Structure of the *N*- and *O*-Glycans of the A-Chain of Human Plasma α_2HS-Glycoprotein As Deduced from the Chemical Compositions of the Derivatives Prepared by Stepwise Degradation with Exoglycosidases†

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ABSTRACT: The structure of the glycans of the A-chain of human plasma α_2HS-glycoprotein was established from the chemical compositions of its derivatives prepared by sequential enzymatic degradation of the carbohydrate moiety, from the determination of the kind and amount of the monosaccharides liberated after each step of the enzymatic digestion, and from the distinct specificity of the highly purified exoglycosidases. The exoglycosidases were three sialidases (*Vibrio cholerae*, fowl plague virus, and *Arthrobacter ureafaciens*), two β-galactosidases (*Streptococcus pneumoniae* and bovine testis), one α-*N*-acetylgalactosaminidase, one β-*N*-acetylglucosaminidase, and one α-mannosidase. Utilizing sialidases with different cleavage specificities, the number of α2–3- and α2–6-linked sialic acid residues could be separately determined. As to the β-galactosidases, the enzyme isolated from *S. pneumoniae* cleaves only β1–4-linked galactose residues, whereas the bovine testes enzyme acts on both the β1–4- and β1–3-linked galactose residues. Jack bean β-*N*-acetylglucosaminidase cleaves β1–2, β1–4, and β1–6 GlcNAc with higher activity for the β1–2. Jack bean α-mannosidase cleaves α1–2, α1–6, and α1–3 Man with greater activity for α1–2 and α1–6. Bovine liver α-*N*-acetylgalactosaminidase cleaves *O*-linked GalNAc. On the basis of these results, the A-chain of α_2HS-glycoprotein was found to possess two biantennary *N*-glycans and two O-linked trisaccharides.

α_2HS-glycoprotein (α_2(HS)[1] (Gejyo et al., 1983; Schwick & Haupt, 1984; Lee et al., 1987) is a normal human plasma protein associated with a number of biological functions. It is one of the negative acute-phase reactants of human plasma and has a significantly decreased blood level during inflammatory states (Lebreton et al., 1979; Baskies et al., 1980). This glycoprotein (gp), which may participate in the regulation of bone metabolism (Colclasure et al., 1988), has been demonstrated to influence the mineral phase of the bone, where it is concentrated up to 300-fold as compared with other plasma gp (Triffitt et al., 1976; Ashton et al., 1976). Fetal bone was reported to contain 10-fold more α_2HS than adult bone (Wilson et al., 1977). Moreover, α_2HS promotes endocytosis by macrophages (Lewis & Andre, 1980), exhibits opsonic properties (van Oss et al., 1974), and may be involved in the development of the brain (Jones et al., 1988). As to its structure, α_2HS was earlier shown to consist of two disulfide-linked peptide chains of different size whose amino acid sequences have been established (Gejyo et al., 1983; Yoshioka et al., 1986). In the initially synthesized protein, these two chains are linked together by a connecting sugar-free fragment (Lee et al., 1987). The shorter chain (B-chain) possesses one O-glycosylation site (Gejyo et al., 1983), whereas the longer chain (A-chain) contains two *N*-glycans linked to residues 138 and 158 and two *O*-glycans linked to Thr 238 and 252. The carbohydrate unit of the B-chain proved to be a trisaccharide (Gejyo et al., 1983).

The present study reports the elucidation of the structure of the four glycans of the A-chain of α_2HS.

MATERIALS AND METHODS

All chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany). Sephadex G-100, Sepharose 6B, and CH-Sepharose 4B were from Pharmacia (Uppsala, Sweden), and Bio-Gel P-2 was from Bio-Rad (Munich, Germany). Major items of equipment were listed earlier (Walsh et al., 1990). 4-Methylumbelliferyl-α-NeuAc was synthesized in our laboratory. α_2-HS was isolated from pooled normal human plasma at Behringwerke AG (Marburg/Lahn, Germany) and generously donated by Professor H. Gerhard Schwick. The chemical and physicochemical properties of this protein were reviewed by Schwick and Haupt (1984). This protein proved to be one of the few plasma glycoproteins that possesses GalNAc (Baenziger, 1984).

