Further Characterization of Type 2 and Type 3 Chain Blood Group A Glycosphingolipids from Human Erythrocyte Membranes†

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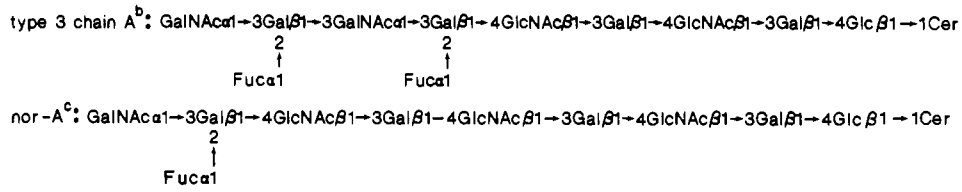
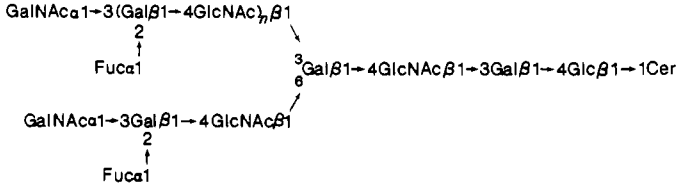
ABSTRACT: Blood group A glycosphingolipids with slow chromatographic mobilities have been separated systematically with an improved chromatographic procedure, and their structures have been analyzed by application of a panel of monoclonal antibodies defining A determinants carried by type 1, type 2, type 3, and type 4 carbohydrate chains as well as by ^{1}H NMR spectroscopy and methylation analysis. Of several A-active fractions, previously termed A^a, A^b, A^c, and A^d, in decreasing order of thin-layer chromatographic mobility, the third fraction (A^c) was characterized as containing one type 3 chain A component and one type 2 chain A component without branching, which have been termed type 3 chain A^b and nor-A^c, respectively.



The major component present in the fourth A-active fraction (A^d) was isolated and characterized as a branched type 2 chain glycolipid formerly termed A^c. The major component in the fifth A-active fraction (A^e) was identified as a branched type 2 chain A previously termed A^d. The structures of A^c (n = 1) and A^d (n = 2) are



Blood group ABH antigens in human erythrocytes are carried on both intrinsic membrane proteins and glycosphingolipids [for a review see Hakomori (1981)]. The blood group A determinant consists of the trisaccharide GalNAcα1→3(Fucα1→2)Galβ; however, it is carried by four distinct types of carbohydrate chains, thus yielding A epitopes with different specificities, each of which is defined by specific monoclonal antibodies. Type 1 chain A structures (Galβ1→3GlcNAcβ1→R) are present on erythrocytes in minor quantities, and their expression is dependent on secretor and Lewis blood group status (Watkins, 1980; Clausen et al., 1985a). Type 2 chain A structures (Galβ1→4GlcNAcβ1→R), in contrast, constitute the bulk of blood group A antigens on erythrocytes with branched and unbranched molecular diversity (Hakomori et al., 1972; Fukuda & Hakomori, 1982). Recently, another A structure, the repetitive A (type 3 chain; Galβ1→3GalNAcα1→R), which is especially abundant in subgroup A_1 erythrocytes, has been isolated and characterized (Clausen et al., 1985b). This structure may immunologically resemble an A determinant linked to an αGalNAc, O-glycosidically linked to serine or threonine of polypeptides (Carlson, 1966; Takasaki et al., 1978) previously termed "type 3 chain A" (Donald, 1980). In addition, A_1 erythrocytes contain trace amounts of globo-A (type 4 chain; Galβ1→3GalNAcβ1→R) (Clausen et al., 1984). Whereas the type 1 and type 4 chain A antigens appear to be carried by only a single glycosphingolipid (Clausen et al., 1984, 1985a), type 2 chain A structures are found on a series of glycosphingolipids with differing length and branching status, termed A^a, A^b, A^c, and A^d (Hakomori et al., 1972; Fukuda & Hakomori, 1982; Clausen, 1985b). Blood group O and B erythrocytes contain similar series of structures, H_1, H_2, H_3, and H_4 and B_I, B_{II}, B_{III}, B_{IV}, and B_V, respectively (Stellner et al., 1973; Watanabe et al., 1975; Fukuda & Hakomori, 1982; Hanfland et al., 1984).

Since a series of type 3 chain A (repetitive A) glycolipids defined by monoclonal antibody TH1 was previously found to have similar TLC mobilities as the type 2 chain A^b, A^c, and A^d components, a systematic reinvestigation was necessary to distinguish the structures of the type 2 chain A and type 3

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chain A glycolipids. Previously, the simplest repetitive A glycolipid, having the same TLC mobility as A^b, was isolated and characterized. In this paper, the isolation and characterization of another repetitive A glycolipid with 11 sugar residues and an unbranched type 2 chain A with 10 sugar residues, both isolated from the A^c fraction, are described. In addition, the isolation and characterization of two branched type 2 chain A analogues, previously called A^c and A^d, are described.

Materials and Methods

Monoclonal Antibodies. A mouse IgG3 monoclonal antibody, HH4, was obtained by immunization with type 2 chain A (A^a) glycolipids adsorbed to acetic acid-treated *Salmonella minnesota* bacteria, followed by fusion of spleen cells with Sp/2-0 myeloma cells (Kohler & Milstein, 1975) (five injections of 2 μg of glycolipid coated on 30 μg of bacteria with 5–7-day intervals, last injection 3 days prior to fusion), and selection of hybridomas by positive reactivity with type 2 chain A but not with other types of A determinants. The reactivity of the hybridoma antibody was tested by solid-phase radioimmunoassay (Kannagi et al., 1983a) and thin-layer chromatography immunostaining (Magnani et al., 1980; Kannagi et al., 1982) with various A glycolipid standards (Table I). The monoclonal antibody TH1, specific for type 3 chain A determinant, was obtained as previously described (Clausen et al., 1985b). In addition, the following monoclonal antibodies were used: AH21 reacting specifically with monofucosyl type 1 chain A (Abe et al., 1984), HH2 and HH3 reacting with difucosyl type 2 and type 1 chain, respectively (Clausen et al., 1985c), and AH16 reacting with all A determinants (Abe et al., 1984).

Isolation and Purification of A-Active Glycolipids. For purification of glycolipids, pooled outdated human whole blood was lysed in ice-cold tap water containing 0.2% acetic acid and membranes were prepared by continuous centrifugation on a Sharpless centrifuge and extracted with 2-propanol–hexane–water (55:25:20 v/v/v). The "upper-phase glycolipid fraction" was prepared as previously described (Kannagi et al., 1982). The neutral glycolipid fraction was separated by DEAE-Sephadex A25 chromatography (Yu & Ledeen, 1972), and the upper neutral glycolipid fraction was fractionated by low-pressure high-performance liquid chromatography (HPLC)[1] on a 1 × 50 cm column of Iatrobeads 6RS-8060 (60 μm particles; Iatron Chemical Co., Tokyo, Japan) (Kannagi et al., 1982). The elution was programmed from 2-propanol–hexane–water 55:40:5 to 55:20:25 (v/v/v) during 200 min with a flow rate of 3 mL/min. Each 6-mL fraction was collected on a fraction collector (total of 600 mL of eluate collected over 100 fractions). Each fraction was analyzed by high-performance thin-layer chromatography (HPTLC) in a solvent mixture of chloroform–methanol–water (56:38:10 v/v/v). Fractions containing glycolipids in A^c (eluted in tubes 57–63), A^d (eluted in tubes 61–67), and A^e (eluted in tubes 67–75) were further purified by high-pressure HPLC on a 0.4 × 50 cm column of Iatrobeads 6RS-8010 (10 μm particles) with a gradient of 2-propanol–hexane–water from 55:35:10 to 55:20:25 (v/v/v) (Watanabe & Arao, 1981). High-pressure HPLC was repeated once or twice until the fractions appeared pure on HPTLC and contained the A-active components as detected by monoclonal antibodies with broad reactivity and our monoclonal antibodies with fine specificity for A glycolipid variants.

