Structure of a Pentahexosylceramide (Forssman Hapten) from Canine Intestine and Kidney*

SUN-SANG J. SUNG, WALTER J. ESSELMAN, AND CHARLES C. SWEELER

From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823

SUMMARY

A pentahexosylceramide isolated from canine kidney and intestine was shown by enzyme hydrolyses, partial acid hydrolysis, permethylation and analysis of the products by gas-liquid chromatography-mass spectrometry, and serological tests to be a Forssman hapten identical in structure with the Forssman glycolipid from horse spleen. The assigned structure was N-acetylgalactosaminyl(α1→3)N-acetylgalactosaminyl(β1→3)galactosyl(α1→4)galactosyl(β1→4)glucosyl(1→1)ceramide. In the course of this work, mass spectrometry was employed to elucidate the structures of partially methylated alditol acetates derived from hexosamines.

In 1911 Forssman (1) demonstrated the formation of sheep blood hemolysins after parenteral administration of extracts of guinea pig organs into rabbits. The antigens that induce the formation of these hemolysins have been called Forssman antigens. They occur in many species of animals and bacteria but not in plants (2). Landsteiner (3) showed that Forssman antigens consist of a specific alcohol-soluble component which he called the hapten and a nonspecific component which was protein in nature. Brunius (2) reported that the purified Forssman hapten from horse kidney was a galactosamine-containing lipid, and Papirmeister and Mallette (4) found that the Forssman hapten from sheep erythrocytes contained hexose, hexosamine, fatty acid, and a base. Makita et al. (5) subsequently proposed the structure, N-acetylgalactosaminyl(α1→3)galactosyl(1→4)galactosyl(β1→4)glucosylceramide, for the Forssman hapten from horse kidney and spleen. In agreement with these results, Mallette and Rush (6) have reported that the Forssman hapten from sheep erythrocytes is also a tetrahexosylceramide with the same composition as that reported for the horse (5). Siddiqui and Hakomori (7) have obtained different results with the Forssman hapten from horse spleen, proposed by Siddiqui and Hakomori (7). A preliminary report of these results has been published (9).

EXPERIMENTAL PROCEDURES

Isolation of Pentahexosylceramide—Small intestines and kidneys were excised from mongrel dogs immediately after they were killed. The kidneys were perfused with distilled water, deassembled, and minced. The intestines were rinsed thoroughly with distilled water and cut into small pieces with scissors. The tissues were then homogenized in a Waring Blender two times with 5 volumes of chloroform-methanol, 2:1, once with 5 volumes of chloroform-methanol, 1:1, and once with 5 volumes of chloroform-methanol, 1:2. The homogenate was filtered through a Buchner funnel each time, and the combined filtrates were evaporated under reduced pressure at a bath temperature of 45°C. Absolute ethanol (0.5 volume) was added, and the solution was again evaporated to dryness in vacuo. Glycolipids were isolated by the method of Saito and Hakomori (10), and GL-5 was isolated from this fraction by thin layer chromatography on 0.3-mm Silica Gel H plates (E. Merck AG, Darmstadt, Germany) with the use of the following solvent systems consecutively, chloroform-methanol-water, 100:42:6; chloroform-methanol-concentrated ammonia, 40:80:25; and chloroform-methanol-water, 65:45:10. Glycosphingolipids were visualized by brief exposure to iodine vapor and by spraying with 2% α-naphthol in ethanol followed by concentrated sulfuric acid and heating at 120°C for 10 min.

Reference Glycosphingolipids and Carbohydrates—Standard glycosphingolipids were isolated from equine kidney, erythrocyte stroma, or plasma as previously described (11). Chondrosine was purchased from Miles Laboratories, Elkhart, Ind., and chitin was obtained from Sigma, St. Louis, Mo. Acetylation of chondrosine (1 mg) was carried out in 2 ml of dry methanol and 0.3 ml of acetic anhydride at room temperature overnight, after which the solvents were removed at reduced pressure.

Estimation of Long Chain Base and Carbohydrate—Long chain glycosphingolipids were isolated from equine kidney, erythrocyte stroma, or plasma as previously described (11). Chondrosine was purchased from Miles Laboratories, Elkhart, Ind., and chitin was obtained from Sigma, St. Louis, Mo. Acetylation of chondrosine (1 mg) was carried out in 2 ml of dry methanol and 0.3 ml of acetic anhydride at room temperature overnight, after which the solvents were removed at reduced pressure.

