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# The structure of the *O*-glycosidic oligosaccharide chains of the major *Zajdela* hepatoma ascites-cell-membrane glycoprotein

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Glycoprotein $MII_2$, the major cell surface glycoprotein (molecular mass 110 kDa) of *Zajdela* hepatoma ascites cells, contains about 25 *O*-glycosidic oligosaccharide chains per molecule. They were released as oligosaccharide-alditols by alkaline borohydride treatment of $MII_2$, and purified by gel filtration on Bio-Gel P-6 followed by high-voltage paper electrophoresis. Four oligosaccharide-alditol fractions (A – D) were obtained in relative yields of 8:6:3:3.

The structure of the components of fractions A – C was determined by 500-MHz $^1$H-NMR spectroscopy in combination with sugar composition analysis, to be as follows.

(A) NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol
($B_1$) NeuAcα(2→3)Galβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol
($B_2$) Galβ(1→3)[NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol
(C) NeuAcα(2→3)Galβ(1→3)GalNAc-ol

On the basis of sugar composition and characteristics on Bio-Gel P-6 filtration, paper electrophoresis and thin-layer chromatography, the structure of the carbohydrate component of fraction D is proposed to be as follows.

(D) NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→6)]GalNAc-ol

Plasma membranes of animal cells contain peripheral and/or transmembrane glycoproteins [1, 2] which play an essential role in the morphological and biological properties of the cells [3 – 6]. Malignant transformation of such cells by viruses or chemicals is accompanied by alterations of these surface glycoproteins [6 – 9]. Transformed cells may differ from their normal analogues in carbohydrate content, glycopeptide profiles, e.g. on ion-exchange chromatography and/or expression of particular glycoproteins [9]. The data obtained vary with cell systems and culture conditions. Nonetheless, it appears as a general feature that plasma membrane glycoproteins of transformed cells contain both *N*- and *O*-glycosidic carbohydrates, while *O*-linked chains are virtually absent from normal cells, except human erythrocytes [10].

Here, we report on the isolation and structural characterization of the *O*-glycosidic oligosaccharides from $MII_2$, being the well-characterized [11] major surface glycoprotein of *Zajdela* hepatoma ascites cells, but absent from homologous normal liver cells.

## MATERIALS AND METHODS

### *Materials*

Bio-Gel P-2 and Bio-Gel P-6 (200 – 400 mesh) and Dowex 50 W-X2 $H^+$ (200 – 400 mesh) were obtained from Bio-Rad Laboratories (USA). Sepharose 6B was from Pharmacia (Sweden), DEAE-cellulose (DE-52) from Whatmann (UK). Silica gel thin-layer chromatography plates (Kieselgel 60 F254) were purchased from Merck (FRG). Tritiated sodium borohydride (specific activity = 555 Ci/mol) and scintillation liquid were purchased from Amersham-Searle (UK).

### *Tumor cells*

*Zajdela* hepatoma cells [12] were originally produced by dimethylaminoazobenzene injection into Sprague-Dawley rats and maintained in the ascites form by intraperitoneal transplantation ($2.5 \times 10^7$ cells/0.25 ml per animal) in 7 – 9-week-old rats, 250 g in weight (Charles River, France). Tumor cells were harvested 7 days after transplantation and washed four times in 0.01 M $NaHCO_3$, pH 7.4, containing 0.15 M NaCl.

Analysis with a Coulter counter showed that 99% of the cells were tumor cells without erythrocyte or lymphocyte contamination.

### *Labeling of cell surface glycoproteins*

The sialyl residues of cell surface glycoproteins were labeled by tritiated sodium borohydride reduction following sodium periodate oxidation as previously described [13]. Briefly, $10^7$ cells/ml were oxidized at a periodate concentration of 0.5 mM for 15 min in the dark at 20°C. After washing twice, the cells were reduced with 1.5 nmol/ml of tritiated

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sodium borohydride. The identification of the labeled compounds was performed by paper chromatography after hydrolysis of the cells in 0.1 M $H_2SO_4$ for 1 h at 80°C as described [13]. Tritium was incorporated into an oxidation product of sialic acid.