The HPLC-purified fractions were finally purified as acetates by preparative HPTLC on Merck plates (Darmstadt, West Germany) in solvent system A (dichloroethane–acetone–water, 40:60:2.5 v/v/v) and solvent system B (chloroform–methanol–water, 89:11:0.01). The homogeneity of the purified fractions was checked by two-dimensional HPTLC as acetates in solvent systems A and B and as native compounds in solvent system C (chloroform–methanol–water, 50:40:10) and solvent system D (chloroform–methanol–water, 50:55:18).

TLC Immunostaining. Immunostaining of glycolipids separated as native compounds or as acetates by two-dimensional HPTLC was performed as previously described (Magnani et al., 1980; Kannagi et al., 1982; Clausen et al., 1985b). In order to identify single components carrying type 3 chain A determinant in the acetylated glycolipid fractions A^c, A^d, and A^e after separation with two-dimensional HPTLC in the two solvent systems A and B, glycolipids were deacetylated in situ by immersing the HPTLC plate in methanol containing 2% sodium methoxide (w/v) (Clausen et al., 1985b). HPTLC plates were thereafter processed according to the method of Magnani et al. (1980) as modified by Kannagi et al. (1982). (The antibody HH4 did not react with glycolipid antigens after in situ deacetylation on HPTLC plates and was therefore only used in solid-phase radioimmunoassay and HPTLC immunostaining of separated native glycolipids.)

Glycolipid Characterization. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded with a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer and pulse programmer, operating in the Fourier-transform mode with quadrature detection. Spectra were recorded at 308 ± 2 K on deuterium-exchanged samples (100–500 μg) dissolved in 0.4 mL of dimethyl-d_6 sulfoxide containing 2% D_2O (Dabrowski et al., 1980) and 1% tetramethylsilane as a chemical shift reference. Other parameters and data treatment were as previously described (Bremer et al., 1984). Glycolipids were methylated (Hakomori, 1964), hydrolyzed, reduced, and acetylated according to published procedures [Björndal et al., 1967; Stellner et al., 1973; Bremer et al., 1984; for a review, see Lindberg and Lönngren (1978)] adapted for analysis of 10–20 nmol of glycolipid (Levery & Hakomori, 1986). Partially O-methylated, N-methylated alditol and hexosaminitol acetates were analyzed by gas chromatography–methane chemical ionization mass spectrometry (Laine, 1980; McNeil & Albersheim, 1977) with selected ion monitoring, under the conditions given in the legend to Figure 9. Further details will be described elsewhere (Levery & Hakomori, 1986).

Results

Specificity of Monoclonal Antibody HH4

The antibody HH4 reacted only with type 2 chain A structures on solid-phase radioimmunoassay and not with type 3 chain A (repetitive A), type 4 chain A (globo-A), type 1 chain A, and ALe^b (Figure 1). As shown in Figure 5, some types of type 2 chain A glycolipids differ in mobility from type 3 chain glycolipids. HH4 stained the A^a, A^b, A^c (weakly), A^d, and A^e regions and unidentified slower-migrating antigens. In contrast, TH1 stained strongly the A^b, A^c, and A^e regions, but not the A^d region (Figure 5B).

Isolation of Glycolipids

A^c Region. Complete resolution of the A^c region was only possible by two-dimensional HPTLC of the acetylated fraction

[1] Abbreviations: HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; ^{1}H NMR, proton nuclear magnetic resonance; GC–MS, gas chromatography–mass spectrometry; DEAE, diethylaminoethyl.

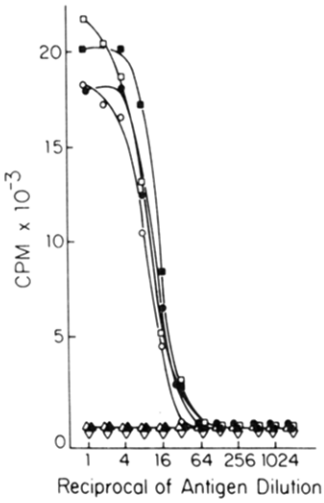


FIGURE 1: Binding specificity of HH4 antibody to purified glycolipids. The initial glycolipid concentration was 100 ng with 300 ng of cholesterol and 500 ng of lecithin. Antibody was used as undiluted culture supernatant. Designations used were as follows: (●) A^a type 2 chain; (■) A^aLe^y; (□) nor-A^c; (○) A^b type 2 chain; (△) A^b type 3 chain; (▽) globo-A (type 4 chain A); (▲) A^c type 3 chain. Additional glycolipids, which gave no binding, where A^a type 1 chain, A^aLe^b, Forssman, and H_2 type 2 chain.

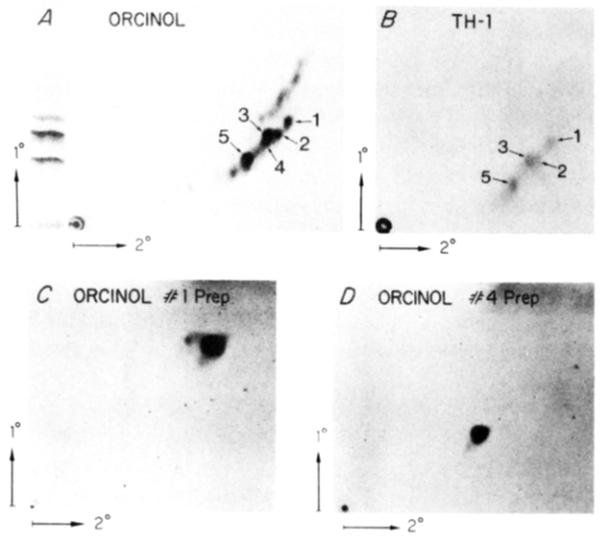


FIGURE 2: Two-dimensional HPTLC of acetylated crude A^c fraction glycolipids: (A) orcinol/H_2SO_4 stain; (B) in situ deacetylation and immunostaining with TH1 (fractions 1, 2, 3, and 5 were reactive); (C) purified component 1; (D) purified component 4. Plates developed in solvent systems A (1°) and B (2°).

(Figure 2A). Five major components were separated, four of which were stained by TH1 on direct immunostaining (Figures 2B and 3C), suggesting that these components carry the repetitive A (type 3 chain A) structure. One component (4) was not reactive with TH1 (Figures 2B and 3C) but was reactive with HH4 (Figure 3D), suggesting that this component carries the type 2 chain A structure. Components 1 and 4 were purified (Figure 2C,D) and subjected to ^1H NMR spectroscopy and methylation analysis for structural determination.

A^d Region. Two major components were partly resolved by two-dimensional HPTLC of the acetylated A^d region (spots 1 and 2 in Figure 4A). TH1 did not react with these components by HPTLC immunostaining of either acetylated or native glycolipids (Figures 4B and 5B), although the TH1 antibody picked up two trace components, possibly from the A^c and A^d region (components 3 and 4). Since the separation of the major components (1 and 2) was difficult, preparative separation of the two was not attempted. The fraction containing the two components (Figure 4C) that were reactive with HH4 was subjected to ^1H NMR spectroscopy and methylation analysis for structural determination.

A^e Region. The A^e region contained two major (1 and 4) and two minor (2 and 3) components (Figure 6A). The minor components were immunostained by TH1 (Figure 6B) but appeared to have a slightly faster mobility as native glycolipids than the major component 4 and the same mobility as component 1 (Figure 7B). Due to the limited quantity of components 1, 2, and 3, these were not analyzed further. Component 4, reactive with HH4, was purified (Figure 6C), and its structure was analyzed.

Chemical Characterization

Each of the purified components was examined first by one-dimensional ^1H NMR spectroscopy, followed by methylation analysis using GC–MS of partially methylated alditol acetates. In each case, a primary structure could be proposed on the basis of comparison of ^1H NMR spectra with data from previously published work (Clausen et al., 1985a,b; Hanfland et al., 1984), which was then confirmed by the results of methylation analysis.

