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The Glycolipids of Dog Intestine*

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ABSTRACT: Preparative silicic acid chromatography of whole lipid extracts of dog intestine gave three fractions which contained glycolipid. The largest fraction contained all of the ceramide oligoglycosides and these were separated from phospholipid and other impurities by solvent fractionation, Florisil chromatography, and dialysis. The ceramide oligoglycosides were then separated into ganglioside and ceramide oligohexoside fractions by DEAE-cellulose chromatography. The latter was partially resolved by cold methanol fractionation followed by silicic acid chromatography. The major component, a ceramide pentaglycoside, was recovered in pure form on the basis of column and thin layer chromatography and on analysis. This is a novel substance giving analytical data and hydrolytic cleavage products consistent with an *N*-acylgalactosaminyl-*N*-acylgalactosaminylgalactosylgalactosylglucosylceramide structure. It contains 30% of the total intestine lipid sugar and most of the galactosamine. The ganglioside fraction was partially resolved on a silicic acid column, yielding a chromatographically homogeneous ganglioside as the major component. Analyses of this substance were consistent with a sialyldigalactosylglucosylceramide structure and with a minor glucosamine-containing impurity.

The sequence of sugars and nature of the long-chain base are not known. Both the ganglioside and the pentaglycoside contain predominantly long-chain fatty acids with average molecular weights of 338 and 321, respectively.

Studies on the chemistry and metabolism of the glycolipids of vertebrate tissues have been largely confined to those of nervous tissues, erythrocyte stroma, spleen, and kidney. All glycolipids isolated from these sources appear to be glycosides of a ceramide with glucose, galactose, galactose sulfate ester, lactose, or oligosaccharides which may contain in addition *N*-acylhexosamine or sialic acid or both. The chemistry and metabolism of these substances have been reviewed (Law, 1960; Svennerholm, 1964; Carter *et al.*, 1965). In this laboratory studies have been carried out on the composition of glycolipids of intestine and liver. These tissues contain some unique ceramide glycosides as well as other lipids containing fatty acyl primary amines. This paper describes the preparation of the ceramide oligoglycoside[1,2] fraction of dog intestine and the isolation and composition of the major component. This substance is a ceramide pentaglycoside which contains two *N*-acylgalactosamines, two galactoses, and one glucose in that order on the ceramide. A description of the isolation and composition of another major glycolipid component, a ganglioside, is also included in this paper.

* From the Department of Biochemistry, University of Alabama Medical Center, Birmingham, Alabama. *Received August 8, 1965; revised November 3, 1965.* This work was supported by a grant from the Life Insurance Medical Research Fund. A major portion of the data in this paper is taken from a dissertation submitted by W. R. Vance to the University of Alabama in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry. A preliminary report of this work has been presented (McKibbin and Vance, 1964).

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[1] In this paper the following terminology is used: "ceramide oligoglycoside" is a ceramide glycoside containing more than three sugar residues. "Ganglioside" is a glycolipid which contains sialic acid. "Ceramide oligohexoside" is a ceramide oligoglycoside which does not contain sialic acid. The composition of all solvent mixtures is expressed on a volume per volume basis.

[2] The following abbreviations are used in this paper: tlc, thin layer chromatography; CPG, ceramide pentaglycoside.

Experimental Section

Analytical Methods. Methods for the determination of phosphorus and primary amine have been described previously (McKibbin *et al.*, 1961). Total N was determined by the method of Koch and McMeekin (1924) modified for lipid material (McKibbin and Taylor 1949b). Long-chain base nitrogen was determined by the method of McKibbin and Taylor (1949b) on aliquots hydrolyzed by refluxing for 3 hr with 2 N aqueous HCl. Chromic acid oxidations were carried out by the method of Bloor (1928), and ester groups by the method of Snyder and Stephens (1959). Sialic acid was determined by the method of Svennerholm (1957) as modified by Miettinen and Takki-Luukkainen (1959) without previous hydrolysis and using a commercial *N*-acetylneuraminic acid (Sigma Chemical Co.) as a standard. Hexosamine was determined by the method of Elson and Morgan (1933) as modified by Blix (1948) on an aliquot hydrolyzed by refluxing 3.5 hr with 2.5 or 3 N HCl. All anthrone-reacting material was determined by the method of Bailey (1958) using a galactose standard and expressed as galactose. Glucose was determined with glucose oxidase (Glucostat, Worthington Biochemical Corp.) on the neutralized hydrolysates prepared by refluxing the aliquot with 2 N HCl for 3 hr. Since the molar color yield from the anthrone reaction with glucose is much higher than with galactose, total hexose was calculated from the galactose values corrected for the glucose content of the mixture. For galactose:glucose ratios of 2, as determined for the lipids considered here, multiplying the galactose values by 0.86 gave true values for total hexose. The glucose values were subtracted from total hexose and the remainder was expressed as "true galactose." Formaldehyde was determined by the method of Frisell and Mackenzie (1958). Total sulfur was determined by benzidine precipitation of sulfate from a Carius digest (Kahn and Leiboff, 1928) followed by determination of the benzidine with the naphthoquinone sulfonate reagent.

