

Characterization of neutral sphingolipids from chicken erythrocytes

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Abstract The neutral sphingolipids from chicken erythrocytes were characterized. The total concentration of neutral sphingolipids was found to be 480 nmol/g of dry stroma. They were isolated and purified by droplet counter-current chromatography, Iatrobeads column chromatography, and preparative thin-layer chromatography. The major neutral sphingolipids were free ceramide, ceramide monohexoside, ceramide dihexoside, and ceramide pentahexoside, which represented 43%, 23.5%, 10.0%, and 3.6% of the long chain bases, respectively. Thus, free ceramide was the most abundant neutral sphingolipid in chicken erythrocytes. Ceramide monohexoside was composed of more galactosylceramide than glucosylceramide. Galabiosylceramide was found in the ceramide dihexoside fraction together with lactosylceramide. Ceramide pentahexoside was a Forssman glycolipid. There were two groups of neutral sphingolipids; one had mainly C₁₆ fatty acid and the other had C₂₂ and C₂₄ fatty acids. In both groups sphingosine (d18:1) was predominant as a long chain base. 2-Hydroxy-C₁₆ fatty acid was a major component of one of the ceramide monohexosides. —Shiraishi, T., and Y. Uda. Characterization of neutral sphingolipids from chicken erythrocytes. *J. Lipid Res.* 1985. 26: 860-866.

Supplementary key words droplet counter-current chromatography • free ceramides • glucosylceramide • galactosylceramide • lactosylceramide • galabiosylceramide • Forssman glycolipid • 2-hydroxy-palmitic acid • long chain bases

Since the isolation of hematoside from equine erythrocytes by Yamakawa and Suzuki (1), glycolipids of erythrocytes of various animals have been analyzed. These analyses revealed that the main glycosphingolipid was characteristic of the respective animal species (2). Such characterization has only been achieved in mammals, and the glycosphingolipids of erythrocytes in fowl have not been reported. In the present study, we describe the characterization of neutral glycosphingolipids from chicken erythrocytes. This report also shows the occurrence of free ceramide in chicken erythrocytes.

Progress in the isolation and structural analysis of glycosphingolipids has been facilitated by the development of chromatographic systems, including thin-layer, ion-exchange, and adsorption chromatographies. Recently, droplet counter-current chromatography has been introduced for separation of animal brain (3) and mouse

erythrocyte (4) gangliosides, and mammalian erythrocyte neutral glycosphingolipids (5). In this study, we employed the droplet counter-current chromatography for the separation and purification of chicken neutral sphingolipids.

EXPERIMENTAL

Materials

Chicken blood was kindly supplied by Toriune Co. Ltd., Niigata, Japan. DEAE-Sephadex A-25 and Iatrobeads(6RS-8060) were from Pharmacia Fine Chemicals, Uppsala, Sweden, and Iatron Laboratories, Tokyo, Japan, respectively. Precoated thin-layer plates (Silica gel 60, 0.25 mm thick) and pyridine-d₅ (d > 99%) for NMR spectrometry were purchased from E. Merck Co., Darmstadt, West Germany. Fig α -galactosidase was prepared according to the procedure reported previously by Li and Li (6). Jack bean β -galactosidase was purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan, and Forssman antiserum was from Difco Laboratories, Detroit, MI.

Extraction and purification of neutral sphingolipids

Fresh blood from decapitated chickens was immediately mixed with a one-tenth volume of saturated ammonium citrate solution. Erythrocytes were washed with saline and then centrifuged. The packed erythrocytes were lysed in 0.01 M Tris-HCl buffer, pH 7.2, containing 0.1 M KCl (7). The stroma was washed with 0.01 M Tris-HCl buffer, pH 7.2, containing 0.05 M KCl and water, followed by lyophilization. The total lipid was extracted from the dried stroma with 20 volumes each of the following mixtures: C-M 2:1, C-M 1:1, and C-M-W 60:35:8. The com-

Abbreviations: C-M-W, chloroform-methanol-water; DCC, droplet counter-current chromatography; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; TMS, trimethylsilyl; NMR, nuclear magnetic resonance; in the Tables, CMH, ceramide monohexoside; CDH, ceramide dihexoside; CPH, ceramide pentahexoside; LCB, long chain base.

