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The Isolation and Partial Structure Identification of a Polar Glycosphingolipid of Rabbit Blood

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**THE ISOLATION AND PARTIAL STRUCTURE
IDENTIFICATION OF A POLAR GLYCOSPHINGOLIPID
OF RABBIT BLOOD**

By

Michael B. Shubeck

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
Degree of Master of Arts

**Western Michigan University
Kalamazoo, Michigan
August 1975**

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Michael B. Shubeck

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LIST OF ABBREVIATIONS

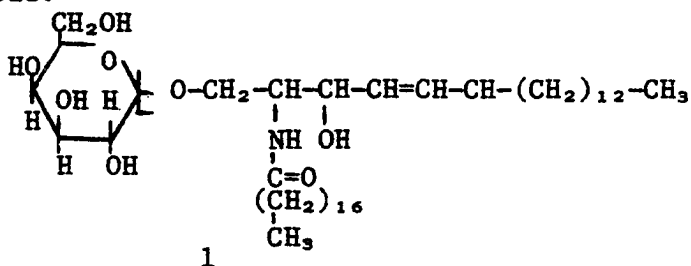
<u>Term</u>	<u>Abbreviation</u>
Chloroform	C
Average Chain Length	ACL
Acetone-Methanol (9:1) Fraction	F-2
Gas Liquid Chromatography	GLC
Unknown Glycolipid	GL-5
Methanol	M
Plasma	P
Pentaglycosylceramide	PGC
Red Blood Cells	RBC
Thin Layer Chromatography	TLC
Water	W

INTRODUCTION

The first reported study of glycosphingolipids was in 1884 by Thudichum¹. He spent the better part of a lifetime working with this unusual class of lipids. Progress in this field was slow. It was not until 1929 that the correct chain length of the sphingosine base (18 carbons) was established by Klenk¹. The correct structure of the sphingosine base (2-amino-1,3-dihydroxyalkene) was established in 1942 by Carter et al.¹.

Due to the high concentration of these lipids in the brain, much work has been done in isolation and structure identification from this tissue. With the recent advent of more sophisticated methods for isolation and structural studies, new classes of sphingolipids have been found using brain tissue as a source material as well as other body tissues and fluids². Of special interest to this investigation is the occurrence of glycosphingolipids in blood³⁻⁸.

Glycosphingolipids consist of long chain bases which are linked by an amide bond at the C-2 position to a long chain fatty acid. The hydroxyl group at C-1 is condensed with a carbohydrate moiety, while a C-3 hydroxyl group is free. The following is an example of a glycosphingolipid.



The common generic name for this compound is cerebroside or galactosylceramide. The accepted IUPAC nomenclature for this compound is N-stearyl-1-O-D-galactosyl-4-sphinganine⁹. Members of this class of compounds vary according to the nature of the fatty acid moiety, the carbohydrate moiety, and the sphingosine base. The fatty acid moiety can have from 12 to 28 carbons with even numbers of carbons predominating. The fatty acid may be fully saturated or have 1 or 2 double bonds. There also may be a hydroxy group at C-2. The carbohydrate moiety may have one or more sugars. The sphingosine base may lack the double bond at position 4 in which case the common 18 carbon base has the IUPAC name sphinganine. The carbon chain length of the base may also vary between 14 and 26 carbons with an even number of carbons predominating.

The objective of this investigation was to partially identify a glycosphingolipid that occurs in rabbit blood. In a previous investigation made by Coles¹⁰ a particularly polar glycosphingolipid was found in rabbit blood but not in porcine blood. The method of isolation involved the Folch partition¹¹ and subsequent purification steps as described by Vance and Sweeley¹². Analysis of this compound by Coles¹⁰ led to the conclusion that it was a mixture of lipids containing a high percentage of hematoside. A hematoside is a glycosphingolipid containing two moieties of hexose, one moiety of sialic acid, one moiety of fatty acid, and one moiety of sphingosine. Coles found the hexose/fatty acid ratio of this compound to be 2/1, a hexose/sialic acid ratio of 2/0.85, and a galactose/glucose ratio of 2/1. These data correspond to the

theoretical ratios of hemaoside except for the galactose/glucose ratio, which should be 1/1.