Preparation of the Glycolipids

Extraction and Purification of the Lipids. All of the intestine samples were taken from dogs immediately at sacrifice. The entire small intestine was slit longitudinally and washed with cold water, and the adhering mesenteric fat was removed. Initially the frozen tissue was powdered and refluxed with ethanol–ether (3:1), and the insoluble residue was extracted with chloroform as described previously (McKibbin and Taylor, 1949a). The residue extraction was not necessary with the intestine. For large-scale preparations the tissue was prepared for reflux by homogenizing in ethanol–ether (3:1) in an explosion-proof Waring Blendor fitted with a clamped top and a sheet Teflon gasket. The volume of extraction solvent was also reduced from 17 ml/g of fresh tissue to 8 ml/g by a two-stage extraction. The homogenate was refluxed initially for 45 min with solvent equivalent to 6 ml/g, then the extract was decanted and the residue was extracted again for 15 min with 2 ml of solvent/g. The extracts in chloroform solution were purified by emulsification with 0.25 M aqueous $MgCl_2$ (McKibbin *et al.*, 1961). The purified lipid extracts in chloroform solution were then fractionated as described below.

Isolation of the Ceramide Oligoglycoside Fraction. The entire fractionation procedure is shown in Figure 1.

SILICIC ACID CHROMATOGRAPHY. Silicic acid (16.7 g) (Mallinckrodt No. 2844) and 16.7 g of Celite (Johns Manville, water washed) were mixed in a chloroform slurry and poured into a 17-mm id column fitted to a suction flask. The column was rinsed with an additional 50 ml of chloroform under slightly reduced pressure. The purified lipids, equivalent to 0.84 mmole of lipid phosphorus, in 60 ml of chloroform solution were added to the column, followed by 30 ml of additional chloroform. The column was eluted with 100 ml of 2%, 200 ml of 10%, 200 ml of 17%, 200 ml of 50%, and 300 ml of 80% methanol in chloroform, respectively. The recovery of lipids in these fractions (designated S2, S10, S17, S50, and S80, respectively) is given in Table I. The ceramide mono-, di-, and trihexosides were contained in the S10 and S17 fractions along with most of the phospholipid. The composition of these glycolipids will be reported later. All of the ceramide oligoglycosides were contained in the S50 and S80 fractions and these were used for further fractionation. For large-scale preparations 41-mm id columns were used with the adsorbent, lipid load, and eluting solvent volumes proportional to cross-sectional area. The elution with 80% methanol in chloroform was omitted for economy.

TABLE I: Fractionation of Dog Intestine Lipids with Silicic Acid.[a]

Fraction	Elution Solvent (% Methanol in $CHCl_3$)	Volume (ml)	% of Total Recovered: Lipid Phosphorus	% of Total Recovered: Lipid Galactose
S2	2	100	0.2	0.3
S10	10	200	41.0	20.6
S17	17	200	18.1	16.0
S50	50	200	22.8	52.5
S80	80	300	12.6	5.7

[a] Silicic acid (16.7 g), 16.7 g of Celite, 17-mm id column, 0.843 mmole of lipid phosphorus, 91.1 μmoles of lipid galactose.

SOLVENT FRACTIONATION OF FRACTION S50. Fraction S50 equivalent to 250 μmoles of lipid galactose was taken up in the solvent system: benzene–petroleum ether (bp 30–60°) (70:30) *vs.* methanol–acetone–water (60:20:20). A five-plate countercurrent extraction was carried out with this system using separatory funnels

