Isolation and characterization of a heptaglycosylceramide from bovine erythrocyte membranes

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Abstract   A heptaglycosylceramide was isolated from bovine erythrocyte membranes. The structure was characterized to be Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-NAc(β1-4)Gal(β1-4)GlcCer. A hexaglycosylceramide that has the same sequence except for the terminal α-galactosyl unit has also been isolated. We have previously found that gangliosides isolated from bovine erythrocyte membranes contain a keratan sulfate type repeating unit $-[3Gal(\beta1-4)-GlcNAc\beta]-_n$. This study shows that the keratan sulfate type repeating unit is also present in the neutral glycosphingolipids of bovine erythrocyte membranes.—**Chien, J-L., S-C. Li, and Y-T. Li.** Isolation and characterization of a heptaglycosylceramide from bovine erythrocyte membranes. *J. Lipid Res.* 1979. **20:** 669–673.

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Due to the fact that erythrocytes are readily available in large quantities and that their plasma membranes can be easily prepared, erythrocyte membranes have been used as a model for the elucidation of the structure and function of cell membranes. In order to appreciate the structure and function of the cell membrane, it is imperative to elucidate fully the nature of its constituents. Since the discovery of globoside and hematoside from human erythrocytes by Yamakawa and Suzuki (1, 2), the glycosphingolipids in erythrocyte membranes have been the subject of great interest (3). Besides simple glycosphingolipids such as glucosylceramide, lactosylceramide, and trihexosylceramide, the glycosphingolipids of mammalian erythrocytes can be classified into two types: *a*) those of human, pig, guinea pig, and sheep which contain *N*-acetylgalactosamine (4, 5); and *b*) those of rabbit (6) and bovine (7, 8) which contain *N*-acetylglucosamine.

Compared with galactosamine-containing glycosphingolipids, glucosamine-containing glycosphingolipids have not been carefully studied. We have made an extensive study of the gangliosides (9) and neutral glycosphingolipids (10) of bovine erythrocyte membranes. The following neutral glycosphingolipids have been isolated and characterized from bovine erythrocytes (10–12): GlcCer, Gal(β1-4)GlcCer, Gal(α1-4)-Gal(β1-4)GlcCer, Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)-GlcCer, and Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcCer. In addition to these glycosphingolipids, we found that bovine erythrocyte membranes also contain neutral glycosphingolipids with a longer saccharide chain. This report describes the isolation and characterization of a heptaglycosylceramide from bovine erythrocyte membranes.

EXPERIMENTAL PROCEDURES

Materials

Fresh bovine blood was obtained from a slaughter house in Covington, LA. Fatty acid methyl esters, sphingosines, 10% DEGS PS on 80/100 Supelcoport, 3% OV-17 on 100/120 Gas Chrom A, 3% OV-275 on 100/120 chromosorb W, and 3% SE-30 on 80/100 Supelcoport were purchased from Supelco, Inc., Bellefonte, PA. Cellex-D and Bio-Sil A (200–325 mesh) were obtained from Bio-Rad Laboratories, Richmond, CA. Precoated silica-gel plates were the products of Brinkmann Instruments, Inc., Westbury, NY. Sephadex LH-20 was from Pharmacia Fine Chemicals, Piscataway, NJ. The following glycosphingolipids were isolated in this laboratory: glucosylceramide from human spleen, and lactosylceramide, trihexosylceramide, and globoside from human blood. Forssman hapten was a gift from Dr. John M. McKibbin of the University of Alabama. The exoglycosidases, α-galactosidase (13), β-galactosidase (14), and β-*N*-acetylhexosaminidase (15) were isolated as previously described.

