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DETERMINATION OF THE STRUCTURE OF A FUCOSE-CONTAINING TRISACCHARIDE MONOPHOSPHATE ISOLATED FROM HUMAN PREGNANCY URINE*

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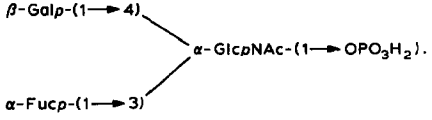
ABSTRACT

A new acidic oligosaccharide, isolated from the urine of a pregnant woman by gel filtration and ion-exchange chromatography, was shown on the basis of sugar analysis, methylation analysis, exo-glycosidase digestion, e.i.-m.s., f.a.b.-m.s., and n.m.r. spectroscopy to have the following structure:



INTRODUCTION

Phosphate-containing carbohydrates occur in many glycans of biological interest. In particular, they are involved in various aspects of glycoconjugate

*Dedicated to Professor N. K. Kochetkov.

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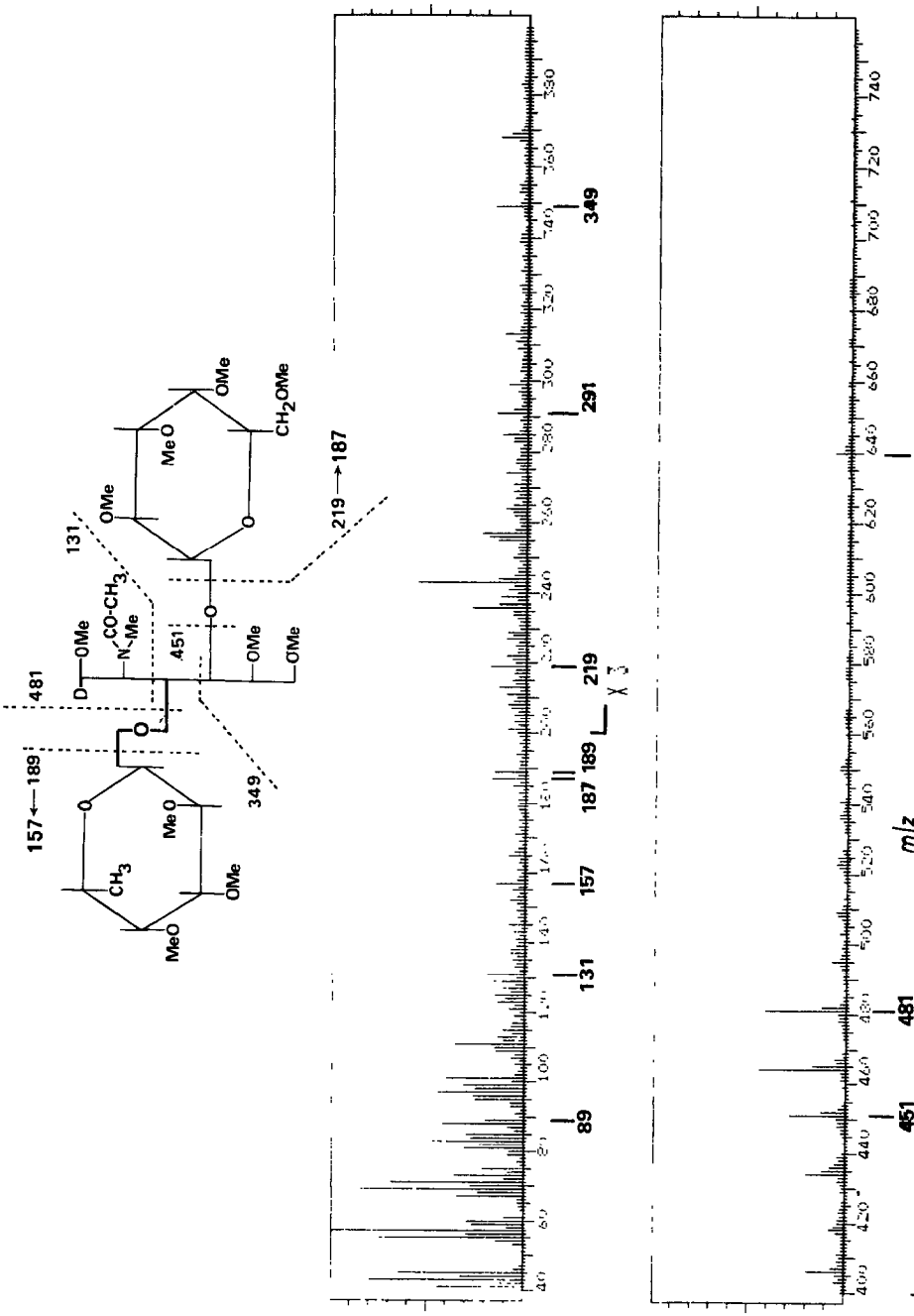


