptide. The M_r value of 1519 fits precisely for a glycopeptide of composition $Fuc_2Hex_3HexNAc_3Asn$, and the presence of aspartic acid/asparagine was confirmed by amino acid analysis.

A limited number of plausible structures can be proposed for a glycopeptide of this composition. To ascertain which structure(s) was correct, f.a.b.m.s. analyses of suitable derivatives were performed. We have previously shown (Dell *et al.*, 1983) that permethylated and peracetylated glycoconjugates cleave predominantly at the glycosidic linkage, particularly aminoglycoside linkages, to yield A-type-sequence ions.

Fraction III was *N*-acetylated [with acetic anhydride/[$^2H_6$]acetic anhydride (1:1, v/v)] and permethylated. The positive f.a.b.m.s. spectrum of the derivative is shown in Fig. 5(*b*). Protonated molecular ions containing the 1:1 isotopic label are present at *m/z* 1926/1929 and 1940/1943, the former corresponding to the expected *NO*-permethyl molecule, the latter containing one additional methyl group, presumably the result of *C*-methylation of asparagine. None of the fragment ions contains the label; hence all fragment ions are derived from the carbohydrate portion of the molecule. Abundant A-type-sequence ions occur at *m/z* 638, 1291 and 1710, corresponding to compositions $FucHexHexNAc^+$, $FucHex_3HexNAc_2^+$ and $Fuc_2Hex_3HexNAc_3^+$ respectively. A very weak signal at *m/z* 842 (accompanied by methanol loss at *m/z* 810), which corresponds to the composition $FucHex_2HexNAc^+$, is likely to be a hexosyl cleavage. Analogous ions were present in the spectrum of the peracetylated derivative. These occurred at *m/z* 848 ($FucHexHexNAc^+$), 1711 ($FucHex_3HexNAc_2^+$) and 2228 ($Fuc_2Hex_3HexNAc_3^+$). All of these ions were abundant, indicating that they were derived from aminoglycoside cleavages. In addition, the acetylated sample yielded ions at *m/z* 331 and 276, which can be assigned to Hex^+ and Fuc^+ respectively. Only two structures, (*a*) and (*b*), are consistent with these data (the major fast-atom-bombardment cleavages of permethyl and acetyl derivatives are indicated by the arrows):



Two pieces of evidence suggest that structure (*a*) is the major, or possibly the sole, component of fraction III. These are (i) the presence of Hex^+ in the spectrum of the acetylated sample, which can only be derived from (*a*), and (ii) the absence of a signal at *m/z* 606 (corresponding to methanol loss from 638) from the spectrum of the permethylated sample (see Fig. 5*b*) and the presence of an abundant signal at *m/z* 432 (corresponding to loss of a fucose unit from 638). We have observed in other work (M. Fukuda, A. Dell & M. N. Fukuda, unpublished work) that molecules containing the Fuc–Hex–HexNAc moiety readily lose methanol from the A-type-sequence ion at *m/z* 638 when the 3-position is not substituted with a sugar residue.