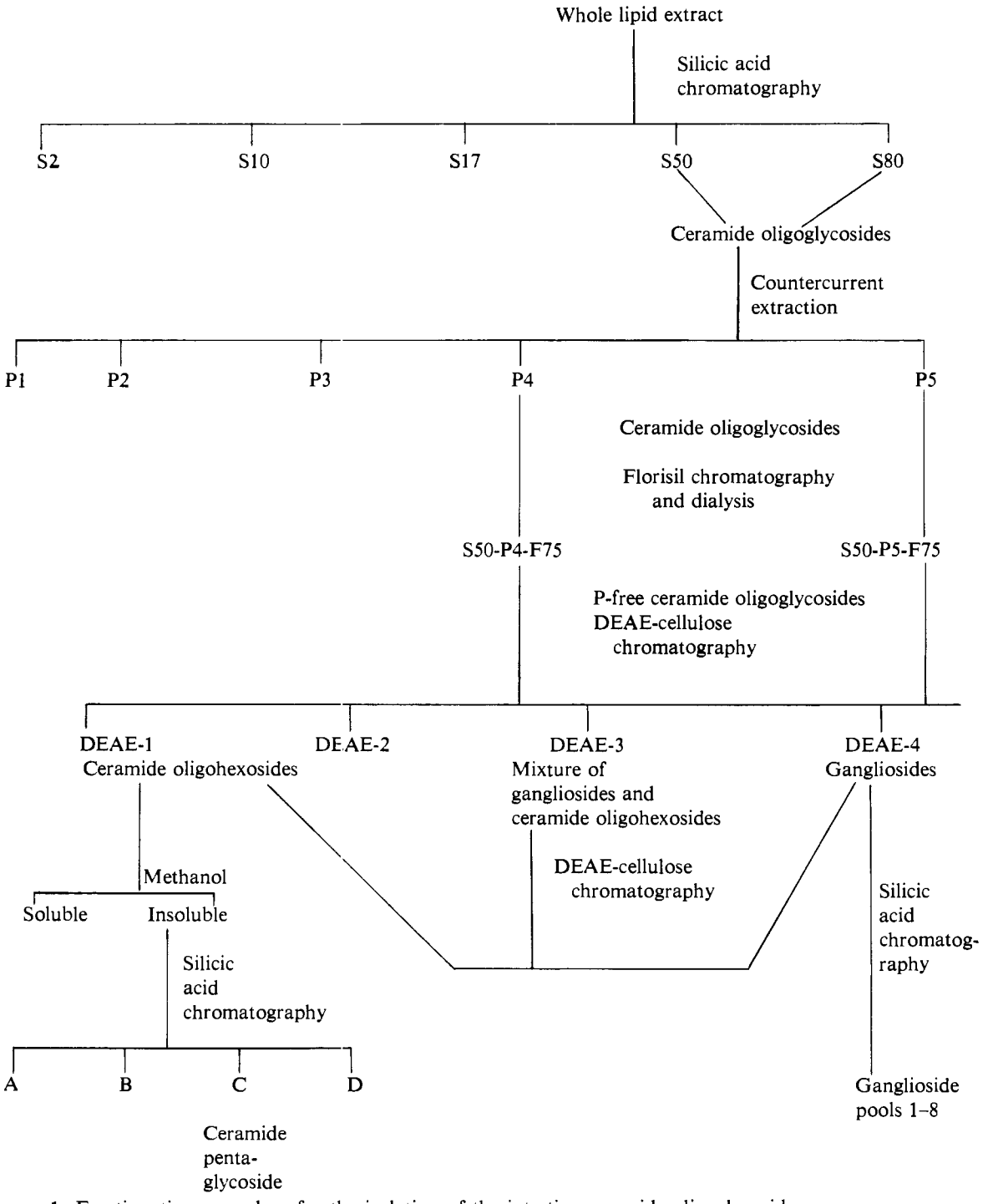

FIGURE 1: Fractionation procedure for the isolation of the intestine ceramide oligoglycosides.

and moving the lower polar phase. The recovery of lipids in these fractions is given in Table II. The glycolipids partitioned strongly to the polar phase and a further removal of phospholipid was achieved. Some differences were found in the composition of the glycolipids in funnels 4 and 5. These were therefore fractionated separately on Florisil columns and designated "P4" and "P5."

FLORISIL CHROMATOGRAPHY OF FRACTION S50-P4. A 17-mm id column was packed with 41 g of Florisil

TABLE II: Countercurrent Extraction of Silicic Acid Fraction S50.[a]

Funnel	% of Total Recovered	
	Lipid Phosphorus	Lipid Galactose
1	47.5	1.5
2	34.7	4.2
3	9.7	8.7
4	3.2	24.2
5	5.1	61.5

[a] Solvent system: benzene–petroleum ether (bp 30–60°) (70:30) *vs.* methanol–acetone–water (60:20:20); lower polar phase moved; fraction S50 equivalent to 0.25 mmole of lipid galactose and 1.01 mmoles of lipid phosphorus.

(Floridin Company, 60/100 mesh) in a chloroform slurry and rinsed with 200 ml more of chloroform. Fraction S50-P4, equivalent to 1350 μmoles of lipid galactose in 200 ml of 35% methanol in chloroform, was then placed on the column, followed by an additional 100 ml of this solvent. This effluent contained only traces of glycolipid. The column was then eluted with 800 ml of 75% methanol in chloroform. About 90% of the glycolipid was recovered from the column in this fraction, designated S50-P4-F75, and this contained only 1–2% phospholipid. Fraction S50-P5 gave essentially the same results with Florisil chromatography.

DIALYSIS. Fraction S50-P4-F75 or S50-P5-F75 equivalent to 1 mmole of lipid galactose was taken up in 35 ml of water, placed in 1-in. Visking tubing, and dialyzed against distilled water at 4° for 24 hr.

Composition of the Ceramide Oligoglycoside Fractions. These preparations contained nearly all of the ceramide oligoglycosides of the original tissue lipid extract. They are free of neutral lipids and nonlipid impurities and contain minimal amounts of phospholipid. The composition of the fractions is given in Table III. The sugars and amino sugars were identified by the following procedure. Suitable aliquots of the fractions were hydrolyzed in 2 N H_2SO_4 in an oven at 100° for 5 hr. The hydrolysates were extracted with chloroform and the aqueous phase was neutralized to pH 5–6 with barium hydroxide. Aliquots of this were then chromatographed on paper using the technique of Masamune and Yosizawa (1953) and locating the sugars by the modified silver nitrate spray method of Trevelyan *et al.* (1950). Only glucose, galactose, and galactosamine were present in these hydrolysates.

Resolution of the Ceramide Oligoglycoside Fraction. The fractions were resolved by use of DEAE-cellulose chromatography, methanol fractionation, and silicic acid chromatography. The following procedure for isolation of the major components is applicable to both the S50-P4-F75 and S50-P5-F75 fractions.

