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STRUCTURAL ANALYSIS OF HEXA- TO OCTA-SACCHARIDE FRACTIONS ISOLATED FROM SHEEP GASTRIC-GLYCOPROTEINS HAVING BLOOD-GROUP I AND i ACTIVITIES

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ABSTRACT

Structural data are presented on six oligosaccharide-fractions (hexa- to octa-saccharides) released from sheep gastric-glycoproteins having blood-group I and i activity by degradation with alkaline borohydride Previous data on two of the oligosaccharides are included for comparison The fractions were analysed, before and after treatment with exo-β-D-glycosidases and an endo-β-D-galactosidase, on Bio-Gel P4 and by p c , by direct-insertion m s (after methylation), and by g l c –m s of the derived, partially *O*-methylated alditol acetates Each fraction contained 1–3 oligosaccharides, each of which had 2-acetamido-2-deoxy-D-galactitol (GalNAc-ol) at the reduced end and involved one of the structures

β-D-Gal-(1→3)-GalNAc-ol, β-D-Gal-(1→3)-GalNAc-ol,
β-D-GlcNAc-(1→6)-GalNAc-ol,

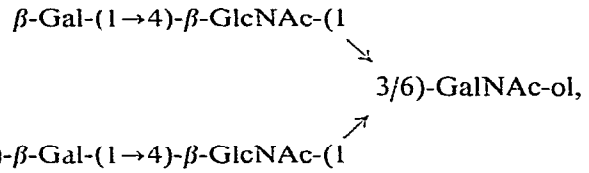
or β-D-GlcNAc-(1→3,6)-GalNAc-ol
β-D-GlcNAc-(1→3,6)-GalNAc-ol

The majority of the oligosaccharides contained the unsubstituted, "type 2" blood-group precursor-chain sequences, β-D-Gal-(1→4)-β-D-GlcNAc-(1→6) and single or repeating β-D-Gal-(1→4)-β-D-GlcNAc-(1→3), which are recognised by various anti-blood-group I and i cold agglutinins The "type 1" sequence, β-D-Gal-(1→3)-β-

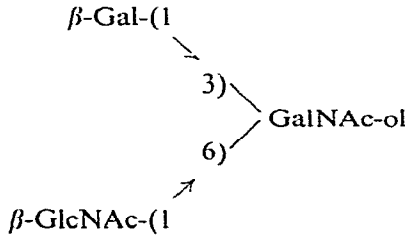
D-GlcNAc-(1→3)-, was not detected The "type 2" sequences were linked to GalNAc-ol either directly or through intervening β-D-Gal-(1→3) or β-D-Gal-(1→3)-β-D-Gal-(1→3) sequences, and formed the backbone structures of the oligosaccharides studied In certain of the oligosaccharides, the backbone structures were substituted with α-L-Fuc-(1→2) (associated with blood-group H), or with α-L-Fuc-(1→2) and α-D-GalNAc-(1→3) (associated with blood-group A) In others, there was evidence for substitution with β-D-GlcNAc-(1→4,3, or 2) or β-D-Gal-(1→3) of unknown antigenic activity The oligosaccharides contain 3 main regions, namely the core region, the backbone consisting of 'type 2" precursor-chains which, when accessible, would react with various anti-i (if linear) and anti-I (if branched) antibodies, and the peripheral region associated with various blood-group isotypes

INTRODUCTION

Gastric mucins of certain sheep are rich sources of blood-group I and i antigens, and the antigenic activity can be enriched by affinity chromatography on an anti-I immunoadsorbent column[2] A glycoprotein preparation thus enriched was degraded with alkaline sodium borotritide, and the released oligosaccharides were fractionated on Bio-Gel P4 as described in the preceding paper[3] The two fractions (K and L) of smallest molecular weight with both blood-group I and i activities were further fractionated by p c and p e , and several tritium-labelled oligosaccharide-fractions were obtained The oligosaccharide (LE_2) obtained in greatest yield was a hexasaccharide with the structure[1]



containing the novel, trihexosamine core-region ($LE_2$1) with two GlcNAc residues linked to GalNAc-ol * In contrast, a second oligosaccharide fraction (LD_2) contained the commoner core-region



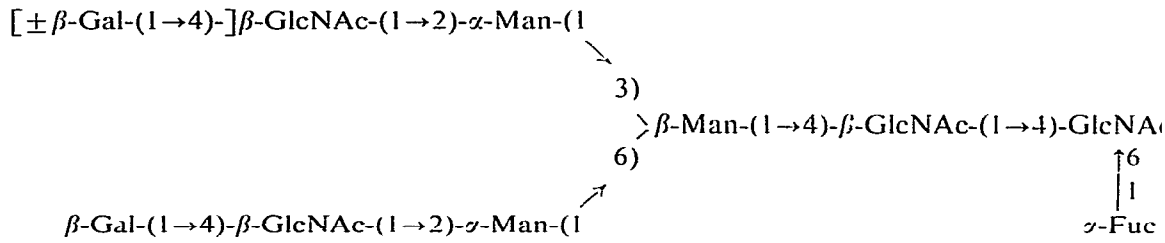
*Except for L-fucose all of the sugars mentioned in this paper are considered to be in the D series

We now report further structural characterisation of LD_2 and of four fractions of higher molecular weight obtained from the Bio-Gel-P4 peak K

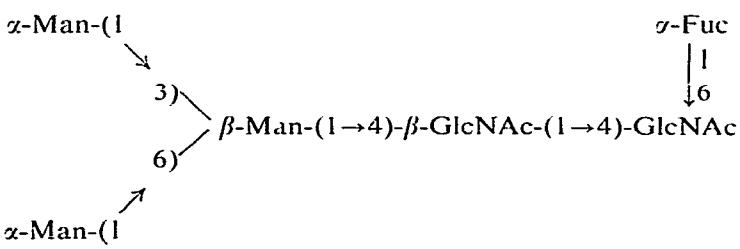
MATERIALS AND METHODS

Oligosaccharide fractions — The preparation and fractionation of tritium-labelled oligosaccharides from sheep gastric-glycoproteins having blood-group Ii activities is described in the preceding paper[3] Sheep gastric-mucin having blood-group Ii activity and weak blood-group A and H activities was enriched for I and i activities by affinity chromatography on an anti-I immunoadsorbent column and degraded with alkaline borohydride/borotritide Chromatography of the dialysable oligosaccharides on Bio-Gel P4 gave 21 fractions (A–W), of which A–L showed both blood-group I and i activities in radio-immunoassays Fractions K and L, the mobility of which on Bio-Gel P4 corresponded to dextran oligosaccharides having 10–12 and 9–10 D-glucose residues, respectively were selected for structural analysis, as they were the shortest oligosaccharides having both I and i activities These fractions were obtained in highest yield (7 and 6 mg, respectively), but were mixtures of several oligosaccharides P c of fraction K yielded 6 sub-fractions (KA–KF), and fraction L yielded 5 sub-fractions (LA to LE) that were subjected to p e Of the 25 fractions thus obtained, four sub-fractions of K (KC_1, KE_2, KF_2, and KF_3a obtained in yields of 120, 200, 160, and 55 μg, respectively) and two sub-fractions of L (LE_2 and LD_2 with yields of 600 and 80 μg, respectively) were analysed further

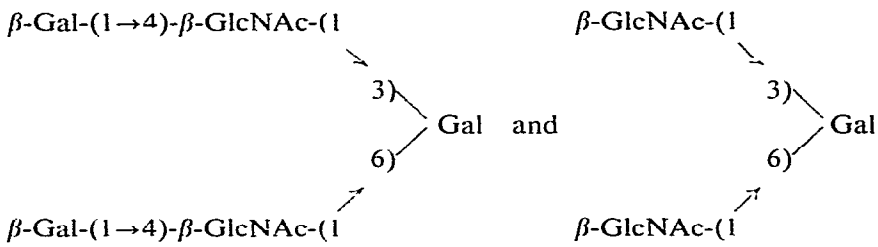
Standard oligosaccharides — The standard of high molecular weight



abbreviated to $(Gal)_{1\ 6}(GlcNAc)_4(Man)_3(Fuc)_1$, was obtained from immunoglobulin glycopeptides[5] by hydrazinolysis[6] The standard



abbreviated to $(GlcNAc)_2(Man)_3(Fuc)_1$, was obtained from the foregoing standard by digestion with exo-glycosidases The tetrasaccharide α-Fuc-(1→2)-β-Gal-(1→4)-β-GlcNAc-(1→3)-Gal was obtained from H_2 glycolipid by digestion with endo-β-D-galactosidase[7] The trisaccharide β-Gal-(1→4)-β-GlcNAc-(1→3)-Gal and the disaccharide β-GlcNAc-(1→3)-Gal were obtained from the tetrasaccharide by digestion with exo-glycosidases These tetra- to di-saccharides were kindly donated by Dr Michiko N Fukuda The branched penta- and tri-saccharides



were kindly supplied by Professor S David (Universite de Paris-Sud, Orsay) All of the standards were labelled[7] by reduction with sodium borotritide