*Preparation of crude membranes*

The labeled cells were suspended in phosphate-buffered saline, disrupted by nitrogen cavitation (azote bomb, Kontron, France) under a pressure of 8 MPa. The homogenate was successively centrifuged at $1000 \times g$ and $9000 \times g$ for 20 min to remove cell fragments and nuclei, and mitochondria, respectively. The last supernatant was centrifuged at $100000 \times g$ for 1 h. The pellet constituted the crude membranes.

*Purification of the major membrane glycoprotein ($MII_2$)*

The purification of glycoprotein $MII_2$ was carried out according to a procedure similar to that described for a trypsinate of these hepatoma cells [11].

The crude membrane fraction was solubilized in 10 mM Tris/HCl buffer, pH 7.8 containing 0.25% sodium deoxycholate. After centrifugation, the solution was applied to a Sepharose 6B column equilibrated in the same buffer. Fractions of 3 ml were collected and the absorbance at 280 nm was determined. Aliquots (0.1 ml) of each fraction were monitored for radioactivity.

Four peaks were obtained, the second of which (MII fraction) contained the majority of the radioactivity; the MII preparation was further fractionated by DEAE-cellulose chromatography.

The homogeneity of the major glycoprotein $MII_2$ was checked by two-dimensional electrophoresis, DEAE-cellulose chromatography, isoelectrofocusing and immunological methods as previously described [11].

*Alkaline borohydride treatment*

*O*-Linked oligosaccharides were released from $MII_2$ glycoprotein by alkaline borohydride treatment in 1 M sodium borohydride, 0.05 M sodium hydroxide aqueous solution at 37°C for 16 h in the dark [14]. The solution was then chilled to 0°C and excess borohydride destroyed by dropwise addition of 4 M acetic acid to pH 5. The solution was diluted with 10 vol. distilled water and applied to a column of Dowex 50 W-X2. The column was washed with 5 vol. water. The eluate was monitored for tritium radioactivity. The combined effluent and washes were neutralized by 1 M pyridine and concentrated to a small volume. Boric acid was removed as methyl borate by the repeated addition and evaporation of methanol.

*Fractionation of oligosaccharide-alditols*

*Gel permeation chromatography.* The $^3H$-labeled oligosaccharide-alditols were dissolved in 1 ml 100 mM pyridine/acetate buffer, pH 5.2 and applied to a column of Bio-Gel P-6 ($100 \times 1$ cm). The column was eluted with the same buffer. Fractions of 1 ml were collected at a flow rate of 4 ml/h and assayed for radioactivity. $^3H$-containing peaks were pooled, lyophilized and desalted on a column of Bio-Gel P-2 ($40 \times 1$ cm) using water as eluent.

*High-voltage electrophoresis.* High-voltage paper electrophoresis was carried out on a Gilson apparatus (Gilson Medical Electronics, model D, France) using Whatmann 3 MM paper in pyridine/acetic acid/water (3/1/387, v/v/v) at a potential of 55 V/cm for 3 h. Markers, *N*-acetylneuraminic acid and sialyl-lactose, were simultaneously subjected to the same conditions of electrophoresis.

The radioactive oligosaccharide-alditols on the electropherogram were detected by scanning with a Packard radiochromatogram, model 7200, eluted with distilled water and subsequently purified on a Bio-Gel P-2 column ($10 \times 0.5$ cm) equilibrated in order to eliminate any paper lint.

*Purity of oligosaccharide-alditols*

The purity of the major oligosaccharide-alditols was checked by thin-layer chromatography on silica gel plates (Merck, FRG) using the solvent *n*-propanol/ammonia/water (6/2/1, v/v/v) [15]. After migration for 2 h, oligosaccharides were located by spraying the plates with the resorcinol reagent [16] and heating them at 100°C for 10 min.