DEAE-CELLULOSE CHROMATOGRAPHY. A 17-mm id column was packed to a height of about 25 cm with DEAE-cellulose in the acetate form as described by Rouser (1962). The column was washed with 110 ml of chloroform and then charged with fraction S50-P4-F75 equivalent to 0.826 mmole of lipid galactose in 100 ml of 15% methanol in chloroform. The column was washed with an additional 100 ml of this solvent mixture and the total effluent was combined as fraction DEAE-1. The column was then eluted with 100 ml of 45% methanol in chloroform (DEAE-2), 140 ml of methanol–glacial acetic acid–chloroform (7:6:7) (DEAE-3), and an additional 160 ml of the latter solvent (DEAE-4). The lipid galactose and sialic acid content of these fractions is given in Table IV. Fractions DEAE-1 and -2 were almost free of gangliosides and were pooled for isolation of the ceramide pentaglycoside as described below. Fraction DEAE-4 was used as described below for the isolation of the gangliosides. Fraction DEAE-3 was a mixture of gangliosides and ceramide oligohexosides and was rechromatographed on an identical DEAE-cellulose column for the complete separation of these two classes of lipids.

TABLE III: Composition of the Dog Intestine Ceramide Oligoglycoside Fractions.[a]

Fraction	S50-P5-F75	S50-P4-F75
Mass (μg)	1875	1520
Total nitrogen (μmoles)	3.40	2.32
Sialic acid as *N*-acetyl (μmole)	0.25	0.43
Galactosamine (μmoles)	1.04	0.82
Other water-soluble nitrogen (μmoles)	1.56	0.44
Anthrone sugar as galactose (μmoles)	3.68	2.91
Fatty acid (μmoles)	1.38	1.40
Chromic acid uptake (μatoms of O)	245	194
Ester groups (μmole)	0.20	0.15
Total phosphorus (μmole)	0.137	0.041

[a] All values are given per micromole of long-chain base.

TABLE IV: DEAE-Cellulose Chromatography of Fraction S50-P4-F75.

Fraction	Elution Solvent	Volume (ml)	% Original Galactose	Sialic Acid/ Galactose Molar Ratio
DEAE-1	15% Methanol in $CHCl_3$	200	28.9	0.009
DEAE-2	45% Methanol in $CHCl_3$	100	33.3	0.015
DEAE-3	Methanol–acetic acid–chloroform (7:6:7)	140	19.6	0.32
DEAE-4	Methanol–acetic acid–chloroform (7:6:7)	160	5.9	0.50

TABLE V: Composition of Pooled Ganglioside Fractions Separated by Silicic Acid Chromatography.

Pool No.	Fraction No.	Galactose (μmoles)	Sialic Acid (μmoles)	Galactose/ Sialic Acid	Nitrogen (μmoles)
1	1–6	14.1	1.4	10.1	—
2	7–9	46.5	13.6	3.4	43.8
3	10–15	112.0	34.5	3.3	—
4	16–20	42.1	15.1	2.8	50.5
5	21–26	23.2	9.6	2.4	29.6
6	27–42	39.4	10.8	3.7	31.5
7	43–62	22.4	4.7	4.7	23.1
8	63–68	138.3	42.6	3.3	—

SILICIC ACID CHROMATOGRAPHY OF THE GANGLIOSIDES. A 17-mm id column was packed with a mixture of 20 g each of silicic acid and Celite from a chloroform slurry. An aliquot of fraction DEAE-4 containing 140 μmoles of lipid sialic acid in 100 ml of 15% methanol in chloroform was placed on the column. The fraction was followed by 100 ml of 15%, 500 ml of 18%, 200 ml of 25%, 165 ml of 35%, and finally 100 ml of 60% methanol in chloroform, respectively. Four 50-ml fractions, four 25-ml fractions, and 64 15-ml fractions were taken to the end of the elution sequence. Anthrone-reacting material was continuously eluted, but 59% of the total was found in tubes 7–20 inclusive and 32% in tubes 63–68. All of the fractions were then combined into eight pools, and these were analyzed for galactose, sialic acid, and total nitrogen (Table V). Aliquots from each of the pools were subjected to thin layer chromatography using silica gel G and the solvent systems chloroform–methanol–water, 60:35:8 (Wagner, 1960) and 65:25:4 (Wagner *et al.*, 1961). In this and in subsequent tests for homogeneity the aliquots contained 0.01–0.04 μmole of lipid galactose and were detected with the naphthoresorcinol–sulfuric acid reagent (Pastuska, 1961). There was a continuous decline in R_F of lipid material progressing from pool 1 to 7. Pool 8, the largest, was the only one giving single spots on these chromatograms (R_F 0.40 and 0.18 for the two systems, respectively). Pool 3, the second in galactose content, contained at least four components.

ISOLATION OF THE CERAMIDE PENTAGLYCOSIDE (CPG).

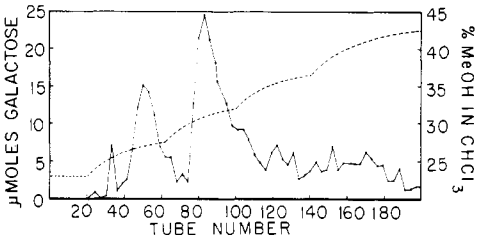

FIGURE 2: Adsorption chromatography of the methanol precipitate fraction of dog intestine ceramide oligohexosides. The column was 31-mm id, contained 250 g of silicic acid, and was charged with 0.78 mmole of lipid galactose. The fraction volume was 25 ml. The gradient was started with tube 20 and the estimated concentration of methanol in chloroform throughout the elution is shown by the dotted line. The solid line represents the micromoles of lipid galactose found in each fraction.