Chromatography — Analytical Bio-Gel chromatography and isolation of fragments formed by digestion with endo- and exo-glycosidases were performed on a column (0 9 × 150 cm) of Bio-Gel P4 (200–400 mesh) at 20–25°, using distilled water at 4 ml/h (2-ml fractions) Oligosaccharides were detected by scintillation counting

Descending p c was performed on Whatman No 1 paper with *A*, ethyl acetate–pyridine–water (10 5 4) for 16 h, or *B*, ethyl acetate–pyridine–acetic acid–water (5 5 1 3) for 40 h Oligosaccharides were detected by radiochromatogram scanning (Model 7201, Packard Instrument Company Inc)

Monosaccharide analysis — Monosaccharide analysis was effected by g l c of trimethylsilylated methyl glycosides[8] Methylation involved the Hakomori method[9] Permethylated oligosaccharides were isolated by chromatography on LH20 columns eluted with acetone–methanol (1 1), and hydrolysed[10] in 0 25M sulphuric acid–90% glacial acetic acid The partially *O*-methylated hexitol acetates and 2-deoxy-2-(*N*-methylacetamido)hexitol acetates were analysed by g l c –m s and identified according to the data described by Bjorndal *et al*[11] and Stellner *et al*[12], respectively Direct-insertion m s of permethylated oligosaccharides was effected on a Finnigan 3300 spectrometer under the conditions described in Table III

Digestion with exo-glycosidases — β-D-Galactosidase and 2-acetamido-2-deoxy-β-D-glucosidase, isolated from Jack-bean meal[13], appeared to be free of contaminating exo-glycosidase activity when assayed by *p*-nitrophenyl glycosides, but some contaminant activity was found when using natural substrates Only very weak α-D-glycosidase activity was detected, when Gal and GlcNAc residues were cleaved, they were therefore considered to have been β-D-linked The β-D-galactosidase of *C. lampas* and the 2-acetamido-2-deoxy-β-D-glucosidase of *T cornutus* obtained from Seikaguku (Miles Laboratories Ltd) were stated to contain <0 5% of contaminant 2-acetamido-2-deoxy-α- or -β-D-glucosidase or α- or β-D-galactosidase activity,

respectively, when assayed by *p*-nitrophenyl glycosides and we found no contaminant activity against natural substrates

Enzyme digestions were performed for 16 h at 37° in 0·02M citrate–phosphate buffer (pH 5) with ~10 units/ml for the Jack-bean enzymes and 0·5 unit/ml for the other enzymes. In analytical experiments, 0·5 nmol of oligosaccharide in a total volume of 20 μl was digested, and labelled fragments were detected by radiochromatogram scanning after p.c. In preparative experiments, 20–40 nmol of substrate in a total volume of 60 μl was digested, and chromatographed on Bio-Gel P4 as described above.

Digestion with endo-β-D-galactosidase. — The endo-β-D-galactosidase of *Escherichia freundii* was isolated as described previously[4] and kindly supplied by Dr. Michiko N. Fukuda. Tritiated, reduced oligosaccharides (0·5 nmol containing ~2 × 10^4 c.p.m.) were treated with 2·5 munit of enzyme in a total volume of 20 μl of 0·1M sodium acetate buffer (pH 5·8, 0·125 unit/ml). After digestion for 16 h at 37°, the enzyme was precipitated by heating at 100° for 1 min and the supernatant solution was subjected to p.c. Fragments containing tritiated reduced termini were detected by radiochromatogram scanning. Digestion with a higher concentration (10×) of enzyme was effected in the same total volume (1·25 unit/ml). Preparative-scale digestion of fraction KE_2 (30 μg) was performed in a total volume of 100 μl with 0·05 unit of enzyme (0·5 unit/ml), the sample was then chromatographed on Bio-Gel P4, and labelled fragments were detected by scintillation counting. One unit of enzyme activity was defined as described previously[14].

RESULTS

General properties of the oligosaccharide fractions. — (*a*) *Chromatography on Bio-Gel P4 and estimation of oligosaccharide size.* Several standard oligosaccharides were used to calibrate the Bio-Gel P4 column. Fig. 1 shows a graph of their elution volume against their size expressed as "hexose units". As defined by Yamashita *et al.*[15], a hexose residue behaves as 1 unit and an *N*-acetylhexosamine residue as 2 units, fucose behaves as 0·7 unit. With standard tetrasaccharides and higher saccharides, a straight-line correlation was obtained and used to estimate the number of hexose units in the unknown oligosaccharides. Thus, three sub-fractions (KE_2, LD_2, LE_2) of K and L of 11, 9–10, and 9 hexose units, respectively, had values which were in good agreement with those obtained by chromatography on the dextran-calibrated column of Bio-Gel P4 described in the preceding paper[3].

Therefore, the remaining sub-fractions (KC_1, KF_2, and KF_3a) were not re-chromatographed, and were regarded as having 10–12 hexose units. As the sub-fractions chromatographed as single peaks on Bio-Gel P4, they were considered to contain oligosaccharides having closely related compositions. The probable compositions of the oligosaccharide sub-fractions were deduced from the g.l.c. data and from the number of hexose units given on Bio-Gel-P4 chromatography.

The calibrated column of Bio-Gel P4 was also used to purify fragments released

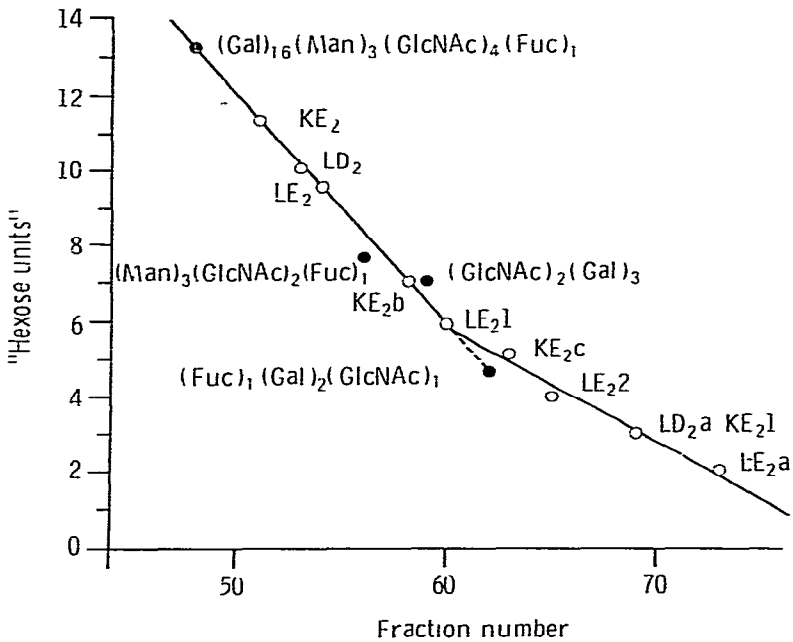


Fig 1 Chromatography of oligosaccharide fractions and their enzyme-degradation fragments on a calibrated column of Bio-Gel P4 ●, standard oligosaccharides, ○ oligosaccharide fractions and fragments characterised in the present studies (see Table I) The column was calibrated[15] in terms of hexose units" on Bio-Gel P4 a hexose residue chromatographs as 1 unit, *N*-acetylhexosamine as 2 units, and fucose as 0 7 unit

after digestion of the oligosaccharide fractions with various glycosidases Their elution volumes (expressed as fraction numbers) are shown in Fig 1 The larger fragments KE_2b and LE_2l (obtained by using endo-β-D-galactosidase with KE_2 and exo-β-D-galactosidase with LE_2, respectively) had elution volumes that fell on the calibration line between the standards with 4–13 hexose units Smaller enzyme-degradation fragments obtained from fractions LE_2, LD_2, and KE_2 had elution volumes that fell on a straight line with shallower slope These fractions were LE_2a, LD_2a, KE_2l, $LE_2 2$, and KE_2c, for which m s . g l c , and p c data (described below and summarised in Table I) suggested the compositions GalNAc-ol (LE_2a), Gal–GalNAc-ol (LD_2a and KE_2l), GlcNAc–GalNAc-ol ($LE_2 2$), and GlcNAc–Gal–GalNAc-ol (KE_2c)