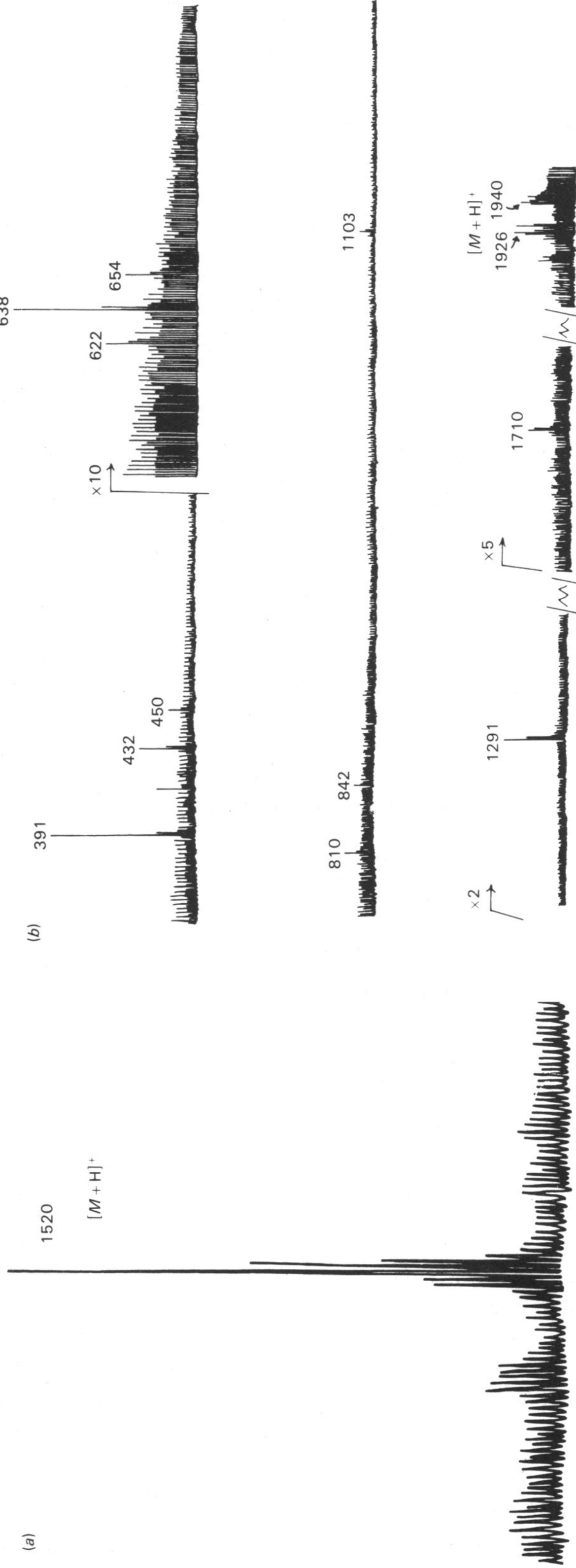
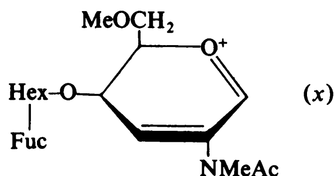


Fig. 5. *F.a.b.m.s. of fraction III separated by gel filtration*

Experimental details are indicated in the Materials and methods section. (*a*) Positive-ion spectrum of unmodified fraction III; (*b*) positive-ion spectrum of *N*-acetylated and subsequently permethylated fraction III.

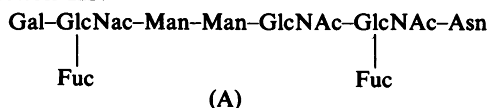
This fragmentation results in the stabilized ion (x):



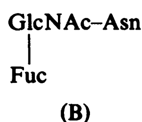
When fucose is substituted at position 3 of N-acetylhexosamine we have observed that a similar fragment ion is formed by the elimination of a fucosyl group (A. Dell, J. E. Oates & M. Fukuda, unpublished work on granulocyte cell-surface glycopeptides) (Scheme 1), affording an ion at m/z 432 from the moiety Hex–HexNAc⁺ (with Fuc attached to HexNAc). The absence of m/z 606 and the presence of an abundant ion at m/z 432 in the spectrum of permethylated fraction III provide strong evidence for the presence of a fucosyl group as a substituent at position 3 of the first N-acetylhexosamine residue in the sequence.

In addition to the cleavages described above, one further fragmentation pathway is usually exhibited by acetyl or permethyl derivatives of oligosaccharides containing fucosyl groups (A. Dell, J. E. Oates & M. Fukuda, unpublished work), namely β-cleavage with a hydrogen transfer to remove a fucosyl group (Scheme 2). This cleavage affords the ions at m/z 450 (loss of fucosyl from 638), 622 (loss of fucosyl from 810), 654 (loss of fucosyl from 842) and 1103 (loss of fucosyl from 1291) in the spectrum of permethylated Fraction III (see Fig. 5b).

