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Major Oligosaccharides in the Glycoprotein of Friend Murine Leukemia Virus: Structure Elucidation by One- and Two-Dimensional Proton Nuclear Magnetic Resonance and Methylation Analysis†

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ABSTRACT: The highly microheterogeneous, *N*-glycosidically linked oligosaccharides in the glycoproteins of Friend murine leukemia virus (as produced by Eveline cells) were liberated with endo-β-*N*-acetylglucosaminidase H and by alkaline hydrolysis. They were fractionated (as desialylated oligosaccharitols) by gel filtration and by concanavalin A affinity chromatography, and the major fractions were analyzed by methylation–gas chromatography–mass spectrometry, by digestion with exoglycosidases, and, especially, by one- and two-dimensional proton nuclear magnetic resonance spectroscopy. Guidelines for qualitative and quantitative analysis of complex oligosaccharide mixtures by NMR were worked out and the results compared with those obtained by methylation analysis. It was found that these major fractions consist of bi-, tri-, and tetraantennary oligosaccharitols of the "complex" type (comprising a minority of species with *N*-acetyllactosamine repeating units), which are, in part, substituted by nonreducing terminal Galα(1→3) and/or bisecting GlcNAcβ(1→4) residues.

The surface of murine leukemia virus (MuLV)[1] particles is—like that of other *Retroviridae* and of enveloped viruses in general—studded with glycoprotein "spikes" protruding to the exterior (Weiss et al., 1982). The amino acid sequences of several MuLV glycoproteins, as occurring on different viral strains, have been established [e.g., Chen (1982), Koch et al.

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[1] Abbreviations: A1, glycoprotein *N*-glycan of the N-acetyllactosaminic ("complex") type; BSA, bovine serum albumin; Con A, concanavalin A; endo H, endo-β-*N*-acetylglucosaminidase H; Gal, galactose; GalOH, galactitol; GLC, gas–liquid chromatography; Glc, glucose; GlcNAc, 2-acetamido-2-deoxyglucose; gp, glycoprotein; M, glycoprotein *N*-glycan of the mixed ("hybrid") type; Man, mannose; MS, mass spectrometry; MuLV, murine leukemia virus; F-MuLV, Friend strain of MuLV; NMR, nuclear magnetic resonance; 1D and 2D, one and two dimensional; NOE, nuclear Overhauser enhancement or effect; ppm, parts per million; SDDS, spin-decoupling difference spectroscopy.

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(1983), Lenz et al. (1982), Linder et al., (1982), and Schinnick et al.(1981)], but their oligosaccharide moieties have to date only been characterized by chromatography of Pronase and/or endo-β-*N*-acetylglucosaminidase digests (Geyer et al., 1982a; Kemp et al., 1979, 1980; Rosner et al., 1980), although an implication of these glycans both in viral tropism and in leukemogenesis has been suggested (Kemp et al., 1980; Ruscetti et al., 1981).

We have therefore taken up the structural analysis of MuLV gp oligosaccharides, starting with the helper-independent Friend strain of MuLV (Troxler et al., 1980) as produced by Eveline cells (Seifert et al., 1975), because this virus–cell system allows the isolation (Schneider et al., 1979, 1980) of viral glycoprotein in amounts large enough for the analysis of its oligosaccharide components also by ^{1}H NMR. Like the glycoprotein obtained from other strains of MuLV (Elder et al., 1977; Krantz et al., 1977), preparations of F-MuLV gp are heterogeneous, consisting, in this case, of (at least) three related glycoproteins (Koch et al., 1983, 1984; Linder et al., 1982; Murray & Kabat, 1979; Schneider et al., 1980). The complete amino acid sequence of the major species, viz., of F-MuLV gp71, has been published and was found to comprise eight potential N-glycosylation sites (i.e., Asn-X-Ser/Thr sequences) (Chen, 1982; Koch et al., 1983). The carbohydrate moieties in the F-MuLV gp's constitute about 19 wt % and consist of the sugars typical for N-glycosidically linked glycoprotein glycans (Berger et al., 1982; Kornfeld & Kornfeld, 1980; Montreuil, 1980), viz., of mannose, *N*-acetylglucosamine, galactose, fucose, and sialic acid (Geyer et al., 1982a).

Since the F-MuLV gp oligosaccharides turned out to be an exceedingly complex mixture of N-acetyllactosaminic ("complex"), oligomannosidic ("high-mannose"), and hybrid ("mixed-type") glycans (Geyer et al., 1982a), we have first analyzed the structures of the major oligosaccharide fractions as obtained after desialylation. Besides methylation–GLC–MS and exoglycosidase digestion, we have especially used one- and two-dimensional ^{1}H NMR techniques, adapted to the analysis of glycan mixtures.

Materials and Methods

Virus. The helper-independent component (F-MuLV) of the Friend murine leukemia virus complex (Troxler et al., 1980), as produced by Eveline cells (Seifert et al., 1975), was used throughout. The cells were propagated in Dulbecco's modified Eagle's medium (Gibco, Paisley, Great Britain), supplemented with 10% (v/v) complement-inactivated (30 min, 56 °C) fetal bovine serum, and the virus particles were harvested by differential centrifugation (Moennig et al., 1974). For in vivo radiolabeling of F-MuLV with D-[6-^{3}H]galactose, the procedure of Geyer et al. (1982a) was employed.

Isolation of Viral Glycoprotein. gp85 "rosettes", comprising the viral constituent glycoproteins as well as non-glycosylated p15E (p12E), were isolated from the F-MuLV particles according to Schneider et al. (1980).

