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PREPARATION AND CHARACTERISATION OF FRAGMENT GLYCOASPARAGINES FROM OVALBUMIN GLYCOPEPTIDES: REFERENCE COMPOUNDS FOR STRUCTURAL AND BIOCHEMICAL STUDIES OF THE OLIGO-MANNOSE AND HYBRID TYPES OF CARBOHYDRATE CHAINS OF GLYCOPROTEINS

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ABSTRACT

Glycoasparagines obtained after exhaustive digestion by Pronase of purified ovalbumin were partially degraded by trifluoroacetic acid or subjected to Smith degradation. The partially degraded glycoasparagines thus obtained were first fractionated according to molecular size on Dowex 50W-X2 and then further fractionated by borate chromatography on a column of Sephadex A-25. For a mixture of glycoasparagines of similar molecular size, the latter procedure fractionates according to increasing content of mannosyl *cis*-2,3-diol. Ten fragment glycoasparagines have been prepared from ovalbumin glycoasparagines, and the structures determined by ^{1}H-n.m.r. spectroscopy and methylation analysis.

INTRODUCTION

During structural studies of "high-mannose" type glycopeptides by high-field, ^{1}H-n.m.r. spectroscopy, it became desirable to prepare their fragment glycopeptides. Ovalbumin is an excellent source of reference glycoasparagines, is available in large amounts, and also contains several structural variants of the carbohydrate chain[1]. The preparation of fragment glycoasparagines was thus attempted by partial hydrolysis with acid or by mild Smith-degradation followed by appropriate chromatographic techniques. Our efforts were directed primarily towards the separation of fragments containing less than seven monosaccharide residues. Possible advantages of this approach were (*a*) fragment glycoasparagines having molecular weight lower than that of the smallest, native, ovalbumin glycoasparagine[2] (GP-VI) should be obtained; (*b*) the proposed fractionation methods should be more efficient and more suitable than paper electrophoresis on a preparative scale; (*c*) fragment glycoasparagines other than those obtainable by glycosidase treatment should be obtained.

Partial hydrolysis of ovalbumin glycoasparagines with acid led to complex mixtures of fragment glycoasparagines. These mixtures were firstly fractionated by

cation-exchange chromatography on Dowex 50W-X2 and secondly by anion-exchange chromatography on DEAE-Sephadex A-25 (borate form) (cf. refs. 3–7). In this way, 10 fragment glycoasparagines were obtained, and subsequently studied by 270-MHz, ^{1}H-n.m.r. spectroscopy. High-field, ^{1}H-n.m.r. spectroscopy has been applied to glycopeptides and glycoprotein oligosaccharides[8–20], and complex carbohydrates have been investigated[9,11–13,15–19]. Furthermore, recent work[15,16,19,20] concerning the structural elucidation of glycoasparagines of the oligo-mannose type prompts us to report our study of the reference and fragment glycoasparagines from ovalbumin.

EXPERIMENTAL

Preparation of the mixture of glycoasparagines. — Glycoasparagines from commercial hen-egg albumin (Difco Laboratories) were prepared[21] after exhaustive Pronase (Kaken Kagaku Co., Tokyo) digestion followed by gel chromatography on Sephadex G-10. Glycoasparagines produced by four cycles of the above procedure were finally purified by elution with aqueous 10% ethanol from a column of Sephadex G-50.

Fragment glycoasparagines. — (*a*) *Partial, acid hydrolysis of ovalbumin glyco-*

