Structures of ceramide tetrasaccharides from various sources: uniqueness of rat kidney ceramide tetrasaccharide

Bader Siddiqui, Jun'ichi Kawanami, Yu-Teh Li, and Sen-itiroh Hakomori*

Departments of Pathobiology and Microbiology, University of Washington, Seattle, Washington 98195; Shionogi Research Institute, Shionogi Pharmaceutical Co., Ltd., Fukushima-ku, Osaka, Japan; and Department of Biochemistry, Tulane University, Delta Regional Primate Research Center, Covington, Louisiana 70433

Abstract Methylation analysis of ceramide tetrasaccharide isolated from human erythrocytes gave acetates of 2,3,6-tri-*O*-methylgalactitol and 2,4,6-tri-*O*-methylgalactitol in a ratio of 1:1, and about 50% of the galactose was oxidized by periodate. Rat kidney ceramide tetrasaccharide gave, in contrast, a much larger proportion of the acetates of 2,4,6-tri-*O*-methylgalactitol (ratio 1:0.3), and less than 20% of the galactose was oxidized by periodate. Sequential degradation by β-*N*-acetylhexosaminidase, α-galactosidase, and β-galactosidase showed ceramide tetrasaccharides to have identical carbohydrate sequences and anomeric structures. The major part of ceramide trihexoside derived from rat kidney ceramide tetrasaccharide migrated on thin-layer chromatography more slowly than that derived from other ceramide tetrasaccharides. The structure of a major part of rat kidney ceramide tetrasaccharide was thus determined to be GalNAcβ(1→3)Galα(1→3)Galβ(1→4)Glcβ-(1→1)Cer, whereas other ceramide tetrasaccharides have Galα(1→4) structure at the penultimate residue.

Supplementary key words human erythrocytes · monkey kidney · horse spleen · bovine kidney · rat kidney · methylation · periodate · glycosidases · Galβ(1→3)Gal · Galα(1→4)Gal

CERAMIDE TETRASACCHARIDES (Cer-Tets) have been isolated from human erythrocytes (1–3), human kidney (4, 5), pig erythrocytes (6, 7), rat lymphosarcoma (8), rat kidney (9), and mouse kidney (10). The carbohydrate compositions of these lipids are identical, having 2 moles of galactose, 1 mole of *N*-acetylgalactosamine, and 1 mole of glucose per ceramide. The glycolipids isolated from human erythrocytes and pig erythrocytes were termed "globosides" (2–4), whereas those of human kidney and rat lymphosarcoma were isolated as cellular lipid hapten and termed "cytolipin K" (5) and "cytolipin R" (8), respectively. The structure of Cer-Tets of human erythrocytes and kidney has been established as GalNAcβ(1→3)Galα(1→4)Galβ(1→4)Glcα(1→1)Cer (3, 4, 11). However, a possible alternative structure (anomeric or positional isomers) has been predicted because the immunological reactivity of a Cer-Tet isolated from rat lymphosarcoma (cytolipin R) was different from that of human kidney Cer-Tet (cytolipin K) (5, 8) and because the nuclear magnetic resonance (NMR) spectrum of a tetrasaccharide obtained from rat kidney Cer-Tet was clearly distinguishable from a tetrasaccharide derived from Cer-Tets of porcine spleen and human erythrocytes (12). Furthermore, it was suggested that a galactosyl 1→3 or 1→2 linkage might be present in cytolipin R in contrast to the presence of a galactosyl 1→4 linkage in human Cer-Tet as judged by mass spectrometry of whole glycolipids.[1]

Abbreviations: C–M, chloroform–methanol; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry or mass spectra; Cer-Tet, ceramide tetrasaccharide having a carbohydrate sequence and composition as GalNAc→Gal→Gal→Glc→Cer (this is the same as "globoside I" of Yamakawa et al. [3]); Cer, ceramide; Cer-Tri, ceramide trihexoside (Gal→Gal→Glc→Cer).