Fig. 1. E.i. mass spectrum (70 eV) of the methylated fucose-containing trisaccharide-alditol-*1-d*.

metabolism. The phosphate substituent may act as an activator in the form of a nucleotide monosaccharide during transglycosylation[1]. For *N*-glycosylated proteins, the glucose-containing oligomannoside-type oligosaccharide is transferred to asparagine residues in the protein core *via* the corresponding phosphodolichol derivative[1]. The unmasking of the mannose-phosphate-monoester constitutes a signal for the recognition and translocation of lysosomal hydrolases by the mannose 6-phosphate receptor[2]. Recently, Parkkinen and Finne[3] reported on the isolation and characterisation of novel phosphate-containing sialyloligosaccharides, terminating with α-GlcNAc-(1→OPO_3H_2) and α-GalNAc-(1→OPO_3H_2), from normal human urine.

We now describe the characterisation of a fucose-containing trisaccharide monophosphate found in human pregnancy urine. Some of these data have been reported in a preliminary communication[4].

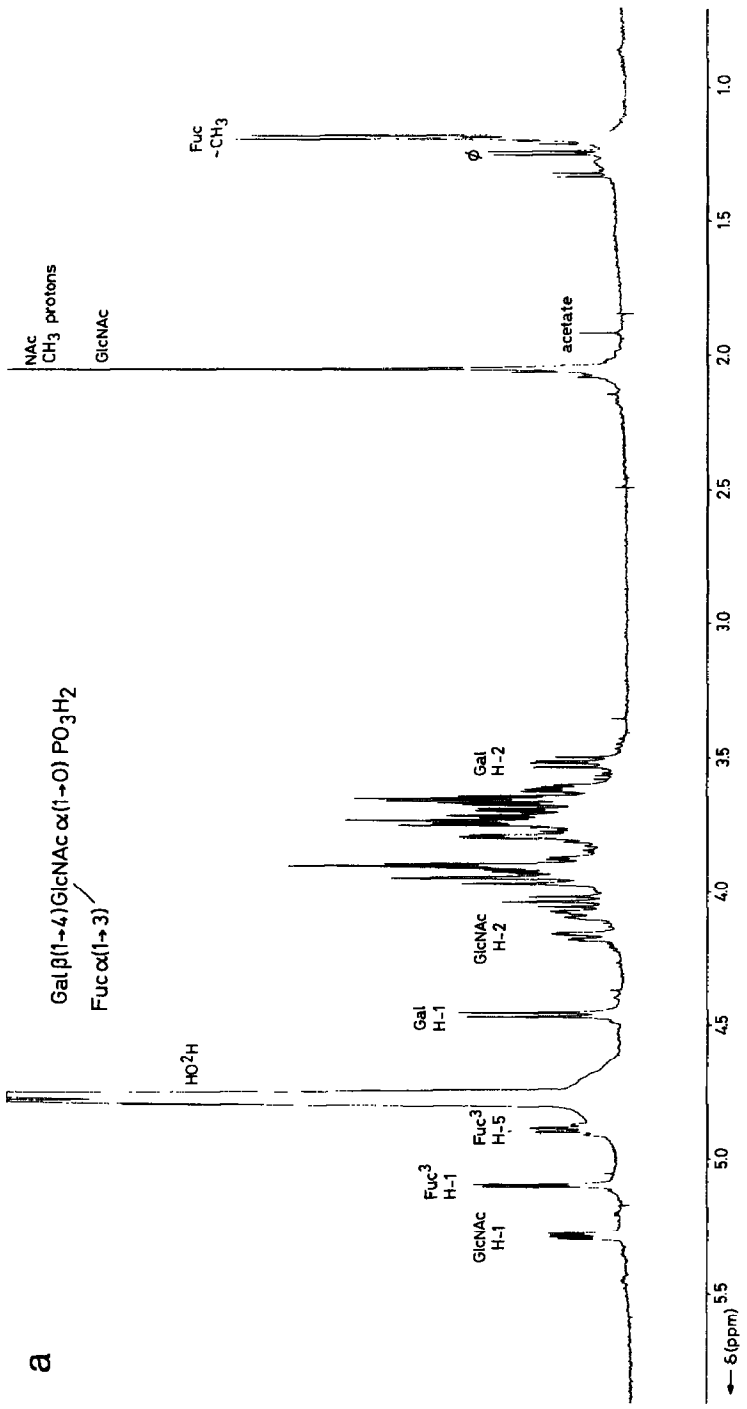
RESULTS

Sugar analysis[5,6] of the isolated oligosaccharide showed the presence of Gal, Fuc, and GlcNAc in the molar ratios 1.0:1.1:0.9. To verify the homogeneity of the oligosaccharide, the corresponding tritium-labelled alditol was analysed by p.c. using four solvent systems (*A–D*); the $R_{Lactose}$ values in the various systems were *A*, 1.5; *B*, 1.1; *C*, 2.6; and *D*, 1.5. The methylation analysis data[7–9] of the methylated oligosaccharide-alditol-*1-d* gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol, and 3,4-di-*O*-acetyl-2-deoxy-1,5,6-tri-*O*-methyl-2-*N*-methylacetamidoglucitol-*1-d* in the molar proportions 0.8:0.9:1.0. These results indicated the urinary oligosaccharide to be a trisaccharide with terminal Gal and Fuc residues and a 3,4-disubstituted GlcNAc residue.