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Isolation of glycosphingolipid from bovine erythrocyte membranes

Stroma from bovine erythrocytes was prepared from fresh blood by the toluene flotation method (16). The acetone-dried stroma (100 g) was suspended and homogenized in four volumes of 0.1 M KCl solution. To the homogenate, four volumes of tetrahydrofuran were added; the mixture was stirred at room temperature for 2 hr. After filtering through a sintered glass funnel, the residue was re-extracted two more times with tetrahydrofuran–0.1 M KCl 6:1. The combined filtrate was dried in a rotary evaporator under reduced pressure. The crude lipid extract was then treated with 0.6 N NaOH in methanol at 37°C for 5 hr and dialyzed. After drying in a rotary evaporator, the residue (1.4 g) was dissolved in chloroform–methanol 2:1 and applied to a DEAE-cellulose column (acetate form) previously equilibrated with chloroform–methanol 2:7. Neutral glycosphingolipids were eluted from the column with the same solvent. The eluate was dried (1.25 g) and applied to a Bio-Sil A column (1.9 × 30 cm) in chloroform. The column was washed with chloroform to remove neutral lipids. Glycosphingolipids were eluted with chloroform–methanol–water 125:42:6. Fractions of 6 ml were collected. Fractions containing hexa- and heptaglycosylceramides were pooled and further purified by preparative thin-layer chromatography. The yields of heptaglycosylceramide and hexaglycosylceramide were 1.1 mg and 0.4 mg, respectively.

Chemical analysis

Pure bovine heptaglycosylceramide was hydrolyzed according to the method of Yang and Hakomori (17). Neutral sugars were quantitated by an automated sugar analyzer (18) while hexosamine was determined by an amino acid analyzer. The sugar composition was also determined by gas–liquid chromatography as alditol acetate derivatives (19). Fatty acid methyl esters were obtained by hydrolyzing the glycolipid with 1.5 M anhydrous methanolic HCl at 80°C for 24 hr. After extracting three times with hexane, they were separated at 190°C on a 10% DEGS column. Sphingosines were converted to trimethylsilyl derivatives and analyzed on a 3% SE 30 column (20).

Sequential degradation with glycosidases

For the treatment of glycosphingolipids with various glycosidases, we followed our previously described procedures (21). After incubation, four volumes of chloroform–methanol 2:1 were added to the reaction mixture to extract glycolipids. After removing the lower layer, the upper layer was washed with four volumes of the theoretical lower layer to remove traces of the glycolipids that remained in the upper layer. Combined lower layers were then analyzed by thin-layer chromatography.

Determination of linkages between sugar residues

The glycosphingolipid (0.5 mg) was methylated with dimethylsulfinyl ion and methyl iodide (22). The reaction mixture was passed through a LH-20 column (1 × 20 cm). The combined glycolipid fractions were hydrolyzed, reduced, and acetylated according to the procedure of Yang and Hakomori (17). Neutral sugar derivatives were separated on a 3% OV-275 Supelcoport column at 180°C. Aminosugar derivatives were analyzed on a 3% OV-17 Supelcoport column with the initial temperature set at 150°C and programmed 2°/min to 200°C (17, 23). The peaks were further identified from their mass spectra which were obtained by using a Finnigan mass spectrometer, model 3300.

RESULTS AND DISCUSSION

The purified glycosphingolipid migrated well below Forssman hapten which was isolated from sheep erythrocytes (**Fig. 1**). This glycolipid contains 4 mol of galactose, 2 mol of N-acetylglucosamine, and 1 mol of glucose per mol of sphingosine. It can only be hydrolyzed by α-galactosidase and remains intact when incubated with other exoglycosidases. The glycolipid derived from the treatment with α-galactosidase migrated at the same rate as hexaglycosylceramide, which was also isolated from bovine erythrocytes. Further treatment of hexaglycosylceramide with β-galactosidase converted it into a pentaglycosylceramide which migrated very closely to Forssman hapten. Alternate incubations of this pentaglycosylceramide with β-N-acetylhexosaminidase and β-galactosidase produced lacto-N-neoteraosyl-, triglycosyl-, lactosyl-, and glucosylceramide (Fig. 1).