^1H NMR Spectroscopy. (A) Type 3 Chain Component from A^c Region. The downfield region of the ^1H NMR spectrum of component 1 from the A^c region is reproduced in Figure 8A. The unique occurrence of four α-anomeric resonances ($^3J_{1,2}$ = 4 Hz) at 4.947, 4.958, 5.062, and 5.331 ppm was immediately identified as characteristic of the type 3 chain A structure reported previously (Clausen et al., 1985b). The β-anomeric resonances ($^3J_{1,2}$ = 7.3–7.9 Hz) found at 4.343, 4.614, and 4.662, along with the αFuc H-5 resonances (broadened quartets at 4.106 and 4.232 ppm) coupled to methyl doublets at 1.077 and 1.024 ppm, respectively ($^3J_{5,6}$ = 6.7 Hz), and the αGalNAc H-2 at 4.085 ppm ($^3J_{1,2}$ = 3.7 Hz; $^3J_{2,3}$ = 11.0 Hz) are all similarly found in the spectrum of the type 3 chain A glycolipid isolated from the A^b region. The only H-1 resonances not previously found are the additional β-anomeric signals at 4.260 and 4.655 ppm, which are characteristic shifts (under these conditions) for unsubstituted

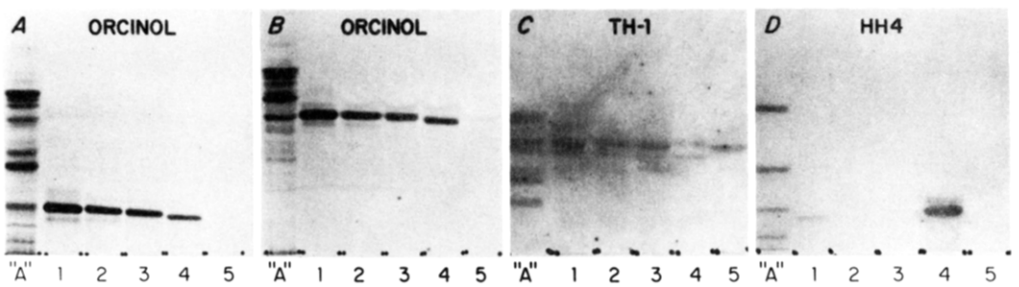


FIGURE 3: HPTLC of purified components from A^c region: (A, B) orcinol/H_2SO_4 stain; (C) immunostaining with TH1 of components 1, 2, 3, and 5; (D) immunostaining with HH4 only of component 4. Plates developed in solvent systems C (A and D) and D (B and C).

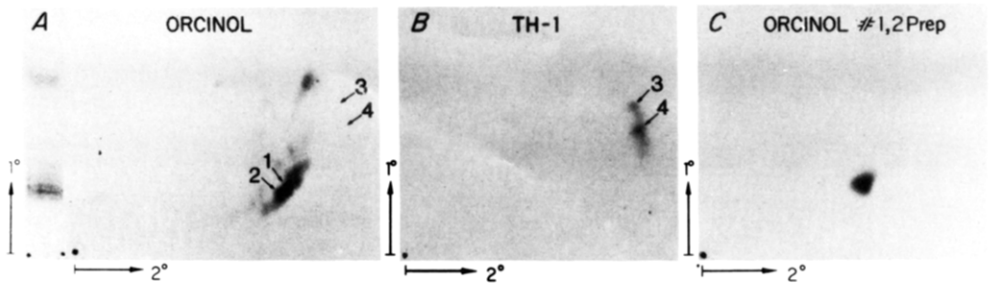


FIGURE 4: Two-dimensional HPTLC of acetylated crude A^d fraction glycolipids: (A) orcinol/H_2SO_4 stain; (B) in situ deacetylation and immunostaining with TH1 [very minor components (3, 4), possibly carry-over from the A^c region, are reactive with TH1]; (C) purified components 1 and 2 mixture. Plates developed in solvent systems A (1°) and B (2°).

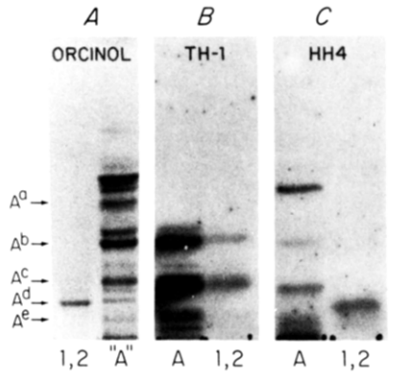
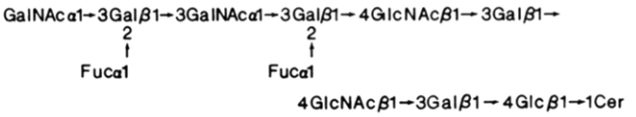


FIGURE 5: HPTLC of purified components from A^d region: (A) orcinol/H_2SO_4 stain; (B) immunostaining with TH1; (C) immunostaining with HH4 of components 1 and 2. Plates developed in solvent system C.

internal →3Galβ1→4GlcNAcβ1→ units of poly(*N*-acetyllactosaminyllactose)-containing glycosphingolipids [see, for example, Dabrowski, U., et al. (1984), Hanfland et al. (1984), Clausen et al. (1985b, 1986b), and type 2 chain A structures presented in this work]. This is further indicated by the presence of an additional GlcNAc NAc at 1.820 ppm. On this basis, the major glycolipid from the A^c region is proposed to have the structure



which is the type 3 chain A glycolipid containing an additional β-*N*-acetyllactosamine unit inserted in its core. The resonances in the ^1H NMR spectrum are summarized and assigned as shown in Tables II and III and in Figure 8. The rationale for assignment of resonances for type 3 chain structures has been discussed previously (Clausen et al., 1985b, 1986b). Currently, work is in progress to assign the spectrum unambiguously by using two-dimensional ^1H NMR spectroscopic methods.

(*B*) *Type 2 Chain Component from A^c Region.* The downfield region of the ^1H NMR spectrum of the minor A-active component 4 from the A^c region (Figure 8B) is qualitatively identical with that previously obtained for the type 2 chain A^b octaglycosylceramide (Clausen et al., 1985b). Integration showed that the β-anomeric resonances at 4.260 and 4.655 ppm represent one more proton each than those in the spectrum of A^b, i.e., an additional β-*N*-acetyllactosamine unit in the core. An additional βGlcNAc NAc is also found

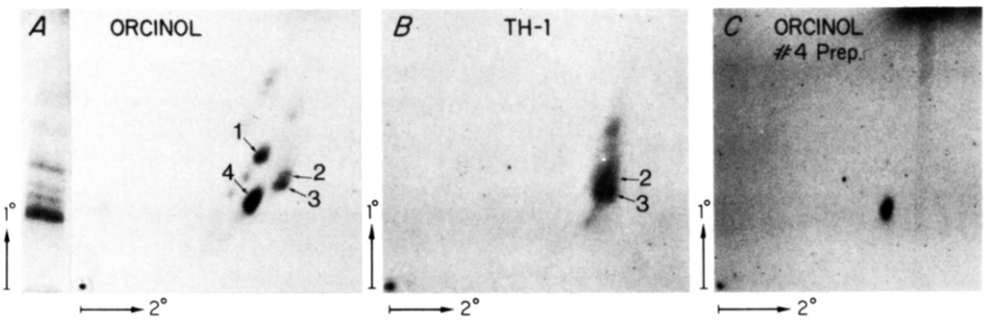


FIGURE 6: Two-dimensional HPTLC of acetylated crude A^c fraction glycolipids: (A) orcinol/H_2SO_4 stain; (B) in situ deacetylation and immunostaining with TH1 of minor components 2 and 3; (C) purified component 4. Plates developed in solvent systems A (1°) and B (2°).