Combining the f.a.b.m.s. data with knowledge of the established structures of fucose containing asparagine-linked oligosaccharides allows us to postulate structure (A) for the major component of fraction III:

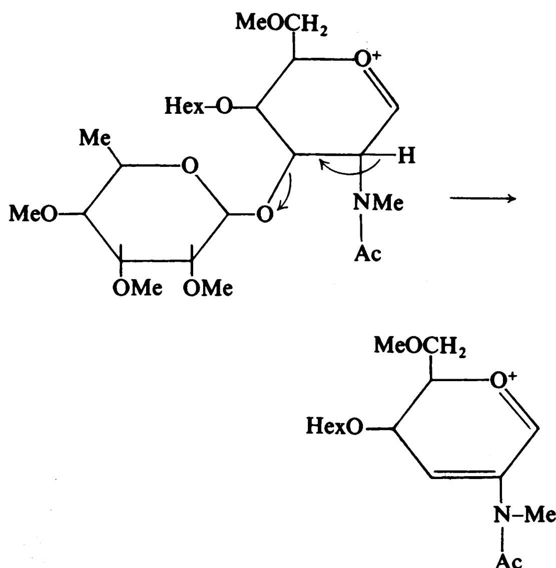


F.a.b.m.s. of fraction IV yielded a protonated molecular ion at m/z 482, consistent with the composition FucHexNAcAsn. Amino acid analysis and f.a.b.m.s. of the acetylated derivative provided corroborative evidence for this composition, and we assign structure (B) to this substance:

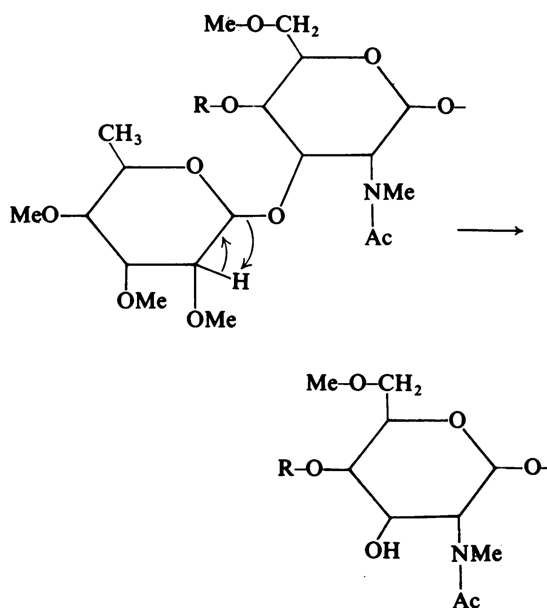


(B)

F.a.b.m.s. of fraction II showed it to be a mixture. Some contamination with fraction III was evident (molecular ion at m/z 1520), and, in addition, two



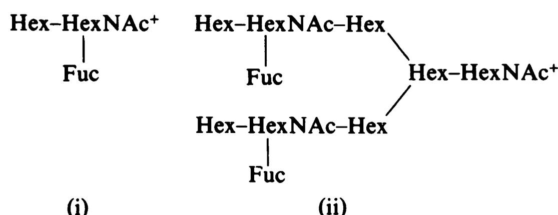
Scheme 1



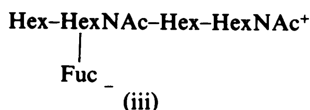
Scheme 2

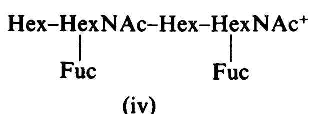
higher-M_r components were present, which afforded ions at m/z 2193 (major) and 2031 (minor). These correspond to glycoasparagines of composition $Fuc_3Hex_5HexNAc_4Asn$ and $Fuc_3Hex_4HexNAc_4Asn$ respectively. F.a.b.m.s. of the N-acetyl-NO-permethyl derivative yielded only two major A-type-sequence ions. These were present at m/z

638 and 2118, and are consistent with structures (i) and (ii) respectively:

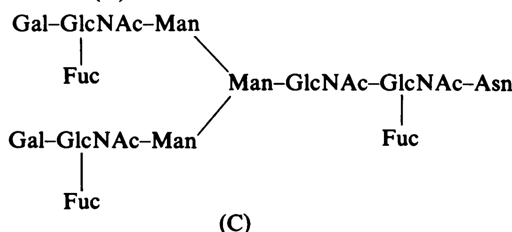


The absence of signals at *m/z* 1087 and 1261 ruled out structures containing sequences (iii) and (iv):





The f.a.b.m.s. data can only be rationalized if the major component present in fraction II has the structure (C):



Also present is the related substance that lacks a hexosyl unit, presumably a terminal galactosyl group.

Discussion

A deficiency of α-L-fucosidase and the accumulation of fucose-containing oligosaccharides and glycopeptides are characteristic of the human lysosomal storage disease fucosidosis. Therefore the demonstration of a deficiency of α-L-fucosidase and accumulation of fucose-containing glycoasparagines in the brains of the two dogs under investigation, together with the pathology and clinical symptoms, justifies the diagnosis of canine fucosidosis in these animals.

The enzymic defect is very similar in dog and man. There is a concomitant deficiency in both species of the two forms of α-L-fucosidase, I and II, separable by ion-exchange chromatography on DEAE-cellulose. There is less than 5% residual α-L-fucosidase in the brains of the affected dogs.

The excessive accumulation of fucose-containing glycoasparagines in the brain in canine fucosidosis suggests that the storage products result from the incomplete catabolism of glycoproteins. In human fucosidosis, the main storage products in the brain were found to be a predominant decasaccharide and a less-abundant disaccharide, Fuc(α1–6)GlcNAc (Tsay & Dawson, 1976). Fucose-containing glycolipids were not stored in the brain to an appreciable extent, reflecting their normal low concentration in this tissue (Margolis & Margolis, 1972). However, human patients do excrete larger amounts of fucose-containing glycopeptides than fucose-containing oligosaccharides in their urine (Nishigaki *et al.*, 1978). Oligosaccharides have been detected in the urine of a dog with fucosidosis (Kelly *et al.*, 1983).

The postulated structures of the glycoasparagines found in canine fucosidosis are consistent with the incomplete catabolism of glycoproteins as the result of a deficiency of α-L-fucosidase. Structures (A), (B) and (C) have been found in the urines of human patients with fucosidosis (Yamashita *et al.*, 1979). The retention of the aspartamido linkage in all the canine storage products, particularly (B), Fuc–GlcNAc–Asn, suggests that the aspartoamidohydrolase cannot hydrolyse this linkage if fucose is still attached to the *N*-acetylglucosamine linked to asparagine. Further, the structures of the larger storage products (A and C) suggest that the endoglucosaminidase cannot hydrolyse the core chitobiosidic linkage if fucose is attached to either an *N*-acetylglucosamine at a non-reducing terminal or to the *N*-acetylglucosamine linked to asparagine. However, component (B) could arise from the action of the endoglucosaminidase on *N*-linked glycans of the high-mannose type, or non-fucose-containing complex, or hybrid types. This suggests that it may be fucose at the non-reducing terminal(s) that blocks the action of the endohexosaminidase. This would imply that lysosomal α-L-fucosidase acts at an early stage in the catabolism of glycoproteins.

The fucose residue at the non-reducing terminal of the stored glycoasparagines always appears to be linked to *N*-acetylglucosamine and not to a hexosyl group (presumably galactose). This structure has been called the X-determinant in humans (Hakomori & Kobata, 1974). The homogeneity of the fucosyl linkage at the non-reducing terminal probably reflects a genetically controlled biosynthetic pathway. The structure of these glycoasparagines indicates that the lysosomal catabolism of glycoproteins by endo- and exoglycosidases is probably a highly ordered process and that the substrate-specificities of the enzymes involved are very precise. The elucidation of the full structures of the storage products should throw more light on this process. Thus canine fucosidosis is a valuable model for studying the corresponding human disorder and the biosynthesis and catabolism of mammalian glycoproteins.

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