(*1*) *Exoglycosidases.* The sources and specificities of *Vibrio cholerae* sialidase (VCS), bovine testes β-galactosidase, bovine liver α-*N*-acetylgalactosaminidase, jack bean β-*N*-acetylglucosaminidase, and jack bean α-mannosidase were described recently [Walsh et al. (1990); see also Table I]. The following glycosidases were also used: *Arthrobacter ureafaciens* and fowl plague virus (kindly supplied by Prof. H. Rott, Giessen, Germany) sialidases and *Streptococcus pneumoniae* β-galactosidase. The employed affinity matrices were described in the mentioned paper. All exoglycosidases were free of other glycosidases and proteases (Walsh et al., 1990) except for the fowl plague virus sialidase, which was used only for analytical purposes.

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[1] Abbreviations: α_2HS, α_2HS-glycoprotein (an α_2-glycoprotein discovered by Heremans and Schmid); Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; gp, glycoprotein(s); Man, mannose; NeuAc, *N*-acetylneuraminic acid; and VSC, *Vibrio cholerae* sialidase.

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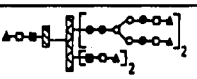
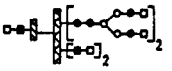
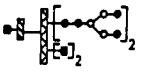
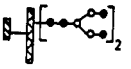
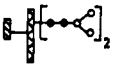
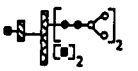
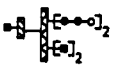
Table I: Monosaccharides Released from Human Plasma α_2HS by Sequential Treatment with Exoglycosidases and the Specificity of These Enzymes

step	enzyme used	specificity	residues released/mol of protein
1a	VCS	α2–3, α2–6, α2–8 NeuAc	6.2 NeuAc (α2–3, α2–6)
1b	*Arthrobacter ureafaciens* sialidase	α2–6 > α2–3 NeuAc	6.2 NeuAc (α2–3, α2–6)
1c	fowl plague virus sialidase	α2–3 ≫ α2–6 NeuAc	2.5 NeuAc (α2–3)
2a	*Streptococcus pneumoniae* β-galactosidase	β1–4 Gal	4.0 Gal (β1–4)
2b	bovine testes β-galactosidase	β1–3, β1–4 Gal	6.6 Gal (β1–3, β1–4)
3	bovine liver α-*N*-acetylgalactosaminidase	α1–0 GalNAc	2.6 GalNAc
4	jack bean β-*N*-acetylglucosaminidase	β1–2 > β1–4, β1–6 GlcNAc	3.4 GlcNAc
5	jack bean α-mannosidase	α1–2, α1–6 ≫ α1–3 Man	3.5 αMan

Table II: Carbohydrate Composition of α_2HS and Its Partially Deglycosylated Derivatives As Determined by Acid Hydrolysis

	derivative	structure of carbohydr moiety[f]	M_r	sugar constituent (residues/mol of protein) NeuAc	Gal	Man	GlcNAc	GalNAc	total carbohydr cleaved (%)
I	native		39 065[a] 53 000[b]	6.2 7[c]	6.6[d] 14[c]	5.9[d]	7.9 9[c]	2.6	0
II	asialo		37 900	0	nd[e]	nd	nd	nd	21
III	agalacto		36 800	nd	0.07	nd	7.2	2.3	43
IV	a-*N*-acetylgalactosamino (prepared from III)		36 200	nd	nd	5.8	8.1	0.4	50
V	a-*N*-acetylglucosamino (prepared from IV)		35 400	nd	nd	6.0	4.5	0.3	63
VI	a-*N*-acetylglucosamino (prepared from III)		36 000	nd	nd	6.0	nd	nd	55
VII	a-α-mannosino (prepared from VI)		35 400	nd	nd	2.8	4.5	2.8	66

[a] Calculated from the structure of the B-chain, the amino acid composition, and proposed glycans of the A-chain and also reported earlier by Schmid and Bürgi (1961) (see also text). M_r of carbohydrate, 6206. [b] Taken from Gejyo and Schmid (1981). This M_r and all subsequent ones were derived from SDS–PAGE. [c] Taken from Schwick and Haupt (1984), who reported a Fuc content of 1 residue. [d] Calculated hydrolysis values (see text). [e] nd, not determined. [f] Key: ▲, NeuAc; □, Gal; ○, Man; ●, GlcNAc; ■, GalNAc; ⊠, protein core of the A-chain; ⊠, protein core of the B-chain.