*Analytical methods*

Sugar analysis was carried out by gas-liquid chromatography of pertrimethyl silylated derivatives of methylglycosides formed by methanolysis in methanol/1.5 M hydrochloric acid at 85°C for 18 h [17] on a Hewlett-Packard model 5710A gas chromatograph, equipped with glass columns (3%, w/w, of SE 30 chromosorb WAW/DMCS, 100–200 mesh). Mannitol was used as an internal reference and a mixture of free sugars as standards.

*$^1H$-NMR spectroscopy*

Prior to $^1H$-NMR spectroscopic analysis, the purified samples A–D were repeatedly treated with $^2H_2O$ (99.96 atom % 2H, Aldrich, USA) at $p^2H$ 7 and room temperature. Then, they were subjected to $^1H$-NMR spectroscopy at 500 MHz, using a Bruker WM-500 spectrometer (SON hf-NMR facility, University of Nijmegen, The Netherlands). Further experimental details have been described elsewhere [18]. Chemical shifts ($\delta$) are expressed downfield from internal sodium 4,4-dimethyl-4-silapentane 1-sulfonate, but were actually measured by reference to internal acetone ($\delta$ = 2.225 ppm in $^2H_2O$ at 27°C).

## RESULTS

*Isolation and purification of oligosaccharide-alditols from $MII_2$*

The *O*-glycosidically linked oligosaccharide chains of $MII_2$, the major glycoprotein of *Zajdela* hepatoma cells, were quantitatively released as reduced oligosaccharides by alkaline borohydride treatment; the latter was confirmed by complete transformation of *N*-acetylgalactosamine originally present into *N*-acetylgalactosaminitol. Oligosaccharide-alditols were fractionated by gel permeation on Bio-Gel P-6. Only the acidic oligosaccharides were studied. Four peaks designated P 6-1 to P 6-4 were described in the included region

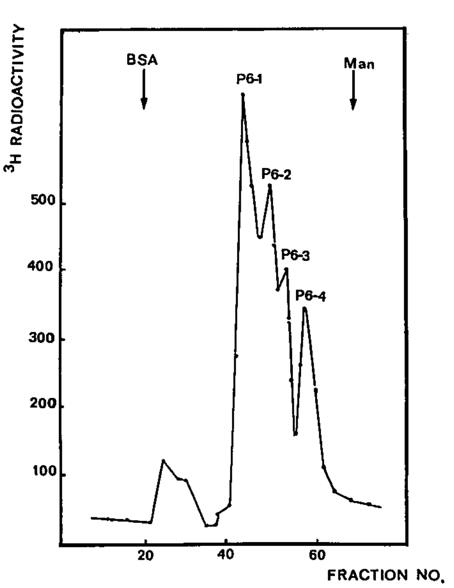



Fig. 1. *Fractionation pattern of the* $^3$*H-labeled oligosaccharide-alditols derived from glycoprotein* $MII_2$ *on a Bio-Gel P-6 column (100 × 1 cm).* The column was equilibrated and eluted with 100 mM pyridine/acetate buffer, pH 5.2. Fractions of 1 ml were collected at a flow rate of 4 ml/h and assayed for $^3$H radioactivity (cpm). The excluded and total volumes of the column were determined with bovine serum albumin (BSA) and tritiated mannose (Man)