* To whom reprint requests should be addressed (Department of Pathobiology, R.D. 98, University of Washington, Seattle, Wash. 98195).

[1] Observation by C. C. Sweeley presented at the 13th International Conference on the Biochemistry of Lipids in Athens, September 1969; quoted in the symposium lecture by John Wherrett at the Symposium of Sphingolipids, American Chemical Society Meeting, September 1970.

In this paper we wish to report the results of structural analysis of Cer-Tets isolated from two different animal organs and of a Cer-Tet derived from Forssman hapten glycolipid (13).

Only rat kidney Cer-Tet contained galactosyl $\alpha(1\rightarrow3)$ linkage in contrast to other Cer-Tets which contained galactosyl $\alpha(1\rightarrow4)$ linkage at the penultimate residue. The sequences of carbohydrates and the anomeric configurations of Cer-Tets so far analyzed were identical.

MATERIALS AND METHODS

Preparations of ceramide tetrasaccharides

Cer-Tets of human erythrocyte stroma (1–3) were prepared as follows. One part of wet tissue was homogenized with 10 vol of absolute ethanol in a Waring Blendor for 2 min, and the homogenate was then heated in a water bath at 80°C. The homogenate was kept at the boiling point for a few minutes and then was filtered on a Büchner funnel while hot; this filtrate was placed in an Erlenmeyer flask at −10°C. After the precipitate had settled, most of the supernatant fluid was aspirated off, and the precipitate was collected in a centrifuge bottle by centrifugation at −10°C. The precipitate was then stirred with 20–30 vol of acetone, centrifuged, and dried in a desiccator. The dry precipitate was stirred in 20–30 vol of diethyl ether (v/w) at room temperature for 30 min, and the ether was removed by centrifugation. The precipitate was extracted three times with diethyl ether in the same way. The ether-insoluble precipitate was extracted with C–M 2:1, and insoluble particles were filtered off. The filtrate was evaporated in a rotary evaporator, and the residue was chromatographed on Bio-Sil A (Bio-Rad Laboratories, Richmond, Calif.). The solvent systems have been described previously (14). Cer-Tet was eluted from the Bio-Sil A column with C–M (75:25–70:30). The preparations were further purified by preparative TLC on 0.5-mm silica gel H layers.

Cer-Tet of rat kidney was prepared from kidney of the Wistar-Sio strain rat (maintained in Shionogi Research Laboratory, Osaka, Japan) according to the method described earlier (9). This method includes saponification of ester lipids and successive chromatographic separations on silicic acid, magnesia–silica gel (Florisil, Floridin Co., Tallahassee, Fla.), and DEAE-cellulose (9). Cer-Tet was also obtained from Forssman antigen by hydrolysis with α-*N*-acetylgalactosaminidase as described earlier (13).

Properties of ceramide tetrasaccharides

Elemental analysis. Preparations from human erythrocytes and rat kidney showed the following carbon, hydrogen, and nitrogen values: C, 59.2; H, 9.49; N, 2.01 for human erythrocytes; and C, 52.24; H, 8.72; N, 1.84 for rat kidney globoside. Calculated for *N*-lignocerylsphingosine-*O*-trihexoside-mono-*N*-acetylhexosaminide, $C_{63}H_{126}N_2O_{23}$ (mol wt 1339.71): C, 60.96; H, 9.48; N, 2.09.

Carbohydrate composition. Carbohydrate composition was analyzed by GLC according to the method described by Yang and Hakomori (15). Ratio of Glc:Gal:$GalNH_2$ was 1:2.1:0.95 for human erythrocyte Cer-Tet and 1:2.2:0.98 for rat kidney Cer-Tet. Hexose values (by anthrone–H_2SO_4) of human erythrocyte Cer-Tet and rat kidney Cer-Tet were 42% and 43%, respectively (as galactose, theoretical value 40.3%).

