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# Structure determination of oligosaccharides isolated from Cad erythrocyte membranes by permethylation analysis and 500-MHz $^1$H-NMR spectroscopy

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Alkaline borohydride reductive cleavage ($\beta$-elimination) of glycophorin A isolated from one individual of the rare blood group Cad, resulted in the release of six acidic oligosaccharide-alditols which were separated by high-performance liquid chromatography (HPLC) on an alkyl amine silicagel column. The structure of four of them has been determined by the application of methanolysis, methylation analysis and $^1$H-NMR spectroscopy at 500 MHz. The structures and relative amounts were as follows: oligosaccharide 1: NeuAc($\alpha$2–3)Gal($\beta$1–3)GalNAc-ol (3.5%); oligosaccharide 3: GalNAc($\beta$1–4)[NeuAc($\alpha$2–3)]Gal($\beta$1–3)GalNAc-ol (10.5%); oligosaccharide 5: NeuAc($\alpha$2–3)Gal($\beta$1–3)[NeuAc($\alpha$2–6)]GalNAc-ol (10.4%); oligosaccharide 6: GalNAc($\beta$1–4)[NeuAc($\alpha$2–3)]Gal($\beta$1–3)[NeuAc($\alpha$2–6)]GalNAc-ol (71.2%). The two other oligosaccharides (2 and 4) were obtained in very low amount. The major pentasaccharide (oligosaccharide 6) carries the blood group Cad determinant and is a potent inhibitor of human anti-Sd$^a$ antibody.

Cad is a rare blood group antigen inherited as a dominant character and first identified on human group O and B erythrocytes showing a strong reactivity with the lectin from *Dolichos biflorus* [1]. Hemagglutination inhibition tests have further suggested that the chief structural determinant of Cad specificity is *N*-acetylgalactosamine [2, 3] a sugar already involved in A, Tn, P and Forssman determinants.

Preliminary investigations have shown that Cad determinants are carried by the main red cell membrane sialoglycoproteins (glycophorins A and B). Following SDS/polyacrylamide gel electrophoresis of red cell membrane polypeptides prepared from a blood group Cad individual, a decreased migration of both glycophorin A and B was noted, suggesting an increase in the apparent molecular mass of these components by 3 kDa and 2 kDa respectively [4].

Analysis of the purified glycophorin A molecule indicated a high content in *N*-acetylgalactosamine (GalNAc) and a normal *N*-acetylneuraminic acid (NeuAc) content as compared to control and suggested that these residues form part of alkali-labile oligosaccharide chains [4, 5]. Moreover the purified glycoproteins reacted strongly with the *D. biflorus* lectin.

The predominant oligosaccharide chain obtained by alkaline borohydride treatment of purified glycophorin A molecules was isolated previously and analyzed [6]. It appeared to be a pentasaccharide-alditol that is an extension of the classical disialotetrasaccharide [7] by a GalNAc residue in $\beta$1–4 linkage to Gal. We present in this paper the separation of six oligosaccharides and the complete structure of four of them obtained by $\beta$-elimination of glycophorin A from Cad erythrocyte membranes.

*Abbreviations.* GalNAc, *N*-acetyl-D-galactosamine, GalNAc-ol, *N*-acetyl-D-galactosaminitol; GlcNAc, *N*-acetyl-D-glucosamine; Gal, D-galactose; Man, D-mannose; Glc, D-glucose; NeuAc, *N*-acetylneuraminic acid; HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate.

## EXPERIMENTAL PROCEDURES

Details of the source of red cells from the original Cad donor and control blood group Cad(–), Sd(a–) donors, isolation of the red cell membrane sialoglycoprotein (glycophorin A) and conditions of alkaline borohydride reductive cleavage ($\beta$-elimination) were all according to previous communications [1, 5, 6].

Affinity purified *Dolichos biflorus* agglutinin was obtained from Serva Laboratories (Le Perray en Yvelines, France). Human anti-Sd$^a$ was kindly provided by Dr L. Messeter (Lund, Sweden) and the $GM_2$ ganglioside, GalNAc($\beta$1–4)-[NeuAc($\alpha$2–3)]Gal($\beta$1–4)Glc-Cer, by Dr K. Stephan (Bonn, FRG). The Sd$^a$ active mucin was isolated from human urine of blood group O individuals as described [8].