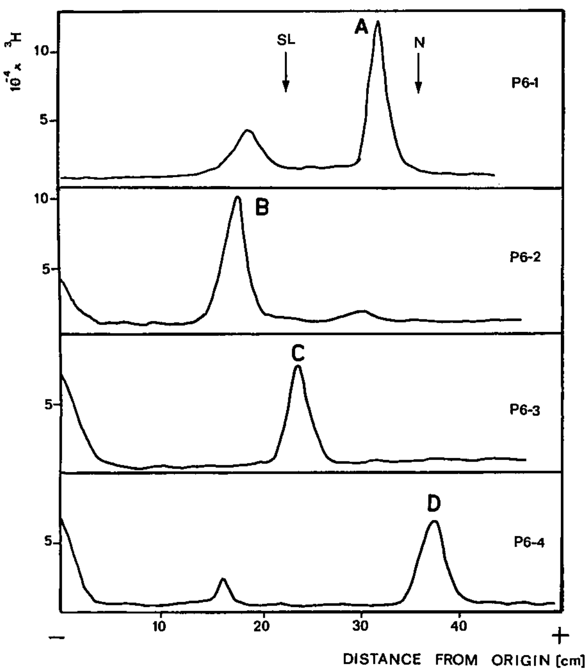



Fig. 2. *High-voltage paper electrophoresis of oligosaccharide-alditol fractions obtained from Bio-Gel P-6 chromatography of* $MII_2$ *β-elimination.* Electrophoresis was carried out as described in the text. The oligosaccharide-alditols were located by a scanner measuring $^3$H (cpm). Arrows indicate the positions of sialyllactose (SL) and *N*-acetylneuraminic acid (N)

(Fig. 1). The relative amounts of these fractions were judged from radioactivity to be 8:6:3:3, respectively. These four peaks were further purified by high-voltage electrophoresis. Their migration was compared to that of two standards, namely sialyl-lactose and *N*-acetylneuraminic acid (Fig. 2).

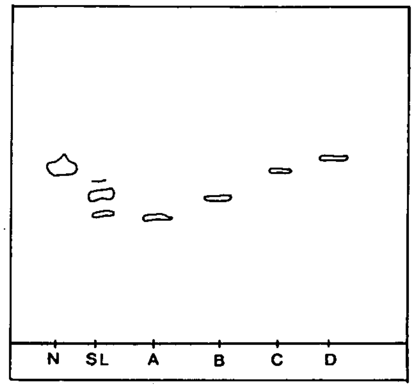



Fig. 3. *Thin-layer chromatography of oligosaccharide-alditols (A, B, C and D) purified by Bio-Gel P-6 filtration and high-voltage paper electrophoresis.* Thin-layer chromatography was performed on silica gel plates in *n*-propanol/ammoniaI$H_2O$ (6:1:2, v/v/v). The plate was sprayed with resorcinol reagent. Sialyllactose (SL) and *N*-acetylneuraminic acid (N) were used as standards

Table 1. *Molar composition of oligosaccharide-alditols A, B, C and D from glycoprotein* $MII_2$*, purified by high-voltage paper electrophoresis*
Molar ratios were calculated relative to one GalNAc-ol residue

| Monosaccharide | Amount in compound | | | |
|---|---|---|---|---|
| | A | B | C | D |
| | mol/mol | | | |
| Man | 0.08 | 0.25 | 0.50 | – |
| Gal | 2.02 | 2.20 | 1.08 | 1.12 |
| GlcNAc | 1.06 | 0.95 | – | – |
| GalNAc-ol | 1 | 1 | 1 | 1 |
| NeuAc | 1.92 | 1.03 | 1.00 | 2.15 |

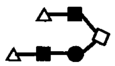
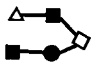
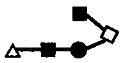
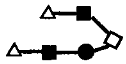
Fraction P 6-1 showed a major peak A which represented 70% of the radioactivity and which migrated faster than sialyllactose. Fraction P 6-2 revealed a major peak B (80%) which migrated slower than sialyl-lactose. Fraction P 6-3 gave oligosaccharide fraction C (60%) which migrated as sialyllactose. Fraction P 6-4 showed a peak D (50%) which migrated faster than *N*-acetylneuraminic acid. After elution, the purity of the A – D oligosaccharide fractions was checked by thin-layer chromatography (Fig. 3).