Thin-layer chromatography. All these preparations were homogeneous on TLC in a silica gel H plate (Merck, distributed by Brinkmann Instruments, Westbury, N.Y.) developed with C–M–water 65:30:8 (lower layer), C–M–water 60:35:8, and C–M–2.5 M ammonia 60:30:8. Chromatograms were revealed with 0.2% orcinol sulfuric acid, iodine vapor, and Tzintzadze's spray (16). Contamination of these preparations with other glycolipids and phospholipids would be less than 5%, if at all, and 50 μg of globosides chromatographed on plates failed to reveal additional spots, aside from globoside, with the above-mentioned reagents.

Optical rotations. The preparation from human erythrocytes was twice precipitated from methanol (globular crystalline materials) and had an optical rotation of $[\alpha]_D^{20} = +19.5$ ($c = 0.50$). Rat kidney Cer-Tet was precipitated by acetone and had $[\alpha]_D^{22} = +21.2 \pm 0.9$ ($c = 0.445$).

Infrared spectra. The preparations were characterized by the presence of three major absorptions at λ_{max}: 1650 cm^{-1} (C:O vibration of —CONH—), 1550 cm^{-1} (N—H bending), and 1450 cm^{-1} (—CH— and CH_2— vibration and deformations). The samples were essentially devoid of infrared absorption at wave number 1750 cm^{-1} (C:O vibration of —COO—). The spectra that were obtained were typical of pure sphingolipids and excluded the possibility of contamination from ester-containing lipids such as glycerophospholipids, cholesteryl esters, etc.

Methylation analysis

The glycolipids were methylated in dimethylsulfoxide–sodium hydride–methyliodide (17) until hydroxyl absorption in the infrared spectra disappeared. The permethylated glycolipids were isolated by successive partition with water and chloroform and were subjected to formolysis. 1 mg of the permethylated product was dissolved in 0.5 ml of 90% formic acid and heated at 100°C for 3 hr. Formic acid was evaporated in a vacuum evaporator, and the residue was hydrolyzed in 0.5 ml of 0.3 N H_2SO_4 at 80°C for 18 hr. The hydrolysate was treated with 300 mg of Dowex 1-X8 (acetate form). The

filtrate from Dowex 1 was reduced with 10 mg of sodium borohydride at room temperature for 4 hr. The reaction mixture was passed through a small column of Dowex 50 (H^+ form). The filtrate and washings were evaporated with methanol in order to eliminate boric acid as methyl-borate. The residue was acetylated with 0.3 ml of pyridine and 0.2 ml of acetic anhydride, and these solvents were evaporated with toluene. The partially methylated alditol acetates were separated on a 6-ft glass column that contained 3% ECNSS-M on Gas-Chrom Q (Applied Science Laboratories, State College, Pa.).

The GLC–MS pattern was measured by a Finnigan 3000-GC peak identifier and compared with the pattern of standard peaks: acetates of 2,3,4,6-tetra-*O*-methyl-galactose and 2,4,6-tri-*O*-methylglucose (obtained from the permethylated lactosylceramide) and acetates of 2,3,6-tri-*O*-methylgalactose and 2,3,6-tri-*O*-methyl-glucose (obtained from the permethylated hematoside).

In addition, GLC–MS analyses were carried out on partially methylated alditol acetates: 2,3,4,6-tetra-*O*-methylglucitol, 2,3,4,6-tetra-*O*-methylgalactitol, 2,3,6-tri-*O*-methylgalactitol, 2,3,6-tri-*O*-methylglucitol, 2,4,6-tri-*O*-methylgalactitol, and 3,4,6-tri-*O*-methylgalactitol. These compounds were obtained as follows. Methyl-α-glucoside and methyl-α-galactoside were, respectively, incompletely methylated by the classical Purdie method (18) in methyliodide and silver oxide for 5, 24, and 48 hr. Aliquots of reaction mixtures were filtered from silver oxide and evaporated to dryness; the residues were subjected to formolysis and then hydrolyzed in 0.3 N H_2SO_4 for 18 hr. The hydrolysates were reduced and acetylated for preparation of alditol acetates as described above. The identification of each peak was based on the relative retention time in GLC and on the MS patterns (19, 20). Although the retention values of partially methylated alditol acetates were not identical with those reported (19), the order of elution was identical.