A. METHANOL PRECIPITATION. Fractions DEAE-1 and DEAE-2 equivalent to 1 mmole of lipid galactose were taken up in 9 ml of hot absolute methanol. The small amount of insoluble material was removed by centrifuging and the supernatant solution was refrigerated overnight. A voluminous white precipitate was formed, separated in the centrifuge, and washed with 2.5 ml of cold methanol. The combined supernatant and washings

TABLE VI: Composition of the Pooled Fractions from Silicic Acid Chromatography of Fractions DEAE-1 and DEAE-.2

Fraction Group	Tube No.	Total Nitrogen (μmoles)	Galactose (μmoles)	Nitrogen/ Galactose Ratio
A	20–43	9.52	18.4	0.516
B	44–70	31.4	59.9	0.525
C	75–110	347	388	0.895
D	111–200	138	214	0.645

TABLE VII: Composition of Dog Intestine Gangliosides.[a]

	Pool 3	Pool 8	Calcd[b]
Mass	1480	1380	1412
Total Hexose	2.40	2.57	3.0
Sialic acid	1.02	0.93	1.0
Galactosamine	0.041	0.147	0.0
Glucose	0.80	0.78	1.0
Galactose	1.60	1.80	2.0
Total nitrogen	2.46	2.32	2.0
Chromic acid uptake of $CHCl_3$ fraction after hydrolysis (μ-atoms of O)	130	121	115
Av mol wt of fatty acids	331	338	—
Distribution of fatty acids (%)			
24:0	38.4	45.8	
23:0 or 22 enes	7.1	7.9	
22:0	18.5	18.8	
20:0	11.8	9.9	
18 enes	2.0	1.7	
18:0	8.1	6.0	
16:0	14.1	9.9	

[a] All values are given in micromoles or micrograms per micromole of long-chain base. [b] Calculated for the magnesium salt of *N*-acetylneuraminylgalactosylgalactosylglucosylceramide containing sphingosine and behenic acid.

were concentrated to about 1.5 ml. The precipitate formed on cooling was washed with 1.5 ml of methanol. The two precipitates contained 795 μmoles of galactolipid and nearly all of the CPG. The supernatant contained 90 μmoles of galactolipid and had a N:galactose molar ratio of 0.88 compared with the ratio of 0.74 in the precipitate.

B. SILICIC ACID CHROMATOGRAPHY. A 31-mm id column was packed with 250 g of silicic acid in a slurry of chloroform–methanol (2:1). The column was then washed with 250 ml of chloroform. The methanol precipitate fraction containing 780 μmoles of lipid galactose was dissolved in 50 ml of 23% methanol in chloroform and placed on the column. The mixing vessel was charged with 2.5 l. of 23% methanol in chloroform and 500 ml of this solvent was placed in the reservoir. The elution was begun and the reservoir successively loaded with 1 l. each of 38, 43, 48, and 53% methanol in chloroform, respectively. Two hundred 25-ml fractions were collected and the column was finally stripped with 600 ml of 80% methanol in chloroform. Galactose determinations were made on every third or fourth tube and the resulting elution pattern is shown in Figure 2. A number of the fractions were tested for homogeneity using thin layer chromatography as described in the fractionation of the gangliosides. The fractions were combined into four groups on the basis of the thin layer chromatograms and the elution curve. The galactose and nitrogen content of these groups, A, B, C, and D, is given in Table VI. The composition of groups A, B, and D will be reported later. The largest, group C, the CPG, was homogeneous and was found by thin layer chromatography and subsequent analysis to be identical with the comparable fraction prepared from

TABLE VIII: Composition of the Ceramide Pentaglycoside.[a]

	CPG-1	CPG-2	Calcd[b]
Mass	1581	1650	1496
Galactosamine	1.88	1.95	2.0
Total hexose	2.85	3.15	3.0
Glucose	1.11		1.0
Galactose	1.74		2.0
Total nitrogen	2.96	3.16	3.0
Chromic acid (μatoms of O consumed)	190		179
Water-soluble nitrogen after hydrolysis	2.08	2.12	2.0
Chromic acid uptake of the $CHCl_3$ fraction after hydrolysis (μatoms of O)	113.8		111
Sialic acid (max)	0.037		0.0
Phosphorus (max)	0.026	0.044	0.0
Total sulfur		0.11	0.0
Av mol wt of fatty acids	321		
Distribution of fatty acids (%)			
24:0	21.7		
23:0 or 22 enes	8.8		
22:0	18.1		
20:0	17.7		
18:0	22.7		
16:0	11.1		

[a] All values are given in micromoles or micrograms per micromole of long-chain base. [b] Calculated for *N*-acetylgalactosaminyl-*N*-acetylgalactosaminylgalactosylgalactosylglucosylceramide with an average fatty acid molecular weight of 321.

the S50-P5 glycolipids. This preparation is designated CPG-1. Another batch of the CPG was prepared in identical fashion using a column load of 1.62 mmoles of lipid galactose. The resolution of the glycolipids with this load was as successful as with the smaller load. This preparation is designated CPG-2.

Composition of the Isolated Lipids.