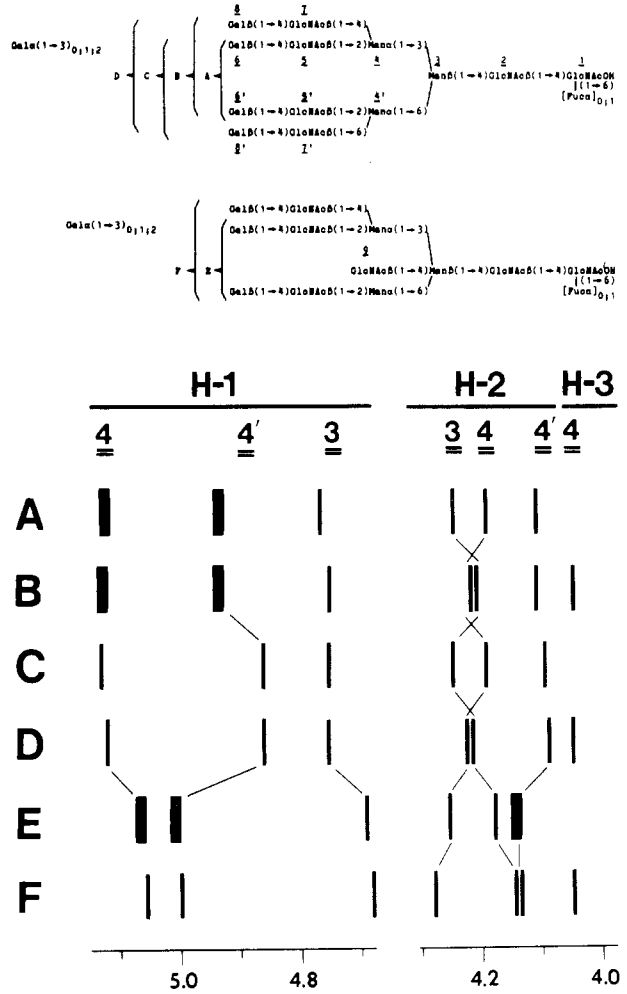
Liberation, in Vitro Radiolabeling, and Fractionation of Glycoprotein Oligosaccharides (as Oligosaccharitols). Except for the following two modifications, the oligosaccharides in F-MuLV gp85 rosettes (80 mg) were liberated, reduced, desialylated, and fractionated as previously described [see Geyer et al. (1982a) and Figure 1]. (i) Instead of $NaBH_4$, $KB^3H_4/NaBH_4$ was used for reduction of the oligosaccharides released by endo H; for experimental details, see Geyer et al. (1983). (ii) The N-acetyllactosaminic glycans were not liberated by hydrazinolysis but by alkaline hydrolysis (Lee & Scocca, 1972) and were radiolabeled with [^{14}C]acetic anhydride: for this purpose, 40 mg of $NaBH_4$ in 400 μL of 5 N aqueous NaOH was added to the endo H resistant Pronase glycopeptides in 600 μL of water, and the mixture was heated at 100 °C for 4 h. After cooling, neutralization (to pH 5) with acetic acid, and lyophilization, the boric acid was chased by repeated evaporation with methanol. For re-N-acetylation, the sample was taken up in 2 mL of saturated aqueous $NaHCO_3$, mixed with 100 μCi of [1-^{14}C]acetic anhydride (60–120 mCi/mmol; Amersham Buchler, Braunschweig, GFR) in 1 mL of toluene, and shaken for 30 min at room temperature. Keeping a pH of 8 by addition of solid bicarbonate, two 100-μL portions of unlabeled acetic anhydride were then admixed, followed by 15 min of shaking each time. After evaporation, the sample was taken up in 1 mL of water, adjusted to pH 5 with acetic acid, and desalted by passage over a column of Bio-Gel P-2 (–400 mesh; 1.6 × 100 cm) with 0.02 wt % aqueous NaN_3. After lyophilization, the sample was finally stored in 0.5 mL of 0.1 N aqueous NaOH at room temperature for 30 min, adjusted to pH 5–6 with acetic acid, and lyophilized again. Essentially the same procedure was used for the alkaline hydrolysis of metabolically labeled glycopeptides, except that re-N-acetylation was carried out with unlabeled acetic anhydride only. After addition of scintillation cocktail, all fractions were monitored for radioactivity with a Packard (Downers Grove, IL) Model 4550 liquid scintillation counter and were pooled and lyophilized where indicated.

Quantitation and Methylation Analysis of Glycoprotein Oligosaccharitol Fractions. For quantitation, the major glycan fractions were hydrolyzed, and the sugar components were determined by GLC of the alditol acetates (Sawardeker et al., 1965; Geyer et al., 1982b); the amounts of the smaller glycan fractions were then estimated from their relative radioactivity. For the methylation studies, the oligosaccharitols were permethylated according to Hakomori (1964) and analyzed by GLC–MS of the partially methylated alditol acetates (Jansson et al., 1976; Stellner et al., 1973). The capillary columns, instrumentation, and experimental conditions used for these procedures at a microscale have previously been described in detail (Geyer et al., 1983).

Standard Oligosaccharides. Oligosaccharides (GalGlcNAc)$_{2,3,3'}$Man$_3$GlcNAc (structures A–C in Chart I, but without the proximal GlcNAc residues), as isolated from the urine of patients with gangliosidosis GM 1 (Strecker & Montreuil, 1979), were kindly donated by G. Strecker (Université des Sciences et Techniques, Villeneuve d'Asq, France). Before use in gel filtration, they were reduced with $KB^3H_4/NaBH_4$.

Digestion with Exoglycosidases. All exohexosidases were purchased from Sigma (Munich, FRG) and they were applied by using, essentially, the conditions described by Courtois & Petek (1966), Li & Li (1972), and Takasaki et al. (1980). The oligosaccharide samples were thus taken up in 100 μL of 50 mM sodium citrate buffer of pH 4.0, of 50 mM sodium citrate buffer of pH 5.0, of 50 mM sodium acetate buffer of pH 4.5 (with 0.2 mM $ZnCl_2$), or of 150 mM sodium phosphate/citrate buffer of pH 6.0, respectively, and β-galactosidase (EC 3.2.1.23; 2–3 nkat), β-*N*-acetylglucosaminidase (EC 3.2.1.30; 4–5 nkat), or α-mannosidase (EC 3.2.1.24; about 25 nkat) from jack beans or α-galactosidase (EC 3.2.1.22; 3–4 nkat) from green coffee beans was added. All mixtures were incubated for 24 h at 37 °C in the presence of 0.02 wt % sodium azide. After this time, the enzyme addition and incubation were once (twice in the case of β-*N*-acetylglucosaminidase) repeated. After 48 (or 72) h, the reactions were terminated by heating the mixtures to 100 °C for 3 min, and the solutions were desalted by passage through a column (0.5 × 7 cm) of

Chart I: General Formulas of Oligosaccharitols A–F and Expected Chemical Shifts (ppm) for Their H-1, H-2, and (Partly) J-3 Resonances of Mannose Residues 3, 4, and 4′, As Compiled from Literature (Vliegenthart et al., 1981, 1983; Carver & Grey, 1981; Grey et al., 1982)



Amberlite MB-3 resin with distilled water and were lyophilized.

Proton Nuclear Magnetic Resonance Spectroscopy. Prior to measurements, the oligosaccharides were exchanged with D_2O with intermediate lyophilization and then dissolved in 0.3 mL of D_2O containing a trace of acetone, which was used as internal reference (δ 2.225 at all temperatures). The 360-MHz ^1H NMR spectra were obtained on a Bruker HX-360 spectrometer by pulsed Fourier-transform techniques with quadrature detection. Digital resolution was 0.27 Hz. The spectrum of Al_2 (180 μg) was measured at 500 MHz on a Bruker WM-500 spectrometer. For resolution enhancement, the free-induction decays were multiplied by the Lorentzian–Gaussian transformation function (Ernst, 1966). The subsequent Fourier transformation was performed with zero filling to 32K data points. The spectra were measured at several temperatures between 293 and 318 K in order to uncover different characteristic signals masked or distorted by the temperature-sensitive HDO peak. The small temperature coefficients of the saccharide signals (0.0001–0.001 ppm/°C) were taken into account when the chemical shifts obtained here were compared with those reported earlier. SDDS spectra (Gibbons et al., 1975; Dabrowski et al., 1980) free of NOE were obtained by inserting delays between scans. NOE difference spectra were recorded as described by Wagner & Wüthrich (1979). A two-dimensional scalar shift-correlated ^1H NMR spectrum (a "COSY", 90–t_1–90 pulse sequence with