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bined lipid extracts were evaporated to dryness and treated with 0.5 M sodium methoxide in C-M 1:1 for 24 hr at room temperature. After being neutralized and dialyzed against water, the content was dried in vacuo. Contaminating phospholipids were removed by peracetylation and chromatography on Florisil (8). The neutral glycosphingolipids and ceramides were obtained by DEAE-Sephadex A-25 (acetate form) column chromatography (9).

The purification of neutral sphingolipids as fractions showing one band on TLC was performed on appropriate combination of preparative TLC, Iatrobeds column chromatography, and DCC. The preparative TLC plates were developed with C-M-W 65:25:4, with visualization with iodine vapor. The columns packed with Iatrobeds(6RS-8060) were eluted with linear gradient systems of C-M or C-M-W mixtures as follows: C-M 98:2 and C-M 90:10 for ceramides; C-M-W 90:9:0.5 and C-M-W 75:25:1 for ceramide monohexosides; and C-M-W 75:23:2 and C-M-W 40:57:3 for ceramide pentahexoside. Ceramide dihexosides were purified by DCC under the same conditions as described below.

Separation of sphingolipids by DCC

The heavier layer of the solvent system of carbon tetrachloride-chloroform-methylene chloride-methanol-water-acetic acid 3:3:4:10:9:7 was used as a mobile phase. The sample was dissolved in a small volume of the lighter layer (stationary phase) at concentrations below 5% and applied to the first several columns through the sample chamber. The fractionation was carried out in the descending mode on a DC-300S-G2 (Tokyo Rikakikai, Tokyo, Japan) equipped with 300 glass columns (40 cm length, 2 mm inner diameter), and started immediately after the beginning of the elution of the mobile phase. The flow rate was controlled so that a continuous descending stream of droplets at intervals of about 1 cm was formed.

Quantitation of long chain bases

Long chain bases were assayed by the spectrophotometric method of Lauter and Trams (10).

Analytical methods

The sugar compositions of the isolated glycosphingolipids were quantitatively determined by GLC on 3% OV-225 on Chromosorb WAW as alditol acetates, which were prepared according to the method described by Yang and Hakomori (11). Methylation analysis was performed as below. The glycosphingolipids were methylated with a methyl sulfinyl-carbanion base and methyl iodide. The permethylated products were hydrolyzed, reduced, and then acetylated according to the method of Stellner, Saito, and Hakomori (12). The partially methylated alditol acetates were analyzed by GLC on 3% OV-225 on Chromosorb WAW. The fatty acids and long chain bases

were determined after hydrolysis in 1 N HCl in 82% aqueous methanol (13). The fatty acid fractions were treated with diazomethane to convert free carboxyl groups into methyl esters, which were separated into normal and 2-hydroxy fatty acid methyl esters on TLC plates developed with n-hexane-ether 85:15. Methyl nonadecanoate was added to the separated acids as an internal standard to determine the proportions of normal and 2-hydroxy fatty acid methyl esters. The 2-hydroxy fatty acid methyl esters were analyzed as their TMS derivatives. The composition of each acid was determined by GLC on two kinds of columns, 5% Shinchrom E-71 on Shimalite AW and 4% OV-1 on Chromosorb WAW. The long chain bases were converted to their TMS derivatives and to the corresponding aldehydes by oxidation with lead tetraacetate (14). The TMS derivatives were analyzed by GLC on 4% OV-1 on Chromosorb WAW, and the aldehydes on 5% Shinchrom E-71 on Shimalite AW.

The mass spectra of ceramides as their TMS derivatives were measured with a Hitachi RMU-7M spectrometer at an ionizing potential of 20 eV, an accelerating voltage of 3 KV and an ion source temperature of 290°C. Two hundred MHz proton NMR analyses of the anomeric structures were performed with a JEOL FX200 spectrometer in pyridine-d₅ solution at 80°C.