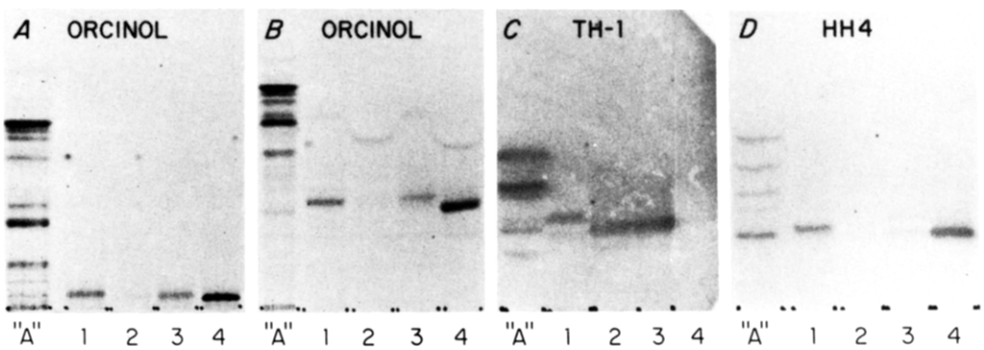


FIGURE 7: HPTLC of purified components from A^c region: (A, B) orcinol/H_2SO_4 stain; (C) immunostaining with TH1 of components 2 and 3; (D) immunostaining with HH4 only of component 4. Plates developed in solvent systems C (A) and D (B, C, and D).

Table I: Blood Group A Active Glycolipids

		antibody	reference
A. Type 1 chain			
$A^{a(a)}$	GalNAcα1→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc 2 ↑ Fucα1	AH21	Abe et al. (1984)
$A^{a}Le^{b(a)}$	GalNAcα1→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc 2 4 ↑ ↑ Fucα1 Fucα1	HH3	Clausen et al. (1985c)
B. Type 2 chain			
$A^{a(b)}$	GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 ↑ Fucα1	HH4	This paper
$A^{a}Le^{y(c)}$	GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 3 ↑ ↑ Fucα1 Fucα1	HH2, HH4	Clausen et al. (1985c)
$A^{b(b)}$	GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 ↑ Fucα1	HH4	
$norA^{c(d)}$	GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 ↑ Fucα1	HH4	
$isoA^{c(b,d)}$	GalNAcα1→3[Galβ1→4GlcNAc]$_1$β1 2 ↘ ↑ $^{3}_{6}$ Fucα1 Galβ1→4GlcNAcβ1→3Galβ1→4Glc ↗ GalNAcα1→3Galβ1→4GlcNAcβ1 2 ↑ Fucα1	HH4	
$A^{d(b,d)}$	GalNAcα1→3[Galβ1→4GlcNAc]$_2$β1 2 ↘ ↑ $^{3}_{6}$ Fucα1 Galβ1→4GlcNAcβ1→3Galβ1→4Glc ↗ GalNAcα1→3Galβ1→4GlcNAcβ1 2 ↑ Fucα1	HH4	
C. Type 3 chain			
$A^{a(e)}$	GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 2 ↑ ↑ Fucα1 Fucα1	TH1, HH5	Clausen et al. (1985b); unpublished
$A^{b(d)}$	GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 2 ↑ ↑ Fucα1 Fucα1	TH1, HH5	
D. Type 4 chain			
$A^{x(f)}$	GalNAcα1→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc 2 ↑ Fucα1	HH5	

[a] Clausen et al., 1985a. [b] Hakomori et al., 1972; Fukuda & Hakomori, 1982; Hakomori, 1981. [c] McKibbin et al., 1982. [d] This paper. [e] Clausen et al., 1985b (note previous designation of the nonasaccharide repetitive A, type 3 chain A^{b}, has been changed to A^{a} type 3 chain). [f] Clausen et al., 1984.

at 1.822 ppm. The somewhat surprising absence of resonances indicative of branching, i.e., →3(→6)Galβ1→ H-1 at 4.30 ppm, →4GlcNAcβ1→6 H-1 in the region 4.42–4.35 ppm [see, for example, Hanfland et al. (1981), Dabrowski, U., et al.

Table II: Glycosyl H-1 Chemical Shifts (ppm from Tetramethylsilane) and $^3J_{1,2}$ Coupling Constants (Hz) of Glycolipids in Dimethyl-d_6 Sulfoxide/2% D_2O (Dabrowski et al., 1980) at 308 ± 2 K

1 GalNAcα1---------→3
Fucα1---→2Galβ1---→4GlcNAcβ1---------→3Galβ1---→4GlcNAcβ1→3Galβ1---→4GlcNAcβ1→3Galβ1----→4Glcβ1→1R

A^a	4.935	5.149	4.387	4.620		--	--	--	--	4.258	4.172
	(4.3)	(3.7)	(7.9)	(7.3)						(7.9)	(7.9)
A^b	4.933	5.146	4.383	4.616		—	--	4.258	4.659	4.258	4.169
	(3.7)	(4.3)	(7.9)	(7.3)				(7.3)	(7.9)	(7.3)	(7.3)
A^c	4.934	5.146	4.385	4.625		4.262	4.659	4.262	4.659	4.262	4.169
	(3.7)	(3.7)	(7.3)	(7.3)		(7.3)	(8.5)	(7.3)	(8.5)	(7.3)	(7.3)

2 GalNAcα1---------→3
Fucα1---→2Galβ1----------→4GlcNAcβ1----------↘6
Galβ1---→4GlcNAcβ1→3Galβ1---→4Glcβ1→1R
GalNAcα1---------→3
Fucα1---→2Galβ1---→4GlcNAcβ1---------→3Galβ1---→4GlcNAcβ1↗3

A^c	4.933	5.146	4.380	4.615	4.342	-	-	4.297	4.656	4.263	4.169
	4.933	5.142	4.380								
	(3.7)	(3.4)	(7.3)	(7.9)	(8.5)			(6.7)	(9.1)	(6.7)	(7.3)
A^d	4.932	5.146	4.383	4.624	4.352	4.261	4.656	4.295	4.656	4.261	4.168
	4.932	5.142	4.383								
	(4.3)	(3.4)	(7.3)	(7.3)	(7.9)	(7.3)	(8.5)	(8.5)	(8.5)	(7.3)	(7.9)

3 GalNAcα1---------→3 3GalNAcα1---------→3
Fucα1---→2Galβ1----------→Fucα1---→2Galβ1---→4GlcNAcβ1→3Galβ1---→4GlcNAcβ1→3Galβ1----→4Glcβ1→1R

A^a	4.947[a]	5.061	4.663	4.955[a]$_b$	5.329$_b$	4.344	4.618	--	--	4.260	4.168
	(4.3)	(3.7)	(7.9)	(4.3)	(4.3)	(7.9)	(7.3)			(7.3)	(7.9)
A^b	4.947[a]	5.062	4.662	4.958[a]$_b$	5.331	4.343	4.614	4.260	4.655	4.260	4.168
	(4.3)	(3.7)	(7.3)	(4.3)	(4.3)b	(7.9)	(7.9)	(8.5)	(7.3)	(7.3)	(7.9)

[a] Assignments may be reversed. [b] Obtained at 328 K.

Table III: Fucosyl H-5,6, GalNAc H-2, and HexNAc NAc Chemical Shifts (ppm from Tetramethylsilane) for Type 2 and Type 3 Chain Glycosphingolipids in Dimethyl-d_6 Sulfoxide at 308 ± 2 K

	GalNAcα1→3		Fucα1→2		GalNAcα2→3		Fucα1→2		GlcNAcβ1→3	
	H-2	NAc	H-5	CH_3	H-2	NAc	H-5	CH_3	NAc	NAc
A^a (2)					4.105	1.849	4.144	1.081	1.824	
A^b (2)					4.106	1.846	4.144	1.080	1.824	1.820
A^c (2)					4.102	1.844	4.142	1.080	1.824	1.820 (×2)
A^c (2, br)					4.101	1.844	4.144	1.080	1.837	1.820
					4.101	1.844	4.132	1.071	1.827	
A^d (2, br)					4.102	1.844	4.142	1.080	1.824 (×2)	1.818 (×2)
					4.102	1.844	4.130	1.080		
A^a (3)	ND[a]	1.843	4.237	1.024	4.084	1.843	4.104	1.076	1.818	
A^b (3)	ND[a]	1.842	4.232	1.024	4.085	1.842	4.106	1.077	1.818	1.818

[a] ND, not determined.