(*2*) *Digestion with Exoglycosidases.* For preparation of partially deglycosylated α_2HS derivatives, which were used for the determination of the glycan structure of this protein, the abovementioned highly purified exoglycosidases were employed. Incubations were performed at 37 °C under the conditions indicated below and in buffers containing 0.02% NaN_3 and 1% pepstatin.

(*a*) *Cleavage of NeuAc.* α_2HS (40 mg) was incubated for 1 day with 100 milliunits of VCS in 1 mL of 0.05 M NaAc buffer (pH 5.5) containing 7 mM $CaCl_2$. At the end of the experiment, 90% of the enzymatic activity remained. Sialidase was removed from the incubation mixture by affinity chromatography as follows (Walsh et al., 1990): after dilution to 10 mL with the above buffer and adsorption on 10 mL of immobilized NeuAc, asialo-α_2HS was eluted from the column whereas the enzyme was retained. Subsequently, the asialo-gp was desalted on Bio-Gel P-2. After lyophilization, 31 mg (yield 81%) of asialo-α_2HS was obtained (derivative II, Table II). The release of NeuAc by treatment with additional sialidases was carried out as follows (steps 1b and 1c, Table I): 2 mg α_2HS was incubated with either 50 or 3 milliunits of *A. ureafaciens* sialidase (Friebolin et al., 1981) in 0.5 mL of 0.05 M NaAc buffer (pH 5.5) or with 0.05 milliunit of fowl plague virus sialidase in 0.5 mL of 0.1 M sodium citrate phosphate buffer (pH 7.0) (Figure 1). Aliquots (10 μL) were removed at 20-min intervals during the first 3 and then after each hour, and the liberated NeuAc was quantified. The experiments with the latter two sialidases were performed for analytical purposes only.

(*b*) *Cleavage of Gal.* Incubation with *S. pneumoniae* β-galactosidase (0.5 unit) was performed using 16.4 mg of asialo-α_2HS in 1 mL of 0.3 M sodium phosphate (pH 6.2) containing 3 mM $CaCl_2$. This protein derivative was obtained by treating native α_2HS with VCS. After 1 day of incubation, 0.5 mL of bovine testes β-galactosidase (0.6 unit) in 0.1 M sodium citrate phosphate buffer (pH 4.3) was added, and the incubation continued for one more day. At the end of the incubation time, 80% of the enzymatic activity remained. Both enzymes were removed by affinity chromatography on 4-aminophenyl thiogalactoside–CH-Sepharose 4B. Salt and Gal were removed on Bio-Gel P-2, and 13 mg (yield 80%) of agalacto-α_2HS was obtained after lyophilization (derivative III, Table II; steps 2a and 2b, Table I).

(*c*) *Cleavage of GalNAc.* Agalacto-α_2HS (14.9 mg) was treated with 1 unit of α-*N*-acetylgalactosaminidase in 1 mL of 0.1M sodium citrate (pH 4.7). After 2 days of incubation, at which time 75% of the enzymatic activity remained, enzyme, salt, and GalNAc were separated from the modified protein by chromatography on Sephadex G-100. Freeze-drying afforded 12.4 mg (yield 89%) of a-*N*-acetylgalactosamino-α_2HS (derivative IV, Table II; step 3, Table I), which was devoid of sugar at the B-chain.

(*d*) *Cleavage of GlcNAc.* (1) From the *a-N-acetylgalactosamino* derivative: a-*N*-acetylgalactosamino-α_2HS (8 mg)

was incubated with 4 units of β-*N*-acetylglucosaminidase in 0.5 mL of 0.1 M sodium citrate (pH 4.7). After digestion for 3 days, 3.4 GlcNAc residues were released (100% of the enzymatic activity remained). Chromatography on Sephadex G-100 afforded 4.7 mg (yield 67%) of a-*N*-acetylglucosaminamino-α_2HS (derivative V, Table II; step 4, Table I). (2) From the *agalacto* derivative: agalacto-α_2HS (13.2 mg) was digested with 10 units of β-*N*-acetylglucosaminidase in 1 mL of 0.1 M sodium citrate (pH 4.7). The release of GlcNAc was followed over an incubation period of 5 days. No loss of enzymatic activity was observed. Subsequent chromatography on Sephadex G-100 afforded 8.2 mg (yield 65%) of a-*N*-acetylglucosamino-α_2HS (derivative VI, Table II; step 4, Table I) with a terminal GalNAc on the B-chain.