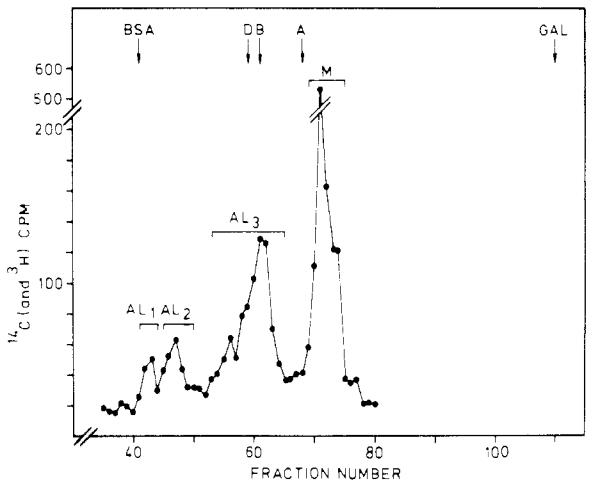


FIGURE 1: Gel chromatogram of desialylated N-acetyllactosaminic and hybrid oligosaccharitols from the glycoproteins in Friend murine leukemia virus. The endo H resistant Pronase glycopeptides and (proximally tritiated) sialylated hybrid oligosaccharitols from F-MuLV gp85 rosettes were treated with aqueous $NaOH/NaBH_4$ at 100 °C, re-N-acetylated with [^{14}C]acetic anhydride, digested with neuraminidase, and chromatographed at hydrostatic pressure through a column (0.6 × 200 cm) of Bio-Gel P-4 (−400 mesh) with 0.02 wt % aqueous sodium azide (0.8 mL/h). Fractions of 10 drops were collected and monitored for radioactivity. Arrows with BSA, GAL, A, B, and D indicate elution volumina of bovine serum albumin, galactose, and bi-, tri-, or tetraantennary standard oligosaccharitols $(GalGlcNAc)_{2\text{-}4}Man_3GlcNAcO^3H$ (i.e., *without* GlcNAc-1; cf. Chart I). $Al_{1\text{-}3}$ and M, fractions of desialylated N-acetyllactosaminic and mixed-type oligosaccharitols. Due to the double radiolabeling, fraction M is overrepresented.

stepwise incremented evolution time, t_1) of Al_{3A} was measured at 360 MHz and 318 K with standard software from Bruker (version 820601.3).

Results and Discussion

Isolation of Oligosaccharide Fractions from Friend Murine Leukemia Virus Glycoproteins. F-MuLV gp85 rosettes (Schneider et al., 1980), comprising the viral spike glycoproteins and the non-glycosylated peptide p15E (p12E), were used as a starting material and were first digested with Pronase. Essentially as described in detail previously (Geyer et al., 1982a), the oligosaccharides were then released from the Pronase glycopeptides by successive treatment with endo H (liberation of oligomannosidic and hybrid glycans) and with aqueous NaOH at 100 °C (liberation of *N*-acetyllactosaminic glycans), and they were fractionated by gel filtration of the oligosaccharitols obtained after reduction, radiolabeling, and desialylation (see Figure 1 for the final chromatogram). Five fractions were thus obtained: (A) oligomannosidic and not sialylated mixed-type F-MuLV gp oligosaccharitols (13–17 wt % of the total glycans). (B) desialylated mixed-type oligosaccharitols (fraction M in Figure 1; 2–4 wt %). (C) small desialylated N-acetyllactosaminic oligosaccharitols (fraction Al_3 in Figure 1; 55–67 wt %). (D) medium-size desialylated N-acetyllactosaminic oligosaccharitols (fraction Al_2 in Figure 1; 16–20 wt %). (E) large desialylated N-acetyllactosaminic oligosaccharitols (fraction Al_1 in Figure 1; 2–4 wt %). Major fractions Al_3 and Al_2, comprising together about 80% of the desialylated viral carbohydrates, were subjected to structural analysis, also after subfractionation of Al_3 into its components with and without affinity to concanavalin A (fractions Al_{3A^+} and Al_{3A^-}, about two- and three-fifths respectively, of Al_3).

Methylation Analysis. The results obtained upon methylation etc. of F-MuLV gp oligosaccharitol fractions Al_3, Al_{3A^+}, Al_{3A^-}, and Al_2 are shown in Table I. Although the

Table I: Methylation Analysis of Major Oligosaccharitol Fractions from the Glycoproteins in Friend Murine Leukemia Virus[a]

item	peracetate of[b]	eq[c]	molar ratio[d] as obtained from fraction			
			$A1_3$	$A1_{3A^+}$	$A1_{3A^-}$	$A1_2$
1	2,3,4-FucOH		0.3[e]	0.4[e]	0.2[e]	0.55
2	3,4,6-ManOH	$2A + B + 2E$	1.5[d]	2.0[d]	1.3[d]	1.2[d]
3	3,6-ManOH	$B + D$	0.3[d]		0.4[d]	0.2[d]
4	3,4-ManOH	D	0.2[d]		0.3[d]	0.6[d]
5	2,4-ManOH	$A + B + D$	0.6	0.8	0.4	0.7
6	2-ManOH[f]	E	0.3		0.5	0.25
7	2,3,4,6-GalOH		1.9	1.6	1.7[g]	2.1
8	2,4,6-GalOH		0.35	0.4	0.3	0.6
9	2,3,4-GalOH					0.2
10	2,4-GalOH					0.1
11	1,3,5,6-GlcN(Me)-AcOH[h]		0.1	0.2	0.15	trace
12	3,4,6-GlcN(Me)-AcOH	E	0.35	0.2	0.8	0.3
13	1,3,5-GlcN(Me)-AcOH[h]		0.2	0.3	0.25	trace
14	3,6-GlcN(Me)-AcOH	$3A + 4B + 5D + 3E$	3.4	2.7	3.65	3.7

[a] The major N-acetyllactosaminic oligosaccharitol fractions were methylated and hydrolyzed, and the methylated component sugars were analyzed by capillary GLC–mass fragmentography of the alditol acetates, extrapolating the peak ratios obtained to the ratios of detection by flame ionization [see Geyer et al. (1983)]. [b] 2,3,4-FucOH, 2,3,4-tri-*O*-methylfucitol; 1,3,5,6-GlcN(Me)AcOH, 2-deoxy-2-(*N*-methylacetamido)-1,3,5,6-tetra-*O*-methylglucitol; etc. [c] Equations describing the molar contribution of oligosaccharides A–E to the amount of each alditol acetate found. For brevity, the symbols *A*, *B*, etc. are used here in the sense of mole fractions, X_A, X_B, etc. Oligosaccharides C and F, which were not found by NMR, were omitted in these calculations (see Methylation Analysis). [d] Peak ratios based on sum of 3,4,6-, 3,6-, and 3,4-ManOH peracetates = 2.0 (mannose residues 4 and 4′ in Chart I). [e] Probably due to evaporation losses of 2,3,4-FucOH peracetate; lower values of fucosylation were generally found by methylation analysis than by ^{1}H NMR spectroscopy (cf. Table III). [f] Corrected for losses during acetolysis (Conchie et al., 1982). [g] Underestimated, as evidenced by the higher value in Al_3 and by ^{1}H NMR spectroscopy (see Table III). [h] Due to degradation of the proximal GlcNAc residue during alkaline hydrolysis, the peracetates of 1,3,5,6- and 1,3,5-GlcN(Me)AcOH are only partly recovered.

substitution position of all constituent sugars and their localtion in molecules A–F are unequivocally identified by the corresponding partially methylated alditol acetates (cf. Chart I), the reconstruction of the composition of the original oligosaccharitol mixture is not possible algebraically. However, the exclusion of structures C and F by NMR (vide infra) renders this task solvable for $A1_3$. By combining the equations concerning the α-mannoses (items 2–4 in Table I) and the 3,4,6-trisubstituted β-mannose (item 6),[2] one obtains 37 mol % A, 10 mol % B, 20 mol % D, and 33 mol % E. Putting these values into the equation for →4GlcNAc (item 14), one obtains 3.5 mol for the sum of GlcNAc-2, -5, -5′, -7, and -7′, in accord with the experimental value of 3.4. However, a few methylation values are obviously too low. Thus, the above percentage of compounds A, B, D, and E requires 2.5 terminal Gal residues whereas 1.9 was found (item 7). This discrepancy is partly explained by assuming that some branches are incomplete, i.e., not galactosylated, as follows from the 0.2 mol of terminal GlcNAc residues found for $A1_{3A^+}$ and from the 0.3 mol excess of such residues estimated for $A1_{3A^-}$.[3] Naturally, these terminal GlcNAc units should also appear in $A1_3$; hence, the experimental value of 0.35 (item 12) almost coinciding with the value of 0.33 for E (i.e., for GlcNAc-9) calculated above, must be viewed as underestimated. Other deviations are explained in footnotes *e*, *g*, and *h* to Table I. As expected, the data for $A1_3$ approaches the weighted average of those found for its subfractions $A1_{3A^+}$ and $A1_{3A^-}$.