The 70-eV e.i.-mass spectrum of the methylated trisaccharide-alditol-*1-d* (Fig. 1) showed, in the high-mass range, a peak at *m/z* 640 corresponding to (M^+ – CHDOMe). The peaks at *m/z* 219 and 189 were attributable to terminal Gal and Fuc residues, respectively (fragment aA_1 for each residue). The branching pattern of GlcNAc-ol-*1-d* was indicated by the peaks at *m/z* 451 and 481 (fragment baA_1 for each residue) (see Fig. 1). The detection of an ion at *m/z* 349 was evidence for the location of Fuc at C-3 of GlcNAc-ol-*1-d*. Based on the presence of a peak at *m/z* 291, it is suggested that the double substitution of the GlcNAc-ol-*1-d* unit involved[10] C-3,4 (see also methylation analysis data). Consequently, Gal ought to be linked to C-4.

Bovine epididymis α-L-fucosidase failed to release Fuc from the tritium-labelled trisaccharide-alditol. However, treatment with 0.1M trifluoroacetic acid for 1 h at 80° released 70% of the Fuc. When the defucosylated disaccharide-alditol, isolated by preparative p.c. (8 h, solvent *A*), was incubated with jack-bean β-D-galactosidase, the single radioactive product was GlcNAc-ol (p.c., solvent *A*).

The foregoing results indicated the structure **1** for the oligosaccharide. However, the behaviour of the urinary trisaccharide on DEAE Sephadex A-25 indicated


a
Gal β(1→4)GlcNAc α(1→O) PO_3H_2
Fuc α(1→3)
HO^2H
Gal H-1
GlcNAc H-2
Gal H-2
NAc CH_3 protons
GlcNAc
Fuc $-CH_3$
acetate
GlcNAc H-1
Fuc^3 H-1
Fuc^3 H-5
δ(ppm)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0