(*b*) *P c. analysis of oligosaccharide composition* Labelled oligosaccharides and fragments released by enzyme digestion were chromatographed simultaneously with standards (di- to penta-saccharides) Estimation of the size of unknown oligosaccharides was made by using the following observations (*i*) the loss of one residue from an oligosaccharide approximately doubles the distance migrated from the origin; (*ii*) this distance is greater if the ratio of *N*-acetylhexosamine (or fucose) to hexose of a particular oligosaccharide is increased For example, a sample with a migration distance that is 1 3 times that of the standard trisaccharide Gal–GlcNAc–Gal-ol is

TABLE I

ELUTION PROFILES ON Bio-Gel P4 AND PROPOSED COMPOSITION OF LABELLED OLIGOSACCHARIDE FRAGMENTS RELEASED BY ENDO- OR EXO GLYCOSIDASE TREATMENT OF FRACTIONS LE_2, LD_2, AND KE_2

Designation	*Source of fragment*	*Bio-Gel P4 fraction number ("hexose units")*	*Proposed composition*	*Confirmatory evidence*
LE_2a[a]	Successive exo β-D galactosidase and exo-2 acetamido-2-deoxy-β-D glucosidase treatment of LE_2	73 (2)	GalNAc ol	Mass spectrum
LD_2a	Successive exo β-D galactosidase and exo-2 acetamido-2-deoxy-β-D glucosidase treatment of LD_2	69 (3)	Gal-GalNAc-ol	Glc
$KE_2$1	Main fragment obtained from exo 2 acetamido-2-deoxy-β-D glucosidase (containing some exo β D galactosidase) treatment of KE_2	69 (3)	Gal-GalNAc-ol	Co chromatography on paper and Biogel P4 with LD_2a
$LE_2$2[a]	Minor fragment obtained from exo β D galactosidase treatment of LE_2	65 (4)	GlcNAc-GalNAc-ol	Paper chromatography
KE_2c	Fragment from endo β D-galactosidase treatment of KE_2	63 (5)	(Gal)(GlcNAc)GalNAc-ol	Mass spectrum
$LE_2$1[a]	Major fragment from exo-β D-galactosidase treatment of LE_2	60 (6)	(GlcNAc)(GlcNAc)GalNAc-ol	Mass spectrum and Biogel-P4 calibration line (Fig 1)
KE_2b	Fragment from endo β D-galactosidase treatment of KE_2	58 (7)	(Gal)(GlcNAc)(GlcNAc)GalNAc ol	Paper chromatography and Biogel-P4 calibration line (Fig 1)

[a]Additional evidence for these fragments has been reported elsewhere[1] $LE_2$2 was considered to have been formed from $LE_2$1 by contaminant 2-acetamido-2-deoxy β D-glucosidase activity in the exo β-D-galactosidase preparation

TABLE II

THE PARTIALLY *O*-METHYLATED ALDITOL ACETATES[a] DERIVED FROM PERMETHYLATED OLIGOSACCHARIDE FRACTIONS

Fraction	*Alditol acetates detected (and their retention times relative to that of 2,3,4,6-tetra-O-methyl-D-galactose)*													
	O-*Methyl-Fuc*	O-*Methyl-Gal*							O-*Methyl GalNAc-ol*			O-*Methyl-GlcNAc*		
	2,3,4- (0.3)	*2,3,4,6- (1.0)*	*2,4,6- (1.5)*	*2,3,6- (1.6)*	*3,4,6- (1.6)*	*2,3,4- (2.1)*	*4,6- (2.2)*	*2,4- (3.2)*	*1,3,4,5,6- (1.4)*	*1,4,5,6- (2.7)*	*1,4,5 (5.1)*	*3,4,6- (4.2)*	*3,6- (5.8)*	*4,6- (6.5)*
KC_1	–	+++	+++	–	+	–	–	+++	–	+++	+++	+++	+++	–
KE_2	–	++	+++	+[b]	–	–	–	–	–	+++	+++	+++	+++	–
KE_2c	–	–	±	–	–	–	–	–	–	+++	–	±	–	–
KF_2	+	+++	+++	+	+	±	–	–	–	–	+	+++	+++	–
KF_2a	+++	++	+++	–	+++	–	+++	–	–	–	+++	+	+++	–
LD_2	–	+++	+++	–	–	–	–	–	–	–	+++	–	+++	–
LE_2	–	+++	+++	–	+++	+	–	–	–	–	+++	–	+++	–
LE_2l	–	–	–	–	–	–	–	–	–	–	+++	+++	–	–
LE_2a	–	–	–	–	–	–	–	–	+++	–	–	–	–	–

[a]Analysis by g.l.c.–m.s., using a column of 3% of OV-225, programmed from 150→230° at 1°/min, m.s. conditions as described in Table III. [b]This alditol acetate was unequivocally demonstrated in component KE_2a rather than the original KE_2. 1,4,5-Tri-*O*-methyl GalNAc-ol was the only other alditol acetate unequivocally demonstrated in KE_2a.

TABLE III

MAJOR FRAGMENTS DETECTED BY DIRECT-PROBE MASS SPECTROMETRY OF PERMETHYLATED OLIGOSACCHARIDE FRACTIONS[a]

Fraction	*Fragment ions* (m/z)[b]													
	219	*260*	*423*	*464*	*521*	*669 (668 + 1)*	*710 (709 + 1)*	*639 (638 + 1)*	*843 (842 + 1)*	*884 (883 + 1)*	*726 (725 + 1)*	*930 (929 + 1)*	*914 (913 + 1)*	*900 (898 + 2)*
KC_1	+	+[c]	−	+	−	+	+	±	−	−	+	+	+	+
KE_2	−	+	−	+	−	+	+	−	−	−	−	−	−	−
$K\Gamma_2$	+	+[c]	−	+	−	+	−	+	+	+	+	−	+	−
KF_3a	−	+	−	+	−	+	+	+	−	+	+	−	−	−
LD_2	+	+	−	+	−	+	−	+	−	−	+	+	+	−
LE_2	+	+	+	+	−	+	−	−	−	−	+	+	−	−
LE_2I	−	+	−	−	+	−	−	−	−	−	−	−	−	−

[a]Analysis by a Finnigan 3300 mass spectrometer with 6100 data-system, mass range, 100–1000 a.m.u., electron energy, 70 eV, ion energy, 3 eV, extractor, 3.5 V, lens, 16 V, emission, 0.5 mA, electron multiplier, 1800 V. [b]Key: +, detected, −, not detected, ±, the intensity of these fragments was low and related "daughter" fragments [*e.g.*, those formed by loss of MeOH (−32)] were lacking, the structures represented by the fragments are shown in Fig. 2 and in the text. [c]The intensity of *m/z* 260 was greater than that of *m/z* 219, indicating that both hexose and *N*-acetylhexosamine were non-reducing, terminal residues. In fractions LE_2 and LD_2, the intensities of these fragment ions were comparable, suggesting that hexose rather than *N*-acetylhexosamine was the non-reducing, terminal residue.