### *Structural characterization of the oligosaccharide-alditols from* $MII_2$

The molar carbohydrate composition of fractions A – D is given in Table 1. The major fractions A and B, which represent more than 70% of total oligosaccharide alditols, contain Gal, GlcNAc, GalNAc-ol and NeuAc in molar ratios 2:1:1:2 and 2:1:1:1, respectively. This suggests that these fractions contain hexa- and penta-saccharides, respectively. The minor fractions C and D do not contain GlcNAc as a constituent monosaccharide. They contain Gal, GalNAc-ol and NeuAC in ratios 1:1:1 and 1:1:2, respectively, indicating that C may contain a tri- and D a tetra-saccharide.

For further structural characterization, fractions A – D were subjected to 500-MHz $^1$H-NMR spectroscopy. The chemical shifts of relevant structural-reporter groups of con-

Table 2. $^1$*H chemical shifts of structural reporter group protons of constituent monosaccharides of the oligosaccharide-alditols A, B and C obtained from glycoprotein MII$_2$ of* Zajdels *hepatoma cells*
Chemical shifts are given downfield from internal 4,4-dimethyl-4-silapentane 1-sulfonate in $^2H_2O$ at 27°C acquired at 500 MHz. In the table heading, structures are represented by short-hand symbolic notation [18–21]: (◇) GalNAc-ol, (■) Gal, (●) GlcNAc, (△) NeuAc α(2→3). ▼ The superscript for each sugar residue indicates to which carbon atom of the adjacent monosaccharide it is linked. For example, Gal$^3$ means: Gal β(1→3)-linked. A second superscript is used to discriminate between identically linked residues, by indicating the type of the next linkage in the sequence. n.d. = not determined; obsc, obscured by a resonance of a non-carbohydrate contaminant

| Residue | Reporter group | Chemical shift in compound | | | | Reference |
|---|---|---|---|---|---|---|
| | | A  | $B_1$  | $B_2$  | C  | [19]  |
| | | ppm | | | | |
| GalNAc-ol | H-2 | 4.383 | 4.389 | 4.389 | 4.389 | 4.387 |
| | H-3 | 4.068 | 4.064 | 4.064 | obsc. | 4.066 |
| | H-4 | 3.439 | 3.435 | 3.435 | n.d. | 3.441 |
| | H-5 | 4.265 | 4.268 | 4.278 | 4.180 | 4.265 |
| | N-Ac | 2.065 | 2.065 | 2.065 | 2.067 | 2.065 |
| Gal$^3$ | H-1 | 4.529[a] | 4.532 | 4.461 | 4.546 | 4.529 |
| | H-3 | 4.112 | obsc. | 3.700 | n.d. | 4.113 |
| GlcNAc$^6$ | H-1 | 4.552[a] | 4.550 | 4.554 | – | 4.550 |
| | N-Ac | 2.062 | 2.065 | 2.061 | – | 2.062 |
| Gal$^4$ | H-1 | 4.546[a] | 4.466 | 4.550 | – | 4.545 |
| | H-3 | 4.112 | 3.700 | obsc. | – | 4.113 |
| NeuAc$^{3,3}$ | H-3*ax* | 1.799 | 1.799 | – | 1.800 | 1.800 |
| | H-3*eq* | 2.775 | 2.778 | – | 2.761 | 2.775 |
| | N-Ac | 2.032 | 2.033 | – | 2.030 | 2.033 |
| NeuAc$^{3,4}$ | H-3*ax* | 1.799 | – | 1.799 | – | 1.800 |
| | H-3*eq* | 2.755 | – | 2.744 | – | 2.755 |
| | N-Ac | 2.031 | – | 2.031 | – | 2.031 |

[a] All three anomeric doublets showed a coupling constant of 8.3 Hz.

stituent monosaccharides of the compounds in A, B and C are listed in Table 2, along with the corresponding data for a pertinent reference hexasaccharide-alditol [19, 20]. Oligosaccharide-alditol fraction D appeared to be contaminated by some non-carbohydrate material of unknown origin and structure, making it impossible to deduce the structure of the D tetrasaccharide(s) by NMR spectroscopy.