Periodate oxidation

Periodate oxidation was carried out in 0.1 M sodium metaperiodate in aqueous C–M solution for 120 hr according to the modified conditions described by Ledeen and Salsman (21). 1 μmole of Cer-Tet was dissolved in 1 ml of C–M 1:5, and 0.2 ml of aqueous solution of 0.6 M sodium metaperiodate was added. The mixture was kept in the dark at room temperature for 120 hr. The excess periodate was reduced by glycerol, and the oxidized Cer-Tet was removed from the reaction mixture by shaking with 10 ml of C–M 2:1 and 2 ml of water. The upper layer was discarded and the lower layer was shaken with C–M–water 1:10:10 three times. The carbohydrate composition of intact Cer-Tet and the oxidized Cer-Tet was determined by GLC. Mannitol acetate was used as the internal standard.

Enzymatic hydrolysis

β-*N*-Acetylhexosaminidase (β-2-acetamido-2-deoxy-D-hexoside acetamidodeoxyhexose hydrolase, EC 3.2.1.30) (22) and β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) (23) were prepared from jack bean meal; α-*N*-acetylgalactosaminidase was prepared from hog liver (24); and β-glucosidase was prepared from ox brain according to the method of Gatt and Rapport (25). The enzymatic hydrolysis and stepwise degradation of glycolipids were carried out according to the method previously described (11). Glycolipids were dissolved in 100 μl of 0.05 M sodium citrate buffer containing 100 μg of sodium taurocholate in small conical test tubes. The glycolipids readily dissolved with warming, agitation on a Vortex mixer (Scientific Products, Evanston, Ill.), and finally, immersion of the test tubes in an ultrasonic cleaning bath (Balsonic, Bausch and Lomb, supplied by Van Waters, Seattle, Wash.). The pH of the citrate buffer was 4.5 for α-galactosidase, 4.0 for β-galactosidase, and 5.0 for β-*N*-acetylhexosaminidase. The enzyme was then added to the solution, which was incubated at 37°C for 18 hr. The activities of the enzymes used were: jack bean β-galactosidase, 0.5 unit/50 μl; jack bean β-*N*-acetyl-hexosaminidase, 2 units/μl; and fig α-galactosidase, 0.6 unit/60 μl.

RESULTS

Under the experimental conditions described above, the hydrolysate of methylated Cer-Tet of human erythrocytes gave about equal quantities of 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol and 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol (Fig. 1 and Table 1). These peaks were identified by mass spectrometry; the

TABLE 1. Ratio of the amount of 2,3,6-tri-*O*-methyl-galactitol relative to the amount of 2,4,6-tri-*O*-methyl-galactitol in the hydrolysate of permethylated ceramide tetrahexoside[a]

	2,4,6-Tri-*O*-methyl-galactitol (T = 2.49 ± 0.03)[b]	2,3,6-Tri-*O*-methyl-galactitol (T = 2.71 ± 0.03)[b]
Human erythrocyte Cer-Tet (sample 1)	1.00	1.06
Human erythrocyte Cer-Tet (sample 2)	1.00	0.99
Horse spleen Forssman Cer-Tet	1.00	0.92
Rat kidney globoside (expt. 1)	1.00	0.35
Rat kidney globoside (expt. 2)	1.00	0.30

[a] For the amount of 2,3,6-tri-*O*-methylglucitol (T = 2.87 ± 0.03) see the text and footnote 2.

[b] T, the relative retention time with 2,3,4,6-tetra-*O*-methyl-glucitol as 1.00.