Enzyme treatment

The carbohydrate structures of ceramide dihexosides were also analyzed by α - and β -galactosidase hydrolysis. The incubation mixture contained the following components in 200 μ l of 50 mM sodium citrate buffer (pH 4.0): ceramide dihexoside, 25 μ g; sodium taurocholate, 400 μ g; and 0.18 units of fig α -galactosidase or 0.21 units of jack bean β -galactosidase. After incubation at 37°C for 20 hr the products were analyzed on borate-impregnated TLC plates (15) developed with C-M-W 65:25:4 and visualized with orcinol reagent.

Immunological identification

Forssman hapten activity of ceramide pentahexosides was investigated by a double immunodiffusion method using commercial Forssman antiserum. The glycolipids were dissolved in saline and placed in the wells of an agar plate (pH 7.2). After the plate had been allowed to stand at room temperature for 5 hr, the precipitin lines were observed.

RESULTS

Isolation of neutral sphingolipids

The total concentration of neutral sphingolipids in chicken stroma was 480 nmol/g of dry stroma, which was determined as long chain bases. The compositions of the

neutral sphingolipids were relatively simple, as shown in **Fig. 1** (A) lane 1 and (B) lane 1. The neutral sphingolipid were composed of three main orcinol-positive components: ceramide monohexoside, ceramide dihexoside, and ceramide pentahexoside. In addition, they contained an orcinol-negative component, ceramide. The percentage distribution of these components was 43.0% ceramide, 23.5% ceramide monohexoside, 10.0% ceramide dihexoside, and 3.6% ceramide pentahexoside.

The total neutral sphingolipid fraction was subjected to DCC under the conditions described in the Experimental section. **Fig. 2** shows the TLC pattern of each fraction on this chromatography. Ceramide (not visible), ceramide monohexoside, and ceramide dihexoside were eluted in fractions 2 to 16. But the two components of ceramide dihexoside, which gave two bands on TLC, were clearly separated from each other by DCC; one was eluted in fractions 2 to 8 and the other in fractions 10 to 16. Thus the DCC was used for the final purification of ceramide dihexoside, and N2-a and N2-b were obtained. The ceramide pentahexoside was broadly eluted and separated into three fractions (102 to 120, 140 to 190, and 210 to 240). In a double immunodiffusion test, as described later, the lipids from these fractions gave single precipitin lines, which fused with the line of Forssman glycolipid.

After the DCC separation, the purified neutral sphingolipids (N0-a, N0-b, N0-c, N1-a, N1-b, N1-c, N2-a, N2-b,

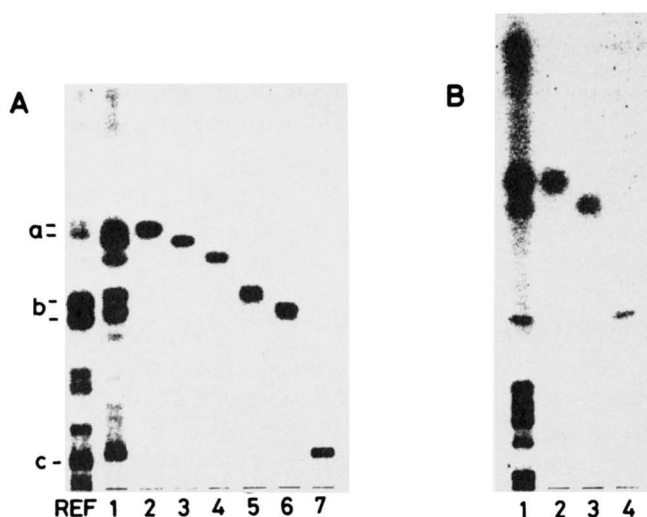


Fig. 1. TLC of neutral glycosphingolipids (A) and free ceramides (B) isolated from chicken erythrocytes. A: The reference neutral sphingolipids (REF) were obtained from bovine erythrocytes (16); a, glucosyl- and galactosylceramide; b, lactosylceramide; and c, IV³- α -galactosyl-neolactotetraosylceramide. Lane 1, total neutral sphingolipids from chicken erythrocytes; lane 2, N1-a; lane 3, N1-b; lane 4, N1-c; lane 5, N2-a; lane 6, N2-b; lane 7, N5. The plate was developed with C-M-W 65:25:4, with visualization with orcinol reagent. B: Lane 1, total neutral sphingolipids from chicken erythrocytes; lane 2, N0-a; lane 3, N0-b; lane 4, N0-c. The developing solvent was C-M 90:10 and spots were visualized with 40% sulfuric acid.

and N5) were obtained by means of thin-layer, Iatrobeds column, and droplet counter-current chromatographies (**Fig. 1** (A) lanes 2 to 7 and (B) lanes 2 to 4).