Oligosaccharide alditols obtained by $\beta$-elimination from Cad glycophorin A were purified on a Bio-Gel P-6 column [6]. Oligosaccharide-alditols contained in fraction III [6] were fractionated by HPLC on an Amino-AS-5A column (5 µm, 0.46 × 25 cm, Touzart et Matignon, France) according to Bergh et al. [9].

Oligosaccharide-alditols were analyzed by thin-layer chromatography (TLC) on 20 × 20 cm Kieselgel plates (Merck, Darmstadt, FRG) in ethanol/water/butanol/pyridine/acetic acid (100:30:10:10:3, v/v) for 6 h at room temperature [10]. Carbohydrates were stained with orcinol/

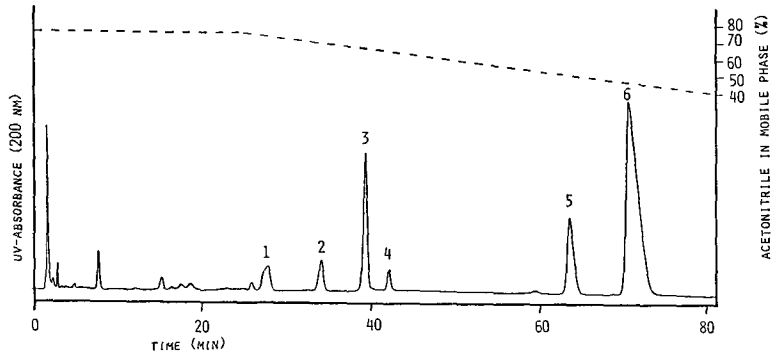


Fig. 1. *Separation by HPLC of the oligosaccharide-alditols (Bio-Gel P-6, fraction III) obtained after β-elimination of glycophorin A from Cad erythrocytes.* Column, Amino AS 5A (0.4 × 25 cm, 5 μm). Isocratic elution for 25 min in acetonitrile/15 mM phosphate buffer pH 5.2 (4:1, w/v) followed by a linear gradient in which the ionic strength of the buffer increased at the rate of 0.6% (v/v) $min^{-1}$; flow 1 ml/min; sugar detection at 200 nm

Table 1. *Carbohydrate composition of glycophorin A and of its β-elimination products prepared from Cad erythrocyte membranes*
Sugar analysis was performed as described by Zanetta at al. [12] using a Varian 1400 gas chromatograph equipped with flame ionization detector. Trifluoroacetyl derivatives of methylglycosides were separated on a glass column (300 × 0.3 cm) filled with OV-210 5% silicon on chromosorb W(HP) DMCS, 100–120 mesh. Nitrogen flux was 7.5 ml/min, the column temperature was raised at 2°C/min, from 100° to 210°C. Fractionation of fractions II and III on Bio-Gel P-6 is described elsewhere [6]. Separation of oligosaccharides 1, 3, 5, 6 by HPLC is given in Fig. 1

| Fraction | | Molar ratio of carbohydrates | | | | | |
|---|---|---|---|---|---|---|---|
| | | Gal | Man | GalNAc | GlcNAc | NeuAc | GalNAc-ol |
| Glycophorin A | | 1 | 0.16 | 1.72 | 0.33 | 1.95 | 0 |
| β-Elimination products: | | | | | | | |
| Bio-Gel P-6: | fraction II | 1 | 0 | 1.2 | 0 | 2.2 | 1.02 |
| | fraction III | 1 | 0 | 0.81 | 0 | 2.14 | 1.19 |
| HPLC of fraction III | oligo 1 | 1 | 0 | 0 | 0 | 0.93 | 0.95 |
| | oligo 3 | 1 | 0 | 1.1 | 0 | 0.71 | 0.61 |
| | oligo 5 | 1 | 0 | 0 | 0 | 1.90 | 0.94 |
| | oligo 6 | 1 | 0 | 1.2 | 0 | 1.92 | 1 |

sulfuric acid reagent at 105°C for 10 min [11]. The sugar composition of oligosaccharide alditols was determined by gas-liquid chromatography after treatment of the oligosaccharides with 0.5 M HCl/methanol for 24 h at 80°C and pertrifluoroacetylation [12]. The oligosaccharide-alditols were methylated according to Finne et al. [13] as modified by Paz-Parente et al. [14]. Identification of methylated sugars was performed after methanolysis (0.5 M HCl/methanol, 24 h at 80°C) and peracetylation (pyridine/acetic anhydride 1:1, 100°C, 30 min) by gas-liquid chromatography/mass spectrometry according to Fournet et al. [15].