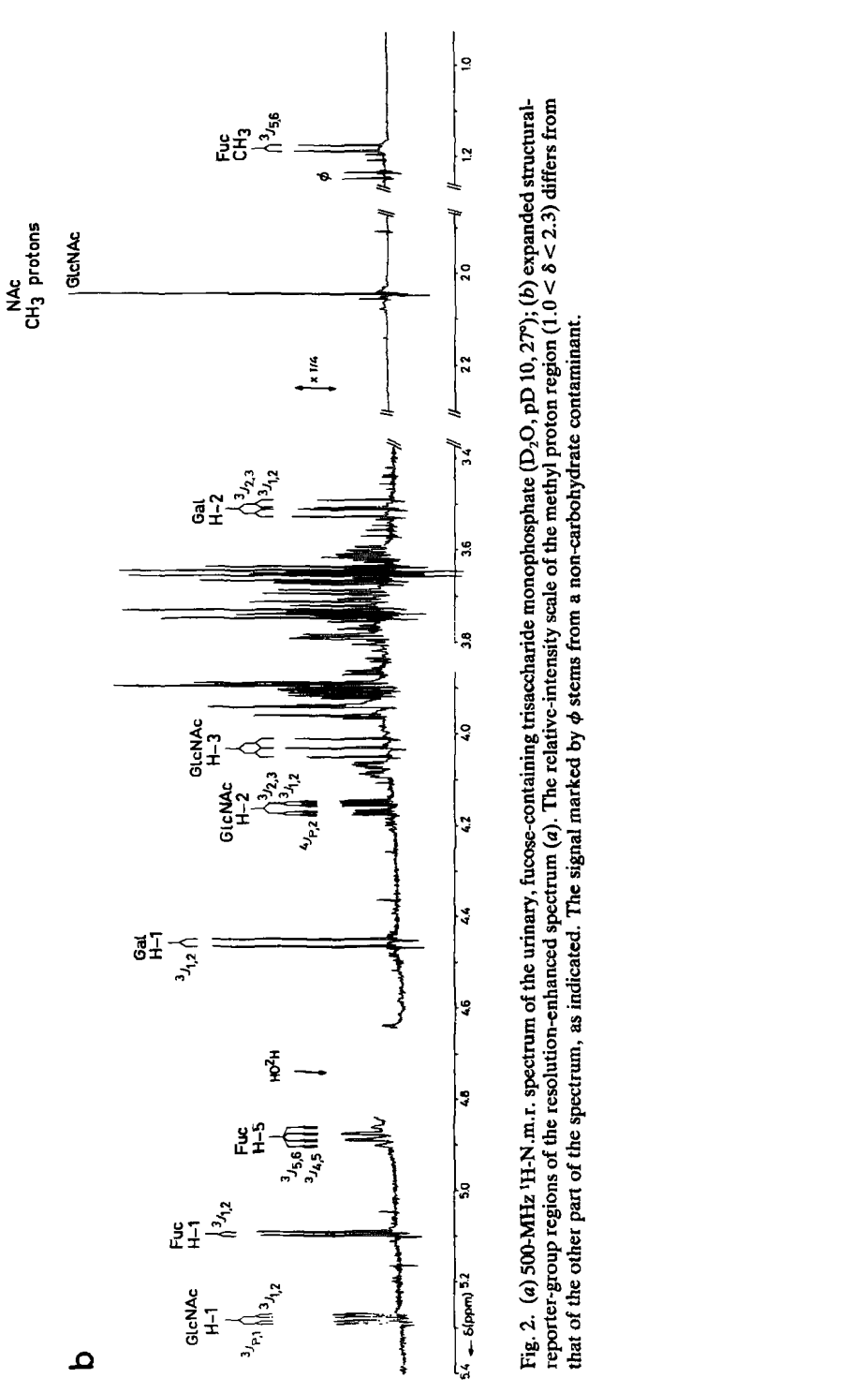


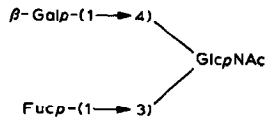
Fig. 2. (*a*) 500-MHz ^{1}H-N.m.r. spectrum of the urinary, fucose-containing trisaccharide monophosphate (D_2O, pD 10, 27°); (*b*) expanded structural-reporter-group regions of the resolution-enhanced spectrum (*a*). The relative-intensity scale of the methyl proton region ($1.0 < \delta < 2.3$) differs from that of the other part of the spectrum, as indicated. The signal marked by ϕ stems from a non-carbohydrate contaminant.