Structures of neutral sphingolipids

The structures of the purified neutral sphingolipids were elucidated on the basis of the sugar composition, analysis of partially methylated aldohexitol acetates, fatty acid and long chain base compositions, proton NMR spectrometry, electron-impact mass spectrometry (for ceramide), enzyme treatment (for ceramide dihexoside), and an immunological method (for ceramide pentahexoside).

Ceramide. N0-a and N0-b contained no sugar moiety. The predominant long chain base in N0-a and N0-b was sphingosine (d18:1) and dihydrosphingosine (18:0) was a minor component (**Table 1**). The major fatty acids were behenic and lignoceric acids in N0-a, and palmitic acid in N0-b. Thus, it was proved that the main molecular species of N0-a were N-behenoyl-sphingosine and N-lignoceroyl-sphingosine; and the main species N0-b was N-palmitoyl-sphingosine. This was supported by the results of mass spectral fragmentation of their TMS derivatives. In the spectrum of TMS-N0-a, three distinct pairs of "molecular weight fragments" (17, 18) were observed at m/z 778 (M-15), 750 (M-15), 703 (M-90), 675 (M-90), 600 (M-90-103) and 572 (M-90-103). This indicated that TMS-N0-a was largely composed of two main species, which had molecular weights of 793 and 765, respectively. The "fatty acid fragment ions" appeared at m/z 555 (M-311 + 73), 527 (M-311 + 73), 482 (M-311) and 454 (M-311). The nature of the constituent long chain bases was also indicated by the ions at 426 (M-366-1 and M-338-1), 336 (M-366-1-90 and M-338-1-90) and 311 (M-482 and M-454) to be mainly sphingosine. The fragment ions from TMS-N0-b were observed at m/z 666 (M-15), 591 (M-90), 488 (M-90-103), 443 (M-311 + 73), 370 (M-311), 426 (M-254-1), 336 (M-254-1-90) and 311 (M-370), and they could be explained in the same way. These mass-spectrometric observations were consistent with the results of chemical analyses of N0-a and N0-b. On the other hand, the structure of N0-c remained obscure, because its mass spectrum showed no distinct ions of molecular weight fragments and fatty acid fragments for the ceramide structure.

Ceramide monohexoside. Each glycolipid, N1-a, N1-b, and N1-c, contained both galactose and glucose in the molar ratios of 2.81:1.00, 12.5:1.00, and 7.00:1.00, respectively (**Table 2**). On permethylation analysis, ceramide monohexosides N1-a, N1-b, and N1-c gave, respectively, two peaks on GLC, which co-chromatographed with the acetates of 2,3,4,6-tetra-O-methylgalactitol and 2,3,4,6-tetra-O-methylglucitol. The proton NMR spectrum of N1-a showed two doublets at 4.75 ppm ($J = 7.6$ Hz) and