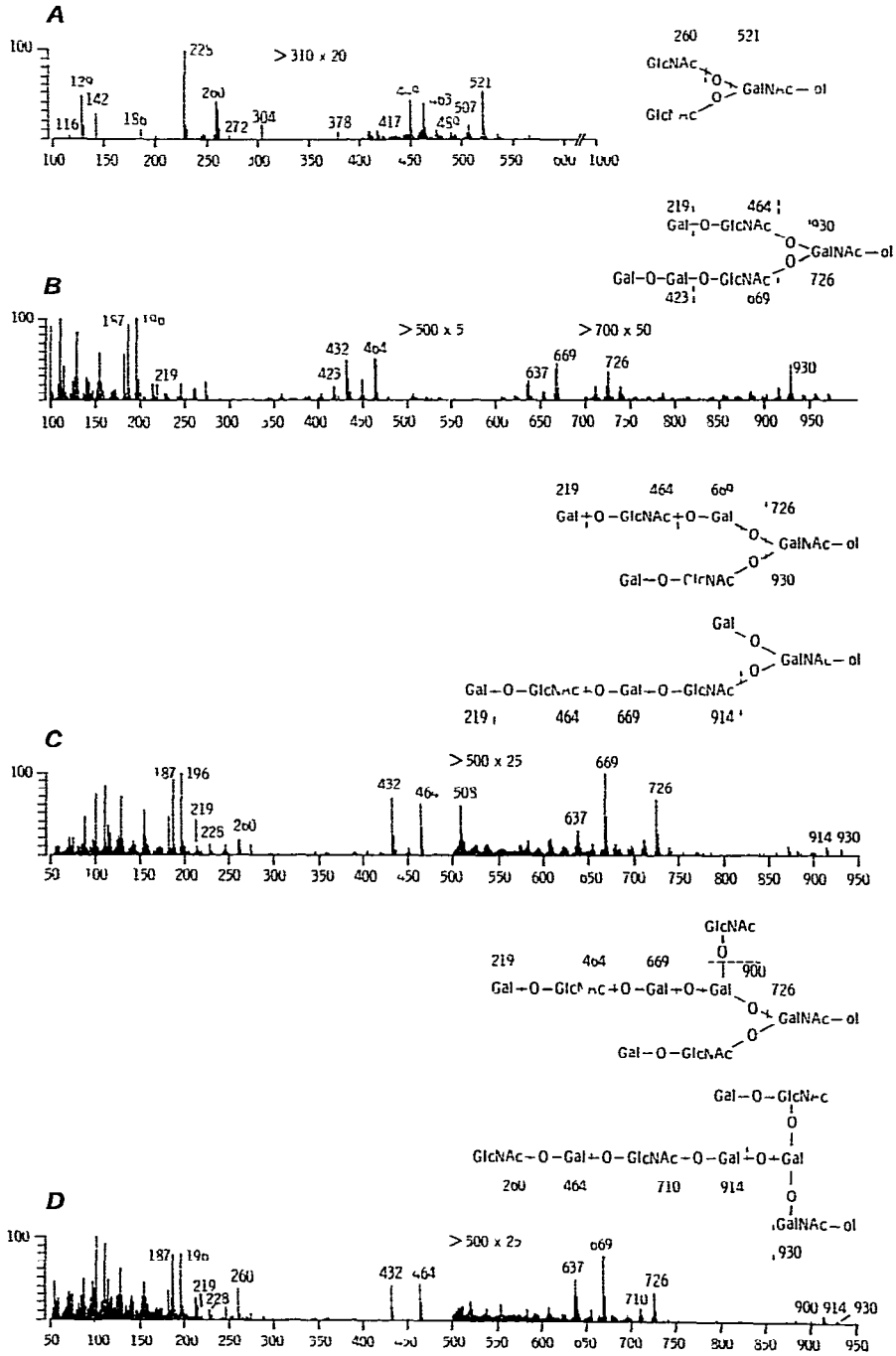
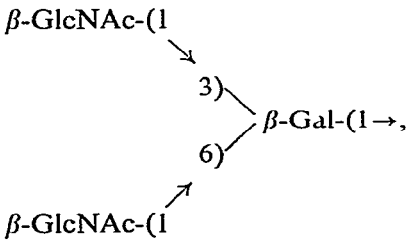


Fig 2 Direct-insertion mass spectra of permethylated oligosaccharides *A*, $LE_2$1, *B*, LE_2, *C*, LD_2, and *D*, KC_1 Spectra *A* and *B* are reproduced from ref 1 with permission

likely to be a fast-running trisaccharide having a higher HexNAc/Hex ratio than the standard, *e g*, with composition $(HexNAc)_1(Hex)_1HexNAc$-ol the HexNAc-ol being the reduced and labelled component detected on radiochromatogram scanning

(*c*) *Methylation analysis* Table II shows the partially *O*-methylated alditol acetates obtained from permethylated oligosaccharide fractions, identified by their retention times in g l c and by their mass spectra Table III shows the major fragments found by direct-insertion m s of the permethylated oligosaccharides The interpretation of the characteristic fragment-ions, shown in the text and in F,g 2, were based on those given by Karlsson *et al* [16] and Watanabe *et al* [17]

(*d*) *Digestion with endo-β-D-galactosidase* The endo-β-D-galactosidase hydrolyses[7] the lactoglycosyl series having the common structure R→β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc(or GlcNAc), where β-Gal-(1→4)-Glc(or GlcNAc) is the susceptible linkage In the erythrocyte glycosphingolipid H_2, α-Fuc-(1→2)-β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-Glc-(1→Cer the middle β-Gal-(1→4) linkage was the most susceptible, being split with 0 125 unit/ml of enzyme, whereas the β-Gal-(1→4)-Glc linkage was only cleaved at 1 25 unit/ml Furthermore, the R→β-Gal-(1→4) linkage was resistant to digestion unless R was GlcNAc attached to O-3 or O-6 of Gal If two GlcNAc residues were attached at positions 3 and 6, as in



digestion occurred with 1 25, but not with 0 125, unit/ml of enzyme

It was considered that 125 munit/ml of endo-β-D-galactosidase (1× enzyme concentration) would cleave similar linkages in the oligosaccharide fractions, in addition, 10× enzyme concentration (1 25 unit/ml) was used to ensure that all susceptible linkages were digested This specificity, together with indications from Bio-Gel-P4 and paper chromatography on the composition of labelled fragments released by the enzyme, enabled predictions to be made on the structures of the core regions of digestible fractions Fractions LE_2, KF_3a, and KC_1 were not digested by 1× or 10× enzyme concentrations Fractions LD_2, KE_2, and KF_2 were partially digested by 1× enzyme concentration and, to the same extent, by 10× enzyme concentration, suggesting that only part of these fractions had digestible linkages

Oligosaccharide structures — (*a*) *Fraction* LE_2 Structural data on LE_2 and the fragments, LE_21, LE_22, and LE_2a (see Table I), formed on digestion with exoglycosidases, have been reported[1] The Bio-Gel-P4 profiles (Fig 1) and data for partially-*O*-methylated alditol acetates (Table II) are shown for comparison with those of the other oligosaccharide fractions The mass spectra of permethylated LE_2

and $LE_2$1 are included in Fig 2 and the characteristic fragments found are included in Table III.

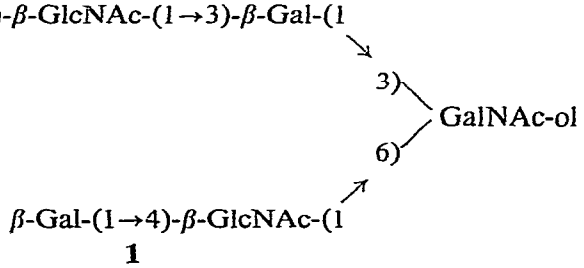
(*b*) *Fraction* LD_2 This fraction chromatographed as a broad peak on Bio-Gel P4, eluting in a position corresponding to 9–10 hexose units (Fig 1), and was estimated to contain hexasaccharide(s) with composition[1] Gal–GlcNAc–GalNAc-ol 3 2 1 Consecutive digestion with the Jack-bean meal exo-glycosidases (β-D-galactosidase followed by 2-acetamido-2-deoxy-β-D-glucosidase) gave a single product, LD_2a (Fig 1, Table I), the composition of which was confirmed as Gal–GalNAc-ol 1 1 by g l c

Digestion of LD_2 with exo-β-D-galactosidase (Jack-bean meal) gave three labelled products detected by p c (solvent *A*) accounting for ~30%, ~30%, and ~40%, respectively, of the radioactivity The first product behaved as a pentasaccharide (1 6× relative to the original hexasaccharide LD_2, and 0 4× relative to standard trisaccharide Gal–GlcNAc–Gal-ol), corresponding to loss of one galactose residue· the second behaved as a fast-running tetrasaccharide (3 7× relative to original hexasaccharide LD_2, and 0 9× relative to standard Gal–GlcNAc–Gal-ol), corresponding to loss of two galactose residues, and the third was considered to be the product of digestion with β-D-galactosidase and contaminant 2-acetamido-2-deoxy-β-D-glucosidase, because it chromatographed close to the standard GlcNAc–Gal-ol (0 9× relative to standard GlcNAc–Gal-ol, and 2 0× relative to standard Gal–GlcNAc–Gal-ol) This product is suggested to be the core oligosaccharide Gal-(1→3)-GalNAc-ol, the β-(1→3) linkage is proposed, because it is difficult to cleave by Jack-bean galactosidase[13] The isolation of this disaccharide, and not the trisaccharide $LE_2$1 or disaccharide $LE_2$2 obtained by similar treatment of LE_2 (Table I Fig 1), suggested that LE_2 and LD_2 had different core-region structures