TABLE I

^{1}H-CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR SOME RELEVANT PROTONS IN THE N.M.R. SPECTRA[a] OF THE URINARY TRISACCHARIDE PHOSPHATE β-Gal*p*-(1→4)[α-Fuc*p*-(1→3)]-α-Glc*p*NAc-(1→OPO_3H_2), THE MECONIUM TRISACCHARIDE β-Gal*p*-(1→4)[α-Fuc*p*-(1→3)]-Glc*p*NAc, GlcNAc, AND α-Glc*p*NAc-(1→OPO_3H_2)

Residue	*Reporter group*	*Chemical shift [and coupling constant (Hz)]*			
		Urinary trisaccharide phosphate (500 MHz, pD 10, 27°)[b]	*Meconium trisaccharide (500 MHz, pD 6.5, 25°)*	*GlcNAc*[18] *(500 MHz, pD 7.0, 27°)*	*α-GlcNAc-(1→OPO_3H_2) (500 MHz, pD 7.0, 27°)*
GlcNAc	H-1	5.278 ($J_{1,2}$ 3.2) ($^3J_{P,1}$ 7.3)	(α) 5.106 ($J_{1,2}$ 4.0) (β) 4.729 ($J_{1,2}$ 8.0)	(α) 5.196 ($J_{1,2}$ 3.6) (β) 4.707 ($J_{1,2}$ 8.4)	5.344 ($J_{1,2}$ 3.1) ($^3J_{P,1}$ 7.5)
	H-2	4.159 ($J_{2,3}$ 10.3) ($^4J_{P,2}$ 1.9)	(α) 4.155 ($J_{2,3}$ 10.0) (β) n.d.	(α) 3.872 ($J_{2,3}$ 10.7 (β) 3.669 ($J_{2,3}$ 10.3)	3.914 ($J_{2,3}$ 10.4) ($^4J_{P,2}$ 1.6)
	NAc	2.045	(α/β) 2.033	(α/β) 2.044	2.058
Gal	H-1	4.455 ($J_{1,2}$ 7.8)	(α) 4.465 ($J_{1,2}$ 7.8) (β) 4.455 ($J_{1,2}$ 7.8)	—	—
	H-2	3.507 ($J_{2,3}$ 9.9)	(α) 3.505 ($J_{2,3}$ 9.9) (β) 3.495	—	—
Fuc	H-1	5.092 ($J_{1,2}$ 4.0)	5.098 ($J_{1,2}$ 3.5)	—	—
	H-5	4.881 ($J_{4,5}$ <1)	4.839	—	—
	CH_3	1.179 ($J_{5,6}$ 6.7)	(α) 1.179 (β) 1.174	—	—

[a]Chemical shifts are in p.p.m. relative to internal DSS (using internal acetone at δ 2.225). [b]Some of the chemical shifts for this compound are strongly pD-dependent, *e.g.*, GlcNAc H-1 (δ 5.310 at pD 6.5) and GlcNAc H-2 (δ 4.188 at pD 6.5). The other δ values do not change more than ±0.02 p.p.m. on going from pD 10 to 6.5. Apparently, $^3J_{P,1}$ and $^4J_{P,2}$ are also pD-dependent (see text).



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the presence of an additional acidic group. Apparently, the treatment with alkaline borohydride caused the loss of this acidic substituent. In order to identify the nature and position of the acidic group, the urinary trisaccharide was further analysed by f.a.b.-m.s. and ^{1}H-n.m.r. spectroscopy.

The negative-ion f.a.b. mass spectrum contained an intense peak at *m/z* 608. Since Gal, Fuc, and GlcNAc are present in the molar ratios 1:1:1, this peak, if considered as the pseudomolecular ion $(M - H)^-$, suggested the presence of a phosphate or sulphate substituent in the trisaccharide. No fragment ions could be observed in the spectrum.

The 500-MHz ^{1}H-n.m.r. spectrum (D_2O, pD 10) of the trisaccharide is depicted in Fig. 2. The chemical shifts of the structural-reporter groups and of some other pertinent protons are compiled in Table I. In interpreting this spectrum, advantage was taken of the ^{1}H-n.m.r. data for the meconium trisaccharide β-Gal*p*-(1→4)[α-Fuc*p*-(1→3)]-GlcNAc[11], GlcNAc, and α-GlcNAc-(1→OPO_3H_2); these data have been included in Table I.