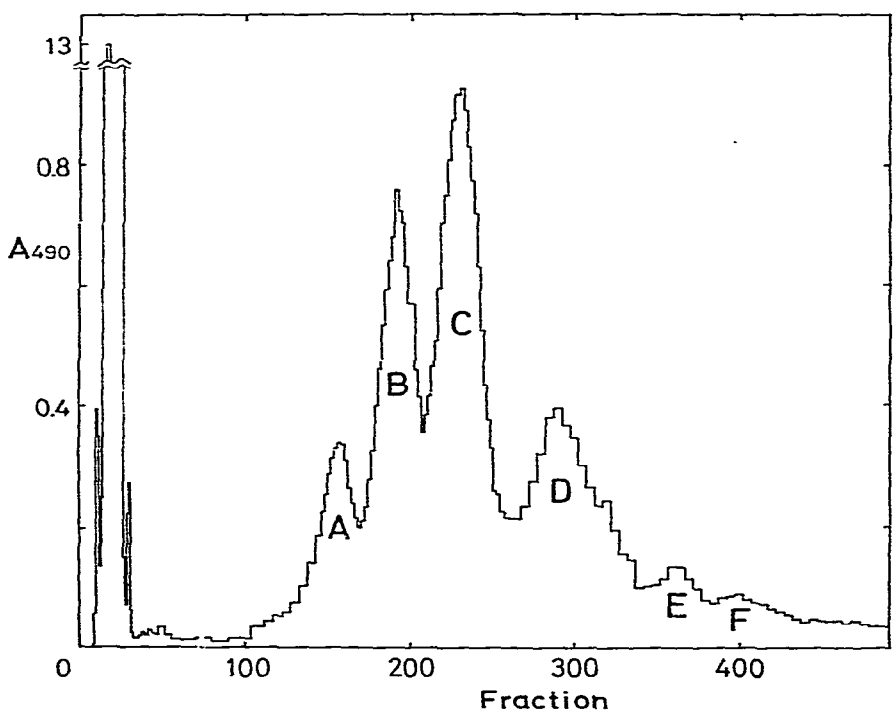


Fig. 1. Chromatography of the partial, acid hydrolysate [trifluoroacetic acid (11 ml), 30 min, 90°] of ovalbumin glycoasparagines (190 mg in Man) on a column (2.8 × 65 cm) of Dowex 50W-X2 with sodium acetate (pH 2.6, mM in Na^+); 14-ml fractions at 55 ml/h assayed by the phenol–sulfuric acid method at 490 nm.

asparagines. A solution of the ovalbumin glycoasparagines (190 mg in Man) in 2.5M trifluoroacetic acid (11 ml) was maintained at 90° for 30 min in a closed container. The solution was cooled and concentrated *in vacuo* at ~30°. Hydrolysis in 2M HCl for 12 min yielded extensively hydrolysed products, including β-GlcNAc-(1→4)-Asn as a major glycoasparagine[22].

(*b*) *Fractionation according to molecular size*[21,23]. The partial, acid hydrolysates were applied to columns of Dowex 50W-X2 resin (200–400 mesh), after being equilibrated with mM AcOH–AcONa (pH 2.6). Glycoasparagines were then eluted with the same buffer solution. The results are shown in Fig. 1. Fractions were assayed by the phenol–sulfuric acid method[24] (490 nm). Sugar and amino acid analyses indicated that the materials in the peaks were glycoasparagines, which were eluted in order of decreasing molecular size. The fractions containing peaks D–F were combined, concentrated, and desalted.

(*c*) *Fractionation according to sugar composition and sequence.* Fragment glycoasparagine fractions A–F (Fig. 1) were not homogeneous. Based on the procedures[3–7] for borate chromatography on DA-X4 resin, we applied borate chromatography on DEAE-Sephadex A-25 to sub-fractionation of fractions A–F. DEAE-Sephadex A-25 was washed with 0.1M NaOH and then water, converted into the borate form with