For $^1$H-NMR spectroscopic analysis the oligosaccharide-alditols were repeatedly exchanged in $D_2O$ (99.96 atom% D, Aldrich, Milwaukee, USA) at room temperature with intermediate lyophilization. Spectra were recorded on a Bruker WM-500 machine controlled by an Aspect-2000 computer (SON NMR facility, Nijmegen University, The Netherlands) essentially as described [6, 16, 17]. However, the probe temperature was kept at 22.0 (± 0.5)°C, allowing the spectral region upfield from 4.75 ppm to be observed undisturbed by the HOD signal.

Agglutination tests of Cad and control red cells by lectins and human anti-Sd$^a$ reagents were performed in $P_i$/NaCl medium (10 mM phosphate buffer pH 7.2 in 150 mM NaCl) by a direct assay and the antiglobulin test respectively, according to standard procedures [18]. Agglutination-inhibition tests were performed as previously described [5].

## RESULTS AND DISCUSSION

The reduced oligosaccharides obtained by β-elimination of 10 mg of lipid-free glycophorin A were purified on a Bio-Gel P-6 column [6]. Two fractions containing carbohydrates (fractions II and III) were isolated and analyzed by TLC. Fraction II (200 μg) consisted of a pure pentasaccharide of which the structure had been determined previously [6]. Fraction III (700 μg) provided several oligosaccharides of which the pentasaccharide present in fraction II is the predominant component. This fraction III was further fractionated by HPLC on an alkyl amine column. Six oligosaccharide-alditols were obtained (Fig. 1) with three major species (oligosaccharides 3, 5 and 6). Yields of oligosaccharide-alditols 1–6 were as follows: 27, 23, 82, 11, 85 and 355 μg, respectively. The composition and molar ratios of carbohydrates present in the purified oligosaccharide-alditols are given in Table 1. Fractions II and III obtained from Bio-Gel P-6 chromatography contained non-reduced GalNAc in addition to Gal, NeuAc and GalNAc-ol residues. The carbohydrate compositions of fraction II and oligosaccharide 6 from HPLC were similar, indicating the presence of a pure pentasaccharide

Table 2. *Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated Cad-glycophorin A oligosaccharide-alditols*

| Alditols | Molar ratio of partially methylated monosaccharides | | | | | |
|---|---|---|---|---|---|---|
| | 2,4,6-$Me_3$Gal | 2,6-$Me_2$Gal | 3,4,6-$Me_3$GalNAcNMe | 4,7,8,9-$Me_4$NeuNAcNMe | 1,4,5,6-$Me_4$GalNAcNMe-ol | 1,4,5-$Me_3$GalNAcNMe-ol |
| Oligosaccharide 1 | 1 | | | 0.85 | 0.79 | |
| Oligosaccharide 3 | | 1 | 0.85 | 1.1 | 0.6 | |
| Oligosaccharide 5 | 1 | | | 1.9 | | 0.73 |
| Oligosaccharide 6 | | 1 | 0.8 | 2.03 | | 0.7 |

Table 3. *$^1H$ chemical shifts of structural-reporter groups of constituent monosaccharides for some oligosaccharide-alditols derived from Cad erythrocyte membranes*

Data were acquired at 500 MHz, for neutral solutions in $D_2O$ at 22°C. Chemical shifts are measured downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate using internal acetone at $\delta = 2.225$ ppm as reference. The structures of oligosaccharides 1, 3, 5 and 6 are given in Fig. 2