Fig. 2A shows the total ion chromatogram of partially methylated alditol acetates obtained from an OV-275 column. Since the sample was found to be contaminated with plasticizers and other unknown substances, the mass chromatogram of m/e 117 was derived from the total ion chromatogram as shown in Fig. 2B. The m/e 117 fragment has been shown to be a primary fragment for alditol acetates that contain acetyl group at C-1 and methyl group at C-2 (24). Thus all of the partially methylated alditol acetates derived from hexoses, except with substitution at C-2, should give this fragment. There are four major peaks that contain m/e 117; however only three peaks, indicated
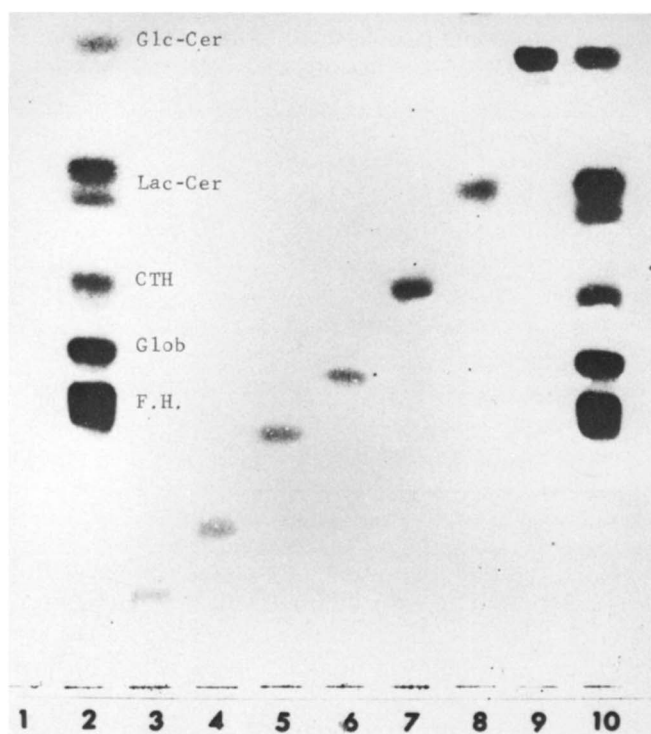
Fig. 1. Enzymatic hydrolysis of bovine stroma heptaglycosylceramide. Lane *1* is the control containing buffers and all of the enzymes used. Lanes *2* and *10* are the standards: from top, glucosylceramide (Glc-Cer), lactosylceramide (Lac-Cer), ceramide trihexoside (CTH), globoside (Glob), and Forssman hapten (F.H.). Lane *3* is heptaglycosylceramide; *4* is *3* + α-galactosidase; *5* is *4* + β-galactosidase; *6* is *5* + β-N-acetylhexosaminidase; *7* is *6* + β-galactosidase; *8* is *7* + β-N-acetylhexosaminidase; and *9* is *8* + β-galactosidase.

by the shading, are derived from carbohydrates as judged by their mass spectra. Fig. 2*C* is the mass spectrum of peak I which was identified to be that of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol. Fig. 2*D* is the mass spectrum of peak II which was identified to be that of 2,4,6,-tri-*O*-methyl-1,3,5,-tri-*O*-acetylgalactitol. Fig. 2*E* shows the mass spectrum of peak III which was identified to be that of 2,3,6,-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol. These identifications were made by comparing the mass spectra with known standards. It is evident that the 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol peak is quite small compared to that of 2,3,4,6-tri-*O*-methyl-1,5,-di-*O*-acetylgalactitol which was derived from the terminal galactose. It has been widely experienced that the sugar unit immediately next to the ceramide moiety is very resistant to the acid hydrolysis.