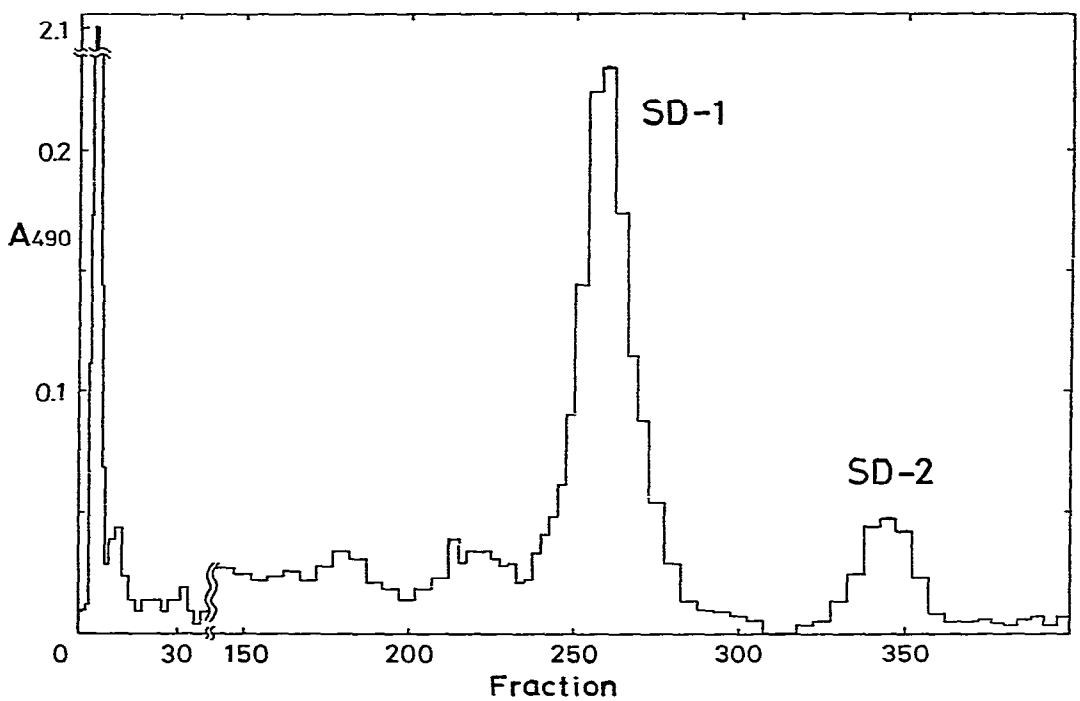


Fig. 2. Chromatography of the Smith-degradation products from ovalbumin glycoasparagines (29 mg in Man) on a column (1.2 × 90 cm) of Dowex 50W-X2 with sodium acetate buffer (pH 2.6, mM in Na^+); 10-ml fractions at 16 ml/h.

sodium borate (pH 9.2), and washed with water. Good resolution of the components in fractions D–F (Fig. 3) was achieved on elution from this ion-exchanger with borate buffer (pH 8.0). See Fig. 3 for details.

Preparation of fragment glycoasparagines by Smith degradation of ovalbumin glycoasparagines. — Ovalbumin glycoasparagines were subjected to Smith degradation under mild conditions[25]. An aqueous solution (13 ml) of ovalbumin glycoasparagines (29 mg) and sodium periodate (560 mg) was kept at 6° for 20 h. 0.45M Sodium borohydride–0.3M borate buffer (pH 8.0) (13 ml) was then added, and, after a further 15 h at 6°, the solution was acidified to pH 1 with sulfuric acid, left at room temperature for 1 day, desalted by Sephadex A-25 (AcO^- form), and subjected to chromatography on Dowex 50W-X2 (Fig. 2).

Methylation analysis of fragment glycoasparagines. — The glycoasparagines were methylated by the Hakomori method[26], the products were desalted, hydrolysed, and reduced[27], and each mixture of partially methylated alditols was acetylated[28] and then subjected to g.l.c. (occasionally to g.l.c.–m.s.). G.l.c. (Shimadzu chromatographs, Models GC-7A and GC-4BMPF, flame-ionisation detection) was performed with nitrogen as carrier gas, glass columns (3 mm × 1.5 m) packed with 3% of OV-17 on Gas Chrom Q (80–100 mesh), and a temperature gradient of 4°/min from 180°→240°. Peak areas were measured with a Shimadzu digital integrator ITG-4A or a Shimadzu Chromatopac C-R1A. For g.l.c.–m.s., a column (2 mm × 1.5 m) of OV-17 (1.5%) was used together with a JEOL JGC-20 Gas Chromatograph–JMS-D300 Mass Spectrometer. G.l.c. was performed at 170°→230° at 4°/min, and the mass spectra were recorded at 70 eV with an ion-source temperature of 200°.

^{1}H-N.m.r. spectroscopy. — Solutions of the glycoasparagines in 99.8% D_2O were concentrated to dryness. This procedure was repeated thrice. Spectra were then recorded with a Bruker WH 270 spectrometer, operating in the Fourier-transform mode with a probe temperature of 60° unless otherwise specified. Chemical shifts are expressed in p.p.m. relative to that of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

RESULTS

Fractionation, and sugar and methylation analyses of fragment glycoasparagines. — After several attempts to fractionate the fragment glycoasparagines, it was found that borate chromatography on DEAE-Sephadex A-25 was more effective, although a complete fractionation could not always be attained. The results obtained with fractions D–F (Fig. 1) are shown in Fig. 3. Where there was partial overlapping of peaks, further chromatography was carried out.