The methylation analysis of $A1_{3A^+}$ is compatible with that of a partially fucosylated biantennary oligosaccharitol (structure A in Chart I), except that, as stated, about 10% of its branches seem to lack outer galactose-6 or -6′ (item 11) while another about 20% of them appear to carry additional terminal Gal residues at position 3 (item 8). These conclusions are in agreement with the Con A affinity of fraction $A1_{3A^+}$ [cf. Cummings & Kornfeld (1982a)] and with the finding (see Figure 2 below) that it coelutes with a structure B standard lacking the proximal GlcNAc-1 residue.

The gel filtration, Con A binding, and methylation results of $A1_{3A^-}$, on the other hand, indicate that this fraction consists of the following, also partially fucosylated species: around 55% of structure E (items 6 and 5 in Table I), ca. 30% of structure D (item 4), and ca. 10% of structure B (items 3 and 4). In the same manner as above, it can then be deduced that, also in $A1_{3A^-}$, some (around 10%) of the antennae seem to carry no outer galactoses, while others (about 15%) are substituted by additional Gal residues.

Fraction $A1_2$, finally, appears to have a composition similar to that of $A1_3$, except that it seems to contain a smaller portion of structure A glycans (item 2 in Table I) and some triantennary oligosaccharitols of type C (items 4 and 3). The larger size of the $A1_2$ components (Figure 1) is accounted for by a higher content of galactose units, which, interestingly, comprise 6- and 3,6-substituted residues in addition to unsubstituted and 3-substituted ones. Since still larger amounts of Gal (3.4, 2.2, 0.2, and 0.9 parts of unsubstituted and 3-, 6-, and 3,6-substituted residues, respectively) and of GlcNAc (7.3 parts) were found in fraction $A1_1$ (not shown in Table I), these results may be taken as an indication that the largest F-MuLV gp oligosaccharitols comprise some oligolactosaminoglycans [cf. Järnefelt et al. (1978) and van den Eijnden et al., (1983)].

Digestion with Exoglycosidases. Oligosaccharitol fractions $A1_{3A^+}$, $A1_{3A^-}$, and $A1_2$, metabolically labeled with [^{3}H]-galactose for this purpose, were digested with exoglycosidases, and the products were analyzed by gel filtration. In addition, the truncated glycans obtained after enzymatic digestion of unsubstituted antennae were subjected to methylation analysis (localization of outer substituents).

Treatment of $A1_{3A^+}$ with β-galactosidase did not liberate all the [^{3}H]galactose residues in this fraction. However, by consecutive exposure to α- and β-galactosidase, 28 and then 72% of the radioactivity was released as a peak coeluting with Gal (Figure 2). Upon methylation of the products obtained from $A1_{3A^+}$ by successive digestion with β-galactosidase, β-*N*-acetylglucosaminidase, and α-mannosidase, 2,4,6-, 2,3,4-, and 2,4-ManOH were obtained in a ratio approaching 3:24:10–11, indicating the proportion of type A oligosaccharitols substituted by α-Gal residues at position 3 of β-Gal-6, of β-Gal-6′, or of both.

Exposure of metabolically labeled $A1_{3A^-}$ to α- and then β-galactosidase (chromatograms not shown) analogously released all radiolabel and confirmed that the additional terminal Gal residues are α-linked also in this fraction. Methylation of $A1_{3A^-}$ after enzymatic digestion of the unsubstituted branches yielded 2,4,6- and 2,3,4-ManOH in a ratio of about 1:4. It thus appears that the antennae linked to position 6 of the core mannose, i.e., galactose residue 6′ (or/and 8′), are preferentially α-galactosylated in $A1_{3A^-}$ as well.

[2] Mole fraction recalculated to 0.33 on the basis of 2,4- and 2-ManOH set equal to 1.0.

[3] Calculated from 0.8 mol of 3,4,6-GlcN(Me)AcOH and 0.5 mol of 2-ManOH.

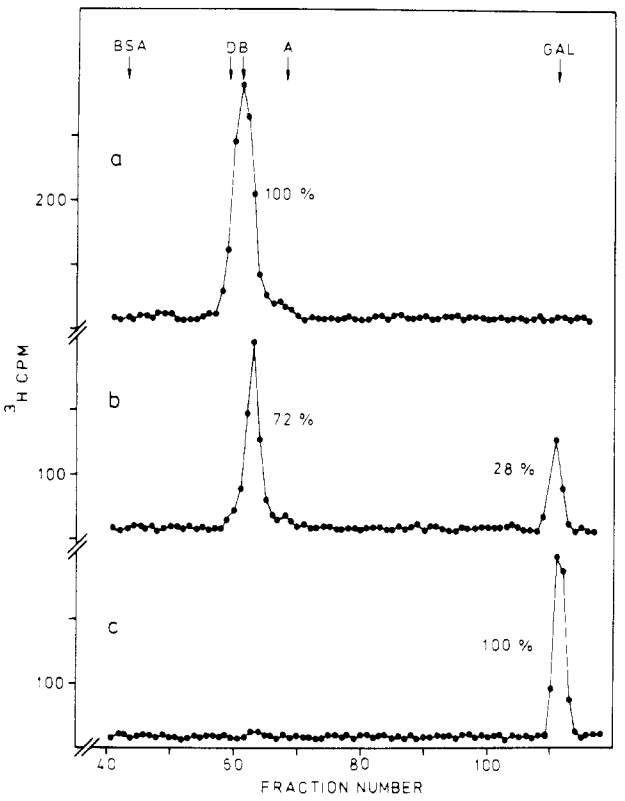


FIGURE 2: Gel chromatograms of F-MuLV oligosaccharitol fraction $A1_{3A^+}$ (metabolically labeled with [^{3}H]galactose) before and after digestion with exogalactosidases: (a) fraction $A1_{3A^+}$; (b) fraction $A1_{3A^+}$ after digestion with α-galactosidase; (c) fraction $A1_{3A^+}$ after successive digestion with α- and then β-galactosidase. Conditions of chromatography and standards are as in Figure 1.

In contrast, digestion with the two galactosidases liberated only 74% of the radioactivity in the metabolically labeled $A1_2$. However, after additional exposure to β-*N*-acetylglucosaminidase, another 13% of the [^{3}H]galactoses in this fraction were rendered accessible to β-galactosidase. These results are in agreement with the assumption that fraction $A1_2$ contains some oligolactosaminoglycans.

^{1}H NMR Spectroscopy. The identification of the particular oligosaccharitols in heterogeneous mixtures by NMR was based mainly on mannose H-1 and H-2 chemical shifts characteristic of different structures [Vliegenthart et al. (1981, 1983) and references cited therein; Carver & Grey, 1981; Grey et al., 1982]. This literature data is compiled in Chart I.