Fig. 3*A* shows the total ion chromatogram obtained from the OV-17 column. Again due to the contaminants, we obtained the mass chromatogram for *m/e* 117 (Fig. 3*B*) as well as *m/e* 158 (Fig. 3*C*). It has been shown that *m/e* 158 is a primary fragment for all derivatives
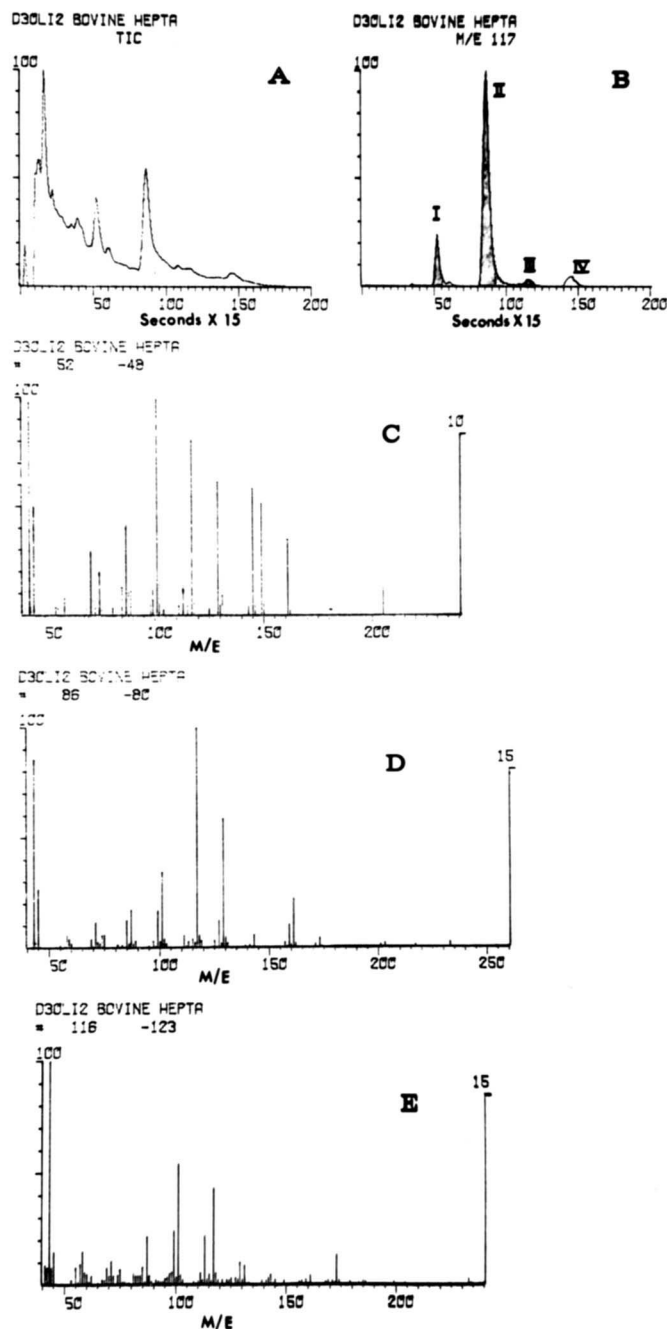

Fig. 2. Analysis of partially methylated alditol acetates by gas–liquid chromatography and mass spectrometry. *A*, Total ion chromatogram of partially methylated alditol acetates derived from the heptaglycosylceramide as analyzed on an OV-275 column; *B*, mass chromatogram of *m/e* 117 derived from *A*; *C*, mass spectrum of peak I; *D*, mass spectrum of peak II; *E*, mass spectrum of peak III. The numbers at the upper left-hand corner of *C*, *D*, and *E* indicate the position where the mass spectrum was taken.