(1984), Hanfland et al. (1984), Kannagi et al. (1983b), and branched glycolipids discussed subsequently] indicates that the glycolipid must be a straight-chain analogue of A^b, as shown by the structure in Figure 8B. All resonances shown were assigned by analogy with A^a and A^b glycolipids (Clausen et al., 1985a,b) as indicated in Tables II and III. Comparison of data for unbranched type 2 chain A structures so far analyzed with that available for B-active glycolipids (Hanfland et al., 1984) demonstrated systematic similarities, as might be expected, as well as some predictable differences. The combined effects of GalNAcβ1→3 and Fucα1→2 glycosylation induced chemical shifts to higher frequency for the terminal Galβ1→4 H-1 ($\Delta\delta$ = +0.175 ± 0.001 ppm) and to lower frequency for the subterminal GlcNAcβ1→3 H-1 ($\Delta\delta$ = −0.04 ± 0.005 ppm), nearly the same values as found for conversion to B terminal by Fucα1→2 and Galα1→3 glycosylation. Also, when spectra of A and B glycolipids were acquired at similar temperatures (Levery and Hakomori, unpublished experiments), values found for Fucα1→2 H-1 and CH_3 of A terminal were found within 0.01 ppm of the corresponding resonances for B terminal. Thus, the GalNAc NAc group appears to be in a position that has only a relatively small influence on the shifts of these protons. By far the major obvious difference is in the location of the αGalNAc H-2 at approximately 4.10 ppm, a large shift to higher frequency as compared with the same proton in αGal ($\Delta\delta$ = 0.45 ppm), as observed previously by Dabrowski et al. (1980). Conversely, the A-terminal αGalNAc H-1 is found shifted to somewhat lower frequency ($\Delta\delta$ = −0.045 ppm) compared with the B-terminal αGal H-1. The shielding component of the 2-*N*-acetamido group with respect to H-1 of the α-galactopyranosyl unit has likewise been previously noted (Dabrowski et al., 1980). Other terminal trisaccharide resonances that appear to be substantially affected by the 2-*N*-acetamido group are the Fucα1→2 H-5 ($\Delta\delta$ = +0.028 ppm, A^b vs. B-II, 308 K) and Galβ1→4 H-4 ($\Delta\delta$ = −0.036 ppm). The above observa-

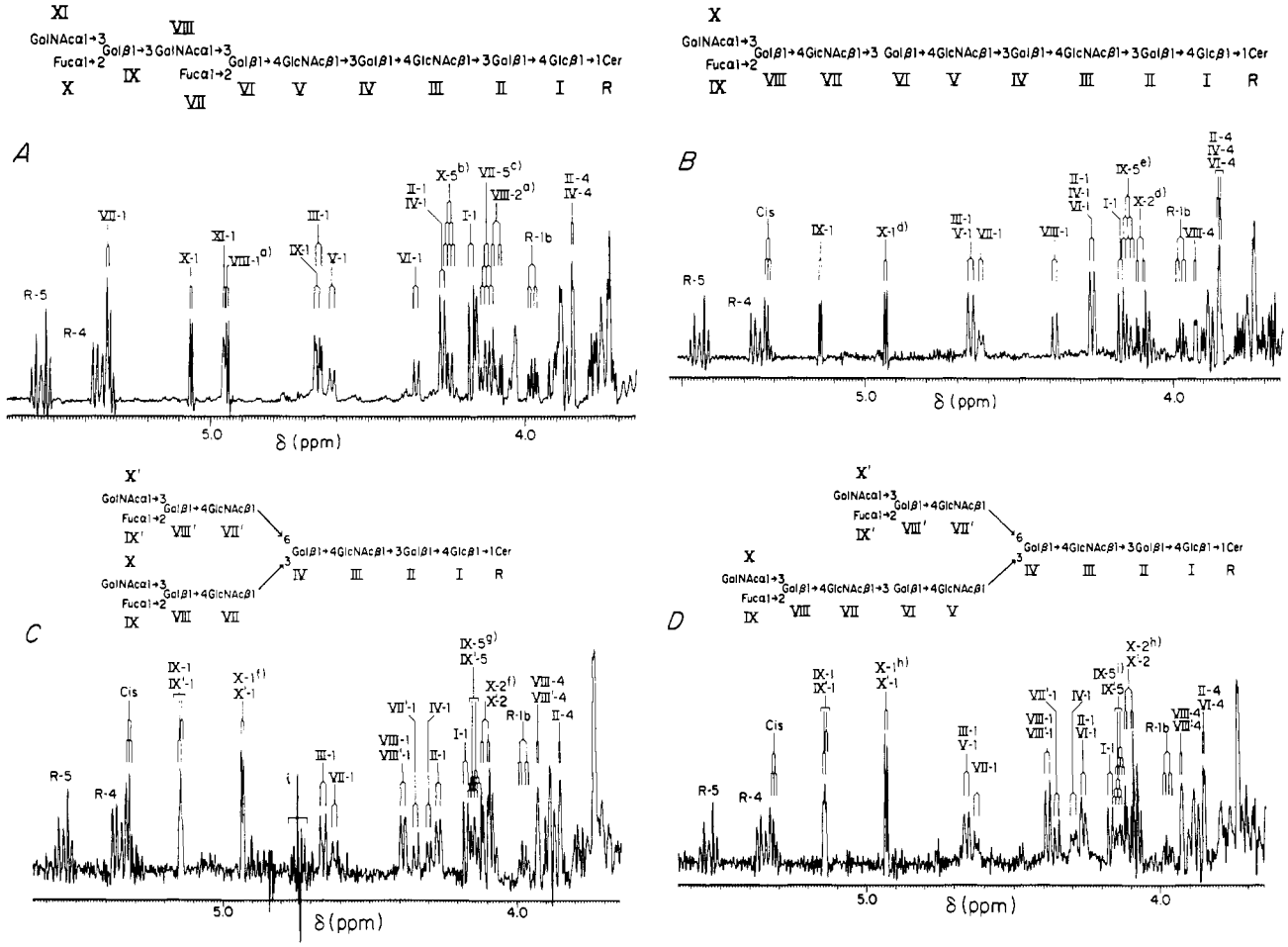


FIGURE 8: Downfield region of resolution-enhanced ^1H NMR spectra of A erythrocyte glycolipids in dimethyl-d_6 sulfoxide/2% D_2O at 308 ± 2 K. (A) Fraction 1 from A^c region (type 3 chain A^b ceramide undecasaccharide). Concentration ~0.5 mM; 3600 scans accumulated. (B) Fraction 4 from A^c region (type 2 chain A^c ceramide decasaccharide). Concentration ~0.3 mM; 6600 scans accumulated. (C) Fractions 1 and 2 from A^d region (branched type 2 chain A^c ceramide dodecasaccharide). Concentration ~0.1 mM; 10 000 scans accumulated. (D) Fraction 4 from A^e region (branched type 2 chain A^d ceramide tetradecasaccharide). Concentration ~0.2 mM; 1500 scans accumulated. Arabic numbers refer to protons of residues indicated by Roman numerals in the structure drawn at the top of each panel. Resonances marked by "R-" are from the sphingosine backbone, while triplets marked "cis" are from *cis*-vinyl protons of unsaturated fatty acids. The bracketed signal marked "i" in (D) is from an impurity. Footnotes: (a, d, f, h) αGalNAc H-1, H-2 connectivities established by decoupling experiments; (b) αFuc H-5 established by decoupling from Me doublet at 1.024 ppm; (c) αFuc H-5 established by decoupling from Me doublet at 1.077 ppm; (e) αFuc H-5 established by decoupling from Me doublet at 1.080 ppm; (g) αFuc H-5 resonances established by decoupling from Me doublets at 1.071 and 1.080 ppm; (i) αFuc H-5 resonances established by decoupling from Me doublets at 1.080 ppm.

tions were helpful in interpreting the spectra of the more complex A-active glycolipids.