Sub-fractions D-1, D-4, D-5, E-3, F-1, and F-3 were shown to be homogeneous by their ^{1}H-n.m.r. spectra and had the following compositions: D-1, $(Man)_3(GlcNAc)_4$-Asn; D-4, $(Man)_4(GlcNAc)_2Asn$; D-5, $(Man)_4(GlcNAc)_2Asn$; E-3, $(Man)_3$-$(GlcNAc)_2Asn$; F-1, $(Man)_2(GlcNAc)_3Asn$; and F-3, $(Man)_3(GlcNAc)_2Asn$.

Glycoasparagines D-2, D-3, and F-2 were not isolated homogeneous. The

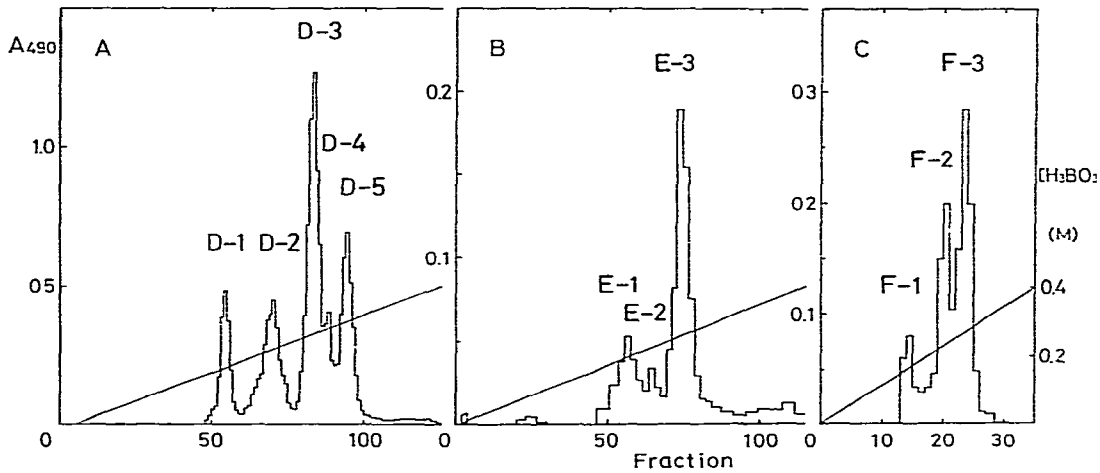


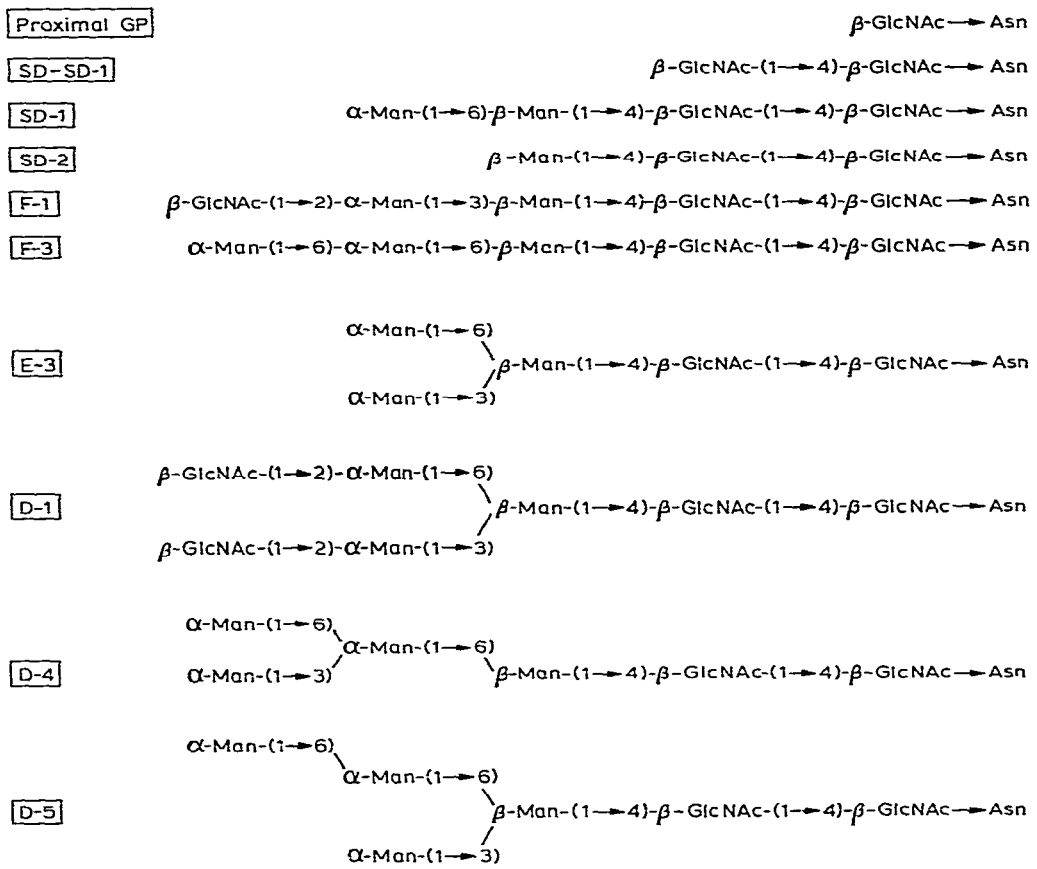
Fig. 3. Chromatography of fractions D–F (Fig. 1) on DEAE-Sephadex A-25 (borate form) in borate buffer: *A*, 0.9 × 72 cm column, linear gradient of sodium borate from 0 (water, 500 ml) → 0.4M (pH 8.0, 500 ml), 8-ml fractions at 12 ml/h; *B*, 0.9 × 100 cm column, linear gradient of sodium borate (pH 8.0) from 0.01 (450 ml) → 0.4M (450 ml), 9.5-ml fractions at 12 ml/h; *C*, 0.9 × 40 cm column, linear gradient of sodium borate from 0 (water, 200 ml) → 0.4M (pH 8.0, 200 ml), 10-ml fractions at 20 ml/h.

TABLE I