Since none of these sets of assigned chemical shifts completely coincides with any other one, the structure of any individual compound of type A–F is uniquely determinable. However, determinability is not that explicit if mixtures are concerned, because many combinations of two, three, etc. of compounds A–F may perfectly mimic other ones. The following guidelines concerning the analysis of mixtures can be derived from Chart I: (i) the binary mixtures AB, AC, AE, AF, BD, BE, BF, CD, CE, CF, DE, and DF are uniquely determined by chemical shifts alone, regardless of relative concentration of their components, though it might be difficult to distinguish AF from BE and CF from DE; (ii) equimolar mixtures AD and BC are indistinguishable from each other and from ABCD, the same applies to such mixtures containing E and/or F in addition; (iii) other mixtures are not discriminable by chemical shifts alone but can be identified with the help of integration. This, in turn, requires well-separated signals and complete absence of interfering contaminations—a hardly realistic condition for complex mixtures of natural origin. It seems that quantitative estimations will be burdened with considerable errors in most cases.

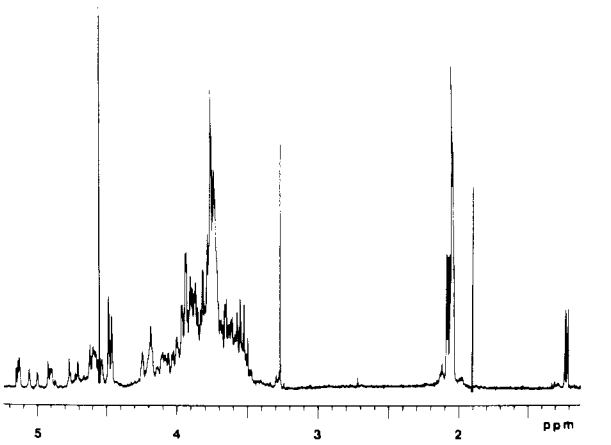


FIGURE 3: Resolution-enhanced 360-MHz ^{1}H NMR spectrum of fraction $A1_3$ (mixture of oligosaccharitols A, B, D, and E) in HDO at 308 K.

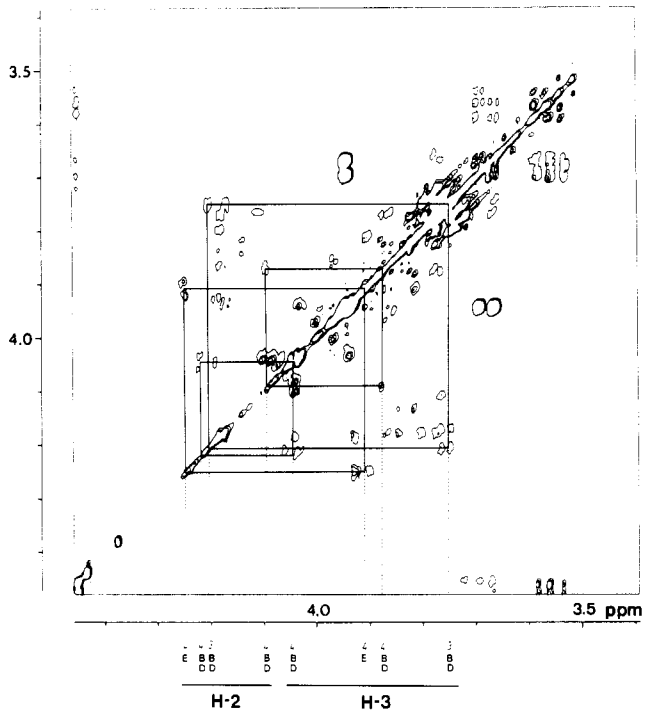


FIGURE 4: The 3.4–4.5 ppm region of the two-dimensional scalar shift-correlated (COSY) spectrum of the fraction $A1_{3A^-}$ (mixture of oligosaccharitols B, D, and E) in HDO at 318 K. The assignments for the H-2 and H-3 mannose residues 3, 4, and 4′ derived from shift correlations labeled (—) are given below the scale.

To avoid possible ambiguities in combining the Man H-1 and H-2 resonances for the F-MuLV oligosaccharitol fraction $A1_3$ into pairs belonging to the same molecular species, it was necessary to experimentally establish the connectivities between them. For this purpose, spin-decoupling difference spectroscopy (SDDS; Gibbons et al., 1975; Dabrowski et al., 1980), two-dimensional ^{1}H-correlated spectroscopy, and NOE difference spectroscopy (Wagner & Wüthrich, 1979; Lemieux et al., 1980; Dabrowski et al., 1981; Hanfland et al., 1981; Carver et al., 1981) were applied. This allowed us to assign the resonances of the constituent sugar residues listed in Table II. The whole spectrum of this fraction is shown in Figure 3. Some ambiguities concerning our assignments were resolved by analyzing the not yet utilized H-3 signals that were found with the aid of 2D correlated spectroscopy (Figure 4).

The integral intensity of the Man-4 H-1 resonance is divided in the proportion of 37:64[4] between the signals at 5.062 and

Table II: Proton Chemical Shifts (ppm[a] at 318 K in ^2H_2O) for Components A, B, D, and E[b] of the Oligosaccharitol Mixture Obtained from Friend Murine Leukemia Virus Glycoproteins

residue[b]	protons	component A[b]	component B[b]	component D[b]	component E[b]
GlcNAc-1	H-2	4.187	4.188	4.188	4.188
GlcNAc-2	H-1	4.641[c]	4.641[c]	4.641[c]	4.641[c]
Man-3	H-1	4.772	4.762	4.773	4.709
	H-2	4.244	4.204	4.204	4.188
	H-3	3.761	3.748	3.748	*d*
Man-4	H-1	5.131	5.132	5.132	5.062
	H-2	4.187	4.214	4.214	4.251
	H-3	3.903	4.04	4.04	3.913
Man-4′	H-1	4.924	4.920	4.872	5.003
	H-2	4.108	4.09	4.09	4.146
	H-3	3.885	3.88	3.88	*d*
GlcNAc-5, -5′	H-1	4.583[c]	4.583[c]	4.583[c]	4.583[c]
GlcNAc-7	H-1		4.545[c]	4.545[c]	
GlcNAc-7′	H-1			4.554[c]	
GlcNAc-9	H-1				4.479
	H-2				3.69
Gal-6	H-1	4.472[e]	4.475[e]	4.475[e]	4.475[e]
Gal-6′	H-1	4.476[e]			
Gal-8, -8′	H-1		4.475[e]	4.475[e]	
Gal-6, -6′, -8, -8′	H-2	3.558	3.558	3.558	3.558
Fuc(F)	H-1	4.901	4.903	4.903	4.903
	H-2	3.804	3.804	3.804	3.804
	H-5	4.072	4.072	4.072	4.072
	H-6	1.227	1.225	1.225	1.225
Gal(G)	H-1	5.149	5.149	5.149	5.149
	H-2	3.865	3.865	3.865	3.865

[a] Relative to acetone set equal to δ 2.225. [b] The shorthand notation in the headings of this table corresponds to the numbering given in Chart I, but F and G denote Fucα(1→6) and Galα(1→3), respectively. [c] Measured at 300 K in the $A1_3$ fraction. [d] Assignment uncertain. [e] Assigned to terminal β-Gal residues; for those α-galactosylated, this resonance is overlapped with the H-1 resonances of GlcNAc-5, 5′, -7, and -7′ residues (see footnote 6).