From the presence of the H-1 signal at δ 5.092 ($J_{1,2}$ 4.0 Hz), in conjunction with the typically shaped signal for H-5 at δ 4.881 and the CH_3-doublet at δ 1.179, it was concluded that the urinary trisaccharide contained an α-Fuc residue (1→3)-linked to GlcNAc that forms part of an *N*-acetyl-lactosamine unit[11,12]. In line with this conclusion, a β-Gal H-1 doublet was found at δ 4.455 ($J_{1,2}$ 7.8 Hz). The singlet at δ 2.045 must be attributed to the NAc group of GlcNAc. Similar chemical shifts have been found for the meconium trisaccharide (see Table I)[11].

A very characteristic feature of the ^{1}H-n.m.r. spectrum of the urinary trisaccharide (Fig. 2) is the signal (dd) at δ 5.278 due to H-1 of GlcNAc. At pD 6.5, this signal was observed at δ 5.310 but was considerably broadened and without any fine-structure. However, at pD 10, the signal was well resolved, showing *J* values of 3.2 and 7.3 Hz due to H-1,2 and H-1,P couplings, respectively. Moreover, the difference in the chemical shift of the signal of H-1 of GlcNAc, as compared to that of the meconium trisaccharide (see Table I), has to be ascribed to the presence of the phosphate group.

By broad-band ^{31}P-decoupling, the signal (dd) at δ 5.278 (Fig. 3a) collapsed to a doublet with a remaining *J* of 3.2 Hz (Fig. 3b). In addition, the pattern of the signal at δ 4.159 (see Fig. 2b) was simplified from 8 to 4 lines, showing couplings of 3.2 and 10.3 Hz. Therefore, $J_{1,2}$ of GlcNAc was 3.2 Hz, indicative of an α-phosphate group. The signal at δ 4.159 ($J_{2,3}$ 10.3, $J_{1,2}$ 3.2, $^4J_{P,2}$ 1.9 Hz) belongs to H-2 of GlcNAc. By selective irradiation of this GlcNAc H-2 resonance at δ 4.159, only the

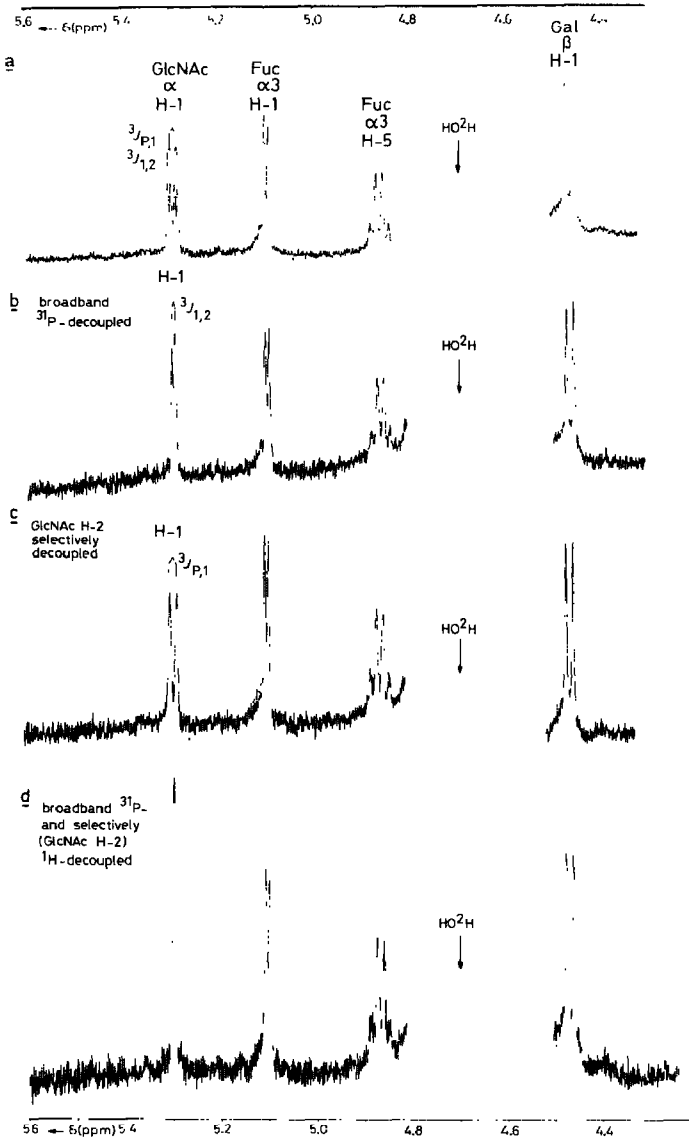
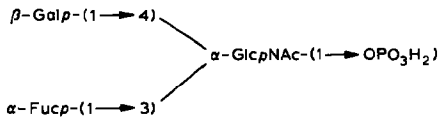