PARTIALLY METHYLATED ALDITOL ACETATES OBTAINED IN THE METHYLATION ANALYSIS OF FRAGMENT GLYCOASPARAGINES AND OVALBUMIN GLYCOASPARAGINE GP-VI

Alditol acetate derived from	*Retention time*[a]	*Relative molar ratios*[b]									
	3% OV-17	*SD-SD-1*	*SD-2*	*SD-1*	*F-1*	*F-3*	*E-3*	*D-1*	*D-4*	*D-5*	*GP-VI*
2,3,4,6-Me_4-Man	1.00		*1.0*	*1.0*		*1.0*	*2.0*		*2.0*	*2.0*	*2.0*
3,4,6-Me_3-Man	1.32				*1.0*			*2.0*			
2,4,6-Me_3-Man	1.43[c]				0.9						1.0
2,3,4-Me_3-Man	1.43[c]			1.2		1.8			1.1	1.0	
2,4-Me_2-Man	1.93						1.1	1.0	1.0	1.1	1.2
3,4,6-Me_3-GlcN(Me)Ac	2.24	*1.0*			1.0			1.5			
3,6-Me_2-GlcN(Me)Ac	2.65	0.7	1.7	1.2	1.5	1.2	1.1	0.9	1.2	1.0	1.1

[a]On a column (0.3 × 150 cm) of 3% OV-17 from 180° at 4°/min. Retention times are relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol. [b]The data are normalised to one or two sugar residues in italics. [c]The isomers 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol were satisfactorily resolved when a glass column (0.3 × 150 cm) containing 2% of OV-101 on Gas Chrom Q from 175° at 3°/min was used; the retention times were 1.37 and 1.47, respectively.