(C) Components 1 and 2 from the A^d Fraction. The downfield region of the ^1H NMR spectrum of components 1 and 2 isolated from the A^d fraction is reproduced in Figure 8C. The finding of two overlapping sets of characteristic resonances for type 2 chain A trisaccharides, along with a resonance characteristic for a branching →3(→6)Galβ1→4 (4.297 ppm, $^3J_{1,2}$ = 6.7 Hz) strongly suggested a tentative identification as the branched type 2 chain glycolipid, formerly assigned as A^c. Consistent with this structure are two βGlcNAc H-1 signals, one unshifted from its position in type 2 chain core structures, the other shifted 0.4 ppm to lower frequency, as expected for H-1 of →4GlcNAcβ1→3 linked to an A trisaccharide. The β-anomeric resonance at 4.342 ppm ($^3J_{1,2}$ = 8.5 Hz) may then be assigned as arising from the →4GlcNAcβ1→6 residue bearing the other A trisaccharide, shifted 0.06 ppm to lower frequency from its position in the spectrum of iso-nLc_8Cer (4.409 ppm at 308 K; Levery and Hakomori, unpublished observation). The larger shift for the β-GlcNAc forming the 1→6 branch was also observed for the analogous B-III glycosphingolipid (Hanfland et al., 1984). Assigning the remaining two β-anomeric resonances to the core 3Galβ1→4Glcβ1→ residues (see Figure 8C) accounts for all the anomeric signals in the spectrum. The proposed structure is further supported by the presence of three βGlcNAc and two αGalNAc NAc singlets in the spectrum (see Table III). Other signals are assigned as shown in Figure 8C. Interestingly, the two αFuc anomeric and H-5 signals are observed at slightly different frequencies. This was not observed in the case of B-III (Hanfland et al., 1984), but may be due to the higher temperature (333 K) or lower field strength (360 MHz) used in that work. The source for this effect is not known, but it may be presumed to arise either from an effect of one chain on the other or from some small intrinsic difference in the conformation of the A trisaccharide on the β1→6 branch from that on the β1→3 branch.

(D) Component 4 from the A^e Fraction. Inspection of the ^1H NMR spectrum of component 4 isolated from the A^e fraction again suggested a branched type 2 chain glycolipid bearing two A trisaccharides. In this case, integration revealed the presence of two additional resonances corresponding to insertion of an unsubstituted →4GlcNAcβ1→3Galβ1→ unit into the core structure of the A^c dodecasaccharide discussed above. The problem of the location of the additional β-*N*-acetyllactosamine unit has been approached already in con-

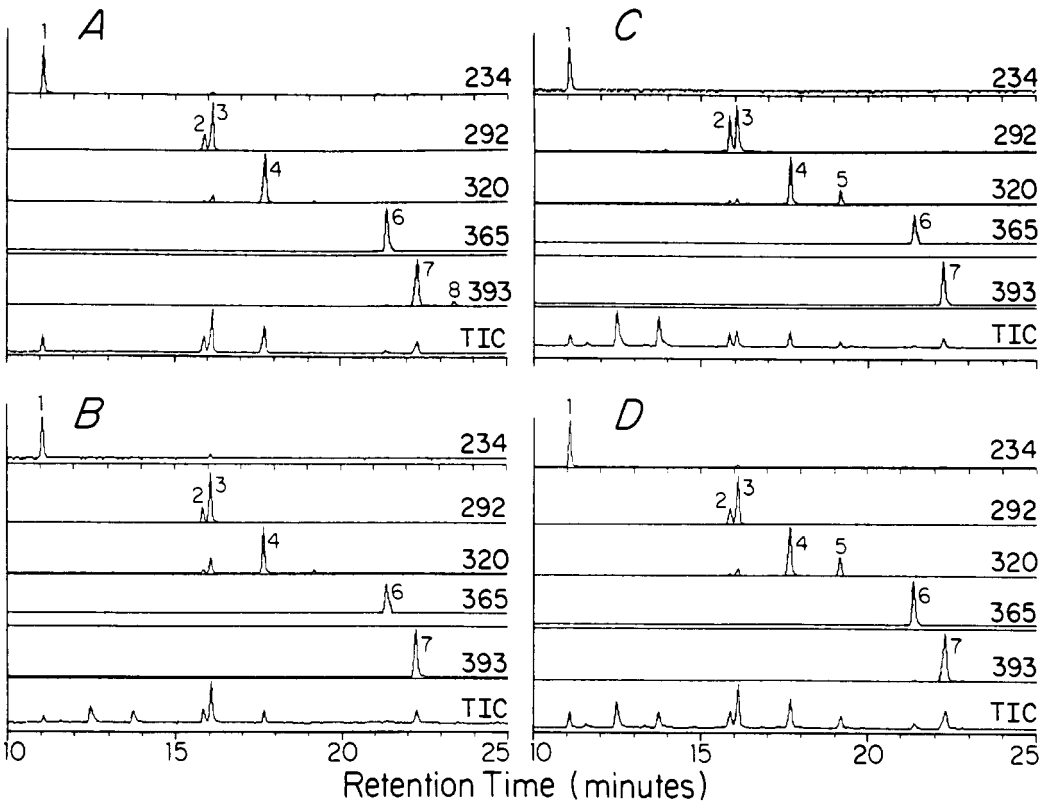


FIGURE 9: Gas chromatography–mass spectrometry of partially O-methylated, N-methylated alditol and hexosaminotol acetates from hydrolysis of permethylated A erythrocyte glycolipids: (A) type 3 chain A^b; (B) unbranched type 2 chain A^c; (C) branched type 2 chain A^c; (C) branched type 2 chain A^d. Ordinate, intensity of each ion at the mass number indicated. Abscissa, scan number. Peaks identified were as follows: (1) 2,3,4-*O*-Me-Fuc; (2) 2,3,6-*O*-Me-Glc; (3) 2,4,6-*O*-Me-Gal; (4) 4,6-*O*-Me-Gal; (5) 2,4-*O*-Me-Gal; (6) 3,4,6-*O*-Me-GalNAcMe; (7) 3,6-*O*-Me-GlcNAcMe; (8) 4,6-*O*-Me-GalNAcMe. Analyses were performed on a Hewlett-Packard HP5985 GC–MS system with a 30M DB-5 bonded-phase fused silica capillary column (J & W Scientific, Rancho Cordova, CA) temperature-programmed from 50 to 140 °C at 10 deg/min and from 140 to 230 °C at 4 deg/min. Source pressure, 400 μm methane; emission current, 0.5 mA; source temperature, 200 °C; injection temperature, 225 °C; transfer line, 250 °C. Twenty ions were monitored (cycle time 1 s), including all relevant MH^+, $(MH-60)^+$ for hexosamine and $(MH-32)^+$, $(MH-60)^+$ for neutral sugar derivatives. A limited number of these are shown in order to simplify presentation.

nection with the structure elucidation of B-IV glycolipid (Hanfland et al., 1984). As pointed out in that work, the βGlcNAc resonances bearing A, B, or H structures are "labeled" by their shift to lower frequency relative to those in the unsubstituted core structure. Thus, the location of the extension in the 1→6 branch is excluded by the absence of a signal for the GlcNAcβ1→6 H-1 at approximately 4.40–4.42 ppm. In the case of B-IV, the otherwise difficult question of whether the additional residues are then in the 1→3 branch or in the unbranched segment between Gal IV and ceramide was settled by fast atom bombardment mass spectrometry of the permethylated intact glycolipid, which unambiguously identified the former case. In the absence of such data, however, it is still possible to reach a conclusion based on the ^1H NMR data. A careful inspection of the spectrum shows that the anomeric protons of the two βGlcNAc residues bearing the A trisaccharides are each shifted 0.01 ppm to higher frequency compared with those of the A^c glycolipid (see Table II). The fact that the same trends are reported for B-IV vs. B-III (Hanfland et al., 1984) supports the idea that these effects are not artifacts due to temperature or concentration differences in obtaining spectra but are due specifically to the extension of the 1→3 branch having a small effect on the shifts of residues remaining in (GlcNAc VII′), or borne out of (GlcNAc VII), the branching region. It would be considerably more difficult to envision these same effects arising from insertion of the additional β-*N*-acetyllactosamine unit into the unbranched core segment. Thus, on the basis of spectroscopic, as well as biological, analogy, the corresponding structure (A^d) can be proposed as shown in Figure 8D. Confirmation of this structure by mass spectrometric methods is in progress.