(*e*) *Cleavage of α-Man.* A-*N*-Acetylglucosamino-α_2HS (4 mg; derivative VI, Table II with O-linked GalNAc) was treated with 4 units of α-mannosidase in 0.5 mL of 0.1 M sodium citrate buffer (pH 4.7) containing 10 mM $ZnAc_2$. Release of Man was followed over an incubation time of 3 days (40% of the enzymatic activity remained), and the cleaved monosaccharide was determined as described by Walsh et al. (1990). Following chromatography on Sephadex G-100, lyophilization afforded 3 mg (yield 78%) of a-α-mannosino-α_2HS (derivative VII, Table II; step 5, Table I).

(*3*) *Evaluation of the Purity of the α_2HS Derivatives.* Each gp derivative was analyzed by SDS–PAGE (Laemmli, 1970). N-Terminal sequence analysis was performed with the aid of a Beckman liquid-phase sequencer (Model 890C). Similar to results obtained in our previous studies (Walsh et al., 1990; Watzlawick et al., 1991), the polypeptide chains of α_2HS also remained intact throughout these procedures.

(*4*) *Chemical Analyses.* The acid hydrolysis conditions for liberating the different sugars from the protein and the methods for quantifying these sugars and proteins were discussed in detail by Walsh et al. (1990) [for specific details of analysis for NeuAc, Gal, Man, GlcNAc, and GalNAc, respectively, see Silver et al. (1990), Kurz and Wallenfels (1974), Gawehn (1974), Preinl (1987), and Watzlawick et al. (1991)]. An interesting problem in determining the GalNAc content was noted. After acid hydrolysis of the gp and reacylation, the liberated GalNAc value was too high. Interference by an acetylated amino acid on the HPLC separation was suspected, so the acetylated amino acids and/or peptides were removed on Dowex 1-X4 prior to sugar measurement. Subsequent determination of GalNAc afforded the expected value.

RESULTS

(*1*) *Asialo-α_2HS.* In step 1a (Table I), VCS was used resulting in almost complete release of sialic acid (98%, 6 h). Therefore, when this enzyme was employed, it was not possible to differentiate between α2–3- and α2–6-linked NeuAc. To determine unambiguously the type of α-ketosidic linkage(s) of NeuAc, two other sialidases with distinct specificities (Table I) were next utilized. As shown in Figure 1, *Arthrobacter ureafaciens* sialidase readily released ~60% of the NeuAc within 1 h at higher enzyme concentration, and almost complete liberation was obtained at low enzyme concentration within 23 h. However, using fowl plague virus sialidase, only 40% of the NeuAc was released within the same period of incubation. After the incubation time was extended to 33 h, no significant increase in the cleavage of NeuAc was observed. With the total NeuAc content of 6.2 residues, these data indicate that 2.5 NeuAc residues are α2–3 linked, whereas the remaining NeuAc (3.7 residues) possess an α2–6 bond.

(*2*) *Agalacto-α_2HS.* For cleavage of the penultimate monosaccharide, Gal, two different β-galactosidases with

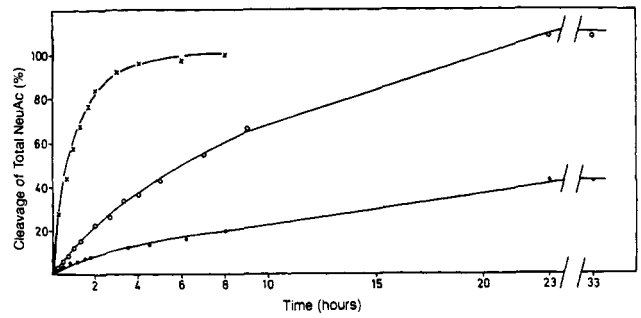


FIGURE 1: Time course of the release of NeuAc during digestion of native α_2HS with 50 (×) or 3 (○) milliunits of *Arthrobacter ureafaciens* sialidase or 0.05 milliunit of fowl plague virus sialidase (●).