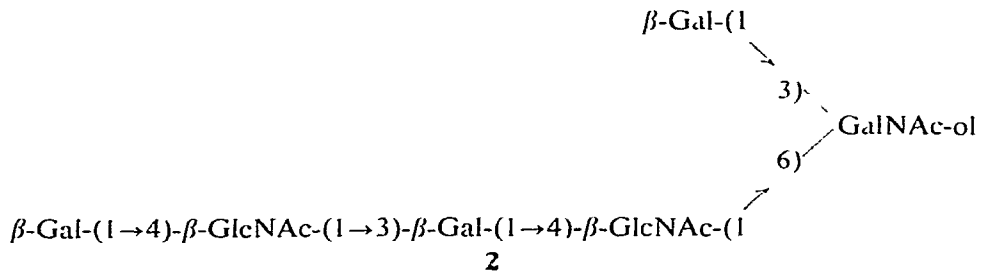
Fraction LD_2 was not digested by 2-acetamido-2-deoxy-β-D-glucosidase from *T cornutus* indicating that Gal was the only terminal, non-reducing residue

Analysis of the partially *O*-methylated alditol acetates derived from LD_2 (Table II) revealed 2,3,4,6-tetra-*O*-methyl- and 2,4,6-tri-*O*-methyl-galactose, 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol, and 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, confirming that galactose was the only non-reducing, terminal residue and indicating that (*i*) internal, 3-substituted galactose residues were also present, (*ii*) the GalNAc-ol had substituents at both positions 3 and 6, and (*iii*) the GlcNAc residues were linked at position 4

From the composition, the exo-glycosidase data, and the partially *O*-methylated alditol acetates derived from permethylated LD_2, two possible isomeric structures (**1** and **2**) can be proposed



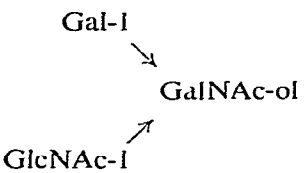
1



2

There was evidence for the presence of both structures from m s of permethylated LD_2 (Fig 2*C*) The three largest fragments (with *m/z* 726 930, and 914) could not be obtained from a single oligosaccharide having the composition proposed for LD_2 therefore, more than one component was present

Structure **1** is suggested to be the main component of LD_2 as ~70% of this fraction was resistant to digestion by the endo-β-D-galactosidase and was therefore lacking the susceptible, internal β-Gal-(1→4)-β-GlcNAc-sequence Digestion at 1× and 10× concentration of the endo-β-D-galactosidase gave a product accounting for ~30% of the labelled GalNAc-ol, with mobility in p c (solvent *A*) 1 4× relative to that of standard Gal–GlcNAc–Gal-ol This migration is compatible with the product being the fast-running trisaccharide



Thus, it can be deduced that ~30% of LD_2 has an oligosaccharide sequence with an internal β-Gal-(1→4)-β-GlcNAc linkage, as shown in the long chain of structure **2**, which gives the permethylated fragment *m/z* 914, and that ~70% of LD_2 has the structure **1**

(*c*) *Fraction KF_3a* This fraction was estimated from g l c to have the composition Gal–GlcNAc–GalNAc–Fuc–GalNAc-ol 2–3 2 1 1 1, consistent with elution from dextran-calibrated Bio-Gel P4 in a position corresponding to 10–12 hexose units

Analysis of the partially *O*-methylated alditol acetates derived from permethylated KF_3a (Table II) gave 2,3,4-tri-*O*-methylfucose, 4,6-di-*O*-methyl-, 2,4,6-tri-*O*-methyl-, and 3,4,6-tri-*O*-methyl-galactose, 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol, and 2-deoxy-3,4,6-tri-*O*-methyl- and 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, indicating the presence of (*i*) terminal fucosyl groups, (*ii*) internal 2-, 3-, and 2,3-substituted galactosyl residues, (*iii*) 3,6-substituted GalNAc-ol, and (*iv*) non-reducing, terminal and internal, 4-substituted GlcNAc residues The major fragments detected by m s of permethylated KF_3a (Table III) can therefore be interpreted as follows

	m/z
HexNAc→	260
HexNAc→Gal→	464
HexNAc→Gal→GalNAc-ol←	726
HexNAc→Gal→ ↑ Fuc	639
HexNAc→Gal→HexNAc→ ↑ Fuc	884
HexNAc→Gal→HexNAc→	710
HexNAc→Gal→Gal→	669

On account of the blood-group A activity[18] of the parent fraction K, it is likely that the GalNAc residue detected by g.l.c. in KF_3a is the terminal, non-reducing residue linked to the fucosylated galactose as follows.

α-GalNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→
↑2
|
1
α-Fuc

The following structure can be proposed as the major component of KF_3a:

β-GlcNAc-(1→3)-β-Gal-(1
↘
3/6)-GalNAc-ol.
↗
α-GalNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1
↑2
|
1
α-Fuc

The relatively low abundance of permethylated fragments with *m/z* 710 and 669 (compared to those of *m/z* 726, 639, and 884) suggested that they were obtained from a minor component of KF_3a having the one chain lacking in fucose [GalNAc-(1→3)-Gal-(1→4)-GlcNAc-(1→] and the other having an additional galactose [GlcNAc-(1→2/3)-Gal-(1→3/2)-Gal-(1→], respectively. The presence of such a minor component would account for the high galactose ratio and for the small proportion of 2-substituted-galactose residues (alditol acetate of 3,4,6-tri-*O*-methylgalactose; Table III).

Digestion with exo-glycosidase was not performed, on account of the limited amount of this fraction available; but, consistent with the proposed structures, this fraction was resistant to digestion by the endo-β-D-galactosidase. From the known specificity of this enzyme, neither of the sequences

α-GalNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→
↑2
|1
α-Fuc
or α-GalNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→

have digestible linkages. If the terminal residue on the longer chain were GlcNAc, the small proportion of unfucosylated GlcNAc-(1→3)-Gal-(1→4)-GlcNAc, which gave the permethylated fragment with *m/z* 710, would have been digested.

(*d*) *Fraction* KC_1. This fraction was estimated from g.l.c. and the elution profile on dextran-calibrated Bio-Gel P4 to be an octasaccharide with composition Gal–GlcNAc–GalNAc-ol 4:3:1.

Analysis of the partially *O*-methylated alditol acetates derived from permethylated KC_1 (Table II) revealed 2,3,4,6-tetra-*O*-methyl-, 3,4,6-tri-*O*-methyl-, 2,4,6-tri-*O*-methyl-, and 2,4-di-*O*-methyl-galactose, 2-deoxy-3,4,6-tri-*O*-methyl- and 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, and 2-deoxy-1,4,5,6-tetra-*O*-methyl- and 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol, indicating the presence of (*i*) internal 2-, 3-, and 3,6-substituted Gal residues, (*ii*) non-reducing, terminal Gal and GlcNAc residues, (*iii*) additional, 4-substituted GlcNAc residues, and (*iv*) 3- and 3,6-substituted GalNAc-ol residues.

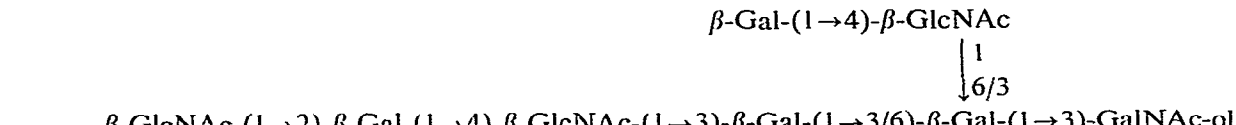
Treatment of KC_1 with the exo-β-D-galactosidase from *C. lampas* and the exo-2-acetamido-2-deoxy-β-D-glucosidase from *T. cornutus*, with p.c. (solvent *B*) of the digests, revealed two major products from the former which migrated 2.5× and 5.8× relative to the original material, and one product from the latter which migrated 2.0× relative to original material. These results were consistent with the loss of one Gal, two Gal, and one GlcNAc, respectively.

Consecutive digestion with Jack-bean meal exo-β-D-galactosidase and exo-β-acetamido-2-deoxy-β-D-glucosidase gave several products, the fastest migrating of which co-chromatographed on p.c. (solvent *A*) with LD_2a and which was assigned the structure β-Gal-(1→3)-GalNAc-ol.