The $^1$H-NMR spectrum of fraction A matches that of the hexasaccharide-alditol NeuAcα (2→3) Galβ (1→3) [NeuAcα (2→3) Galβ (1→4) GlcNAcβ (1→6) ] GalNAc-ol, previously obtained from human plasma galactoprotein [20] and from human platelet glycocalicin [19]. To illustrate the identity of the compounds involved, the chemical shift data for a reference hexasaccharide have been included in Table 2. It is interesting that the two NeuAc residues which are both α(2→3)-linked to a β-Gal residue can be distinguished from each other on the basis of the typical chemical shift value for H-3*eq*. The NeuAc$^{3,3}$ residue, i.e. the one linked α(2→3) to the Galβ(1→3) unit, shows its H-3*eq* at $\delta$ = 2.775 ppm, whereas NeuAc$^{3,4}$ that is α(2→3)-linked to Galβ(1→4) shows its H-3*eq* at $\delta$ = 2.755 ppm (Table 2).

The $^1$H-NMR spectrum of sample B resembles closely that of A in that it contains structural-reporter group signals at the same positions and in the same intensity ratios. In addition, however, two doublets (intensity 1:1) are observed at $\delta \approx$ 4.46 ppm, which are attributed to the H-1 atoms of non-reducing, terminal β-Gal residues [18]; furthermore, the *N*-acetyl region of the spectrum of B contains the signals at $\delta$ = 2.065 ppm and $\delta$ = 2.061 ppm in intensity ratio 3:1, instead of 1:1 as was observed for A. These features are accounted for by sample B consisting of two isomeric pentasaccharide-alditols, denoted $B_1$ and $B_2$, in ratio 1:1. The structure of compounds $B_1$ and $B_2$ differs from that of A in the absence of either NeuAc$^{3,4}$ or NeuAc$^{3,3}$, respectively. It should be noted that the NMR spectrum of the pure pentasaccharide identical to compound $B_1$ (obtained from cow colostrum κ-casein) has been reported previously [21]. This facilitated the listing of the chemical shift data for compound $B_2$ (Table 2). The compound missing NeuAc$^{3,4}$ (that is, $B_1$) possesses the *N*-acetyl singlet of GlcNAc$^6$ at $\delta$ = 2.065 ppm while $B_2$ has this signal at $\delta$ = 2.061 ppm; this assignment is in accord with the well-established effect of attachment of a NeuAc residue in α(2→3)-linkage to an *N*-acetyllactosamine residue on the chemical shift of the GlcNAc *N*-acetyl signal ($\Delta\delta \approx -$ 0.003 ppm) [18].

The $^1$H-NMR spectrum of fraction C revealed the presence of the linear trisaccharide-alditol NeuAcα(2→3)Galβ (1→3)GalNAc-ol. The chemical shifts of the structural-reporter groups of C (Table 2) match those of the same trisaccharide isolated from a number of other glycoproteins (see, e.g., [19–21]).

Fraction D is proposed to contain the tetrasaccharide-alditol NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→6)]GalNAc-ol. This interpretation fits the sugar composition (Table 1), as well as the chromatographic properties of D described in the previous section.

The composition analysis and NMR spectroscopic characterization of oligosaccharide-alditols A–D confirm that all GalNAc present in $MII_2$ has been quantitatively converted into GalNAc-ol by the alkaline borohydride treatment. This leads to the conclusion that all GalNAc (content 5.6%, see [11]) present in the original $MII_2$ is peptide-linked. Combination of this data with the molecular mass of the glycoprotein $MII_2$ (110 kDa [11]) leads to the conclusion that there are about 25 oligosaccharides *O*-glycosidically attached to the protein.