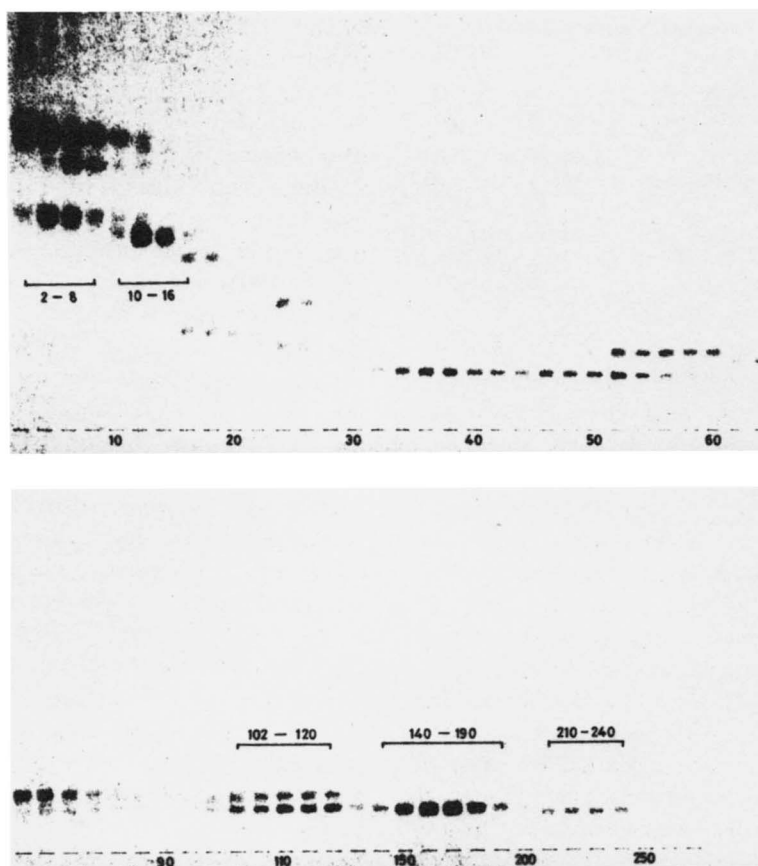


Fig. 2. TLC of the fractions from droplet counter-current chromatography. Separation of sphingolipids by DCC was carried out with the following solvent system, carbon tetrachloride-chloroform-methylene chloride-methanol-water-acetic acid 3:3:4:10:9:7, in the descending mode with 300 glass columns (40 cm length, 2 mm inner diameter). Fractions of 4 g (1 to 10) and 9 g (11 to end) were pooled and analyzed by TLC with development with C-M-W 65:25:4. The lipids were visualized with orcinol reagent.

4.83 ppm ($J = 7.6$ Hz), which were ascribed to the anomeric protons of β -galactose and β -glucose, respectively. The proton NMR spectra of N1-b and N1-c showed doublets at 4.75 ppm ($J = 7.6$ Hz) and 4.73 ppm ($J = 7.6$ Hz), respectively, which indicated the presence of a β -proton of galactose. But distinct anomeric signals due to β -glucose were not seen in these spectra. This was con-

sistent with very low glucose content values of N1-b and N1-c. Consequently, N1-a, N1-b, and N1-c were deduced to be composed of galactosylceramide and glucosylceramide.

Ceramide dihexoside. The sugar contents of N2-a and N2-b were made up to galactose and glucose in the molar ratios of 2.82:1.00 and 3.50:1.00, respectively, as shown in

TABLE 1. Long chain base and fatty acid compositions^a of neutral sphingolipids isolated from chicken erythrocytes

		Ceramide			CMH			CDH		CPH
		N0-a	N0-b	N0-c	N1-a	N1-b	N1-c	N2-a	N2-b	N5
LCB	d18:1	97.0	94.9	99.8	95.2	96.8	93.0	99.1	98.5	99.2
	d18:0	3.0	5.1	0.2	4.8	3.2	7.0	0.9	1.5	0.8
Fatty acid	16:0	2.6	82.5	53.8	13.3	81.5	6.8	5.1	94.1	91.1
	18:0	3.3	8.7	46.2	6.0	8.5	4.9	2.5	4.8	7.1
	20:0				4.4	3.0		1.4	0.2	0.9
	22:0	35.4	5.9		37.9	5.0	0.6	25.4	0.9	0.9
	23:0	10.5			1.5			1.9		
	24:0	48.2	2.9		36.9	2.0		63.7		
	16h:0 ^b									87.7

^aMolar percentages.

^bh, 2-Hydroxy acid.