Fraction KC_1 was resistant to endo-β-D-galactosidase. The mass spectrum of permethylated KC_1 is shown in Fig. 2D, with an interpretation of the fragments found. All of the data are consistent with the following structure for the major component of KC_1:

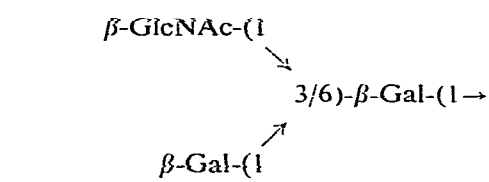
β-GlcNAc
|1
↓6/3
β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→3/6)-β-Gal-(1
3)↘
GalNAc-ol.
6)↗
β-Gal-(1→4)-β-GlcNAc-(1

This structure does not account for the permethylated fragments with *m/z* 710, 914, and 930, or the presence of 2-substituted Gal and 3-substituted GalNAc-ol in KC_1. These would be accounted for by the following structure



This latter structure has an internal β-Gal-(1→4)-GlcNAc linkage, but the 2-substitution of the Gal residue by GlcNAc could account for the resistance of KC_1 to endo-β-D-galactosidase. No evidence was found by mass spectrometry for the presence of Fuc residues or Gal substituted at position 4 in KC_1 (Tables II and III) and, therefore the non-reducing, terminal linkage GlcNAc-(1→2)-Gal-(1→ is proposed. This linkage has recently been reported in hog gastric-mucin[19].

The unusual branched structure



is also proposed in both isomers of KC_1, particularly to account for the identification by m.s. of permethylated fragments with *m/z* 669, 710, and 914 (Fig. 2*D*). The carbohydrate chain of the major component of KC_1, giving the fragment *m/z* 669, could not have the alternative sequence Gal-1→Gal-1→GlcNAc-1→, as this would be inconsistent with the products of exo-galactosidase action (no fragment with a migration compatible with that of a fast-running pentasaccharide was obtained); the alternative sequences giving *m/z* 914 (Gal-1→GlcNAc-1→Gal-1→GlcNAc-1→ or Gal-1→Gal-1→GlcNAc-1→GlcNAc-1→) are not present in the minor component of KC_1, as these do not give rise to the fragment *m/z* 710.

(*e*) *Fraction* KE_2. This fraction was eluted from calibrated Bio-Gel-P4 in a broad peak corresponding to 11 hexose units (Fig. 1), and was estimated by g.l.c. to contain heptasaccharides having the composition Gal–GlcNAc–GalNAc-ol 3 3 1.

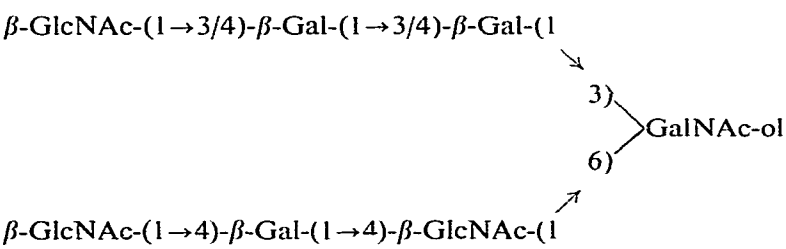
Analysis of the partially *O*-methylated alditol acetates derived from KE_2 (Table III) revealed 2-deoxy-3,4,6-tri-*O*-methyl- and 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, 2,4,6-tri-*O*-methyl- and 2,3,6-tri-*O*-methyl-galactose, and 2-deoxy-1,4,5,6-tetra-*O*-methyl- and 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol, indicating that (*i*) GlcNAc was the only non-reducing, terminal sugar; (*ii*) internal, 4-substituted GlcNAc residues were also present, (*iii*) Gal residues were 3- or 4-substituted; (*iv*) two types of GalNAc-ol were present, 3- and 3,6-substituted. The characteristic fragments found on mass spectrometry of permethylated KE_2 (Table III), with *m/z* 260, 464, 669, and 710, could therefore be interpreted

as the sequences GlcNAc→, GlcNAc→Gal→, GlcNAc→Gal→Gal→, and GlcNAc→Gal→GlcNAc→, respectively

Treatment of KE_2 with the 2-acetamido-2-deoxy-β-D-glucosidase preparation from Jack-bean meal (containing some contaminant β-D-galactosidase) gave several products, one of which ($KE_2$1) co-chromatographed on Bio-Gel P4 (Table I) and paper with LD_2a (see above), which was assigned the structure β-Gal-(1→3)-GalNAc-ol This linkage is, therefore, proposed for KE_2

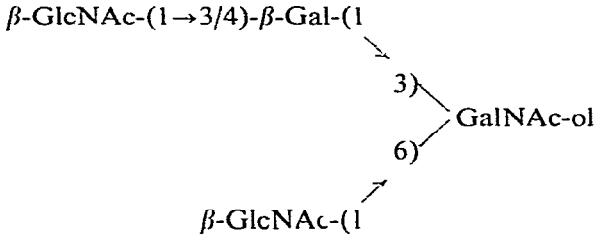
Of fraction KE_2, ~30% was digested by 1× and 10× concentrations of endo-β-D-galactosidase, chromatography of the digest on calibrated Bio-Gel-P4 gave three labelled peaks KE_2a, which co-chromatographed with the original material and contained ~70% of the radioactivity, and two labelled fragments, KE_2b and KE_2c

The analysis of the partially *O*-methylated alditol acetates derived from methylated, non-digestible material KE_2a (Table II) indicated that it contained some 4-substituted Gal and that the GalNAc-ol residue was 3,6-substituted Together with the data given above for the parent fraction the following structure can be proposed for KE_2a, the major component of fraction KE_2.

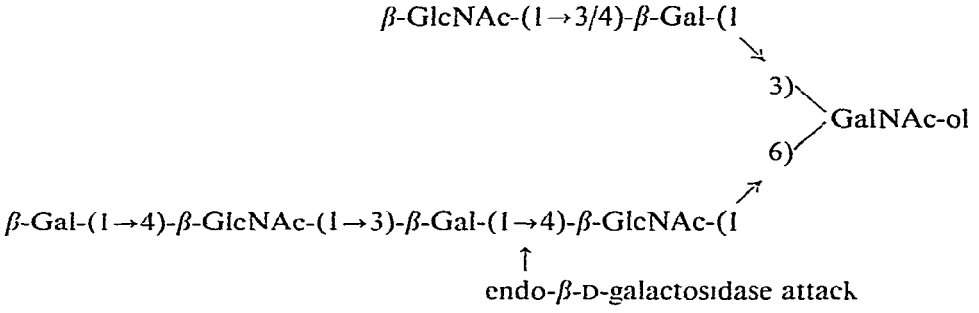


In the GlcNAc–Gal–GlcNAc chain of this structure, the substitution of the galactosyl residue at O-4, rather than at O-3, by the GlcNAc residue could account for the resistance of KE_2a to endo-β-D-galactosidase In the GlcNAc–Gal–Gal chain, the galactosyl residues could be 3- or 4-substituted This chain is similar to that found in KC_1, in the absence of the branch (GlcNAc-1→ or Gal-1→GlcNAc-1→) at galactose

From analysis of the original fraction KE_2 and of the labelled fragments KE_2b and KE_2c, obtained from the reduced and tritiated end of the molecule after digestion with endo-β-D-galactosidase, structures could be proposed for two minor components Fragment KE_2b migrated on calibrated Bio-Gel P4 in a position corresponding to 7 hexose units (Fig 1, Table I), and in p c (solvent *A*) as a fast-running tetrasaccharide (1 1× relative to standard Fuc–Gal–GlcNAc–Gal-ol and 0 8× relative to standard Gal–GlcNAc–Gal-ol) Fragment KE_2b was, therefore, thought to have the composition $(GlcNAc)_2(Gal)_1GalNAc$-ol From the known specificity of the endo-β-D-galactosidase and the data from analysis of KE_2 reported above, the following was considered the most likely structure for KE_2b

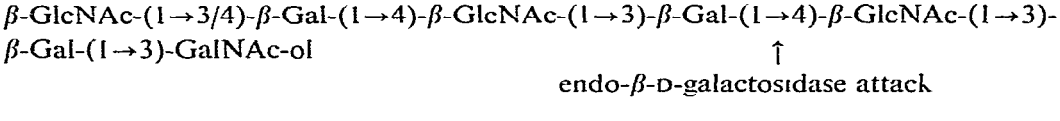


This would be derived from such a structure as the following, which has the enzyme-susceptible sequence and the composition and linkages proposed for KE_2



The second fragment (KE_2c) migrated on Bio-Gel P4 in a position corresponding to 5 hexose units (Fig 1 and Table I), and in p c (solvent *A*) as a fast-running trisaccharide (1 8× relative to standard Gal–GlcNAc–Gal-ol and 0 8× relative to standard GlcNAc–Gal-ol) Analysis of the partially *O*-methylated alditol acetates derived from KE_2c (Table II) showed 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)glucose, 2,4,6-tri-*O*-methyl-galactose, and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)galactitol, consistent with the following structure for KE_2c GlcNAc-(1→3)-Gal-(1→3)-GalNAc-ol