Methylation Analysis of Components 1 and 4 from the A^c Fraction, Components 1 and 2 from the A^d Fraction, and Component 4 from the A^d Fraction. Analysis of the partially methylated alditol acetates produced after permethylation, hydrolysis, reduction, and acetylation of the glycolipids was consistent in each case with the structure proposed (Figure 9). The terminal A trisaccharides were represented by the finding of derivatives of 2,3,4-*O*-Me-Fuc, 3,4,6-*O*-Me-GalNAcMe, and 4,6-*O*-Me-Gal; repeating type 2 chain *N*-acetyllactosamine units by 2,4,6-*O*-Me-Gal and 3,6-*O*-Me-GlcNAcMe; 1→3(1→6) branched galactose by 2,4-*O*-Me-Gal; and core 1→4-linked glucose by 2,3,6-*O*-Me-Glc. Characteristic of type 3 chain structures was the finding of 4,6-*O*-Me-GalNAcMe from the terminal A hapten linked through the βGal unit to the 3 position of GalNAc of the internal A hapten (Clausen et al., 1985b). Quantitation of galactose and fucose derivatives by integration of selected ion peaks showed the molar ratios expected for the structures indicated, when compared with those produced by pure samples of A^b type 2 chain and A^a type 3 chain used as standards. Thus, as shown in Table IV, type 2 chain A^c decasaccharide differed from type 2 chain A^b octasaccharide by an additional 1 mol of 2,4,6-*O*-Me-Gal, while type 3 chain A^b differed from type 3 chain A^a by an additional 1 mol of 2,4,6-*O*-Me-Gal and from type 2 chain A^b by 1 mol each of 2,3,4-*O*-Me-Fuc and 4,6-*O*-Me-Gal from the additional A hapten. The branched A^c dodecasaccharide also produced twice as much of these derivatives as A^b type 2 chain while yielding a mole less of 2,4,6-*O*-Me-Gal and producing a mole of 2,4-*O*-Me-Gal. The branched A^d

Table IV: Molar Ratios of Partially Methylated Neutral[a] Sugars from A-Active Erythrocyte Glycosphingolipids after Permethylation, Hydrolysis, Reduction, and Acetylation

	derivative				
glycolipid	2,3,4-*O*-Me-Fuc	2,3,6-*O*-Me-Glc[b]	2,4,6-*O*-Me-Gal	4,6-*O*-Me-Gal	2,4-*O*-Me-Gal[c]
A^a (3)[d]	2.00	1.0	1.00	2.00	
A^b (2)[e]	1.00	1.0	2.00	1.00	
A^b (3)	1.89	1.0	1.84	1.87	
A^c (2)	1.14	1.0	2.29	0.98	
A^c (2, br)	1.84	1.0	1.03	1.66	0.70
A^d (2, br)	2.11	1.0	2.06	2.37	1.02

[a] Quantitation of amino sugar derivatives by GC–MS was not sufficiently reliable. [b] All values normalized to 1.0 mol of 2,3,6-*O*-Me-Glc. [c] Response factor used same as for 4,6-*O*-Me-Gal. [d] Standard. Response factors (average of three injections) for derivatives from type 3 chain glycolipids calculated to reflect integral composition given here. [e] Standard. Response factors (average of three injections) for derivatives from type 2 chain glycolipids calculated to reflect integral composition given here.

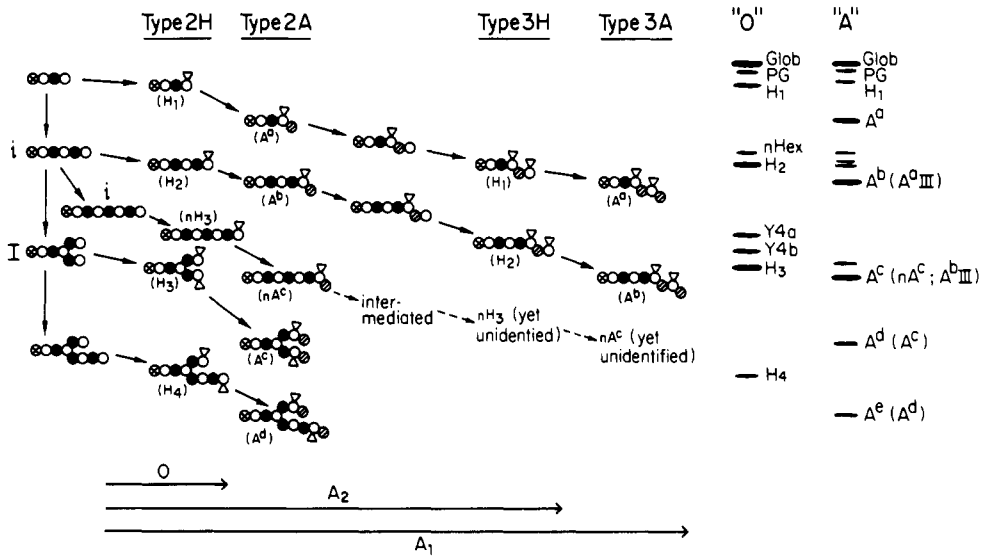


FIGURE 10: Depicted synthetic scheme for blood group A glycolipids in erythrocytes. Type 2 chain polylactosamine core structures may elongate, forming i structures, or branch, forming I structures, by addition of lactosamine structures. This process may be arrested by either sialylation (not shown) or fucosylation to form H structures. Further substitution to form A structures not only involves the addition of a GalNAc residue, but involves an additional three synthetic steps. The type 2 chain A structures are substituted with a galactose residue, a fucose residue, giving rise to the type 3 chain H or A-associated H antigen, and finally the addition of another galactosamine residue. Whether elongation of branched type 2 chain structures occurs was not clarified. Structures are represented in order of their TLC mobility, and reference structures from O and A total upper neutrals are indicated. Symbols: ⊗ = Glc, ○ = Gal, ● = GlcNAc, ⊘ = GalNAc, ▽ = Fuc.

tetradecasaccharide produced 1 mol more of 2,4,6-*O*-Me-Gal than branched A^c. These results are in qualtitative and quantitative agreement with the structures proposed on the basis of ^{1}H NMR spectroscopy, HPTLC mobility, and immunoreactions.

Discussion

The presence of multiple A-active glycolipid fractions has been detected by analysis of the anti-A inhibitory activity of glycolipid fractions obtained from DEAE-Sephadex chromatography. Four such fractions were called A^a, A^b, A^c, and A^d, and the major A-active glycolipid isolated from each fraction was analyzed by methylation analysis and enzymatic degradation. On the basis of the pattern of partially *O*-methylated sugars identified by GC–MS in the hydrolysate of permethylated glycolipids (Hakomori et al., 1972, 1977; Fukuda & Hakomori, 1982), the fractions were determined to contain mainly type 2 chain A glycolipids with or without branched core structure. With the introduction of monoclonal antibodies directed to various A determinants, immunostaining applied on HPTLC, and improved separation and analytical techniques, particularly ^{1}H NMR spectroscopy, it has become apparent that the polymorphism of blood group A antigens in blood cell membranes is much more complex than originally envisioned.