Composition of the Gangliosides. The composition of the lipid of pool 8 is given in Table VII together with that of pool 3 which is included for comparison. The fatty acid distribution of the gangliosides was determined by the following procedure. An aliquot was refluxed at 58–62° for 2.5 hr in 6 ml of 0.3 N HCl in chloroform–methanol 2:1 (Sweeley and Klionsky, 1963). The hydrolysate was concentrated in the cold to 0.5 ml under reduced pressure and then partitioned between 7 ml each of chloroform and water. The chloroform-soluble fraction was refluxed for 8 hr in 6 ml of 3:1 methanol–concentrated HCl. The mixture was then diluted with 5 ml of methanol and refluxed again for 1 hr. The hydrolysate was concentrated under reduced pressure and then partitioned between 6 ml each of chloroform and water. An aliquot of the chloroform-soluble fraction was used to determine long-chain base. The rest was evaporated to dryness under reduced pressure and extracted three times with 3-ml portions of petroleum ether. The petroleum ether extracts contained the fatty acid methyl esters and very little long-chain base. Analysis of the mixture of esters was carried out by gas–liquid partition chromatography using columns with 25% succinate polyester in acid-washed Chromosorb W at 209° and with 10% succinate polyester at 184 and 200.5°.

Composition of the Ceramide Pentaglycoside (CPG). The composition of the CPG is given in Table VIII.

CHLOROFORM–METHANOL–HCl HYDROLYSIS OF THE CPG. An aliquot of the CPG was hydrolyzed with this reagent as described for the gangliosides. The composition of the water- and chloroform-soluble fractions is given in Table IX. A portion of the chloroform-soluble fraction was subjected to preparative thin layer chromatography using silica gel G with the solvent system chloroform–methanol–water (65:25:4). Three major homogeneous lipid components with R_F values of 0.77, 0.59, and 0.40 were eluted from the gel with chloroform–methanol (1:4). These lipids were hydrolyzed by refluxing with 7 ml of 2 N HCl for 2.5 hr on the sand bath. The hydrolysates were then extracted with 10 ml of chloroform. Total nitrogen was determined on the chloroform-soluble fraction, and total hexose and glucose were determined on the aqueous fraction. The

TABLE IX: Composition of the Solvent–HCl Hydrolysate Fractions of the Ceramide Pentaglycoside.[a]

	Chloroform-Soluble	Aqueous
Total nitrogen	6.29	13.4
Galactose	15.3	9.46
Free galactosamine	—	0.0
After rehydrolysis with 2 N HCl–free galactosamine	0.15	11.2
Total hexose	10.4	8.80
Glucose	5.3	1.7
True galactose	5.1	7.1
Chloroform-soluble nitrogen	6.26	0.0
Hexose identification by tlc	Glucose + galactose	Galactose only

[a] All values are given in micromoles.

aqueous fraction was also examined for sugars by thin layer chromatography using silica gel G and the solvent systems 1-propanol–ethyl acetate–water (3:2:1) and 2-propanol–acetic acid–water (3:1:1) (Prey *et al.*, 1963). The sugars were detected with the naphthoresorcinol reagent. The composition of these three lipids is given in Table X.

The water-soluble fraction of the 0.3 N HCl–chloroform–methanol hydrolysate was deacidified on an anion-exchange column (Amberlite IR-45, OH^- form). The effluent was concentrated under reduced pressure at 65° to about 0.5 ml. The solution was applied in a narrow streak to 20 × 20 cm Whatman No. 1 filter paper and chromatographed in the solvent system 2-propanol–glacial acetic acid–water (3:1:1). Thin strips were cut from the chromatogram to serve as markers and developed with the anisidine–phthalate reagent (Schweiger, 1962). Two distinct components were separated with R_F values of 0.64 and 0.44. The chromatograms were then cut to include these substances: a strip from R_F 0.31 to 0.55 and a second from 0.55 to 0.76. The papers were eluted with water giving eluate A (R_F 0.64) and eluate B (R_F 0.44). The two eluates were chromatographed on silica gel G using the same solvent system used above. Eluate A revealed a single spot with the naphthoresorcinol reagent at R_F 0.73. Eluate B gave two components in this system: a major at R_F 0.63 and a minor at R_F 0.73. A 0.7-μmole aliquot of eluate A in 1 ml of sodium phosphate buffer, pH 7.0, was oxidized with 0.5 ml of 0.075 M sodium periodate. After 2.25 hr at room temperature the reaction was terminated by the addition of sodium metabisulfite. An aliquot of this mixture was hydrolyzed for the determination of galactosamine. An aliquot of this hydrolysate was chromatographed on tlc plates of silica gel G using propanol–ethyl acetate–water (3:2:1) and developed with the naphthoresorcinol–sulfuric acid reagent. Authentic samples of galactose and galactosamine were used for reference. The oxidation mixture contained a single chromatographic component having the mobility of galactosamine. The composition of the eluates and the products of periodate oxidation are given in Table XI.

PERIODATE OXIDATION OF THE CPG. Periodate oxidations of the intact CPG (1–6 μmoles) were carried out at pH 4 with sodium acetate buffer and at pH 6–7 with sodium phosphate buffer as described above. Aliquots of the mixture were then treated with 1 ml of 0.05 M sodium arsenite and the excess was titrated iodometrically with a microburet (Wingo, 1964). Other aliquots of the oxidation mixture were reduced with sodium metabisulfite for the determination of formaldehyde and for hydrolysis and determination of galactosamine. After 2 hr about 3 μmoles of periodate had been consumed/μmole of CPG. No formaldehyde was formed in this oxidation and 0.7–0.8 μmole of galactosamine/μmole of CPG had been destroyed. Periodate uptake continued slowly after 2 hr, reaching a maximum of approximately 3.5 μmoles/μmole of CPG at 4 hr.