Scheme 1. The structure of the fragment glycoasparagines obtained from ovalbumin glycopeptides

minor components E-1 and E-2 were not investigated because of the small quantities. The fragment glycoasparagines were subjected to methylation analysis, and the results are summarised in Table I. Using the methylation analysis and ^{1}H-n.m.r. data, structures were established for the carbohydrate chains of 10 fragment glycoasparagines (see Scheme 1).

270-MHz, ^{1}H-N.m.r. spectra of fragment glycoasparagines. — Typical spectra for solutions in D_2O are shown in Fig. 4 for E-3, D-5, and D-1. The ^{1}H-n.m.r. data can be utilised to determine the sequence and linkage of the component monosaccharides because, for aqueous solutions, chemical shifts of anomeric protons are affected by the nature of the aglycon and by substitution on the ring[11,13,18,19]. The anomeric region (4.4–5.2 p.p.m.) of the spectra of E-3, D-5, and D-1 contained, respectively, five, six, and seven signals of equal intensity, consistent with penta-, hexa-, and hepta-saccharide structures.

Assignment of the resonances for the glycoasparagines was made primarily

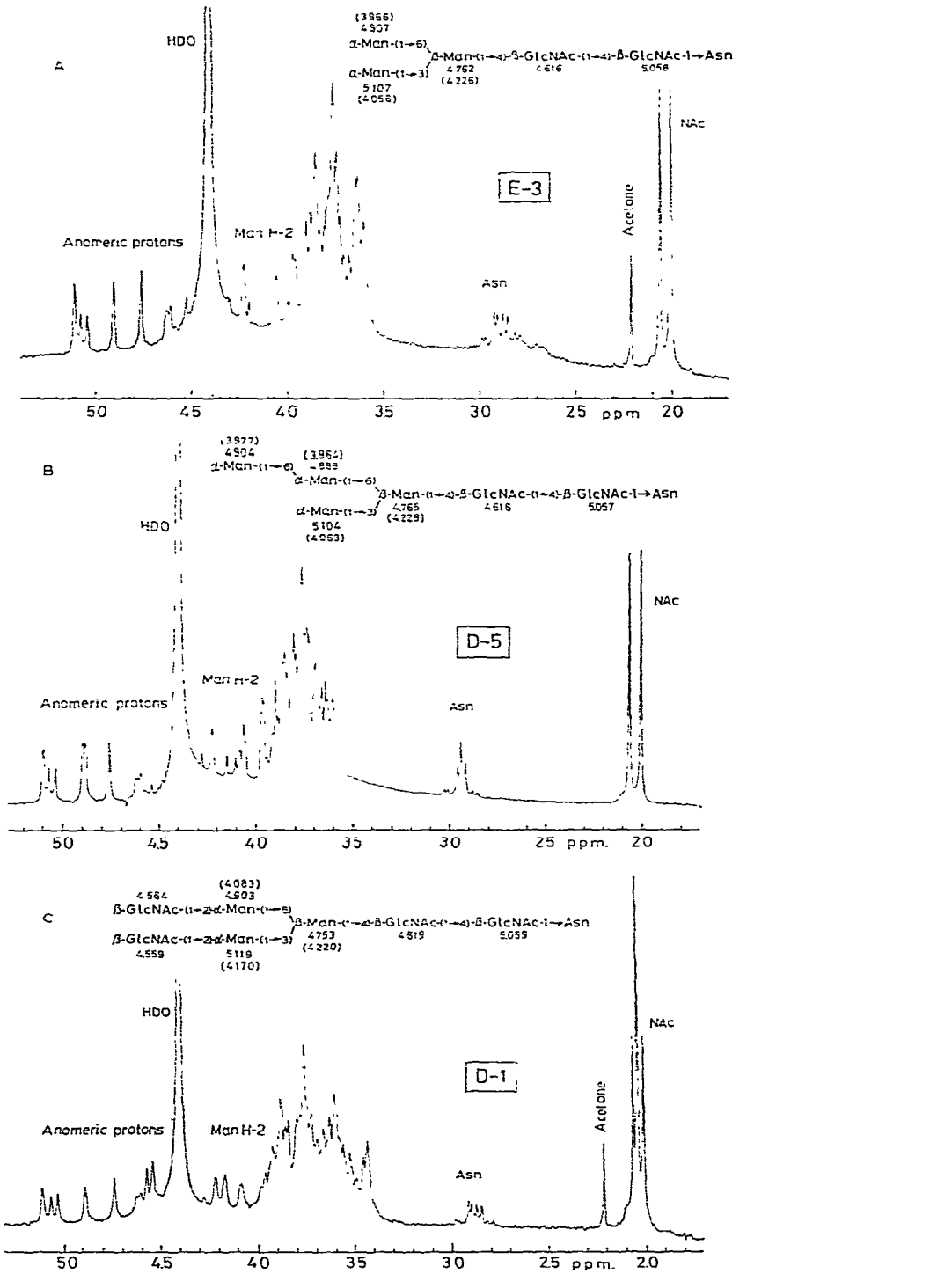
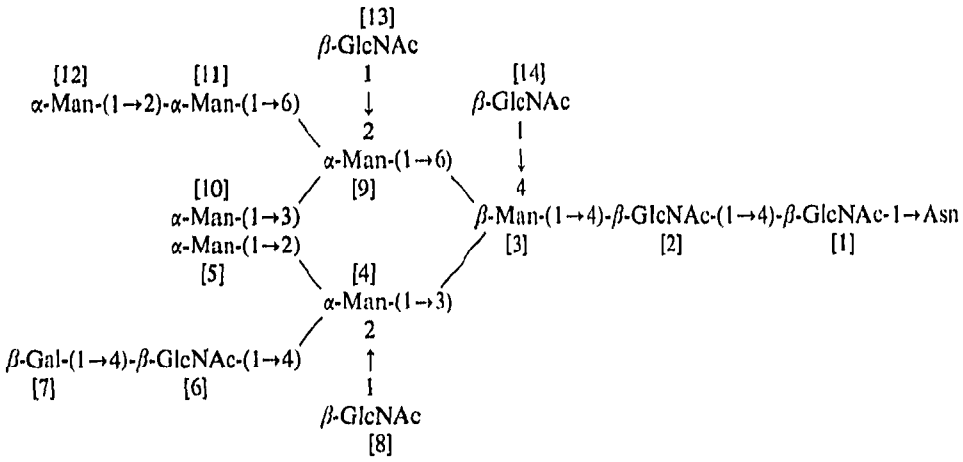