At present, four types of A determinants with different core structures have been identified in human blood cell membranes, each detectable with specific monoclonal antibodies, as shown in Table I. Both type 1 and type 4 chain A represent only one glycolipid structure each, i.e., ceramide hexa- and heptasaccharides, in contrast to the large degree of polymorphism found in type 2 and type 3 chain A glycolipid structures. Since a series of type 3 chain structures detected by monoclonal antibody TH-1 showed similar TLC mobilities as the fractions previously assigned as A^b, A^c, and A^d, a systematic investigation has been performed in order to distinguish between well-known type 2 chain and the newly established type 3 chain structures. In order to perform this study, an antibody probe that reacts specifically with type 2 chain A structures was needed. Initial effort was therefore focused on establishing such an antibody. On TLC immunostaining, antibody HH4 specifically stains type 2 chain A glycolipids, in contrast to antibody TH1, which exclusively stains type 3 chain A glycolipids.

Two-dimensional thin-layer chromatography of acetates and preparative HPTLC purification of glycolipids as acetates made it possible to separate type 2 and type 3 chain blood group A active glycolipids in the A^b, A^c, A^d, and A^e fractions. The pattern of polymorphism in the fractions can be summarized as follows: (1) The A^b fraction contained type 2 chain

A^b and type 3 chain A^a, as previously described[2] (Clausen et al., 1985b). (2) The A^c fraction was found to contain type 3 chain A^b as the major component. In addition, an A determinant carried by an unbranched type 2 chain octasaccharide core was found as a relatively minor component and is hereby called nor-A^c. Interestingly, this component has been demonstrated to be blood group i-active (Hirohashi et al., 1986). We were unable to isolate branched type 2 chain A^c, as previously described (Fukuda & Hakomori, 1982), from this fraction. (3) The A^d fraction contained the classical branched type 2 chain A, previously termed A^c, as the major component (branched ceramide dodecasaccharide). (4) A slower-migrating A^e fraction was determined to contain the branched ceramide tetradecasaccharide previously termed A^d.

The discrepancies between the present results and those reported previously from our laboratory (Fukuda & Hakomori, 1982) can be attributed to the great improvements in methods for isolation and analysis of complex mixtures of glycolipids. On methylation analysis, a mixture of type 3 chain and type 2 chain glycolipids would give a qualitatively identical pattern of partially methylated hexitols and hexaminitols as a type 2 chain except for the presence of 4,6-*O*-Me-GalNAcMe, the yield of which is extremely low and can easily be missed. Complete characterization of type 3 and type 2 chain antigens has only been made possible by the application of highly specific monoclonal antibodies, the use of ^1H NMR spectroscopy, and the use of more sensitive GC–MS techniques in methylation analysis (i.e., capillary columns, chemical ionization, mass fragmentography).

The present concept of the structural relationship of type 2 and type 3 chain glycolipid structures in erythrocytes is presented in Figure 10. Interestingly, it has not been possible to identify difucosylated type 2 chain A structures in erythrocytes (Clausen et al., 1985c), which were previously suggested to be present (Gooi et al., 1985a). In addition to the type 2 and 3 chain A antigens, minor amounts of type 1 and 4 chain A structures have been found in erythrocytes (Clausen et al., 1985b; Clausen et al., 1984). The blood group A antigen, thus, consists of at least six structural variants carrying the terminal "A trisaccharide" [GalNAcα1→3(Fucα1→2)-Galβ1→R] in addition to the polymorphism generated by differences in branching and elongation status of type 2 and 3 chain antigens (Table I). Additional blood group A associated antigens are the A-associated H antigens: type 3 chain H [Fucα1→2Galβ1→3GalNAcα1→3(Fucα1→2)Galβ1→R] and its immediate precursor [Galβ1→3GalNAcα1→3-(Fucα1→2)Galβ1→R] (Clausen et al., 1986a, unpublished results). Since the development of the hybridoma technique (Köhler & Milstein, 1975), a large number of mouse monoclonal antibodies directed to the blood group A antigens have been described (Abe et al., 1984; Clausen et al., 1985b,c; Furukawa et al., 1985; Chen & Kabat, 1985; Gooi et al., 1985b). However, although diversity of the binding specificities of these antibodies has been demonstrated, it has not always been possible to demonstrate the distinct structural basis of the specificity. This has largely been due to a lack of well-characterized monovalent antigens (Chen & Kabat, 1985; Gooi et al., 1985b). Although improvements of immunoassays for oligosaccharides have been obtained (Magnani, 1985; Tang et al., 1985), the immunoassays available for glycosphingolipids, including solid-phase radioimmunoassay, liposome inhibition assays, and HPTLC immunoassays, make glycosphingolipids ideal for the characterization of the structural specificity of anti-carbohydrate antibodies. Through the use of highly purified and well-defined glycolipid antigens, it has become possible to generate and characterize a number of mouse monoclonal antibodies with strict specificity for various blood group A variants (see Table I). This panel of antibodies is very useful in studying the expression of blood group A antigens in relation to A subgroups (Clausen et al., 1985a,b, 1986b), epithelial differentiation (Dabelsteen et al., 1982), and transformation (Clausen et al., 1986a).

Registry No. A^b, 104335-76-4; nor-A^c, 104325-01-1; A^c, 104335-77-5; A^d, 104350-58-5.

[2] This component was previously called type 3 chain A^b because it overlaps with the type 2 chain A^b fraction. However, since the structure of this component is more related to A^a and it is the simplest form of type 3 chain A, it is hereby called type 3 chain A^a.

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Electrostatic Analysis of the Interaction of Cytochrome *c* with Native and Dimethyl Ester Heme Substituted Cytochrome b_5†

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ABSTRACT: The stability of the complex formed between cytochrome *c* and dimethyl ester heme substituted cytochrome b_5 (DME-cytochrome b_5) has been determined under a variety of experimental conditions to evaluate the role of the cytochrome b_5 heme propionate groups in the interaction of the two native proteins. Interaction between cytochrome *c* and the modified cytochrome b_5 was found to produce a difference spectrum in the visible range that is very similar to that generated by the interaction of the native proteins and that can be used to monitor complex formation between the two proteins. At pH 8 [25 °C (HEPPS), I = 5 mM], DME-cytochrome b_5 and cytochrome *c* form a 1:1 complex with an association constant K_A of 3 (1) $\times 10^6$ M^{-1}. This pH is the optimal pH for complex formation between these two proteins and is significantly higher than that observed for the interaction between the two native proteins. The stability of the complex formed between DME-cytochrome b_5 and cytochrome *c* is strongly dependent on ionic strength with K_A ranging from 2.4×10^7 M^{-1} at I = 1 mM to 8.2×10^4 M^{-1} at I = 13 mM [pH 8.0 (HEPPS), 25 °C]. Calculations for the native, trypsin-solubilized form of cytochrome b_5 and cytochrome *c* confirm that the intermolecular complex proposed by Salemme [Salemme, F. R. (1976) *J. Mol. Biol. 102*, 563] describes the protein–protein orientation that is electrostatically favored at neutral pH. For the interaction between cytochrome *c* and the less negatively charged DME-cytochrome b_5, an alternative interaction geometry was identified that is electrostatically isoenergetic with the original model at neutral pH. This alternative complex involves different amino acid residues at the intermolecular interface and positions the heme prosthetic groups in a non-coplanar orientation. Further electrostatic calculations indicate that the difference in stability of these complexes for either pair of proteins is a kilocalorie or less depending on pH. Therefore, we conclude that the nature of protein–protein complexes formed is in part dependent on the solution conditions and that an ensemble of protein–protein complexes exists in solution as precursors to an efficient electron-transfer geometry.

Several studies have shown that rates of electron transfer between proteins that function as physiological electron-transfer partners depend strongly on ionic strength. These observations have led to the conclusion that electrostatic interactions are important in facilitating the formation of a productive protein–protein electron-transfer complex. The reaction between cytochrome b_5 and cytochrome *c* is a particularly attractive model for studying the electrostatic interactions involved in protein–protein recognition during

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