| Residue | Type of linkage | Reporter group | Chemical shift in compound | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 3 | 5 | 6 |
| | | | ppm | | | |
| GalNAc-ol | | H-2 | 4.389 | 4.380 | 4.378 | 4.361 |
| | | H-3 | 4.070 | 4.062 | 4.064 | 4.047 |
| | | H-4 | 3.501 | 3.510 | 3.524 | 3.500 |
| | | H-5 | 4.193 | 4.157 | 4.237 | 4.180 |
| | | H-6 | 3.5–3.9[a] | 3.5–3.9[a] | 3.469 | 3.447 |
| | | NAc | 2.045 | 2.048 | 2.041 | 2.039 |
| Gal | β1–3 | H-1 | 4.548 | 4.565 | 4.539 | 4.560 |
| | | H-2 | 3.5–3.9[a] | 3.411 | 3.5–3.9[a] | 3.416 |
| | | H-3 | 4.120 | 4.162 | 4.118 | 4.162 |
| | | H-4 | 3.927 | 4.095 | 3.930 | 4.089 |
| GalNAc | β1–4 | H-1 | – | 4.725 | – | 4.714 |
| | | H-4 | – | 3.925 | – | 3.913 |
| | | NAc | – | 2.028 | – | 2.025 |
| NeuAc | α2–3 | H-3*ax* | 1.802 | 1.935 | 1.800 | 1.933 |
| | | H-3*eq* | 2.774 | 2.682 | 2.771 | 2.680 |
| | | NAc | 2.032 | 2.034 | 2.031 | 2.032 |
| NeuAc | α2–6 | H-3*ax* | – | – | 1.691 | 1.706 |
| | | H-3*eq* | – | – | 2.724 | 2.734 |
| | | NAc | – | – | 2.031 | 2.032 |

[a] Resonance located in the bulk of sugar-skeleton proton signals; its chemical shift could not be determined more accurately, just by inspection

with the same carbohydrate composition. Oligosaccharides 3 and 5 are both tetrasaccharides, the former with one non-reduced GalNAc and one NeuAc residues and the latter with two sialic acid residues. Oligosaccharide 1 is a trisaccharide with one residue each of Gal, NeuAc and GalNAc-ol. These oligosaccharides were further studied by methylation analysis and $^1H$-NMR spectroscopy.

The molar ratios of the various partially methylated monosaccharides obtained after methanolysis and acetylation of the permethylated oligosaccharide-alditols are given in Table 2. Methylation patterns of oligosaccharides 1 and 5 were found to be identical to those described in the sugar part of κ-caseins from cows milk [19]. Oligosaccharide 1 gives 2,4,6-$OMe_3$Gal, 4,7,8,9-$OMe_4$NeuAcMe and 1,4,5,6-$OMe_4$GalNAcMe-ol with molar ratios of 1:0.85:0.79, respectively. The 'classical' tetrasaccharide 5 with two sialic acid residues [7] gives a 1,4,5-$OMe_3$GalNAcMe-ol derivative. The additional NeuAc residue is glycosidically linked to GalNAc-ol by a 2–6 linkage. Oligosaccharides 3 and 6 carry an additional GalNAc residue. These two oligosaccharides give 2,6-$OMe_2$Gal, 3,4,6-$OMe_3$GalNAcMe and 4,7,8,9-$OMe_4$NeuAcMe. From the substitution patterns of GalNAc-ols, it can be concluded that these residues are substituted at C-3 and C-3, C-6 respectively in oligosaccharide 3 and oligosaccharide 6.

In order to elucidate the complete primary structure of the oligosaccharide-alditols 1, 3, 5 and 6 obtained from Cad erythrocyte membranes, these fractions were analyzed by 500-MHz $^1H$-NMR spectroscopy. The chemical shifts of the structural-reporter-group protons of the constituent monosaccharides for compounds 1, 3, 5 and 6 have been compiled in Table 3. Compound 1 consisted of a pure trisaccharide-alditol, namely, NeuAc(α2–3)Gal(β1–3)GalNAc-ol. The $^1H$-NMR parameters of oligosaccharide 1

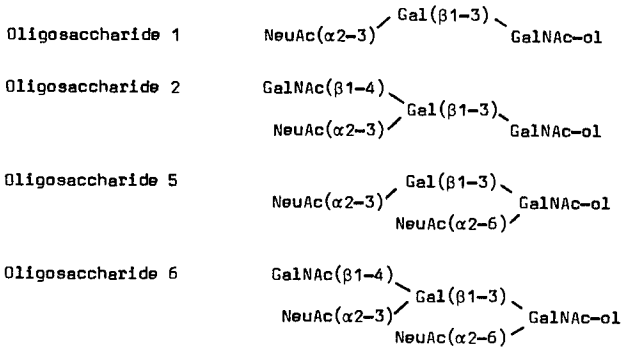


Fig. 2. *Structures of the four identified oligosaccharide-alditols isolated from Cad-erythrocyte membranes*

(Table 3) were found to be identical to those described [16] for a compound of the same structure derived from cow's milk κ-casein. The set of chemical shifts of H-2 and H-5 of GalNAc-ol is indicative of the mono-substitution of GalNAc-ol by Gal in β1 – 3 linkage [20]. The chemical shifts of H-3*ax* and H-3*eq* of NeuAc, in conjunction with those of H-1 and H-3 of Gal, point to the NeuAc(α2 – 3)Gal(β1 – .) sequence [16].