Fig. 4. 270-MHz, ^{1}H-N.m.r. spectra (D_2O, 60°) of glycoasparagines: *A*, E-3; *B*, D-5 (assignments of the peaks at 3.977 and 3.964 p.p.m. are tentative and may be interchangeable); *C*, D-1 (assignments of the peaks at 4.564 and 4.559 p.p.m. are tentative and may be interchangeable). Signals given are for H-1, and those in parentheses are for H-2 of Man.

TABLE II

^{1}H-N.M.R. DATA OF ANOMERIC PROTONS AND H-2 OF MANNOSE FOR TEN FRAGMENT GLYCOASPARAGINES



Compound	*H-1 of residue*									*H-2 of residue*				
	[1]	*[2]*	*[3]*	*[4]*	*[8]*	*[9]*	*[10]*	*[11]*	*[13]*	*[3]*	*[4]*	*[9]*	*[10]*	*[11]*
Proximal GP	5.087													
SD-SD-1	5.061	4.601												
SD-2	5.060	4.619	4.748							4.050				
SD-1	5.060	4.619	4.746			4.905				4.066		3.962		
F-1	5.056	4.604	4.764	5.123	4.556					4.224	4.183			
F-3	5.058	4.618	4.749			4.888		4.904		4.067		3.970		3.970
E-3	5.058	4.616	4.762	5.107		4.907				4.226	4.056	3.966		
D-1	5.059	4.619	4.753	5.119	4.559	4.903			4.564	4.220	4.170	4.083		
D-4	5.064	4.607	4.748			4.865	5.087	4.899		4.063		4.131	4.063	3.973
D-5	5.057	4.616	4.765	5.104		4.888		4.904		4.229	4.063	3.964		3.977