distinct specificities were utilized: β-galactosidases from *Streptococcus pneumoniae*, which hydrolyzes Galβ1–4GlcNAc but neither Galβ1–3GlcNAc nor Galβ1–6GlcNAc (Paulson et al., 1978), and bovine testes β-galactosidase, which hydrolyzes both the β1–4 and β1–3 linkages of various substrates, Galβ1–4GlcNAc, Galβ1–3GlcNAc, and Galβ1–3GalNAc but not Galβ1–6GlcNAc or Galβ1–2GlcNAc (Distler & Jourdian, 1973). Digestion of asialo-α_2HS with the former galactosidase resulted in the release of 4 residues of Gal/mol of protein, indicating that these Gal residues are β1-4 linked to GlcNAc. Subsequent digestion with bovine testes β-galactosidase cleaved 3 (actually 2.6) further Gal residues. Therefore, asialo-α_2HS possesses 4 β1–4-bound Gal, characteristic of N-glycosidic chains, and 3 β1–3-linked Gal which were assigned to the O-linked oligosaccharides.

(*3*) *A-N-acetylgalactosamino-α_2HS.* Treatment of agalacto-α_2HS with α-*N*-acetylgalactosaminidase led to the release of 2.6 GalNAc residues. αGalNAc residues are known to exist only in O-glycosylated proteins (Baenziger, 1984). With the release of approximately 3 GalNAc residues, confirmation of the presence of 3 O-glycosylation sites was obtained. This enzyme reaction afforded α_2HS devoid of O-chains.

(*4*) *A-N-acetylglucosamino-α_2HS.* To cleave GlcNAc, which is the terminal sugar of the N-chains of agalacto-α_2HS (derivative III, Table II) and of a-*N*-acetylgalactosamino-α_2HS (derivative IV, Table II), jack bean β-*N*-acetylglucosaminidase was utilized. This enzyme hydrolyzes GlcNAcβ1–4Man 3 times slower and GlcNAcβ1–6Man 4 times slower than GlcNAcβ1–2Man (Takasaki & Kobata, 1986). The release of GlcNAc was monitored over a prolonged period of enzyme treatment; 60% of the GlcNAc was released within 24 h, while the remaining GlcNAc residues were liberated during the subsequent 4 days (total 3.4 residues). Moreover, since 4 GlcNAc residues were released from the agalacto and the a-*N*-acetylgalactosamino derivatives, these 4 GlcNAc residues must reside with two *N*-glycans, indicating a biantennary structure of the two N-chains. It should be noted that α_2HS has been reported to be sulfated but to a minute degree (0.1 mol/mol of protein) (Hortin et al., 1986). However, the sulfate content did not influence the amount of released sugar.

(*5*) *A-α-mannosino-α_2HS.* The time course of the liberation of Man from α-*N*-acetylglucosamino-α_2HS indicated that ~50% of αMan was easily released by the jack bean α-mannosidase. As this enzyme readily hydrolyzes Manα1–2Man and Manα1–6Man but cleaves the Manα1–3 linkage approximately 15-fold slower (Fournet et al., 1981), ~50% of the αMan appears to be α1–6 or α1–2. However, the latter linkage does not occur in the trimannosyl core. The other

αMan was released very slowly, a finding that can be explained by an α1–3 linkage. After an incubation period of 3 days, 3.5 Man residues were liberated. These results agree with the notion that 4 αMan are contained in the two biantennary N-chains, of which 2 αMan are linked in α1–6 linkage and the other two in α1–3 linkage.

(*6*) *Integrity of the Polypeptide Chain.* SDS–PAGE of the derivatives of α_2HS did not demonstrate the presence of any low molecular weight polypeptide fragments (not shown). Also, N-terminal amino acid sequencing did not reveal any amino acids in addition to those expected from the native protein.