TABLE 2. Sugar compositions of neutral glycosphingolipids isolated from chicken erythrocytes^a

Sugar	CMH			CDH		CPH
	N1-a	N1-b	N1-c	N2-a	N2-b	N5
Glucose	1.00	1.00	1.00	1.00	1.00	1.00
Galactose	2.81	12.50	7.00	2.82	3.50	1.90
N-Acetylgalactosamine						1.79

^aMolar ratios.

Table 2. On permethylation analysis of N2-a and N2-b, the three acetates of 2,3,6-tri-O-methylgalactitol, 2,3,6-tri-O-methylglucitol, and 2,3,4,6-tetra-O-methylgalactitol were identified on GLC. The proton NMR spectra of N2-a and N2-b showed, respectively, four doublets at 4.73 ppm ($J = 7.6$ Hz), 4.77 ppm ($J = 7.6$ Hz), 4.91 ppm ($J = 8.1$ Hz), and 5.57 ppm ($J = 3.9$ Hz), and at 4.74 ppm ($J = 7.3$ Hz), 4.78 ppm ($J = 7.9$ Hz), 4.92 ppm ($J = 7.8$ Hz), and 5.57 ppm ($J = 3.9$ Hz), as shown in Fig. 3. These spectral data for anomeric protons indicated that there were four kinds of glycosidic linkages in each glycolipid fraction, three of which were β and one, α . The above results of chemical analysis and NMR measurement of ceramide dihexoside suggested the presence of ceramide dihexosides having a lactosyl moiety and other disaccharides, like the galactosyl(α 1-4)galactosyl(β 1-) moiety (19). This was well confirmed by the results of enzyme treatment (20) as shown in Fig. 4. Fig α -galactosidase acted on N2-a to yield galactosylceramide, whereas jack bean β -galactosidase treatment gave glucosylceramide (Fig. 4 A). In the case of N2-b similar results were obtained (Fig. 4 B).

Thus, it was proved that both N2-a and N2-b were composed of Gal(β 1-4)Glc (β 1-1)Cer and Gal(α 1-4)Gal(β 1-1)Cer.

Ceramide pentahexoside. As mentioned before, one of the three fractions of ceramide pentahexoside was purified from pooled fractions 140 to 190 after DCC (Fig. 2). Analysis of the sugar composition showed that the purified ceramide pentahexoside N5 contained glucose, galactose, and N-acetylgalactosamine in a ratio of 1:2:2 (Table 2). In addition, permethylation analysis of N5 gave the same GLC pattern as that of dog intestinal Forssman glycolipid (21). Proton NMR spectrum measurement showed the presence of three β -anomeric protons at 4.78 ppm ($J = 7.6$ Hz), 4.94 ppm ($J = 7.6$ Hz), and 5.03 ppm ($J = 8.1$ Hz), and two α -anomeric protons at 5.47 ppm ($J = 4.2$ Hz) and 5.54 ppm ($J = 3.9$ Hz). Moreover, N5 reacted with Forssman antiserum to form a straight precipitin line, which fused in symmetrical arcs with that of dog intestinal Forssman glycolipid (Fig. 5). Therefore, N5 was provided to be a Forssman glycolipid. The amounts of the two other fractions (102 to 120 and 210 to 240), whose R_f values were slightly different from that of N5, were too small for analysis. However, they also reacted with Forssman antiserum as shown in Fig. 5. This suggested that these fractions contained Forssman glycolipid with ceramide structures different from that of N5.

DISCUSSION

The concentrations (nmol/g of dry stroma) of the neutral sphingolipids were as follows: free ceramide (N0-c not included), 206; ceramide monohexoside, 113; cera-