As compared to the NMR spectrum of compound 1, that of compound 3 revealed an additional H-1 doublet at 4.725 ppm and also an additional NAc singlet at 2.028 ppm. These signals are attributed to the GalNAc residue found to be present in compound 3 by carbohydrate analysis. The aforementioned chemical shift for H-1 in conjunction with its coupling constant ($J_{1,2}$ = 8.1 Hz) points to a β-glycosidic linkage for this residue. The combination of the chemical shift values for the GalNAc, Gal and NeuAc reporter groups for compound 3 (Table 3), in particular the relatively downfield position of H-3*ax* of NeuAc (1.935 ppm), is known [6] to be unique for the sequence GalNAc(β1 – 4)[NeuAc(α2 – 3)]Gal-(β1 – .), comprising the so-called internal sialic acid residue; this was first discovered for oligosaccharides derived from gangliosides [6]. The chemical shift of GalNAc-ol H-2 (4.380 ppm) in conjunction with the rather upfield position of its H-5 ($\delta < 4.20$ ppm, compare [20]) is indicative of mono-substitution of GalNAc-ol by Gal in β1 – 3 linkage. Therefore, tetrasaccharide-alditol 3 can be conceived as an extension of trisaccharide-alditol 1 with a GalNAc residue in β1 – 4 linkage to Gal. Apparently, the attachment of GalNAc affects considerably not only the chemical shifts of Gal H-1, H-2, H-3 and H-4, and NeuAc H-3*ax* and H-3*eq* (Table 3), but results also in a marked upfield shift of H-5 of GalNAc-ol ($\Delta\delta$ = – 0.036 ppm).

Compound 5 contained another pure tetrasaccharide-alditol. This also can be conceived as an extension of trisaccharide-alditol 1, namely, by a second NeuAc residue in α2 – 6 linkage to GalNAc-ol, resulting in the 'classical' tetrasaccharide-alditol NeuAc(α2 – 3)Gal(β1 – 3)[NeuAc(α2 – 6)]GalNAc-ol [7]. This could be readily inferred from comparison of the NMR data for compound 5 (Table 3) with those described for the compound possessing the same structure isolated from cow's milk κ-casein [16, 22]. It should be noted that the set of chemical shifts for H-3*ax* and H-3*eq* of the (α2 – 3)-linked NeuAc and that for the (α2 – 6)-linked NeuAc residue differ clearly from each other. Moreover, the chemical shifts of H-5 and H-6′ of GalNAc-ol are significantly influenced by the attachment of the (α2 – 6)-linked NeuAc (compare with compound 1, Table 3).

Compound 6 consisted of the pure Cad-specific pentasaccharide-alditol described previously [6], that is, an extension of trisaccharide-alditol 1 by GalNAc in β1 – 4 linkage to Gal, as well as by NeuAc in α2 – 6 linkage to GalNAc-ol. Considering compound 6 as an extension of compound 5, the effects of introduction of GalNAc traceable from Table 3 are identical to those seen in the step from compound 1 to compound 3. Analogously, the effects of attachment of NeuAc in α2 – 6 linkage to GalNAc-ol, when comparing compounds 6 and 3, are similar to those observed between compounds 1 and 5. Thus, the characteristic NMR features of compounds 3 and 5 are assembled in the spectrum of compound 6. It is noteworthy that the upfield shift effect on H-5 of GalNAc-ol due to introduction of GalNAc at C-4 of Gal and the downfield effect due to branching of GalNAc-ol at C-6 by NeuAc, appear to be independent and additive. This results in a chemical shift value for H-5 of GalNAc-ol (4.18 ppm), which is exceptional in view of the 3,6-disubstitution of this residue: so far, δH-5 = 4.24 – 4.29 ppm has been considered as indicative of the branching GalNAc-ol [16]. Most probably, this deviation is due to steric hindrance of (α2 – 6)-linked NeuAc by the other terminal residues.