A pentahexosylceramide with a similar carbohydrate sequence was observed as the major glycolipid in dog intestine by Vance et al. (8). Our studies on the structure of this glycosphingolipid indicate that it is a Forssman hapten with the same structure as that of horse spleen, proposed by Siddiqui and Hakomori (7). A preliminary report of these results has been published (9).

The abbreviations used are: GL-1, monohexosylceramide; GL-2, dihexosylceramide; GL-3, trihexosylceramide; GL-4, tetrahexosylceramide (globoside); GL-5, pentahexosylceramide (Forssman hapten); GlcNAc, N-acetylneuraminylgalactosylglycosylceramide (hematoside) (GM4 is according to the Svennerholm nomenclature).
bases were estimated colorimetrically, following acid-catalyzed methanolation, by the method of Lauter and Trams (12). The methyl glycosides liberated by the same procedure were measured by the gas chromatographic method of Vance and Sweeley (11) and Chambers and Clamp (13).

**Glycosphingolipid Hydrolyses**—A partially purified fraction of α-N-acetylgalactosaminidase was obtained from hog liver by the method of Weissmann and Hinrichsen (14). Jack bean was used as a source for the isolation of β-N-acetylhexosaminidase and β-galactosidase (15, 16), and α-galactosidase was obtained from ficin (17). Conditions for enzymatic hydrolyses of glycosphingolipids with these hydrolases have been described elsewhere (18). Partial acid hydrolysis was performed by the method of Sweeley et al. (19).

**Preparation and Analysis of Partially Methylated Alditol Acetates**—Permethylation of 1-mg samples was carried out by the procedure of Hakomori (20), after which the glycolipid was hydrolyzed by the method of Yang and Hakomori (21). The dried residue was dissolved in water and reduced with 1 ml of sodium borohydride (1% in aqueous solution) for 2 hours at room temperature, and the partially methylated alditols were acetylated by the method of Griggs et al. (22). Gas-liquid chromatography of partially methylated alditol acetates of neutral sugars was performed with a Hewlett-Packard model 402 gas chromatograph equipped with a glass column (2 m x 2 mm) packed with a mixture of 0.2% ethylene glycol succinate, 0.2% ethylene glycol adipate, and 1.4% XE-60 on 100- to 120-mesh Gas-chrom P (Applied Science Laboratories, Inc., State College, Pa.). Partially methylated alditol acetate derivatives derived from the amino sugar residues of GL-5, globoside, and reference carbohydrates were analyzed with 3% OV-210 on 80 to 100 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.) isothermally at 160° for 15 min and then programmed from 160-250° at 5° per min with nitrogen as carrier gas. Mass spectra were obtained with an LKB 9000 gas chromatograph-mass spectrometer at 70 e.v. with an ion source temperature of 250° and were interpreted with the aid of reference mass spectral data of Björndal et al. (23, 24) and Puro (25).

**Periodate Oxidation**—Periodate oxidation of GL-5 was performed as described elsewhere (7), and the carbohydrate composition of the product was analyzed by gas-liquid chromatography (11, 13) after methanolysis.

**Serological Tests**—Forssman reactivity of GL-5 was kindly performed by Drs. M. M. Rapport and L. Graf, Albert Einstein College of Medicine, by means of the method of complement fixation (26). Globoside and Forssman activity were assayed with antigloboside and anti-Forssman antisera by the Ouchterlony double diffusion method, with the use of thin films of agarose on microscope slides fitted with plastic templates, according to the method described by Sharpless and Lo Grigpo (27). Antigloboside antiserum, anti-Forssman antiserum, and horse spleen Forssman glycolipid were gifts of Dr. B. Siddiqui, Michigan State University; the antisera were prepared by the method of Koscielak et al. (28). Results

**Composition of Purified Pentahexosylceramide**—A thin layer chromatogram of the mixture of glycosphingolipids from canine kidney and intestine is shown in Fig. 1. Monohexosylceramide (GL-1), trihexosylceramide (GL-3), and hematoside (GM3) were the major components of kidney, while GL-3 and the pentahexosylceramide (GL-5) were the major constituents of intestine (Table I). Gas chromatographic analysis of the trimethylsilyl derivatives of methyl glycosides from GL-5 (Fig. 2) indicated that the oligosaccharide moiety consisted of glucose, galactose, and N-acetylglactosamine in a molar ratio of 1.21:2.0. Colorimetric analysis of the long chain base liberated from GL-5 by methanolysis indicated approximately 1 mole of base per mole of glucose.