Coles used thin layer chromatography (TLC) to compare the unknown compound with authentic hemaoside isolated from human spleen¹³. Two thin layer chromatography systems were used: chloroform-methanol-water (100:42:6 v/v/v) and 70% propanol in water. The unknown glycosphingolipid migrated with hemaoside in both systems.

A pentaglycosylceramide reported by Yamakawa et al.¹⁴ to be the major glycosphingolipid in rabbit blood was also compared with the unknown glycosphingolipid. This compound has a galactose/glucose ratio of 3/1 and also contains N-acetyl-glucosamine. For these reasons it was concluded that the unknown glycosphingolipid could not be identical to Yamakawa's compound.

The present investigation was undertaken to determine whether the unknown glycosphingolipid was identical to that reported by Yamakawa, or whether the two isolation procedures actually purified different substances. To this end, both lipid extraction methods, Sweeley¹², and Yamakawa¹⁴, were employed in this study using whole rabbit blood, erythrocytes (RBC), and plasma (P), along with several analytical methods to elucidate the components of this glycosphingolipid.

The specific determinations made with whole blood, RBC, and plasma using both methods were as follows:

- (1) Quantitative determination of the unknown glycosphingolipid (GL-5)

(2) Quantitative and qualitative analysis of fatty acids, hexose, hexosamine, sialic acid and sphingosine.

This includes determining ratios of various moieties of GL-5.

MATERIALS AND METHODS

All reagents used were reagent grade. Chloroform was distilled before use. Methanol was purchased glass-distilled.

Blood Fractionation

Blood used for the isolation of glycosphingolipids was from mature rabbits (Pel-Freeze Biologicals, Inc.). Plasma was obtained by centrifuging whole blood at 3,000 x g for 15 minutes and decanting the supernatant liquid. It was stored under N₂ at -20°C until used. The buffy coat layer was removed from RBC with suction. RBC were resuspended in isotonic saline solution and centrifuged again at 3,000 x g for 15 minutes. The supernatant was removed and the RBC were washed again as before with isotonic saline. RBC were stored under the same conditions as plasma.

Lipid Extraction by Aqueous Partition

Following Sweeley's method¹², samples of whole blood, RBC, or plasma in 50 ml aliquots were mixed with 300 ml methanol. To this was added 600 ml chloroform and stirred with a magnetic stirrer for 15 minutes. The mixture was filtered and the residue washed with 100 ml chloroform-methanol (2/1 v/v). For whole blood and RBC, the residue was reextracted with 350 ml chloroform-methanol (2/1 v/v) by refluxing for 2 hr. and the two extracts combined. This crude extract was shaken with 0.2 its volume of water and the

mixture allowed to separate overnight. The upper layer was removed by suction and discarded. The lower layer was taken to dryness on a rotary evaporator. This residual material was termed crude lipid.

The crude lipid was placed on a column (1 cm I.D. x 30 cm) containing 4 g activated silicic acid and was eluted in a stepwise fashion using 100 ml chloroform, and 200 ml acetone-methanol (9:1 v/v). The acetone-methanol fraction (F-2) was taken to dryness and termed crude glycosylceramides.

Phospholipids were removed from the crude glycosphingolipids by alkaline hydrolysis and by extraction of the resulting glycerol phosphate into an aqueous phase¹². The organic phase was taken to dryness, redissolved in a minimum amount of chloroform-methanol (1:1) and applied as a streak to a TLC plate coated with activated silical gel G. The plate was placed in a tank with paper liner and a solvent mixture of chloroform-methanol-water (100:42:6 v/v/v). Individual lipids were located on the developed plate by brief exposure to I₂ vapor. The material, appearing as a streak, between Rf .055 - .18 was removed, and eluted from the silica gel with chloroform-methanol-water (7:7:1). This material was termed GL-5 as was also done by Coles.