Fig. 3. Anomeric region of the resolution-enhanced, 500-MHz ^{1}H-n.m.r. spectrum of the urinary, fucose-containing trisaccharide monophosphate (D_2O, pD 10, 45°): (*a*) reference spectrum, (*b*) result of broad-band ^{31}P-decoupling, (*c*) result of selective GlcNAc H-2 (δ 4.159) ^{1}H-decoupling, (*d*) result of simultaneous selective homonuclear (GlcNAc H-2) ^{1}H- and broad-band heteronuclear ^{31}P-decoupling.

$^3J_{P,1}$ remained clearly observable at the GlcNAc H-1 resonance (Fig. 3c), whereas simultaneous H-2 selective-proton and broad-band ^{31}P-decoupling resulted in a singlet at δ 5.278 (Fig. 3d).

Thus, the presence of an α-GlcNAc 1-phosphate residue is responsible for the acidic nature of the trisaccharide. Its presence explains both the downfield shift

(δ 5.278 instead of 5.106) of the signal for H-1 of GlcNAc and the second coupling [*cf.* α-GlcNAc-(1→OPO_3H_2) in Table I]. Thus, the urinary oligosaccharide has structure **2**, and the phosphate group is released under the conditions routinely used for the conversion of reducing oligosaccharides into the corresponding alditols.



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The glycan part of the fucose-containing trisaccharide monophosphate is identical to the structure of non-reducing termini of some fucosylated carbohydrate chains of glycoproteins[13–15] and glycolipids[16]. In a similar way, the glycan parts of the sialic acid-containing trisaccharide monophosphates α-Neu5Ac-(2→6)-β-Gal-(1→4)-α-GlcNAc-(1→OPO_3H_2), α-Neu5Ac-(2→3)-β-Gal-(1→4)-α-GlcNAc-(1→OPO_3H_2), and α-Neu5Ac-(2→3)-β-Gal-(1→3)-α-GalNAc-(1→OPO_3H_2), recently isolated from normal human urine[3], are identical with common sialyloligosaccharide end-groups of glycoproteins and glycolipids. There have been several suggestions about the origin of this type of compound[3]. Their excretion in urine suggests the existence of an as yet unknown pathway in the metabolism of complex carbohydrates.

EXPERIMENTAL

Materials. — Human third-term pregnancy urine was collected from a single, non-secretor donor (blood group A) not subject to any dietary restriction. The urine was kept at −18° until used. Tritium-labelled sodium borohydride (167 mCi/mmol) was supplied by Amersham.

Isolation of the acidic oligosaccharide. — Concentrated urine samples were filtered through a column of Sephadex G-25. The fractions obtained were desalted[5] by passage through columns of AG 50W-X8 (H^+) and AG 3-X4A (HO^-) resins (100–200 mesh). Carbohydrate material was fractionated[5] on a column of DEAE-Sephadex A-25, whereby the acidic oligosaccharide was excluded by elution with 0.5M pyridine acetate buffer (pH 5.3). Further purification[5] was carried out on a column of Bio-Gel P-2 (200–400 mesh), using 0.05M pyridine acetate buffer (pH 5.3); 5 mg of oligosaccharide was obtained from 15 L of urine.

Paper chromatography. — Descending p.c. was performed on Schleicher and Schüll 2043-b paper, using *A*, 5:5:1:3 ethyl acetate–pyridine–acetic acid–water; *B*, 2:1:2 ethyl acetate–pyridine–water (upper phase); *C*, 3:1:1 ethyl acetate–acetic acid–water; and *D*, 10:4:3 ethyl acetate–pyridine–water; and detection with the

silver dip reagent[17]. Radioactive compounds were located with a Packard radiochromatogram scanner (Model 7200).