On the basis of permethylation and NMR analysis the structures for the carbohydrate chains of oligosaccharide-alditols 1, 3, 5 and 6 are as proposed in Fig. 2.

500-MHz $^1$H-NMR spectroscopy proved again to be a very suitable method for structural characterization of mucin-type oligosaccharide-alditols. Even in the case of compound 1, where less than 20 nmol of oligosaccharide were available for NMR analysis, the sensitivity of this technique enabled the primary structure of the compound involved to be delineated.

In Table 4 we have compared the biological activity of various glycoconjugates for Sd$^a$ activity as deduced by agglutination-inhibition assays. The glycophorin A isolated from Cad erythrocyte membranes was a strong inhibitor of the human anti-Sd$^a$ antibody as well as of the *Dolichos biflorus* lectin, whereas the glycophorin purified from control erythrocytes was not inhibitory. Comparatively, the mucin prepared from human group O, Sd(a+) urine was a better inhibitor, presumably because of its high sugar content [8]. The main oligosaccharide-alditols 5 and 6 obtained from Cad erythrocyte glycoproteins were also tested for blood group activity as reported in Table 4. The pentasaccharide-alditol 6 was a potent inhibitor of both the lectin and the human antibody, while the sialotetrasaccharide-alditol 5 had no activity. The $GM_2$ ganglioside, which shares the same terminal oligosaccharide structure with the Cad pentasaccharide (component 6), did not inhibit the human anti-Sd$^a$ antibody. However, we have found that this ganglioside inhibited strongly the agglutination of $A_1$ erythrocytes by the *D. biflorus* lectin.

Unfortunately not enough material could be obtained from the oligosaccharide-alditol 3 and from desialylated structures 3 and 6 for agglutination-inhibition assays and it is not known whether or not the sialic acid residues are required for the Cad/Sd$^a$ blood group activity of these structures. Recently however, another closely related pentasaccharide, GalNAc(β1 – 4)[NeuAc(α2 – 3)]Gal(β1 – 4)GlcNAc(β1 – 3)Gal, obtained by endo-β-galactosidase cleavage of the Tamm-Horsfall glycoproteins from Sd(a+) individuals was shown to be a strong inhibitor of human anti-Sd$^a$ [23], thereby indicating further similarity between the blood group Cad and Sd$^a$ determinants. The tetrasaccharide obtained by desialylation of the above structure failed to inhibit anti-Sd$^a$ [23] suggesting that both the terminal *N*-

Table 4. *Biological activity of glycoconjugates and oligosaccharides*

| Inhibitor | Minimum amount of substance giving complete inhibition of four hemagglutinating doses of | |
|---|---|---|
| | anti-Sd$^a$/SD(a+) | *D. biflorus*/$A_1$ |
| | µg/40 µl | |
| Glycophorin A from blood group B donor | > 50 | > 50 |
| Glycophorin A from blood group Cad donor | 0.09 | 0.01 |
| Urinary mucin from Sd(a+) donor | 0.003 | 0.2 |
| NeuAc(α2−3)Gal(β1−3)[NeuAc(α2−6)]GalNAc-ol (compound 5) | >110 | >110 |
| GalNAc(β1−4)[NeuAc(α2−3)]Gal(β1−3)[NeuAc(α2−6)]GalNAc-ol (compound 6) | 2−4 | 1 |
| GalNAc(β1−4)[NeuAc(α2−3)]Gal(β1−4)Glc-Cer (ganglioside $GM_2$) | > 50 | 0.04 |
| GalNAc | >100 | > 6 |

acetylgalactosamine and *N*-acetylneuraminic residues are required for optimal binding.

In conclusion we have identified here two new oligosaccharides (3 and 6) attached by an *O*-glycosidic bond to glycophorin A from Cad erythrocytes. One of them (oligosaccharide 6) has been previously analyzed [6]. We have shown in addition that more than 10% of the remaining alkali-labile sugars attached to this glycoprotein belong to the classical type identified by Thomas and Winzler [7] demonstrating therefore that the postulated β-4-*N*-acetylgalactosaminyltransferase which forms the Cad-specific structures does not convert *in vivo* all the 15 *O*-glycosidic chains of glycophorin. It is possible also that the biosynthesis of Cad structures follows an ordered pathway which remains to be established. It is interesting to note that the trisaccharide GalNAc(β1−4)Gal(β1−3)GalNAc-ol has not been found so far.

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