TABLE X: Composition of the Isolated Chloroform-Soluble Components of the Ceramide Pentaglycoside Hydrolysate.

Component	R_F	Chloroform-Soluble Nitrogen after Hydrolysis (μmoles)	Glucose (μmoles)	True Galactose (μmoles)	Hexose: Nitrogen Molar Ratio	Hexose Identification by Tlc	Chromic Acid Uptake of the Intact Lipid (μatoms of Oxygen)
A	0.77	1.60	1.67	0.02	1.05	Glucose only	—
B	0.59	3.64	3.50	3.53	2.01	Glucose and galactose	550
C	0.40	2.71	2.84	5.16	2.82	Galactose and glucose	460

TABLE XI: Composition and Properties of the Oligosaccharides Isolated from the Water-Soluble Fraction of the CPG Hydrolysate.

	Eluate A	Eluate B
R_F paper (2-propanol–acetic acid–water, 3:1:1)	0.64	0.44
R_F silica gel G (2-propanol–acetic acid–water, 3:1:1)	0.73	0.63 and 0.73
True galactose (μmoles)	6.7	4.6
Galactosamine before hydrolysis	None	None
Primary amine before hydrolysis	None	None
Galactosamine after hydrolysis (μmoles)	13.3	1.8
Galactosamine:galactose molar ratio	1.99	0.39
Formaldehyde released by periodate oxidation (μmoles)	7.18	—
Galactosamine remaining after periodate oxidation (μmoles)	5.76	—
Sugars detected by tlc after periodate oxidation	Galactosamine only	—

Results

Composition of the Gangliosides. The ganglioside preparations (pools 3 and 8, Table VII) obtained have a similar chemical composition despite their chromatographic differences on tlc plates and on silicic acid columns. The pool 8 gangliosides, although homogeneous in tlc, are obviously contaminated with small amounts of a component containing galactosamine. The analyses indicate a ganglioside with two galactose, one glucose, and one sialic acid units to the ceramide. The lower than theoretical values for total hexose and higher values for nitrogen may be due to substitution of a galactosamine for a hexose in one of every seven ganglioside molecules. The low values for glucose are probably due to small losses incurred in hydrolysis. Neither the nature of the long-chain base nor the sequence of the glycosidic units to the ceramide has been determined.

Composition of the Ceramide Pentaglycoside. The CPG-1 and CPG-2 preparations gave generally good analytical data for a di-*N*-acetylgalactosaminyldigalactosylglucosylceramide (Table VIII). However, the values obtained for mass are 5.7 and 10.3%, respectively, higher than the theoretical. Some of this extra mass is due to the small amounts of contaminants which contain sulfur, phosphorus, and sialic acid. Preparation CPG-2 was dialyzed, but this may not have removed the small amounts of silicic acid and Florisil usually present in the parent fractions. However it is doubtful if there are organic constituents present in the lipid other than those given in Table VIII.

The CPG is a white solid, easily soluble in water and mixtures of chloroform and methanol. It is somewhat soluble in chloroform, carbon tetrachloride, and hot methanol and is insoluble in petroleum ether, ethyl ether, and acetone. It has been rechromatographed on silicic acid without change in composition or properties. On paper chromatograms, it gives a yellow-orange fluorescence of moderate intensity with rhodamine G. It has no electrophoretic mobility at pH 6.5 and is homogeneous in this respect. The infrared absorption spectrum is generally characteristic of glycolipid spectra. A peak at 980 cm^{-1} is probably due to a *trans* double bond, presumably in the long-chain base.

Hydrolysis of the lipid with 0.3 N HCl in chloroform–methanol occurred preferentially between the two galactose residues as indicated by the relative molar yields of ceramide glycoside fragments (Table X) and the free oligosaccharides (Table XI). However, significant hydrolysis also occurred at the glucose-galactose and at the galactosamine-galactose linkages, yielding enough of the ceramide mono- and trihexoside fragments for analysis and tentative identification. It is significant that galactosamine has never been found in the chloroform-soluble (ceramide) fraction of these hydrolysates. The trisaccharide isolated from the aqueous phase contained 2 moles of acylated galactosamine/mole of galactose. Periodate oxidation left only one intact galactosamine, placing this in the middle of the chain. The galactose is considered to be at the reducing end of this trisaccharide since (a) the ceramide trihexoside fragment contained two molecules of galactose, (b) galactosamine is not found in the ceramide fraction of these hydrolysates, and (c) periodate oxidation of the intact CPG destroys most of 1 mole of galactosamine, placing it at the nonreducing end of the CPG oligosaccharide.

Discussion

The glycolipids are minor constituents of the lipoproteins of many animal tissues. Although the amount of glycolipid may equal that of phospholipid in brain myelin, the lipid sugar:P molar ratios of other tissues are usually well below 0.10. In a series of dog tissues studied, the small intestine ranked second in proportion of glycolipid found with a ratio of 0.11 (J. M. McKibbin and H. W. Faulkner, 1962 unpublished). Several samples of small intestine including the musculature contained an average of 10.3 μmoles of lipid galactose and

95.3 μmoles of lipid P/g of dry lipid-free tissue. This was equivalent to 1.50 μmoles of lipid galactose and 13.9 μmoles of lipid P/g of fresh tissue. Two samples of intestinal mucosa contained 18 and 22 μmoles of lipid galactose and 137 and 214 μmoles of lipid P/g of dry lipid-free tissue. Heart muscle contained only 3.6 μmoles of lipid galactose and 144 μmoles of lipid P/g. It may be assumed, therefore, that the major portion of the intestinal glycolipid is derived from mucosal lipoproteins.