This would be derived from such a structure as the following, which has the enzyme-susceptible sequence and the composition and linkages proposed for KE_2



(*f*) *Fraction* KF_2 This fraction was estimated by g l c and by chromatography on dextran-calibrated Bio-Gel P4 to have the composition Gal–GlcNAc–GalNAc-ol 3 3 1 with a small proportion of fucose

The analysis of the partially *O*-methylated alditol acetates derived from KF_2 (Table II) revealed 2,3,4-tri-*O*-methyl-fucose, 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and 3,4,6-tri-*O*-methyl-galactose, 2-deoxy-3,4,6-tri-*O*-methyl- and 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, and 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol, indicating the presence of (*i*) non-reducing, terminal Fuc, Gal, and GlcNAc residues, (*ii*) internal Gal residues

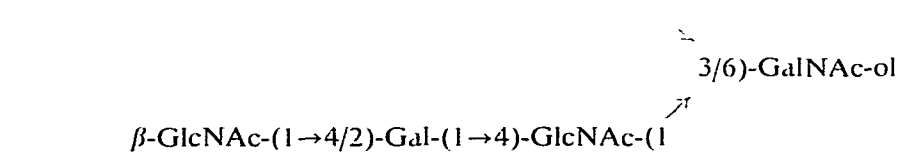
linked at positions 3, 4, or 2, (*iii*) a 3,6-substituted GalNAc-ol residue With these data, the major fragments detected on mass spectrometry of permethylated KF_2 (Table III) could be interpreted as follows

	m/z
Gal→	219
GlcNAc→	260
Gal→GlcNAc→ and GlcNAc→Gal→	464
Gal→GlcNAc→Gal→	669
GlcNAc→Gal→GlcNAc→	710
GlcNAc→Gal→GalNAc-ol←	726
Fuc→Gal→GlcNAc→	639
Fuc→Gal→GlcNAc→Gal→	843

The presence of terminal Fuc, Gal, and GlcNAc was further suggested by treatment of KF_2 with specific exo-glycosidases and analysis by p c (solvent *B*) On treatment with *C lampas* exo-β-D-galactosidase, only a part of the fraction was digestible, giving one or more products chromatographing as a broad peak 2× relative to original (and undigested) material, and 0 9× relative to standard pentasaccharide $(Gal)_2(GlcNAc)_2$Gal-ol This result is consistent with the formation of fast-running hexasaccharide(s) with composition $(GlcNAc)_3(Gal)_2$GalNAc-ol by loss of one galactose residue from the non-fucosylated components and no digestion of fucosylated components Treatment of KF_2 with the 2-acetamido-2-deoxy-β-D-glucosidase from *T cornutus* gave a major product which migrated 1 5× relative to the original material, and 0 75× relative to standard pentasaccharide $(Gal)_2(GlcNAc)_2$ Gal-ol This result is consistent with the loss of one GlcNAc residue, to give a slower running hexasaccharide than that obtained by digestion with the *C lampas* β-D-galactosidase

Of fraction KF_2, ~30% was digested by 1× and 10× concentration of the endo-β-D-galactosidase Analysis of the digests by p c gave three labelled peaks KF_2a which co-chromatographed with the original material and accounted for ~70% of the radioactivity, and two labelled fragments KF_2b and KF_2c

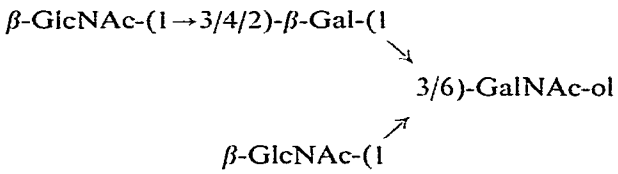
The following structure is proposed for KF_2a the major component of KF_2



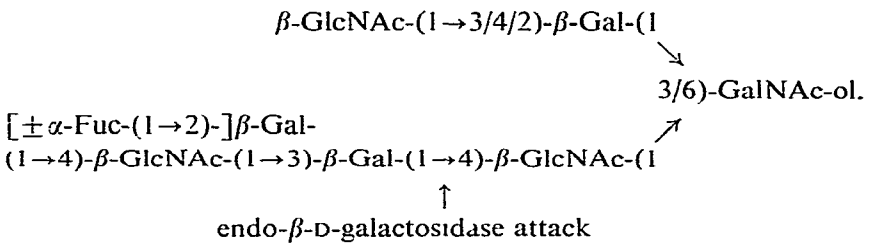
This structure would give all the major permethylated fragments (except *m/z* 726, which was obtained in relatively small amounts) and the partially *O*-methylated alditol acetates obtained from permethylated KF_2 It is proposed that, in the GlcNAc–Gal–GlcNAc chain, the galactosyl residue is 2- or 4-substituted, as this could account for the resistance of this oligosaccharide to endo-β-D-galactosidase From the methyla-

tion-analysis data, the galactosyl residues in the Fuc–Gal–GlcNAc–Gal- chain could be 2-, 4-, or 3-substituted but, on account of the known blood-group H activity in the original glycoprotein, the α-Fuc-(1→2)-Gal linkage is likely and the remaining galactose would be the (1→3)-linked residue detected

From analysis of the original fraction KF_2 and of the labelled fragments KF_2b and KF_2c, obtained from the reduced and tritiated end of the molecule after digestion with endo-β-D-galactosidase, structures could be proposed for two minor components Fragment KF_2b co-chromatographed with KE_2b obtained by treatment of KE_2 with endo-β-D-galactosidase and was therefore suggested to have the structure

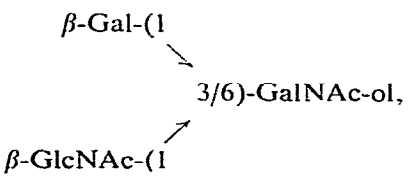


This would be derived from such a structure as the following, which has the enzyme-susceptible sequence and the composition and linkages proposed for KF_2

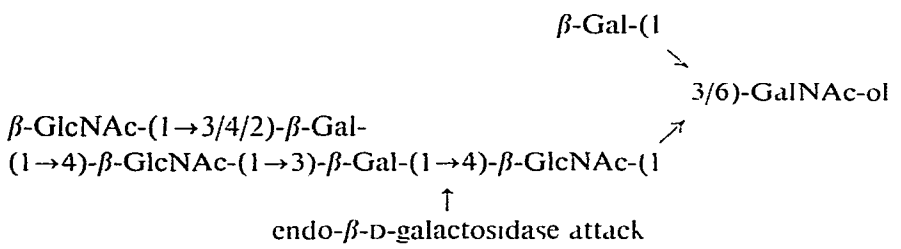


This structure has the sequence giving the permethylated fragment with *m/z* 726 detected in fraction KE_2 (Table III), and only one terminal β-GlcNAc residue and one possible, terminal β-Gal residue which were released on treatment with the specific exo-β-D-glycosidases

Fragment KF_2c co-chromatographed with the fragment obtained by digestion of fraction LD_2 (isomer **2**, see above) with endo-β-D-galactosidase and was therefore considered to have the structure



this would be obtained by digestion of a third component of fraction KF_2 with the structure



DISCUSSION

In these studies, considerable structural information was obtained on oligosaccharide fractions which were available only in 50–500-μg amounts This was made possible by the use of calibrated columns of Bio-Gel P4 for estimation of the size and composition of oligosaccharides and their enzyme-degradation products This information was supplemented by applying calibration rules to p c The presence of tritium-labelled, reduced, terminal GalNAc-ol enabled sensitive detection of the oligosaccharides Also, important structural data were deduced by the use of an endo-β-D-galactosidase known to cleave specifically certain internal β-D-galactosyl linkages[7], and extensive use was made of direct-insertion m s of permethylated oligosaccharides to indicate the number and size of branches present These techniques were used in conjunction with the more classical methods of exo-glycosidase digestion and g l c –m s of partially *O*-methylated alditol acetates derived from permethylated oligosaccharides