(*7*) *Monosaccharides Released by Glycosidases.* The amount of monosaccharides released by sequential treatment of α_2HS with exoglycosidases (Table I) agrees with the carbohydrate composition of α_2HS determined by acid hydrolysis (Table II). When the results from the present study were compared with the carbohydrate content reported for α_2HS (Schwick & Haupt, 1984), the occurrence of 1 Fuc residue was noted. Bovine testes β-galactosidase does not act on Gal[Fuc]GlcNAc (Distler & Jourdian, 1973); however, digestion of asialo-α_2HS with this enzyme yielded complete release of Gal, as judged by the Gal content determined by the acid hydrolysis method. Therefore, Fuc must be attached to the chitobiose core of the glycans. For the evaluation of the degree of sialylation and galactosylation, it should be realized that every other α_2HS molecule has only 2 *O*-glycans. Therefore, the sialylation is achieved to a degree of 95% and that of galactosylation to 100% (Kornfeld & Kornfeld, 1976).

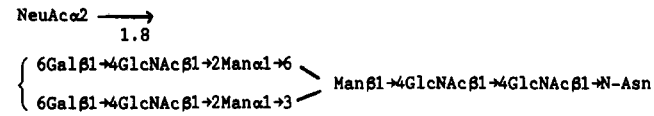
(*8*) *Chemical and Physicochemical Properties of α_2HS and Its Derivatives.* The carbohydrate compositions of α_2HS and its derivatives are compiled in Table II. As to the enzymatic cleavage of sugars of this gp, NeuAc was completely cleaved and Gal was cleaved to the extent of 94%. Removal of GalNAc was achieved to ~85% in the presence of the GlcNAc of the 2 N-biantennas. Subsequent removal of GlcNAc was also performed to about the same extent. The next step involving cleavage of the αMan was effected to ~88%. The total amount of carbohydrate cleaved was 66%. The methods and their sensitivities have been described in detail (Walsh et al., 1990; Watzlawick et al., 1991).

The M_r of α_2HS calculated from its composition (A-chain with 2 N-biantennary glycans including 1 Fuc and 2 *O*-trisaccharides and B-chain with 1 *O*-trisaccharide) was ~39 065. This value compares well with a M_r value of 40 000 derived from physicochemical properties of the protein (Schmid & Bürgi, 1961). The M_r of the α_2HS derivatives, as determined by SDS–PAGE, decreased at each step by approximately the M_r of the sugar residues cleaved. The exception is the NeuAc which, as it is well-known, causes the native protein to exhibit an increased apparent electrophoretic mobility.

DISCUSSION

Sequential degradation of a native glycoprotein with exoglycosidases of defined specificity enables one to determine the type of anomeric configuration of the different monosaccharides, the monosaccharide sequences, and the linkages between sugars. When this procedure was applied to native α_2HS and the number of O- and N-glycosylation sites in the amino acid sequence (Gejyo et al., 1983) and the carbohydrate composition of each gp derivative were considered, the following conclusion was reached: the A-chain of α_2HS contains 2 biantennary N- and 2 O-linked glycans.

(*1*) *Structure of* the *N-Glycans.* The 4 (actually 3.7) α2–6 NeuAc residues must be assigned to the *N*-glycan (for discussion of this point, see the section on *O*-glycans). As to Gal, the *N*-glycans always have these residues in β1–4 linkage (Montreuil, 1982; Vliegenthart et al., 1985). Hence, the 4 Gal residues released by the β1–4-specific β-galactosidase must be assigned to the N-glycans (see also the section on *O*-glycans). Digestion of agalacto-α_2HS with β-*N*-acetylglucosaminidase yielded essentially 4 (3.4) GlcNAc residues. Further evidence for 2 biantennary *N*-glycans were obtained from the enzymatic (α-mannosidase) determination of the fourth sugar, αMan. A triantennary structure could be excluded because only 4 Gal and 4 GlcNAc residues were removed by the appropriate enzymes. The structure of the *N*-glycans is as follows:



As indicated above, only 3.7 residues of α2–6 NeuAc rather than 4.0 such residues were identified, suggesting that the *N*-glycans are partially (~90%) sialylated.