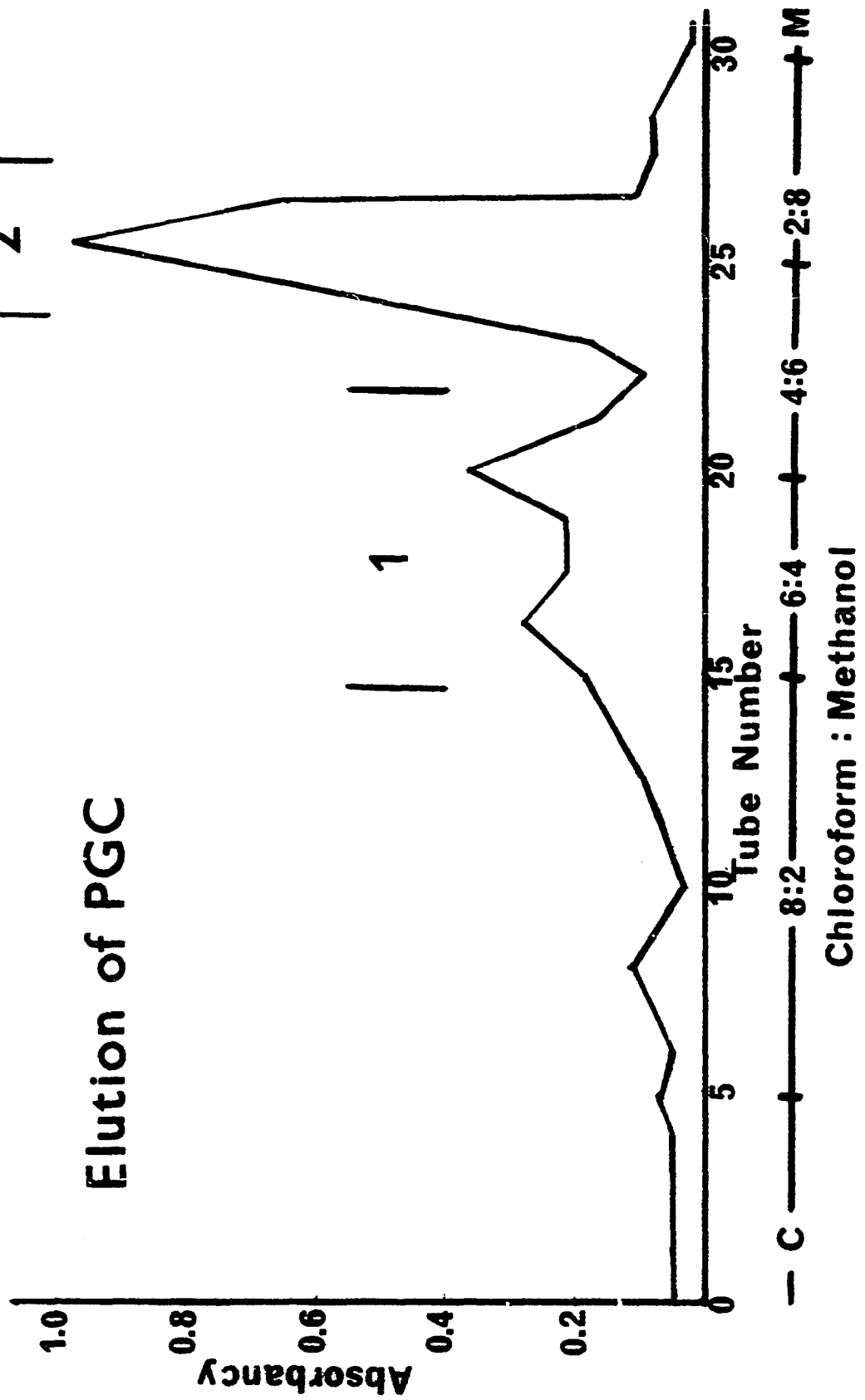
Isolation of Pentaglycosylceramide

Whole blood or RBC was used in this extraction method; both were treated the same, following Yamakawa's method¹⁴. Samples of 200 ml were dehydrated by three treatments with 10 volumes of acetone. This removed some lipids and other water soluble materials.