Of the 12 oligosaccharide structures proposed, all had GalNAc-ol at the reduced end In the majority of the oligosaccharides, this residue was shown to be 3 6-disubstituted, forming a branched-core region. in two oligosaccharides, the core region was unbranched with GalNAc-ol substituted at position 3 only The mass spectrum obtained for the partially-*O*-methylated alditol acetate of the disubstituted GalNAc-ol confirmed that reported by Wrann and Tuppy[19] for 3.6-di-*O*-acetyl-2-deoxy-1,4.5-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol The appropriate spectrum for 2-deoxy-1,3,4,5.6-penta-*O*-methyl-2-(*N*-methylacetamido)galactitol was also obtained on analysis of the fragment LE_2a which was the product of sequential digestion of fraction LE_2 by exo-β-D-galactosidase and exo-2-acetamido-2-deoxy-β-D-glucosidase (Tables I and II) 1,3,6-tri-*O*-acetyl-2-deoxy-4,5-di-*O*-methyl-, 1,6-di-*O*-acetyl-2-deoxy-3,4,5-tri-*O*-methyl-, or 1-*O*-acetyl-2-deoxy-3,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-galactitol, which would be derived from *O*-demethylation of the terminal GalNAc-ol residue, were not detected *O*-Demethylation or *N*-demethylation has been reported to occur during the preparation of partially-*O*-methylated alditol acetates from permethylated oligosaccharides[20–22]

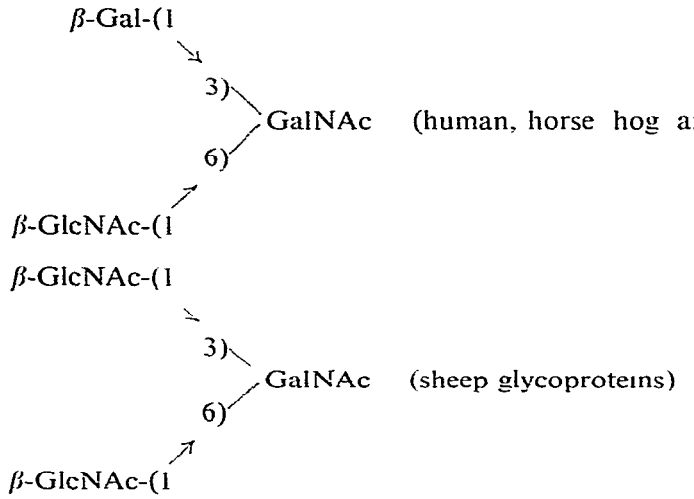
From our studies of sheep gastric-mucins and work from other laboratories on human ovarian-cyst glycoproteins[23,24] and the gastric mucins of man[25], horse[26], and hog[27], some generalisations can be made regarding the structures of oligo-

saccharide chains linked *via* GalNAc-*O*-serine/threonine to protein The oligosaccharides have core and backbone sequences as follows

Unbranched cores

β-Gal-(1→3)-GalNAc (human horse hog, and sheep glycoproteins)
β-GlcNAc-(1→3)-GalNAc (horse gastric-mucin)

Branched cores



(human, horse hog and sheep glycoproteins)

(sheep glycoproteins)

To these may be added the disaccharide sequence α-GalNAc-(1→3)-GalNAc isolated from pig[28] and human ovarian-cyst[24] blood-group substances However, this sequence was not substituted further and may represent a chain-termination point rather than an oligosaccharide-core structure

Backbone structures

β-Gal-(1→3)-β-GlcNAc-(1→3)
β-Gal-(1→4)-β-GlcNAc-(1→3/or 6)

These are commonly known as 'type 1' and 'type 2' precursor-chain sequences, respectively[29] In ovarian-cyst and gastric glycoproteins of man it appears that the preponderant linear-backbone sequences are of type 1 and that (1→4,1→6)-linked, type 2 chains occur at branch points. In the gastric mucins of hog, horse and sheep, the preponderant sequences detected have been of type 2 joined by (1→3) linkages in linear chains and (1→6) linkages at branch points Evidence has been presented for the presence of some type 1 chains in hog gastric-mucin[30 31] As in glycosphingolipids of erythrocyte membranes[17], the (1→4.1→6)-linkage sequence has not been detected in the absence of branching, suggesting that the (1→3,1→3) and (1→4, 1→3) linkages are the preferred (or the first synthesised) sequences

Branched and linear chain sequences of type 2 are antigenic determinants of the blood-group I and i antigens, respectively[17 32 33] In the present studies, the individual purified oligosaccharides could not be tested for antigenic activities on

account of the limited amounts available However, the parent fractions K and L inhibited the anti-I antibodies Ma and Step and the anti-i antibody Den in radioimmunoassays, and sub-fraction LE (of which LE_2 was the major component) was a potent inhibitor of anti-I Ma[3] Anti-I antibody Ma specifically reacts[34-36] with the sequence β-Gal-(1→4)-β-GlcNAc-(1→6)- and substitution of the terminal galactose with α-Fuc-(1→2) blocks this reactivity[33 34], whereas substitution with α-Gal-(1→3) or α-sialic acid-(2→3) does not[17 32] The following structure proposed for LE_2 is consistent with reactivity with anti-I Ma

β-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1 ↘
3/6)-GalNAc-ol
β-Gal-(1→4)-β-GlcNAc-(1 ↗

However it is not known whether the non-reducing, terminal β-Gal-(1→3) substitution would block reactivity with anti-I Ma Thus, it is not possible, from the inhibitory activity of fraction LE, to deduce whether the galactose is on the β-Gal-(1→4)-β-GlcNAc-(1→6) or the β-Gal-(1→4)-β-GlcNAc-(1→3) chain

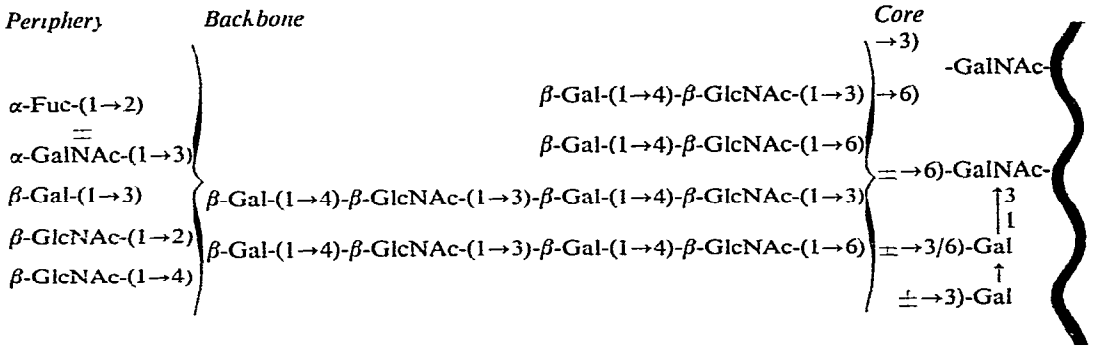
An unsubstituted Ma-specific sequence was also found in fraction LD_2 (isomer **1**) and proposed in fraction KC_1 Fraction LD_2, which was the second, major component of fraction L, contains, in addition, the sequences β-Gal-(1→4)-β-GlcNAc-(1→3)-Gal- (LD_2, isomer **1**) and β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-GlcNAc- (LD_2, isomer **2**) These sequences are involved in the antigenic determinants recognised by anti-I antibodies, other than those of 'Ma type', and the majority of anti-i antibodies[17 32 33], and would account for the antigenic activity of fraction L with anti-I Step and anti-i Den[3]

There was no evidence for the presence of type 1 sequences in any of the oligosaccharides of the present studies It will be of interest to determine whether the lack of type 1 chains is related to the selection by affinity chromatography of glycoproteins having blood-group Ii activities or whether the gastric mucins of sheep differ from human mucins in having exclusively oligosaccharides with type 2 sequences

In certain of the oligosaccharide fractions, there was evidence for substitution of the terminal galactose of the backbone structure with blood-group-H-associated α-Fuc-(1→2) (fraction KF_2) or blood-group-A-associated α-Fuc-(1→2) and α-GalNAc-(1→3) (fraction KF_3a) In other fractions, there was evidence for substitution with β-GlcNAc-(1→4), β-GlcNAc-(1→2), or β-Gal-(1→3) of unknown antigenic specificities The terminal, non-reducing sequence α-GlcNAc-(1→4)-Gal has been found in human ovarian-cyst glycoproteins[24] and hog gastric-mucin[27], and terminal, non-reducing GlcNAc-(1→2)-Gal has been reported previously in hog gastric-mucin[19] It will be of interest to investigate to what extent GlcNAc-(1→2) and GlcNAc-(1→4) substitutions (with α or β configuration) mask the reactivities with various anti-I and anti-i antibodies

From our studies of hexa- to octa-saccharides of sheep gastric-mucins having

blood-group I activities, the following structural model can be proposed, which consists of (*a*) a core region, (*b*) a backbone region having (1→3)- and (1→6)-linked *N*-acetyl-lactosamine [β-Gal-(1→4)-GlcNAc] branches with I activities, and linear, repeating, (1→3)-linked *N*-acetyl-lactosamine units with i activities, and (*c*) a peripheral region with blood-group isotype activities



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