(*2*) *Structure of the O-Glycans.* Employing sialidases with different specificity, the number of α2–3-linked NeuAc was found to be 2.5 (also partially sialylated). The total NeuAc content of 6.2 residues, which essentially agrees with the maximum possible 7 NeuAc residues of the protein, consists of 4 residues from the 2 *N*-glycans and 3 residues from the *O*-glycans. As the *O*-glycans are known to contain, with few exceptions (Rana et al., 1987), α2–3-linked NeuAc residues (Montreuil, 1982; Baenziger, 1984; Berger et al., 1982; Kornfeld & Kornfeld, 1985), the 2.5 α2-3 NeuAc residues are assigned to the 3 *O*-glycans. The remaining 3.7 α2–6 NeuAc residues must, therefore, be assigned to the *N*-glycans. Further, the 3 β1–3 Gal must be assigned to the *O*-trisaccharides, because as far as is known, all *O*-trisaccharides possess only Galβ1–3 (Montreuil, 1982; Baenziger, 1984) and the biantennary *N*-glycans always have Gal in β1–4 linkage (Montreuil, 1982; Vliegenthart, 1985). The amount of GalNAc (Tables I and II) liberated after the release of Gal indicated the presence of 3 *O*-glycans in agreement with the finding of 3 sites for *O*-glycosylation (Yohioka et al., 1986) identified during amino acid sequencing (Yoshioka et al., 1986) and the release of 3 GalNAc residues found in the present study. From these data it was concluded that all 3 *O*-trisaccharides have the structure NeuAcα2–3Galβ1–3GalNAcα1–0.

(*a*) *To exclude the presence of an O-tetrasaccharide and another trisaccharide* with the structures NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAcα1–O, commonly occurring in glycoproteins (Montreuil, 1982; Baenziger, 1984; Berger et al., 1982), and Galβ1–3[NeuAcα2–6]GalNAc, respectively, digestion of α_2HS with a mixture of fowl plague virus sialidase, bovine testes β-galactosidase, and α-*N*-acetylgalactosaminidase yielded equal amounts [2.5 (not 2.6) residues] of NeuAc, Gal, and GalNAc residues. These results exclude a branching at GalNAc by an α2–6-bound NeuAc residue (see Table I for specificity of virus sialidase). Further, this experiment confirms the linear structure of the trisaccharide and the absence of a branched trisaccharide (Galβ1-3[NeuAcα2–6]-GalNAc).

(*b*) *To exclude the presence of the O-hexasaccharide* NeuAc2–3Galβ1–3[NeuAcα2–3Galβ1–4GlcNAcβ1–6]GalNAcα1–O), which was found to be the main glycan of galacto-

gp (Akiyama et al., 1984), the following finding should be noted: after enzymatic treatment for cleavage of NeuAc and Gal, the resulting branched trisaccharide Galβ1–3[(GlcNAcβ1–6)]GalNAc would remain. From such a structure, however, no GalNAc could have been released in the third step, whereas in the present study, after treatment of α_2HS with VCS and β-galactosidase, α-*N*-acetylgalactosaminidase released GalNAc. Hence, the absence of the *O*-hexasaccharide is evident.

(*c*) *To exclude the presence of O-disaccharides* with the structures Galβ1–3GalNAc or NeuAcα2–6GalNAc as occurring in submaxillary mucins (Eckhardt et al., 1987), the following findings would be expected: NeuAc would be cleaved with sialidase from the latter disaccharide. But using β-galactosidase in the second step, no Gal would be released from this disaccharide, contrasting the finding described above. If the disaccharide Gal–GalNAc is assumed to be present, the sialidase step would yield no sugar in contrast to the experimental finding, thus excluding the presence of these two disaccharides.

(*3*) *Characterization of the Various Derivatives* of α_2*HS*. These derivatives can readily be defined in terms of their sugar compositions and in terms of the linear structure of their glycans using the amount and type of monosaccharide released at each degradation step and, equally important, the specificity of the enzymes employed. It should be noted first that, under the conditions employed for the preparation of these derivatives, the integrity of the polypeptide chain is maintained and, second, that only milligram amounts of starting gp are required for such analysis.

The validity of this approach has recently been shown by studying the structure of fibrinogen isolated from a patient with an hereditary dysfibrinogenaemia (Dempfle et al., 1989). Furthermore, such an investigation allows assessment of the biological function of the protein due to the various terminal sugars of the carbohydrate moiety of the different derivatives. Thus, it is possible to correlate the essential monosaccharide of the glycans, unmasked by sequential enzymatic degradation of the carbohydrate moiety of the protein, with biological activity (Bennett & Schmid, 1980; Thotakura et al., 1990; Grinnell et al., 1991).[2]

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