5.132 ppm that are characteristic of bisected (E and F) and nonbisected (A–D) structures, respectively, and the same applies approximately[4] to the Man-3 H-1 signals at 4.709 and 4.762/4.772 ppm (see Table III). Similarly, the intensity of the Man-4′ H-1 resonance is distributed between the signal due to bisected structures at 5.003 ppm and the group of signals in the 4.87–4.93 ppm region characteristic of nonbisected structures. By subtracting the H-1 signal of Fucα(1→6) occurring within this region at 4.903 ppm, one obtains a ratio of 35:64,[4] in excellent agreement with the above value.

For each of the H-1 and H-2 mannose signals of nonbisected oligosaccharides at least two alternative assignments are possible (see Chart I). Nevertheless, the components of the mixture can be identified and their percentage determined by comparing and integrating the spectra of $A1_3$ and of the subfractions $A1_{3A^+}$ and $A1_{3A^-}$ containing the biantennary and the remaining oligosaccharitols, respectively (Figure 5a–c). Thus, it is apparent that in the original mixture the main component among the nonbisected oligosaccharitols must be A because the signals of H-1 of Man-4 (δ ~5.13) and -3 (δ ~4.77), which are common for A–D, and that of Man-4′ (δ ~4.92), which is common for A and B, exhibit much lower intensities in the spectrum of $A1_{3A^-}$. The question of whether these peaks of reduced intensities are due to some residual amount of A or rather to other oligosaccharitols can be solved by analyzing the spectral changes near 4.25 ppm. The unresolved signal centered at 4.244 in the spectrum of $A1_3$ is much weaker in that of $A1_{3A^-}$, its maximum being shifted to 4.251 ppm. Although this residual signal obviously corresponds to Man-4 H-2 of the bisected biantennary oligosaccharitol E, as evidenced by its connectivity with the H-1 signal at 5.062 ppm established by NOE, it might bury some residual intensity related to the original δ 4.244 resonance attributed to Man-3

Table III: Molar Ratios of Sugar Components in Oligosaccharitol Fractions[a] from Glycoproteins of Friend Murine Leukemia Virus: Analysis by ^1H NMR

sugar residue[b]	in structure[b]	ratio of signal integrals[c] as obtained with fraction $A1_3$	$A1_{3A^+}$	$A1_{3A^-}$
α-Fuc(F)	A–E	0.64	0.71	0.60
α-Man-4	A	0.64	1.02	0.44
	B			
	D			
	E	0.37		0.54
α-Man-4′	A	0.51	0.98	
	B			0.28
	D	0.14		0.21
	E	0.34		0.53
β-Man-3	A	0.64	0.96	0.45
	B			
	D			
	E	0.35		0.51
β-GlcNAc(OH)-1, -2, -5, -5′, -7, -7′, -9	A–E	4.49	*d*	*d*
β-Gal-6, -6′, -8, -8′	A–E	2.65	1.98	2.97
α-Gal(G)	A–E	0.48	0.48	0.50

[a] The F-MuLV gp oligosaccharitol fractions were analyzed by ^1H NMR, and the relevant signals were integrated as described in the text. [b] Compare Chart I and Table II. [c] Based on sum of mannose residues 4 and 4′ = 2.00; see footnote 4. [d] Signals of admixtures precluded a reliable integration.

[4] Note that the sum of these two integrals is not exactly 1.00 due to the fact that H-1 signals of both the Man-4 and -4′ residues were taken as a basis for calculations by setting the sum of their integrals equal to 2.00.

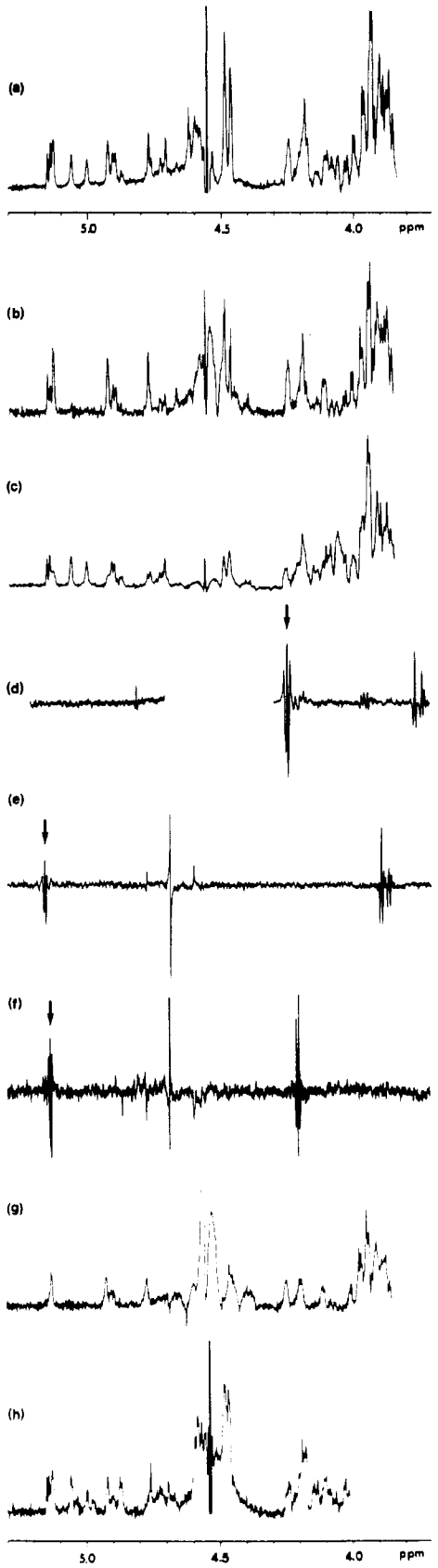


FIGURE 5: Region of characteristic sugar ring proton resonances of the ^1H NMR spectra of several oligosaccharitols examined in HDO. (a–c, g, h) Resolution-enhanced basic spectra of fractions $A1_3$ [(a) 360 MHz, 318 K], $A1_{3A^+}$ [(b) 360 MHz, 318 K], $A1_{3A^-}$ [(c) 360 MHz, 318 K], $A1_{3A^+}$ after the α-galactosidase treatment [(g) 360 MHz, 318 K], and $A1_2$ [(h) 500 MHz, 318 K]. (d–f) The 360-MHz SDDS spectra of the $(GalGlcNAc)_3Man_3GlcNAcOH$ standard oligosaccharitol [(d) 318 K] and fraction $A1_3$ oligosaccharitol mixture [(e and f) 308 K]; irradiation by a second radio-frequency field is indicated in the SDDS spectra by arrows.

H-2 of A. Besides, triantenna C, if present, would also exhibit the Man-3 H-2 signal near 4.25 ppm. In spite of these coincidences, the absence of A, and simultaneously C, can be inferred from the failure to detect the connectivity between this residual signal and the potential H-3 signal of Man-3 anticipated at $\delta \sim 3.75$ according to our decoupling experiments with oligosaccharide standards of types A and C (Figure 5d) and the recent 2D NMR data on type A oligosaccharide from human serum transferrin (Homans et al., 1983). Since negative evidence might seem unconvincing, it should be emphasized that detection of H-2/H-3 connectivities for mannoses is unproblematic, as demonstrated by the following: (a) this same connectivity was easily found by SDDS for $A1_3$; (b) a contour of Man-3 H-3 is clearly seen at 3.748 ppm in the 2D spectrum of $A1_{3A^-}$ (Figure 4), but it correlates exclusively with a resonance at 4.204 ppm corresponding to Man-3 H-2 of B and/or D (Chart I); (c) the Man-4 H-2 resonance of E at 4.251 ppm, whose connectivity with H-1 was mentioned above, shows also the connectivity, both in the SDDS and the 2D spectrum, with the H-3 resonance at 3.913 ppm, i.e., within the region where H-3 signals of 2-glycosylated α-Man residues were found for several oligosaccharides (Bock et al., 1982; J. Dabrowski, unpublished results).