**Sequence and Anomeric Configuration of Carbohydrate Units**—Partial acid hydrolysis of GL-5 liberated glucosylceramide and a dihexosylceramide with a 1:1 molar ratio of galactose to glucose.
Fig. 2. Gas-liquid chromatography of trimethylsilylated methyl glycosides of standard sugars, canine GL-5, and porcine GL-4. Peaks were identified as methyl galactosides (A), methyl glucosides (B), and methyl N-acetylgalactosaminides (C). Standards consist of galactose, glucose, and N-acetylgalactosamine mixed in a 2:1:1 molar ratio. These analyses were performed on a column (2 m x 2 mm) of 3% SE-30 on 80 to 100 mesh Supelcoport with an initial temperature of 100°C programmed at 2°C per min to 210°C with the use of nitrogen as a carrier gas.

indicating a partial internal sequence of galactosylglycosylceramide. Enzymatic hydrolysis of GL-5 was carried out by the following mixtures of glycosidases on a scale (200 μg each) sufficient for analysis by thin layer chromatography (Fig. 3): (a) α-N-acetylgalactosaminidase alone, and (b) α-N-acetylgalactosaminidase and β-N-acetylgalactosaminidase. GL-3 derived from partial acid hydrolysis or enzymatic hydrolysis was treated with (a) α-galactosidase, and (b) α-galactosidase and β-galactosidase. The products were a tetrahexosylceramide, a trihexosylceramide, a dihexosylceramide, and a monohexosylceramide, respectively. The sequence and stereochemistry of the glycosidic linkages of GL-5 were therefore (α-N-acetylgalactosaminyl)-(β-N-acetylgalactosaminyl)-(α-galactosyl)-(β-galactosyl)-(glucosyl)ceramide. These results confirm the arrangement of carbohydrate units proposed by Vance et al. (8).

Linkages of Carbohydrate Units—Gas chromatograms of partially methylated alditol acetates, obtained from the neutral sugars of canine GL-5 and porcine globoside, are shown in Fig. 4. The three peaks from GL-5 corresponded in their relative areas and retention times to those of porcine globoside. Mass spectra provided evidence that they were 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglactitol (Peak A), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglactitol (Peak B), and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol (Peak C), respectively. Enzymatic degradation of 5 mg of GL-5 by a mixture of α-N-acetylgalactosaminidase and β-N-acetylgalactosaminidase (as described above) gave a trihexosylceramide which was isolated by preparative thin layer chromatography. Permethylation and mass spectral analysis of the products after acid hydrolysis, borohydride reduction, and acetylation gave evidence for the presence of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglactitol, and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol. The internal linkages were therefore galactosyl(1→4)galactosyl(1→6)glucosyl(1→1)ceramide. When considered along with the results of permethylation of the intact GL-5, presented above, it was concluded that the penultimate N-acetylgalactosamine residue must have a 1→3 glycosidic linkage to galactose.

Gas-liquid chromatography of the partially methylated alditol acetates derived from the neutral and amino sugars from GL-5 is shown in Fig. 5. Peak C is identical in retention time with the amino sugar derived from globoside, N-acetyl-N-methyl-1,5-di-O-acetyl-3,4,6-tri-O-methylglactosaminitol (Structure 1). Mass spectra of the authentic sample, from globoside, and that from the unknown are shown in Fig. 6. Primary fragment ions at m/e 158, 161, 202, and 205 can be found in both spectra. The base peak at m/e 116 is probably derived from m/e 158 by the loss of ketene (42 a.m.u.). Prominent peaks at m/e 98, 144, and 146 are assumed to be formed from m/e 158, 202, and 205, respectively, by the loss of acetic acid (60 a.m.u.). A significant peak at m/e 129 is related to m/e 205 by the loss of either CH₃OAc or N-methylacetamide (73 a.m.u.).