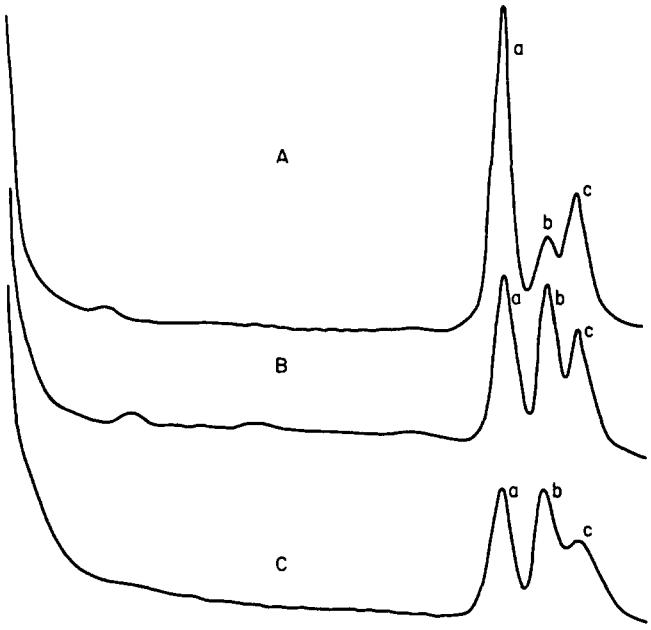

FIG. 1. GLC of partially methylated alditol acetates present in the hydrolysates of the permethylated ceramide tetrasaccharides. *A*, rat kidney Cer-Tet; *B*, Cer-Tet of Forssman glycolipid; *C*, human erythrocyte Cer-Tet. Peak *a*, 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol; peak *b*, 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol; peak *c*, 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol.

MS patterns of peaks *a*, *b*, and *c* in Fig. 1 were indistinguishable from those of 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol, 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol, and 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol, respectively (see Fig. 2). In striking contrast, the hydrolysate of a permethylated Cer-Tet from rat kidney gave a completely different pattern; namely, the amount of 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol relative to the amount of 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol was 0.3:1, and the major component was 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol (Fig. 1 and Table 1). The amount of 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol was, in general, less than the theoretical value.[2] No peak for 3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetylgalactitol, which had a slightly lower retention time than 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol, was detected in any preparation.

Only 10–20% of the galactose residues of rat kidney Cer-Tet was destroyed by periodate oxidation, although all of the *N*-acetylgalactosamine and over 90% of the glucose was destroyed under conditions similar to those described by Ledeen and Salsman (21). In contrast, about 50% of the galactose, corresponding to 1 mole of galactose per ceramide, over 90% of the glucose, and 100% of the *N*-acetylgalactosamine of human Cer-Tet were destroyed (Table 2).

All Cer-Tets, including rat Cer-Tet and a Cer-Tet isolated from the hydrolysate of horse spleen Forssman antigen by an α-*N*-acetylgalactosaminidase, were hydrolyzed by jack bean β-*N*-acetylgalactosaminidase and quantitatively converted to a ceramide trihexoside. The resulting ceramide trihexosides (Cer-Tri) obtained from human Cer-Tet and a Cer-Tet obtained from Forssman glycolipid hapten had migration rates on TLC identical with that of Cer-Tri of human erythrocytes, whose structure was identified as Galα(1→4)Galβ(1→4)-Glc(1→1)Cer. In contrast, the Cer-Tri obtained from rat kidney Cer-Tet had a slightly, but definitively, slower migration rate on TLC than the Cer-Tri obtained from other Cer-Tets and Cer-Tri of human erythrocytes. However, a small quantity of Cer-Tri corresponding to Cer-Tri of human erythrocytes was detected in addition to a large quantity of a slower migrating Cer-Tri in the hydrolysate of rat kidney Cer-Tet.