The elimination of A and C as possible components of $A1_{3A^-}$ has two further consequences. First, another nonbisected oligosaccharitol, signalized by the Man-4′ H-1 resonance at 4.870 ppm, must be the tetraantennary one, D. Second, the residual intensity at 4.920 ppm can only correspond to the triantennary nonbisected oligosaccharitol B. The Man-4 H-3 resonances of B and D characteristically occurring at ~4.05 ppm are not directly visible in the spectra of Figures 5a,c because of signal overlap, but the 2D spectrum contains correlated contours in this region, namely, at 4.214/4.04, as expected for these structures. Of the possible components of $A1_3$, F should also be eliminated since there is no signal at ~4.28 ppm, where the Man-4 H-1 resonance has been found for several bisected, multiply-branched structures [Vliegenthart et al. (1983) and references cited therein; Paz-Parente et al., 1983].

Fucose, found in submolar amounts by methylation analysis, is clearly α(1→6) linked. Our values for its H-1, H-5, and H-6 chemical shifts (δ 4.903, 4.072, and 1.225, respectively) differ slightly from those reported by Vliegenthart et al. (1981) for the -GlcNAcβ(1→4)[Fucα(1→6)]GlcNAcβ(1→)Asn moiety (δ 4.876, 4.126, and 1.206, respectively), but this can readily be explained by the different aglycon effect in the compounds investigated here, viz., -GlcNAcβ(1→4)[Fucα-(1→6)]GlcNAcOH. There is no 1→3-linked fucose residue since irradiation at the frequency of the only fucose methyl signal at δ 1.225 produced exclusively the H-5 difference signal at δ 4.072, whereas no response was observed near δ 4.830, where the H-5 resonance of Fucα(1→3) is expected to occur [Vliegenthart et al. (1981) and references cited therein]. Therefore, the doublet at δ 5.149 ($^3J_{1,2}$ = 3.9 Hz), which could otherwise be mistaken for H-1 of Fucα(1→3) (δ 5.127; loc. cit.), must be attributed to another α-sugar residue. In keeping with the results of the methylation and exoglycosidase treatment (Table I and Figures 2 and 5g) and taking into account that the H-2 chemical shift of this α-sugar unit discovered by SDDS (Figure 5e; δ 3.865)[5] does not differ much from that found for α-galactosyl of melibiose (δ 3.83; DeBruyn et al., 1975), we ascribe the above doublet to the terminal 1→3-linked α-galactose residue. Recently, this H-2 resonance was found by NOE at δ 3.863 for the related trisaccharide Galα-(1→3)Galβ(1→4)GlcNAc (Van Halbeek et al., 1983). In-

tegration shows that about 20% of the arms are α-galactosylated, but there is no specific NMR information about the distribution of α-Gal residues between the particular arms. Compared with the methylation analysis, the total amount of α-Gal units determined by NMR is somewhat higher (0.48 vs. 0.35). Since one of the two practically equal components of the α-Gal H-1 doublet is well separated and can be exactly integrated, we believe that this value is more reliable.

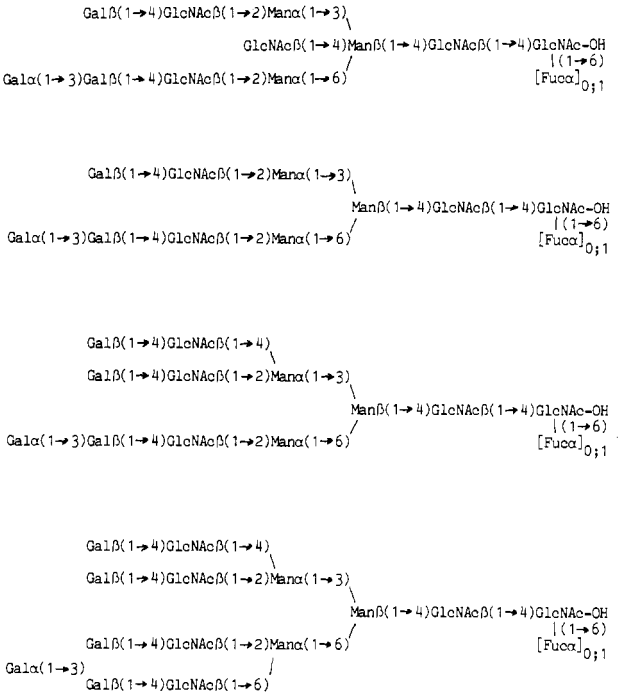
The elimination of the components C and F from further consideration greatly simplifies the quantitative evaluation of the NMR data (Table III). The percentage of the bisected biantenna E determined from three independent sources (H-1 of Man-4, -4′, and -3) is 35 mol % on the average. The amount of tetraantenna D (14 mol %) was directly evaluated from the integral of the Man-4′ H-1 signal at 4.872 ppm. However, the integrable Man H-1 signals are grouped in such a way that the quantitative information on compounds A and B is inseparable (cf. Chart I and Table III). On the other hand, the percentage of A is formally determinable from the integral of the common H-2 signal of Man-4 of E and Man-3 of A at ~4.25 ppm. Unfortunately, for the mixture investigated here, this signal is partly overlapped by the H-1 one of GlcNAc-OH-1 [cf. Paz-Parente et al. (1983)]. Another possibility to determine the contents of A and B is given by the Man-4′ H-1 signals in the spectrum of $A1_{3A^-}$, wherefrom the ratio B/D can be obtained for the calculation of the A/B ratio in $A1_3$. The composition of 32 mol % A, 19 mol % B, 14 mol % D, and 35 mol % E thus resulting is in satisfactory overall agreement with the results discussed under Methylation Analysis, if the uncertainties involved in both methods and the larger relative errors for the minor components are taken into account. The number of GlcNAc-5, -5′, -7, and -7′ residues calculated for this composition is 2.47. The quantitative evaluation of the GlcNAc H-1 resonances requires an explanation. According to Vliegenthart et al. (1983) and references cited therein, the H-1 signals of GlcNAc-5, -5′, -7, and -7′ occupy the 4.54–4.60 ppm region and the one of GlcNAc-9[6] occurs between 4.46 and 4.47 ppm. The latter signal overlaps with those of Gal-6, -6′, -8, and -8′ (4.46–4.48 ppm),[6] and at the same time, the signals of anomeric proton resonances of α-galactosylated Gal-6, -6′, -8, and -8′ residues are expected to be shifted into the 4.54–4.60 ppm region (Van Halbeek et al., 1983). Therefore, one can calculate the GlcNAc-5, -5′, -7, and -7′ contents from the total integral of the 4.54–4.60 ppm region by subtracting the integral of these α-galactosylated β-galactose residues, which, of course, must be equal to the integral of the H-1 signal of α-Gal at 5.149 ppm. The value of 2.60 units thus calculated is only slightly higher than the value of 2.47 (vide supra) or 2.50 units (3.50–1.00—by definition—for GlcNAc-2) obtained indirectly from the integration of mannose signals or evaluation of methylation data on mannose residues, respectively. From the integral of the methyl signals of GlcNAc acetyl groups, one obtains an even smaller value of 2.14 units (4.49 total, minus 2.00—by definition—for GlcNAc-1 and -2, and minus 0.35 for GlcNAc-9), but this is not unexpected since the GlcNAc-1 residues of oligosaccharides prepared by alkaline hydrolysis are known to be partly degraded.