Peak D (Fig. 5) had the same retention time as N-acetyl-N-methyl-1,3,5-tri-O-acetyl-4,6-di-O-methylglactosaminitol (Structure 2) derived from N-acetylcaldrosine, and mass spectra of the two peaks, shown in Fig. 7, B and C, indicate their identity.
Fig. 4. Gas-liquid chromatography of partially methylated alditol acetates of neutral sugars from canine GL-5 (upper) and porcine red cell globoside (lower). Peaks A, B, and C are 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol, and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol, respectively. Analysis on a column (2 m X 2 mm) containing a mixture of liquid phases consisting of 0.2% ethylene glycol succinate, 0.2% ethylene glycol adipate, and 1.4% XE-60, on 100 to 120 mesh Gas-chrom P with an initial temperature of 155° and programmed at a rate of 2° per min with nitrogen as carrier gas. Retention time is given in minutes.

Fig. 5. Gas-liquid chromatography of partially methylated alditol acetates of GL-5 on a column (2 m X 2 mm) of 3% OV-210 on 80 to 100 mesh Supelcoport operated isothermally at 160° for 15 min and then programmed from 160-250° at 5° per min with nitrogen as carrier gas. Retention time is given in minutes. Peak A was shown by mass spectrometry to be 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol, while Peak B was shown to be a mixture of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol. Mass spectra of Peaks C and D are shown in Figs. 6 and 7.

Fig. 6. Mass spectra of partially methylated alditol acetates of amino sugars. A, mass spectrum of N-acetyl-N-methyl-1,4,5-tri-O-acetyl-2,3,6-di-O-methylglucosaminitol (Structure 3) from porcine globoside; B, mass spectrum of Peak C in Fig. 5, derived from canine intestinal GL-5.

Structures 1 to 3

Significant peaks from primary ions were found at m/e 188 and 318 (M-73). The base peak was at m/e 116, as observed with other partially methylated alditol acetates of amino sugars permethylated by the Hakomori procedure as outlined by Björndal et al. (9). Secondary fragments were found at m/e 170, assumed to be formed from the ion at m/e 230 by the loss of acetic acid; m/e 242, derived from m/e 346 by the loss of acetic acid and ketene; and m/e 272, derived from m/e 346 by the loss of ketene and methanol.

The mass spectrum (Fig. 7A) of N-acetyl-N-methyl-1,4,5-tri-O-acetyl-2,3,6-di-O-methylglucosaminitol (Structure 3) prepared from chitin, is clearly different from Peak D derived from GL-5. A similar pattern of ions was observed at m/e 74, 98, 116, and 133, but significant ions characteristic of this amino sugar were observed at m/e 202 and 283. Furthermore, fragment ions at m/e 161, 170, 248, 272, and 318 in the spectrum of Structure 2 were either absent or of very low intensity in this spectrum.

Glucose, galactose, and N-acetylglucosamine in a molar ratio of 1:1:1 were recovered after periodate oxidation of GL-5, showing that the terminal N-acetylglucosamine residue is not 1 →6 linked to the penultimate sugar. The glucose of GL-5 is periodate-resistant under these particular conditions (7).
be concluded from analyses of permethylation products and periodate oxidation products that GL-5 has a 1→3 glycosidic linkage between the two N-acetylgalactosamine residues.

**Serological Tests**—The pentahexosylceramide from kidney and intestine had strong Forssman activity when tested by the complement fixation test (26). Hydrolysis with α-N-acetylgalactosaminidase gave a product which showed globoside activity by Ouchterlony double diffusion and provided additional evidence for the assignment of the configurations of the internal glycosidic linkages of GL-5. A single precipitation band was observed when GL-5 was tested by Ouchterlony double diffusion with anti-Forssman antiserum prepared against horse spleen Forssman antigen. Furthermore, the precipitation band was fused with the horse spleen Forssman precipitation band with no overlap.

**Conclusion**—With the data presented above, we have demonstrated that canine GL-5 isolated from both kidney and intestine is a true Forssman hapten which is chemically and immunologically identical with that of horse spleen Forssman hapten (7), that is, N-acetylgalactosaminyl(α1→3)N-acetylgalactosaminyl(β1→3)galactosyl(α1→4)galactosyl(β1→4)glucosyl(1→1)cera-ramide.

**DISCUSSION**

Siddiqui and Hakomori established the internal linkages of the pentahexosylceramide (Forssman antigen) from horse spleen by showing that the product of hydrolysis with α-N-acetylgalactosaminidase precipitated with antigloboside antiserum (7). In addition they observed that the product was chromatographically indistinguishable from globoside I from human erythrocytes, and permethylation yielded products which were identical with those from globoside. The terminal N-acetylgalactosamine residue was proposed to be linked through a 1→3 glycosidic linkage to the globoside moiety in the pentahexosylceramide since one N acetylgalactosamine residue in the intact glycolipid was resistant to periodate oxidation.