by using established strategies[18,19,29,30] and is explained here only for E-3 (Scheme 1). In the spectrum in Fig. 4A, the resonances of the anomeric protons (H-1), H-2 of some mannose residues, the asparagine methylene protons, and NAc groups are separated from those of the other carbohydrate protons (3.5–4.0 p.p.m.). The chemical shifts of the signals for H-1 and NAc groups are compared with those of the reference compounds, β-GlcNAc→Asn and β-GlcNAc→β-GlcNAc→Asn. The signals at 4.616 and 5.058 p.p.m. are assigned to H-1 of GlcNAc-2 and GlcNAc-1, respectively (see Table II for the residue numbering). Three additional signals with $J_{1,2} \leqslant 1$ Hz appear downfield of the HDO resonance and represent the three Man residues. The chemical shift of the signal for H-1 of a given α-Man residue is affected by the position of its linkage to the next Man residue. Thus, linkage to HO-3 or HO-6 shifts the H-1 signal to higher field [α-Man-(1→3)-, ~5.11 p.p.m.; α-Man-(1→6)-, ~4.905 p.p.m.]. The chemical shifts for the H-1 signals of D-mannopyranosides are also affected by substitution of HO-3 by another mannosyl group, namely, 0.015–0.023 p.p.m. upfield for an α-Man residue and ~0.016 p.p.m. downfield for a β-Man residue. Thus, for E-3, the signals at 5.107 and 4.907 p.p.m. are assigned to the non-reducing, terminal α-Man-(1→3) and α-Man-(1→6) groups, respectively. The signal at 4.762 p.p.m. indicates the sequence α-Man-(1→3)-β-Man and can be assigned to Man-3. Our results on E-3 are compatible with the common inner-core sequence comprising two α-Man residues attached to a β-Man residue.

The resonance at 3.9–4.3 p.p.m., depending on linkage position, could be assigned to H-2 of Man residues, as indicated in Fig. 4, by specific proton-decoupling. The n.m.r. data for the ten fragment glycoasparagines are summarised in Table II.

DISCUSSION

As for the fractionation[21] of native glycoasparagines, prepared from ovalbumin, chromatography on Dowex 50W-X2 under acidic conditions was used to fractionate the fragment glycoasparagines according to molecular size. In order to separate glycoasparagines of similar molecular size, we employed borate chromatography on DEAE-Sephadex A-25, which has not hitherto been reported for this purpose. The use of borate chromatography on DA-X4 resins for the fractionation of sugar mixtures is well known. As an alternative to the strongly basic anion-exchanger, we have used a weakly basic anion-exchanger, DEAE-Sephadex A-25, and found it to be useful for the separation of relatively small quantities of glycopeptides having a similar molecular size but differing in complex-forming ability. It was anticipated[31,32] that the greater the number of available, vicinal *cis*-diol groups, the more firmly the glycoasparagine–borate complex would be bound to DEAE-Sephadex A-25. Other structural features may also contribute to complex-formation, but the present results provide an apparent relation between elution sequence and the number of *cis*-diol groups present in glycoasparagine molecules.

Thus, the 10 fragment glycoasparagines shown in Scheme 1 were obtained. Incidentally, the glycoasparagines SD-1 and D-4 (Scheme 1) correspond to M_2GP

and M_4GP isolated[15] from the urine of a patient suffering from Gaucher's disease. The glycoasparagines in Scheme 1 have proved useful as model compounds in the analysis of spectra–structure relationships for glycopeptides, and augment previous relationships[19,20]. Although we do not claim that all of our assignments are unequivocal, we believe that the general method for studying the primary structure of the oligo-mannose and hybrid types of carbohydrate chains is reliable. In general, α-Man-(1→2)-, α-Man-(1→3), α-Man-(1→6)-, and β-Man-(1→4)- units give H-1 resonances at approximately 5.05, 5.10, 4.90, and 4.745 p.p.m., respectively, which are diagnostic. Also, substitution at HO-3 of one α-D-Man residue by another α-D-Man residue results in an upfield shift of the H-1 signal of 0.015–0.023 p.p.m., which is in the opposite direction to the H-1 shifts for a β-Man-(1→4) unit due to this substitution. Specific effects of 4-*O*-substitution of the Man-3 residue of the pentasaccharide core by a β-GlcNAc group have been discussed[13]. The disadvantage of the n.m.r. method continues to be the need for relatively large amounts of materials (~ 1-mg samples were used in the studies reported here).

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