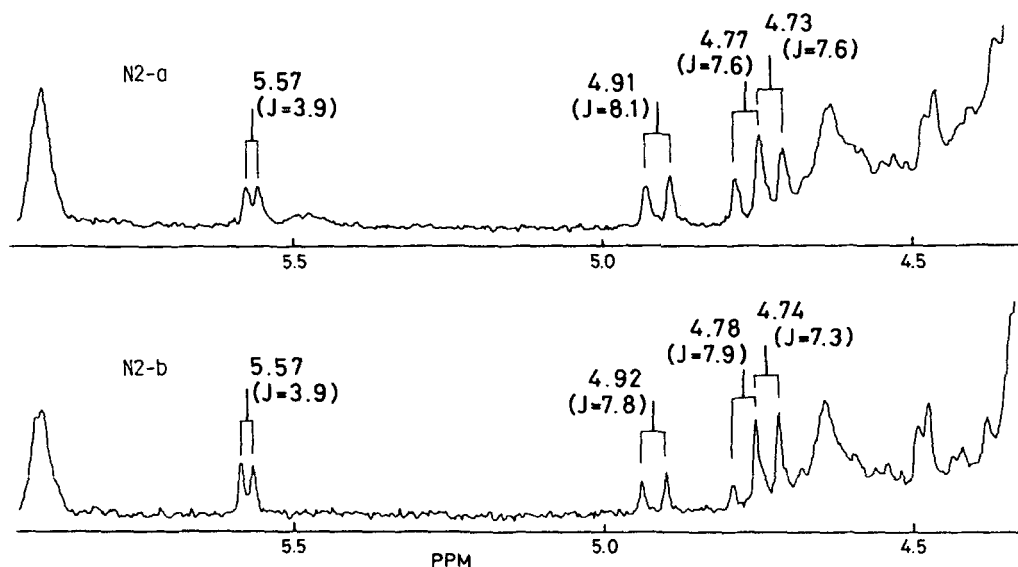


Fig. 3. Two hundred MHz proton NMR spectra of ceramide dihexosides. The samples were dissolved in 0.45 ml of pyridine- d_5 , and the spectra were recorded at 80°C (N2-a: 1.5 mg, 200 pulses; N2-b: 1.0 mg, 150 pulses).

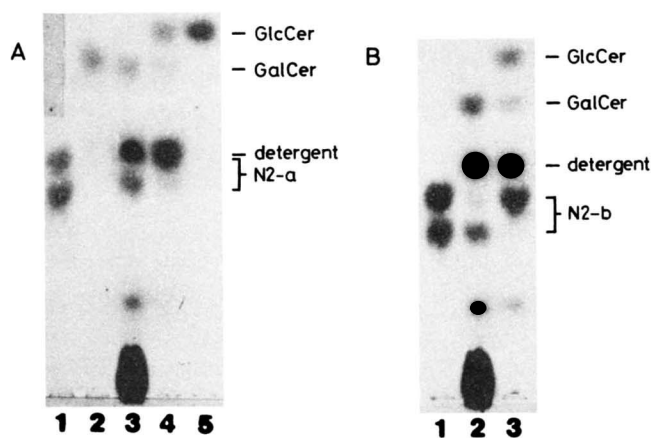


Fig. 4. Enzyme treatment of ceramide dihexoside N2-a (A) and N2-b (B). Each lipid (25 μ g) was treated with fig α -galactosidase (0.18 units) and jack bean β -galactosidase (0.21 units) in 200 μ l of 50 mM sodium citrate buffer (pH 4.0) containing 400 μ g of sodium taurocholate at 37°C for 20 hr. The enzyme digests were analyzed on borate-impregnated TLC plates, which were developed with C-M-W 65:25:4; visualization was with orcinol reagent. A: Lane 1, N2-a; lane 2, galactosylceramide; lane 3, the products of N2-a after treatment with fig α -galactosidase; lane 4, the products of N2-a after treatment with jack bean β -galactosidase; lane 5, glucosylceramide. B: Lane 1, N2-b; lane 2, the products of N2-b after fig α -galactosidase treatment; lane 3, the products of N2-b after jack bean β -galactosidase treatment.

amide dihexoside, 48.0; and ceramide pentahexoside, 17.3. Thus, free ceramide was the most abundant neutral sphingolipid in chicken erythrocytes. Recently, free ceramide has been demonstrated to be a component of the human erythrocyte membrane, and the second sphingolipid in concentration after globoside (22).