## DISCUSSION

The characteristics of $MII_2$, the major surface glycoprotein of *Zajdela* hepatoma ascites cells [11] indicate that most of the $MII_2$ glycans are *O*-glycosidically linked through GalNAc to serine or threonine residues of the polypeptide chain. Here, we have described how the *O*-glycosidic oligosaccharide chains of $MII_2$ were released from protein and were structurally characterized. The release was quantitative, as was judged from the yield of GalNAc-ol (recovery 95%) obtained after alkaline borohydride treatment, and the virtual absence of GalNAc in the β-elimination product. Subsequently, the acidic oligosaccharide-alditols were purified and the structure of their major representatives was determined.

Combination of $^1$H-NMR spectroscopy, quantitative sugar composition analysis, thin-layer chromatography, gel filtration and paper electrophoresis revealed the structures of the major components to be as follows.

(A) NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→3)Galβ(1→4)-GlcNAcβ(1→6)]GalNac-ol

($B_1$) NeuAcα(2→3)Galβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)] GalNAc-ol

($B_2$) Galβ(1→3)[NeuAcα(2→3)Galβ(1→4) GlcNAcβ(1→6)]GalNAc-ol

(C) NeuAcα(2→3)Galβ(1→3)GalNAc-ol

(D) NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→6)GalNAc-ol

Taking into consideration the molecular mass of glycoprotein $MII_2$ (110 kDa), the presence of only one GalNAc residue per chain and the relative abundance of the various oligosaccharide chains (A/B/C/D ≈ 37%/40%/12%/10%), it was calculated that $MII_2$ possesses 25 *O*-glycosidic carbohydrate side chains per molecule, 9 of which are hexasaccharides of structure A, 10 are pentasaccharides of structure B, while there are about 3 tetra- and trisaccharides of structure C and D.

As revealed by $^1$H-NMR, the pentasaccharide fraction B appeared to contain two isomers ($B_1$ and $B_2$) which are distinct from each other only in the site of sialylation. These isomers represent different products of sialylation, and could be the result of the action of two different sialyltransferases which may also be involved in the biosynthesis of the hexasaccharide A (and, thus, are not mutually exclusive). However, the possibility that these isomers $B_1$ and $B_2$ were generated by partial desialylation of hexasaccharide A (and, *mutatis mutandis*, trisaccharide C by desialylation of tetrasaccharide D) under the conditions of the alkaline borohyride treatment and subsequent purification steps, can not be excluded.

From a structural point of view, it should be noted that the structures of compounds C and D have been found previously for numerous glycoproteins. GlcNAc-containing *O*-glycosidic oligosaccharides occur somewhat more rarely; they have, however, been reported for various types of mucins, including gastric [22, 23], submaxillary [24] and ovarian-cyst [25] mucins, where they may be quite large (containing up to 20 monosaccharide residues), are often rich in fucose and may contain more than one GlcNAc residue per chain. Smaller structures like hexa- and penta-saccharides A and B have been found for the soluble proteoglycan from Swan rat chondrosarcoma [26], human plasma galactoprotein [20] and the soluble glycoprotein κ-casein from ewe or cow colostrum [21, 27]. In cell membrane glycoprotein, GlcNAc-containing oligosaccharides were found in structures which are different from those of $MII_2$ hexa- and penta-saccharide. Thus, in epiglycanin [28] and in human mammary cancer cells [29], the oligosaccharide structure is linear. In human rectal adenocarcinoma cells [30], the GlcNAc residue is directly linked to NeuAc and GalNAc-ol and the oligosaccharide does not contain galactose. The branched structure of A and B oligosaccharides of $MII_2$ have been observed in human platelet glycocalicin [19] and AH 66 hepatoma ascites cells [31]. However, it should be noted that in this latter case, the oligosaccharides were obtained from whole cell membrane, and not from a selected, well-defined glycoprotein as $MII_2$ of *Zajdela* hepatoma ascites cells.

Why and how the biosynthetic routes for making *O*-glycosidic chains are activated upon malignant cell transformation, leading to such markers as oligosaccharides A to D on glycoprotein $MII_2$, is a question that will be addressed in a future investigation.

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