The dried acetone powder was then extracted with 19 volumes (w/v) of chloroform-methanol (2/1 v/v) by gentle refluxing for 4 hours. The extract was concentrated to a small volume under reduced pressure and treated with methanolic NaOH¹⁵. This mixture was then acidified with dilute HCl and dialyzed against distilled water for 4 days. Hydrolysis and dialysis removed alkali labile glycerophospholipids and low molecular weight contaminants. The dialyzed material was evaporated to dryness under reduced pressure and then redissolved in a small volume of chloroform-methanol (2/1 v/v). A crude glycosphingolipid fraction was obtained from this by precipitation with 10 volumes of acetone.

The precipitate was suspended in a minimal amount of chloroform and loaded onto a column (2 cm I.D. x 25 cm). The column was prepared from a thin chloroform slurry of 15 g silicic acid (unactivated) and Hyflo-Supercel (Johns-Manville) (2:1 w/w). Stepwise elution was performed with increasing concentrations of methanol in chloroform (see Figure 1). 10 ml fractions were analyzed for hexose using the anthrone method¹⁶. Tubes were combined to form two fractions (1 and 2) as shown in Figure 1. Fraction 2 was taken to dryness and then redissolved in 1 ml of chloroform-methanol (2:1 v/v). Ten ml acetone was added and the resulting precipitate was collected. The precipitate was further purified by TLC or by mild alkaline methanolysis. The product was termed pentaglycosylceramide (PGC).

Figure 1



Isolation of Hematoside from Human Spleen

Hematoside was isolated from human spleen as described by Svennerholm¹⁷. This involved a chloroform-methanol (2:1 v/v) extraction of acetone-dried tissue by refluxing and then an additional reflux extraction of the first residue with chloroform-methanol (1:2 v/v). The second extract was taken to dryness. The chloroform-methanol (2:1 v/v) fraction was then extracted 5 times, once with a 0.1% saline solution then 4 times with chloroform-methanol-water (1:10:10 v/v/v). The upper phases were combined and dialyzed against distilled H₂O. The dialyzed lipid and the dried chloroform-methanol (1:2 v/v) extract were combined and termed crude gangliosides. Hematoside was isolated from the crude gangliosides by silicic acid chromatography and TLC¹⁸.

ANALYTICAL METHODS

Methyl esters of fatty acids were obtained from GL-5, PGC, or hematoside by methanolysis with 1.0 N HCl in methanol for 24 hours at 80°C. This released fatty acid methyl esters, methyl glycosides, and sphingosine bases. The methyl esters were extracted into hexane¹². An internal standard of methyl arachidate was added and the solvent was removed under a stream of N₂.

Fatty Acids

Samples of fatty acid esters were injected onto a 1.8 m column (diethylene glycol succinate as the liquid phase) at 160°C with a temperature program increasing temperature at 4°/minute up to 220°C. The identities of individual peaks were assigned by comparing the retention times of all the major peaks with those of a mixture of authentic fatty acid standards. The internal standard served as a reference for quantitative determination of the various components on the basis of the areas under the peaks.

Glucose, Galactose, and Sphingosine

Glucose and galactose, in the form of methyl glycosides, and sphingolipid bases were obtained after acid catalyzed methanolysis of GL-5, PGC, or hematoside with 1.0 N HCl in methanol. All samples were treated the same. Mannitol was added as an internal standard. After the extraction of fatty acid methyl esters the solution was