of 2-deoxy-2-*N*-methylacetamidohexitol (25). As shown in Fig. 3*B*, the mass chromatogram of *m/e* 117 contains three major peaks (shaded) which correspond to 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol
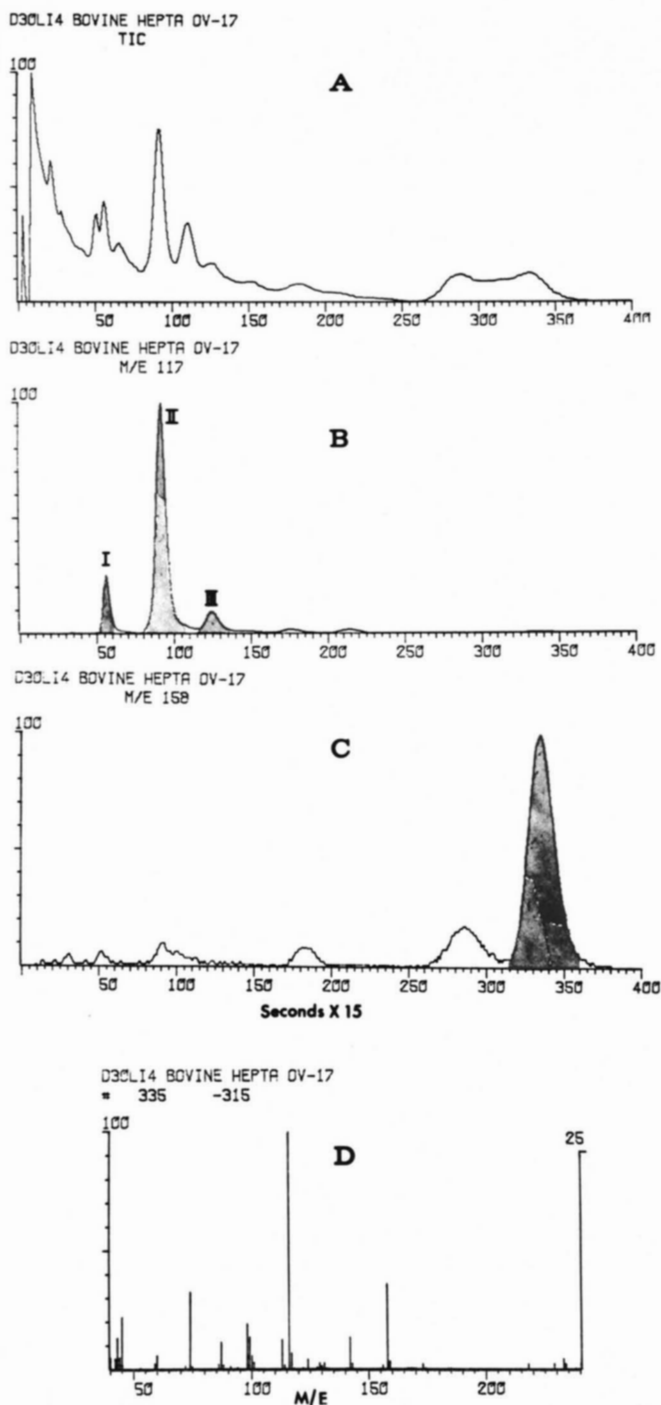
Fig. 3. Analysis of partially methylated 2-deoxy-2-N-methylacetamidohexitol derived from the hexaglycosylceramide by gas–liquid chromatography and mass spectrometry. *A*, Total ion chromatogram of partially methylated alditol acetates including *N*-acetamido sugars as separated by an OV-17 column; *B*, mass chromatogram of *m/e* 117 derived from *A*; *C*, mass chromatogram of *m/e* 158 derived from *A*; *D* mass spectrum of the peak containing *m/e* 158 (shaded). The numbers at the upper left-hand corner indicate the position where the mass spectrum was taken.

TABLE 1. Fatty acid and sphingosine composition of heptaglycosylceramide

	Content (%)
Fatty acid	
C16:0	7.3
C16:1	0.5
C18:0	3.8
C18:1	7.6
C20:0	0.5
C22:0	14.6
C23:0	5.8
C24:0	50.4
C24:1	9.5
Sphingosine	
dC18:0	10.0
dC18:1	89.5
tC18:0	0.5

(peak I), 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol (peak II), and 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol, respectively. The mass chromatogram of *m/e* 158 (Fig. 3*C*) contains one major peak (shaded). Fig. 3*D* shows the mass spectrum of this peak. By comparing with the known standard, it was identified to be that of 3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol.

These results, combined with those of enzymatic hydrolysis, establish the structure of this glycolipid to be Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc-(β1-3)Gal(β1-4)GlcCer. Fatty acids are mainly composed of docosanoate and tetracosanoate. The long chain base consists of only C_{18} (**Table 1**). The quantity of the hexaglycosylceramide isolated was not enough for complete characterization. It is of interest that Gal-(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)-GlcCer has been reported to possess blood group Ii activity (26).

Careful examination of the structure of the heptaglycosylceramide revealed that this glycolipid contains two repeating units of -3Gal(β1-4)GlcNAcβ-. It is probable that bovine erythrocyte membranes may contain glycosphingolipids with three or more such repeating units. However, this type of glycosphingolipids may be too hydrophilic to be extracted by tetrahydrofuran. It is intriguing to find the presence of the keratan sulfate type repeating unit, -3Gal(β1-4)Glc-NAcβ-, in the glycosphingolipids of bovine erythrocytes. So far, this keratan sulfate type repeating unit has been found only in the connective tissues such as cartilage and cornea. Biological significance for the presence of the keratan sulfate type repeating unit in the glycosphingolipids remains to be explained.∎

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