DCC had already been used for the separation of gangliosides by Otsuka and Yamakawa (3, 4), and neutral glycosphingolipids by Otsuka, Suzuki, and Yamakawa (5). In the present study, we also applied the DCC to the separation of chicken erythrocyte neutral sphingolipids with a solvent system different from that described by Otsuka et al. (5). These conditions were not necessarily sufficient to separate simultaneously the total neutral sphingolipid into individual component lipids. However, ceramide dihexoside was distinctly separated into two portions, N2-a and N2-b. C_{22} and C_{24} fatty acids were mainly found in N2-a, in contrast to C_{16} fatty acid for N2-b. It can be seen that the separation of these lipids by DCC depended on a difference in the fatty acid composition. Moreover, according to the results of an immunodiffusion test, the ceramide pentahexoside fraction appeared to be separated into three portions on DCC. A similar finding had been reported for the separation of globoside I (5).

Thus far, two kinds of ceramide dihexosides have been demonstrated. Lactosylceramide is widely distributed in various organs and tissues, and galabiosylceramide has been found in human kidney (23) and neutrophils (24), and kidney of various strains of mice (25). The latter

glycolipid accumulates in the kidneys of patients with Fabry's disease (26), but it has never been reported in the erythrocytes of animals. Our study demonstrated that galabiosylceramide was present in chicken erythrocyte ceramide dihexosides N2-a and N2-b together with lactosylceramide. The molar ratios of lactosylceramide and galabiosylceramide were determined on the basis of the sugar composition values to be as follows; 1.0:0.9 for N2-a and 1.0:1.25 for N2-b.

Some Forssman-positive animals such as the guinea pig do not express Forssman antigen in their erythrocytes, but it is contained in other tissues or organs (27). It had been reported that the chicken, which is also Forssman-positive, possesses Forssman glycolipid in the small intestine (28) and muscle (29). In addition, the present report shows that the same glycolipid is present in erythrocytes as well.

2-Hydroxy- C_{22} or C_{24} fatty acids are usual components of glycolipids in brain, kidney, and intestine, whereas 2-hydroxy- C_{16} fatty acid is minor (30). In chicken erythrocytes, the 2-hydroxy fatty acid found in N1-c was exclusively a C_{16} species. This resembles the case of ganglio-N-triaosylceramide of murine lymphoma cells (31).

The individual neutral sphingolipids, namely, free ceramide N0-b, ceramide monohexoside N1-b, ceramide dihexoside N2-b, and ceramide pentahexoside N5, had similar ceramide structures (Table 1), which were chiefly composed of sphingosine and palmitic acid. This suggests a biosynthetic correlation among them. Also, free ceramide N0-b and ceramide monohexosides N1-b and N1-c were quite alike in ceramide structure except for the 2-hydroxy group of the fatty acid residue of N1-c. These findings made us suspect direct 2-hydroxylation of the

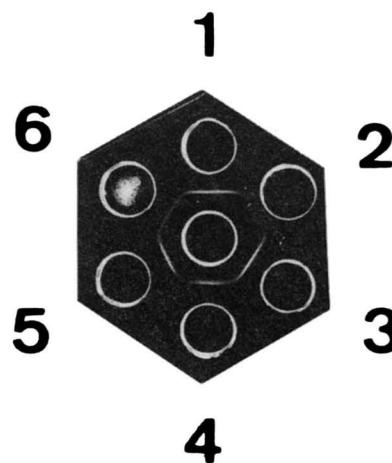


Fig. 5. Double immunodiffusion test on the ceramide pentahexoside fraction from DCC. Glycolipids were dissolved in saline and put in the wells of an agar plate (pH 7.2); 1, 3, and 5, dog intestinal Forssman glycolipid; 2, fractions 102 to 120; 4, N5 purified from fractions 140 to 190; 6, fractions 210 to 240. Forssman antiserum was placed in the center well. After incubation at room temperature for 5 hr, the precipitin lines were observed.

fatty acid residue on N0-b or N1-b. Quite recently, supportive evidence for the direct hydroxylation has been reported (32). On the other hand, Kishimoto (33) explained that the hydroxy group was introduced into the 2-position of the fatty acid residue prior to the formation of ceramide. The introduction of a 2-hydroxy group into a fatty acid residue is therefore of interest. ■

This work was supported in part by the Japan Association of Chemistry.

Manuscript received 26 December 1984.

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