The initial objective of this study was to identify the glycolipids present in this tissue and to determine their quantitative distribution. The recovery of lipid throughout the procedure should bear heavily on the significance of the findings. A large number of preparations of this fraction have been made from a combined total of 25 kg of dog intestine. Recovery of phospholipid by this procedure is about 87% of that with the larger solvent volumes (McKibbin *et al.*, 1961). The recovery of ceramide oligoglycoside through the extraction and $MgCl_2$ purification procedures is uncertain. However, this procedure gives about fourfold greater recovery of liver ceramide oligoglycoside than the method of Folch *et al.* (1957).

The most difficult aspect of the preparation of the glycolipid fraction in high yield is the removal of phospholipid. Some investigators have used cold alkali saponification to assist in this removal on the assumption that these lipids contain no ester linkages (Svennerholm and Svennerholm, 1963; Martensson, 1963). Makita (1964) prepared the glycolipid fraction of human kidney by cold ether precipitation. The fraction was then chromatographed on a silicic acid–Hyflo Supercel column and these fractions were further resolved on Florisil columns. The remaining 10–20% phospholipid impurity was then removed by additional Florisil chromatography. Six glycolipid classes were obtained with a rough estimate of yield totaling 6.55 g for the five major substances from 30 kg of fresh tissue. Assuming average sugar percentages for these substances, the yield of lipid galactose was about 13 mmoles. This is only a third of the amount we have recovered from dog kidney by use of the procedure described above for intestine (J. M. McKibbin and H. W. Faulkner, 1962, unpublished). Aside from species differences, it seems certain that the procedure of Makita does not give good recovery of glycolipid although the lipids recovered may be representative of the total. The large-scale procedure developed in this laboratory for intestine and liver avoids the hazard of alkali treatment and has given reasonable recovery of glycolipid.

The silicic acid and Florisil chromatograms are highly reproducible from preparation to preparation. This is also true of the solvent fractionation step, although there is some variation in the distribution of glycolipid between funnels 4 and 5. The dialysis was necessary to remove the large amounts of free taurine which had persisted through the purification and fractionation procedures. Free taurine was found in the original tissue lipid extract and represents an artifact of extraction rather than one of hydrolysis.

The relatively high ratio of ceramide oligoglycoside to cerebrosides, cytosides, and trihexosides is a characteristic of small intestine, heart, lung, pancreas, and liver glycolipids and is in marked contrast to the distribution of these classes in myelin (J. M. McKibbin and H. W. Faulkner, 1962, unpublished). In the intestine the major component of the ceramide oligoglycoside group is the CPG which contains most of the hexosamine and about 30% of the hexose of intestine glycolipids. The gangliosides contain about 25% of all lipid hexose. The composition of the other lipids of this group will be reported later.

The structure of the CPG may be considered to be *N*-acetylgalactosaminyl-*N*-acetylgalactosaminylgalactosylgalactosylglucosylceramide. The sequence of the sugar residues is indicated by the products formed in the solvent–HCl hydrolysis. The glucosylceramide, galactosylglucosylceramide, and galactosylgalactosylglucosylceramide indicate the sequence from the ceramide end; the *N*-acetylgalactosaminyl-*N*-acetylgalactosaminylgalactose confirms this sequence from the nonreducing end of the oligosaccharide. The relative yield of the products favors an unbranched oligosaccharide. The periodate uptake of 3 moles/mole of intact CPG indicates at least one 1→3 linkage, but the exact nature of the linking must be established with methylation. The absence of formaldehyde from this oxidation mixture indicates no sugar residue with free C-5 and C-6 hydroxyl groups.

The intestine CPG is unique among the known glycolipids. In over-all composition it differs by addition of one *N*-acylgalactosamine residue from a widely distributed tetraglycoside class which contains *N*-acylgalactosamine, galactose, and glucose in the ratio of 1:2:1. Two such substances have been characterized and have the same sugar sequence from the ceramide as that of the CPG (Yamakawa *et al.*, 1963; Makita *et al.*, 1964). The CPG oligosaccharide is unique and its component *N*-acylgalactosamine disaccharide is also unique, although Burton and Carter (1964) isolated a glycolipid from *Escherichia coli* which contained a disaccharide of glucosamine as the only carbohydrate component. Hakomori and Jeanloz (1964, 1965) have also isolated a ceramide pentaglycoside from human adenocarcinoma. This substance, fucosylgalactosylglucosaminylgalactosylglucosylceramide, was not found in significant quantities in normal gastric mucosa.

The intestine gangliosides are distinguished from brain gangliosides of all species in their low content of stearic acid, preponderance of C_{22} and C_{24} acids, and low content of hexosamine. Although most of the ganglioside fractions are not chromatographically homogeneous, it is doubtful if significant amounts of other glycolipids are present. If so, the consistent ceramide:sialic acid ratios of unity indicate that there are no di- or trisialogangliosides in dog intestine lipoprotein.

Acknowledgment

The authors are indebted to Dr. Robert C. Caldwell

for the sugar chromatograms, to Dr. James W. Woods for guidance and assistance with the gas–liquid partition chromatographic analyses, and to Miss Martha Deanna Fendley for valuable technical assistance.

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