In a similar way, the number of β-Gal residues can be calculated from the integral measured between 4.46 and 4.48 ppm by adding the integral of α-Gal and subtracting the integral of H-1 of either Man-4′ or Man-3 of the bisected oligosaccharide E (at 5.003 or 4.709 ppm, respectively), which correspond, for obvious reasons, to the integral of GlcNAc-9. The resulting number of 2.45 residues can be compared with that based solely on integrals of mannose signals, which is, of course, the same as for GlcNAc-5, -5′, -7, and -7′, i.e., 2.47 residues. It should be added that a lower value would rather be expected since some of the branches seem to be incomplete (cf. Methylation Analysis).

It is thus evident that the quantitative NMR data are mutually consistent, the deviations lying within reasonable limits. The correspondence with the methylation analysis is also satisfactory, except for fucose, galactose, and GlcNAc-9 residues.

Fraction $A1_2$, which also consisted of a mixture of several glycans, was only available in a quantity of 0.18 mg (<100 nmol) and contained admixtures preventing exact integration. In spite of higher sensitivity and dispersion at 500 MHz (Figure 6h), it was not possible to obtain good-quality SDDS or NOE spectra. For all these reasons, the results must be considered as tentative, although some of them are undoubtedly reliable. Thus, the amount of the bisected biantenna E and of α-Gal residues is approximately the same as in $A1_3$. The contents of Gal and GlcNAc residues are higher, confirming the presence of additional lactosamine units inferred from methylation and from exoglycosidase digestion. Another inference from methylation data, viz., the presence of the triantenna C, is in accord with the high integral intensity of the signal at 4.87 ppm (cf. Chart I).

Chart II



Conclusions

It may thus finally be estimated that fraction $A1_3$, comprising 55–67 wt % of the F-MuLV gp asialoglycans, contains roughly 22, 21–22, 10, and 10 mol %, respectively, of structures E, A, B, and D in Chart I. In addition, however, about 10, 7–8, 4–5, and 4–5 mol % of the singly α-galactosylated species shown in Chart II are present.

At the average, around two-thirds of each glycan are fu-

[5] The comparison of this spectrum with the one shown in Figure 5f demonstrates the excellent selectivity of the SDDS technique.

[6] Confirmed by our SDDS and 2D measurements (Table II and Figure 4).

cosylated, and the residual about 10 mol % of fraction $A1_3$ consist mainly of the same basic structures carrying outer α-Gal(1→3) units at more than one antenna, or only at β-Gal-6 (or -8). Since about 10% of the branches were additionally found to lack the outer β-Gal units, the $A1_3$ oligosaccharitol mixture is, in fact, still more heterogeneous. The results on the $A1_2$ glycans, finally, indicate that the larger F-MuLV asialooligosaccharides (18–24 wt %) consist of a similar mixture, except that they seem to comprise type C structures in addition, and that some of the antennae appear to be oligolactosamine chains.

In brief then, the major F-MuLV gp asialoglycans consist of common N-acetyllactosaminic oligosaccharides (Berger et al., 1982; Kornfeld & Kornfeld, 1980; Montreuil, 1980), which, however, characteristically carry some Galα(1→3)-Galβ(1→4)GlcNAc and, probably, some oligolactosamine antennae. It may be considered an interesting hint to a possible role of these outer structures as tumor markers that the former were previously detected on plasma membrane glycoproteins from Ehrlich ascites tumor (Eckhardt & Goldstein, 1983) and from murine (Stern et al., 1983), as well as from human (Ozawa et al., 1983), teratocarcinoma cells, besides, of course, on blood group B gp's (where they are additionally fucosylated) and, for instance, on bovine thyroglobulin [Dorland & Vliegenthart, cited by Cummings & Kornfeld (1982b)]. Oligolactosaminoglycans, on the other hand, are known to occur on several animal cells, notably on human erythrocytes and erythroleukemia cells [e.g., Fukuda & Fukuda (1981)] and, probably, on Novikoff ascites tumor cells (van den Eijnden et al., 1983).

Acknowledgments

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Registry No. Glycan component A, 92344-08-6; glycan component B, 92269-97-1; glycan component D, 92284-08-7; glycan component E, 92269-96-0.

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Indirect Inactivation of Rabbit Reticulocyte Initiation Factor eIF-2 by Helenalin and Bis(helenalinyl) Malonate†

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ABSTRACT: Helenalin and bis(helenalinyl) malonate, sesquiterpene lactones that react primarily with exposed sulfhydryl groups, were shown to be equally effective inhibitors of endogenous protein synthesis in rabbit reticulocyte lysates. By use of partially fractionated systems, it was possible to show that helenalin preferentially inhibited the conversion of the ternary initiation complex to the 48S preinitiation complex. Previous experiments have shown that this preferential inhibition is due to selective inactivation of eIF-3 [Williams, W. L., Chaney, S. G., Willingham, W., Considine, R. T., Hall, I. H., & Lee, K.-H. (1983) *Biochim. Biophys. Acta 740*, 152–162]. Bis(helenalinyl) malonate was much less active as an inhibitor of 48S complex formation than helenalin and clearly did not possess sufficient activity in that assay to explain its effectiveness as a protein synthesis inhibitor in whole lysates. Kinetic studies also showed a clear difference between the mechanism of action of these two drugs. Bis(helenalinyl) malonate inactivated protein synthesis in reticulocyte lysates only after a lag of 10 min, and the inhibition of protein synthesis could be completely reversed by the addition of 5 mM cAMP. Helenalin showed more complex kinetics. While full inhibition only occurred after a lag of 10–15 min, a partial inhibition was observed from very early times. cAMP at 5 mM was only partially able to reverse inhibition by helenalin. Phosphorylation studies showed that both helenalin and bis(helenalinyl) malonate were equally effective at activating eIF-2α kinase and indirectly causing phosphorylation of eIF-2. Furthermore, both drugs were able to activate eIF-2α kinase at low enough concentrations to account for their effectiveness as protein synthesis inhibitors in whole lysates. These data were interpreted to indicate that the activation of eIF-2α kinase was the primary mode of action of bis(helenalinyl) malonate as a protein synthesis inhibitor. Helenalin probably acts preferentially at the level of eIF-2α kinase activation in vivo. However, at sufficiently high concentrations it also directly inactivates eIF-3.

The biologically active sesquiterpene lactones usually contain an α-methylene γ-lactone, an α,β-unsaturated cyclopentenone ring, or an α-epoxycyclopentenone ring system (Lee et al., 1971; Hall et al., 1978; Kupchan et al., 1971). These are all electrophilic reactive centers that are capable of alkylating sulfhydryl compounds by a rapid Michael-type addition. While they are, in theory, capable of reacting with any nucleophile, several lines of evidence suggest that the sesquiterpene lactones exert their cytotoxic effects primarily through inactivation of essential sulfhydryl groups. Several studies with model biological nucleophiles have shown that thiols are the only nucleophiles alkylated to any significant extent by sesquiterpene lactones (Kupchan et al., 1970; Hall et al., 1977; Lee et al., 1977). The α-methylene γ-lactone moiety has also been shown to react with exposed sulfhydryl groups on the enzymes phosphofructokinase (Hanson et al., 1970) and glycogen synthetase (Smith et al., 1972). For both enzymes

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