The ceramide trisaccharides, irrespective of their parent ceramide tetrasaccharides, were quantitatively hydrolyzed by fig α-galactosidase but not by jack bean β-galactosidase. They were converted to a ceramide dihexoside which had a migration rate on TLC identical with that of lactosylceramide of human erythrocytes. The resulting ceramide dihexosides, irrespective of their parent Cer-Tet sources, were then hydrolyzed by jack bean β-galactosidase but not by a α-galactosidase and were converted to a ceramide monohexoside (glucosylceramide). The resulting glucosylceramides, derived from human erythrocyte Cer-Tet and rat kidney Cer-Tet, were hydrolyzed by brain β-glucosidase (ceramide glucoside β-glucosidase) (25).

In all the experiments, the enzymatic hydrolysis of the glycolipids was quantitative, and the TLC pattern of the products was essentially the same as reported before (11).

[2] The ratio of 2,4,6-tri-*O*-methylgalactitol to 2,3,6-tri-*O*-methylglucitol in various Cer-Tets was from 1:0.7 to 1:0.37, depending on the subtle differences of the hydrolysis conditions. This indicates that complete hydrolysis of a permethylated glucosyl residue that is directly linked to a ceramide is extremely difficult to attain without "demethylative degradation" of peripheral sugar residues. In contrast, the recovery of 2,3,6-tri-*O*-methylgalactitol and 2,4,6-tri-*O*-methylgalactitol under the given hydrolytic conditions was near the theoretical ratio of 1:1 from Cer-Tets of various sources except that of rat kidney (Fig. 1 and Table 1).

TABLE 2. Recovery of galactose after exhaustive periodate oxidation of ceramide tetrasaccharides relative to recovery of galactose from intact ceramide tetrasaccharides

	% Galactose	% Glucose	% Galactosamine
Rat kidney Cer-Tet	85	3	0
Human erythrocyte Cer-Tet	50	5	0

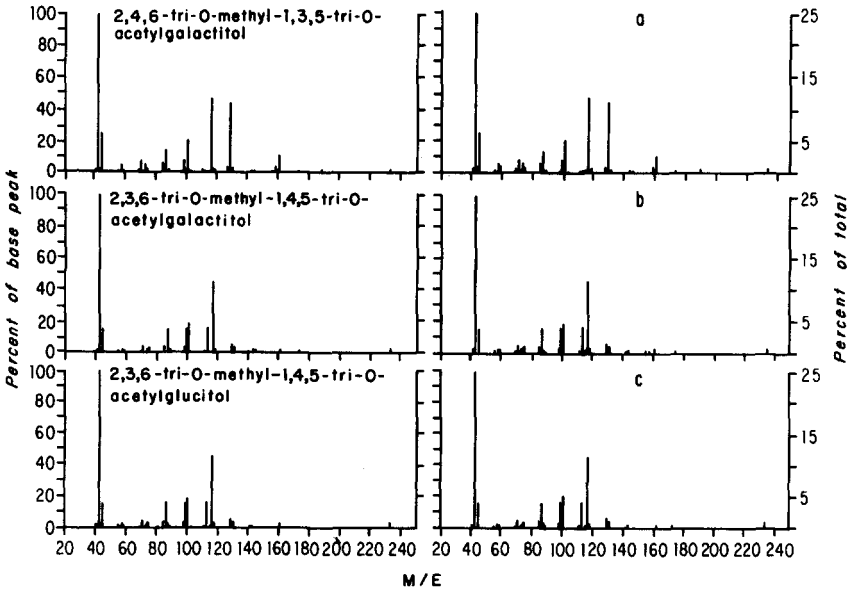

FIG. 2. Mass spectra of partially methylated alditol acetates separated by GLC. *Upper left*, standard of 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol; *middle left*, 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol; *lower left*, 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol. *Upper right*, peak *a* of *A* in Fig. 1; *middle right*, peak *b* of *A* in Fig. 1; *lower right*, peak *c* of *A* in Fig. 1. The mass spectra of peaks *a*, *b*, and *c* in *B* and *C* of Fig. 1 were essentially the same as shown above.