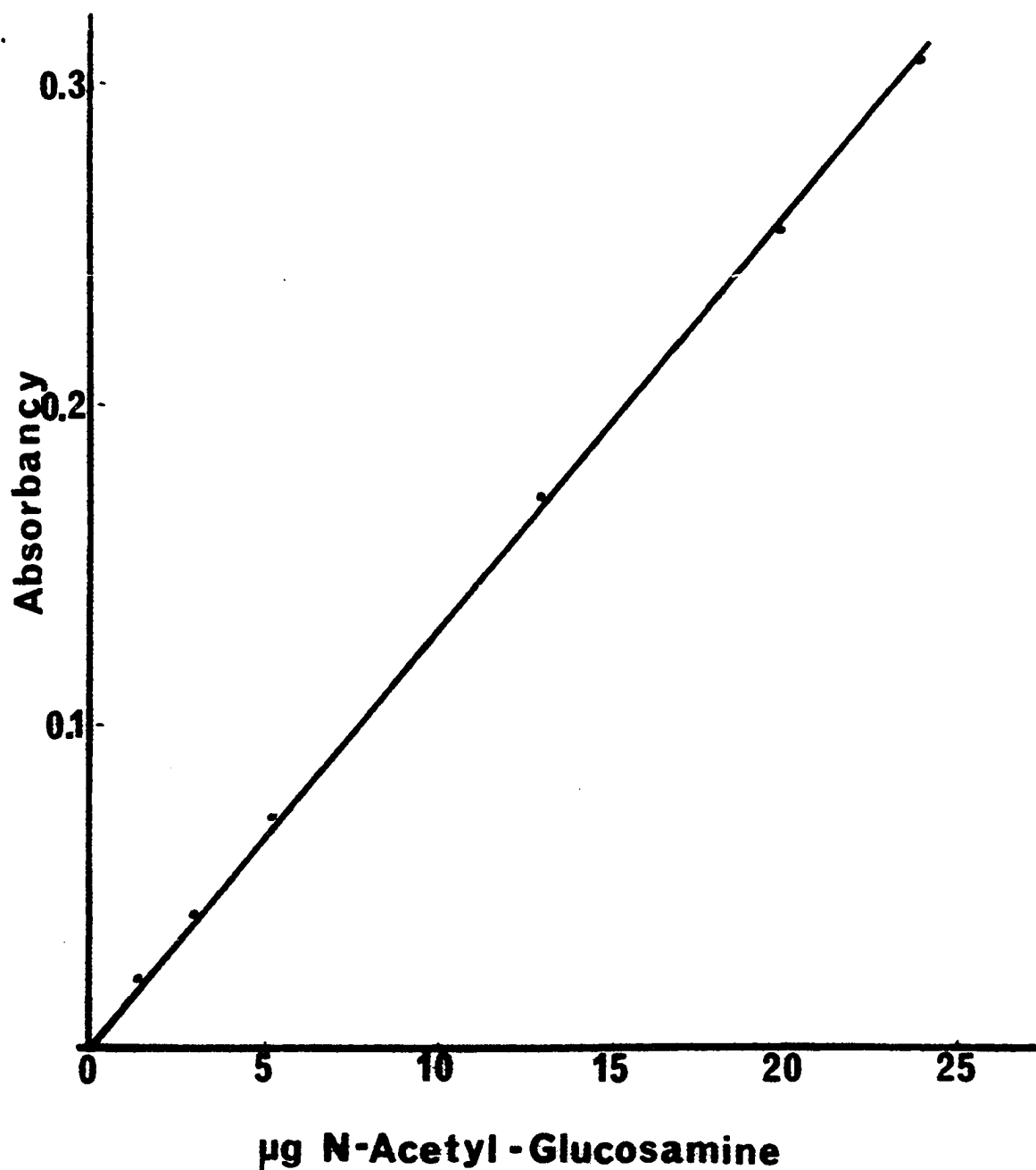
percolated through 1 gram of Amberlite IR 45CP resin (OH phase) packed in a small column. The resin was then washed with methanol. Solvent was removed in vacuo.

Trimethylsilyl derivatives were prepared from the dry residue by the addition of 1 ml Sil-Prep (Applied Science Laboratories, Inc.) silylating reagent. After 15 minutes an aliquot was injected onto a 1 m column containing 2% SE-30 silicone liquid phase (Applied Science Laboratories, Inc.) at 160°C. After 10 minutes the temperature was increased at the rate of 10°C/minute up to 200°C¹². Peaks representing glucose, galactose, and sphingosine were identified by comparing retention times with peaks of authentic standards. The mannitol was used for a quantitative measure of these components.