The results of our structural and immunochemical studies of the pentahexosylceramide hapten from canine kidney and intestine are in agreement with those of Vance et al. (8) and Siddiqui and Hakomori (7), and we have concluded that the structures of the horse and dog Forssman glycolipids are identical. More conclusive evidence for the position of the glycosidic linkage between the two N-acetylgalactosamine residues was provided by mass spectral analyses of the permethylation products of the amino sugars. Previously this technique was restricted to use with neutral sugars (23, 24), due probably to the fact that the products from hexosamines have extremely long retention times under the usual gas chromatography conditions employed with partially methylated alditol acetates. Even with a mixture of liquid phases (see under "Experimental Procedures") with a higher maximum operating temperature (240°C) than that described in earlier work it was not possible to obtain peaks from the hexosamine derivatives in a reasonable time. The partially methylated alditol acetates from N-acetylgalactosamine residues in N-acetylgalactosamine, chitin, and the canine pentahexosylceramide were eluted in 30 to 40 min when the 3% OV-210 column was programmed linearly from 160→250°C after the products from neutral sugars had eluted.

Similar results have been reported recently by Stoffel and Hanfland (29) and by Stoffler et al. (30) for the analysis of amino sugar-containing glycosphingolipids, and by Wolfe et al. (31) for a complex oligosaccharide assumed to be derived from glycoprotein. This improvement in the technique enables determination of the positions of all glycosidic linkages in a complex neutral glycosphingolipid by mass spectral analysis of permethylation products as reduced acetyl derivatives. In combination with studies of anomeric configuration and sequence using specific glycosidases, complete structures can be deduced at the micromole level. Furthermore, it is likely that mixtures of very closely related glycosphingolipids can be detected by this procedure; preliminary results with the GL-3 fraction from canine kidney and intestine indicate that it consists of a mixture of galactosyl-(α1→4)galactosyl(β1→4)glucosylceramide and an isomer, galactosyl(α1→3)galactosyl(β1→4)glucosylceramide, which is related to cytolipin R, previously shown to be the predominant tetrahexosylceramide in rat kidney (18).

It is unlikely that there are Forssman haptens with only one N-acetylgalactosamine moiety, as proposed by Makita et al. (5) and Mallette and Rush (6). Low hexosamine values are expected, due to relatively high stability of the glycosidic linkage between the two hexosamine residues, particularly after removal of the N-acetyl group from the terminal N-acetylgalactosamine moiety (32). Furthermore, the yield of hexosamine is always less than that of neutral sugars when methanolysis products are examined by gas-liquid chromatography (33).
The function of Forssman hapten is not well defined. Increased amounts are observed in contact-inhibited NIL cells (34, 35), while the surface reactivity of cells to anti-Forssman glycolipid antiserum is significantly reduced (36). In virally transformed NIL cells Forssman activity is absent (34, 35), but in BHK transformants the activity increases (38-42). These results suggest that Forssman antigen may play a significant role in the metabolism and function of cell surfaces. The content of Forssman hapten in the dog intestine is remarkably high (81, and this organ may be particularly convenient for studies on the metabolism and function of this interesting glycosphingolipid.

Acknowledgments We would like to thank Drs. M. M. Rapport and L. Graf for kindly performing serological tests on the pentahexosylceramide, Dr. B. Siddiqui for his generous gifts of antigloboside antiserum, anti-Forssman antiserum, and horse spleen Forssman hapten, Mr. Jack Harten for running the mass spectra, and Dr. R. A. Laine for his helpful suggestions.

REFERENCES
1. FORSSMAN, J. (1911) Biochem. Z. 37, 78-115
2. BRUNIUS, F. E. (1936) in Chemical Studies on the True Forssman Hapten, the Corresponding Antibody, and Their Interaction, Aktiebolaget Faktoria Bank, Stockholm
3. LANDSTEINER, K. (1921) Biochem. Z. 119, 294-306
6. MALLETTE, M. F., AND RUSH, R. L. (1972) Immunochemistry 9, 809-820
20. HAKOMORI, S. (1964) J. Biochem. 59, 205-208
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