DISCUSSION

The results of sequential hydrolysis by various glycosidases indicated that all Cer-Tets under investigation had a carbohydrate sequence and anomeric structure identical with that of human Cer-Tet reported previously (11); only the penultimate galactosyl residue was α-glycosidic. The results also indicated that the essential difference between rat kidney Cer-Tet and other Cer-Tets was the difference of the penultimate α-galactosyl linkage. This is because the ceramide trihexoside derived from rat kidney globoside was different from those derived from other Cer-Tets, but the ceramide dihexosides were identical irrespective of the source of Cer-Tet.

The results of methylation studies indicated, however, that about 50–60% of both galactosyl residues of rat kidney Cer-Tet must be substituted at the C-3 position, and only 40% of Cer-Tet was substituted at the C-4 position because 2,4,6-tri-*O*-methylgalactitol was the major component appearing in the hydrolysate of permethylated rat Cer-Tet. This was in striking contrast to the other Cer-Tets, which gave about equimolar amounts of 2,3,6-tri-*O*-methylgalactitol and 2,4,6-tri-*O*-methylgalactitol. Therefore, one galactose was substituted at C-3, and the other galactose was substituted at C-4. In Cer-Tets of human erythrocytes and kidney, C-3-substituted galactose was located at the next internal position (3, 4). Ceramide tetrasaccharides of monkey and bovine kidney and of horse spleen seemed to have the same structure as human Cer-Tet.[3]

I. GalNAcβ(1 → 3)Galα(1 → 3)Galβ(1 → 4)Glcβ(1 → 1)Cer

II. GalNAcβ(1 → 3)Galα(1 → 4)Galβ(1 → 4)Glcβ(1 → 1)Cer

FIG. 3. Structures of ceramide tetrasaccharides. Structure I, major part of rat kidney ceramide tetrasaccharide; structure II, minor part of rat kidney ceramide tetrasaccharide, human erythrocyte ceramide tetrasaccharide, and Cer-Tet from horse spleen Forssman pentasaccharide.

The results of methylation studies were further assessed by periodate oxidation. About 85% of the galactose residues of rat Cer-Tet and only 50% of the galactose residues of other Cer-Tets were unaffected by prolonged periodate oxidation.

All of these results have indicated that the major part of rat kidney Cer-Tet was *N*-acetylgalactosaminosylβ(1→3)galactosylα(1→3)galactosylβ(1→4)glucosyl-β(1→1)ceramide. This is in contrast to Cer-Tets of other sources studied, which have a common structure: *N*-acetylgalactosaminosylβ(1 → 3)galactosylα(1 → 4)-galactosylβ(1→4)glucosylβ(1→1)ceramide (see Fig. 3).

A unique structure of rat kidney globoside that contains galactosyl 1→3 linkage as the penultimate structure must be a basis of the immunological uniqueness of rat globoside assessed by Rapport et al. (8). This structure also suggests that rat tissue must have a unique enzyme that transfers galactosyl residue as α(1→3). However, results of methylation study and periodate oxidation indicate that a minor portion (about 40–45%)

[3] Siddiqui, B., and S. Hakomori, Unpublished results.

of rat kidney Cer-Tet (Wistar-Sio strain) must be an ordinary Cer-Tet having an $\alpha(1\rightarrow4)$ linkage. The proportion of a minor component was calculated to be smaller (about 20–30%), based on the intensities of spots of two ceramide trihexosides incompletely separated on TLC after enzymatic hydrolysis. Attempts to separate rat kidney Cer-Tet into components having, respectively, Gal$\alpha(1\rightarrow3)$ and Gal$\alpha(1\rightarrow4)$ linkages have so far been unsuccessful. Lack of adequate amounts of rat kidney ceramide tetrasaccharide have made it impossible to study the separation of the two oligosaccharides in order to determine the exact ratio.

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