Sugar analysis. — Monosaccharides were determined by g.l.c. of trimethylsilylated methyl glycosides, obtained by methanolysis (methanolic 1.5M HCl, 85°, 18 h) and subsequent derivatisation[5,6].

Borohydride reduction. — The acidic oligosaccharide was treated with sodium borohydride ($NaBH_4$, $NaBD_4$, and tritium-labelled $NaBH_4$) in 0.05M NaOH for 2 h at room temperature. The mixture was then neutralised with glacial acetic acid, sodium ions were removed using Dowex AG 50W-X8 (H^+) resin (100–200 mesh), and boric acid was removed as trimethyl borate by reaction with methanol.

Enzymic degradations. — The tritium-labelled oligosaccharide (40 nmol, 507 c.p.m.) was incubated with α-L-fucosidase (EC 3.2.1.51, 0.1 unit) from bovine epididymis (Sigma) in 0.1M sodium citrate buffer (pH 6.5) containing 0.15M NaCl, for 24 h at 37°. The defucosylated tritium-labelled oligosaccharide was incubated with β-D-galactosidase (EC 3.2.1.23, 1 unit) from jack bean (Sigma) in 0.4M sodium citrate/phosphate buffer (pH 3.5) for 16 h at 37°. To inhibit bacterial growth, a drop of toluene was added to each incubation mixture. Incubations were stopped by heating for 5 min at ~100°. After dilution, samples were desalted on small columns of AG 50W-X8 (H^+) and AG 3-X4A (HO^-) resins, and analysed by p.c. (solvent *A*).

Methylation analysis. — The $NaBD_4$-treated oligosaccharide was methylated[7] with potassium *tert*-butoxide as the base[8]. The partially methylated alditol acetates[9] were identified by g.l.c.–m.s. (e.i. mode), using a Hewlett–Packard 5993-B spectrometer operated with 5885 software. G.l.c. was carried out on a CPsil5 WCOT fused-silica capillary column (25 m × 0.25 mm i.d., Chrompack).

Mass spectrometry. — E.i.-m.s. of the methylated oligosaccharide-alditol-*1-d* was carried out on a Riber 10-10 apparatus, using direct insertion: ionising current, 200 μA; electron energy, 70 eV; accelerating voltage, 8.2 kV; and ion-source temperature, 130°.

F.a.b-m.s. of the non-derivatised oligosaccharide was carried out on a VG Analytical ZAB-HF mass spectrometer. The primary beam was composed of xenon atoms with a maximum energy of ~7.6 keV. The compound was dissolved in glycerol–5% acetic acid. The sputtered ions were extracted and accelerated with a potential of 7 kV. The spectrum was obtained with a u.v. chart-recorder.

N.m.r. spectroscopy. — The urinary oligosaccharide was repeatedly treated with D_2O at pD 10 at room temperature, with intermediate lyophilisation, finally using 99.96% D_2O (Aldrich). The pD was kept at ~10 by adding ND_4OD after each lyophilisation step.

250-MHz ^{1}H-N.m.r. spectra were recorded with a Bruker SY-250 spectrometer (ERA-CNRS 557, Paris, France) operating in the F.t. mode at a probe temperature of 25° under the control of an Aspect-2000 computer. Acquisition para-

meters: pulse width, 3 μs (corresponding to a flip angle of ~30°); spectral width, 2500 Hz; and data points, 32 K.

500-MHz ^{1}H-N.m.r. spectra were recorded with a Bruker WM-500 spectrometer (SON hf-n.m.r. facility, Department of Biophysical Chemistry, University of Nijmegen) operating in the F.t. mode under the control of an Aspect-2000 computer[12]. The probe temperature was 27 or 45°. For ^{31}P-decoupling, a continuous broad-band decoupling level of 1.2 W was applied, using the resonance frequency of trimethyl phosphate (202.5 MHz) as offset for the decoupler. Operating conditions: 5-mm sample tube; pulse width, 20 μs (~90° flip angle); spectral width, 2600 Hz; 16 K data points for FID; Lorentzian-to-Gaussian transformation followed by zerofilling to 32 K points, for resolution enhancement.

Chemical shifts (δ) are expressed in p.p.m. downfield from the signal for internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), and measured by reference to internal acetone (δ 2.225).

ACKNOWLEDGMENTS

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