Hexosamine

A standard curve was prepared for the colorimetric determination of hexosamine using authentic N-acetyl-glucosamine (Figure 2). The standards and samples were treated identically¹⁹. Hydrolysis was brought about in screw cap tubes with teflon lined caps; 2 N HCL was added and the tubes sealed and placed in an oven at 75-80°C for 20 hours. After cooling, the tubes were opened, the contents neutralized with 2 N Na₂CO₂, followed by the addition of acetylacetone, ethanol, and Ehrlich's reagent. The absorbancy was measured at 530 nm against a reagent blank.

Figure 2
Absorbancy vs. N-Acetyl-
Glucosamine



RESULTS

Analysis of Glycosphingolipids from Rabbit Blood

The quantities of crude lipids and glycosphingolipids obtained using Sweeley's method are presented in Table 1. Each entry represents an average of at least five runs. The values for crude glycosylceramide are average amounts applied to TLC plates. The values for GL-5 are average amounts of GL-5 recovered from TLC plates. Values for crude lipid are slightly higher than those reported by Coles. Normal blood was not analyzed for GL-5 by Coles. The only GL-5 from Coles' study was from rabbits on a 180-day diet of 1% cholesterol and 2.8% lard. For RBC 0.898 $\mu\text{mol}/100 \text{ ml}$ RBC was found, and for plasma, 0.115 $\mu\text{mol}/100 \text{ ml}$ plasma. This can be compared with the values for GL-5 in this investigation, keeping in mind the high fat diet.

The amounts of crude glycosphingolipids and pentaglycosylceramides from rabbit blood using Yamakawa's method are presented in Table 2. Yamakawa's value for RBC crude glycosphingolipids was 13.2 $\text{mg}/100 \text{ ml}$ and pentaglycosylceramide was 1.15 $\mu\text{mol}/100 \text{ ml}$. Whole blood was not analyzed by Yamakawa.

Fractions of GL-5, PGC, and hematoside were subjected to acid methanolysis. This released fatty acid methylesters, methyl glycosides and sphingosine bases. These components were analyzed by GLC and the ratios of glucose to fatty acid, galactose to glucose,

TABLE 1

Crude Lipids and Glycosphingolipids from
Rabbit Blood

Sweeley Method

Source	Crude lipids mg/100 ml	Crude Glycosylceramide mg/100 ml	GL-5 umol/100 ml
RBC	860 (110) [*]	40.5 (7.0)	1.2 (0.1)
Plasma	390 (50)	21.1 (3.6)	0.18 (0.2)
Whole Blood	990 (140)	39.0 (7.5)	1.3 (0.2)

^{*} Standard Deviation

TABLE 2

Glycosphingolipids and Pentaglycosylceramides from
Rabbit Blood

Yamakawa Method

Source	Crude Glycosphingolipids mg/100 ml	Pentaglycosylceramide umol/100 ml
RBC	25.8 (4.5) [*]	3.72 (0.8)
Whole Blood	24.0 (4.5)	2.89 (0.8)

^{*} Standard Deviation

and galactose to sphingosine were calculated. The results are presented in Table 3. Though hexosamine was found in GL-5 and PGC by colorimetric methods, no peaks were seen for hexosamine on GLC. Hexosamine was not found in hematoside using either method.

Table 4 summarizes the fatty acid composition found in whole blood GL-5 and PGC, along with fatty acids found in spleen hematoside. Fatty acids are denoted as chain length: number of double bonds. The amounts are given as a percentage of the total area measured on gas chromatograms. Peak areas were measured by planimetry. As can be seen, the fatty acid distribution of GL-5 and PGC predominant fatty acid for spleen hematoside was 18:0.

Thin Layer Chromatography of Glycosylceramides

Preparative TLC was performed routinely for the isolation of GL-5 from whole blood, RBC, or plasma. All plates were essentially the same except for relative intensity of the bands. Figure 3 is a picture of a typical TLC plate. GL-5 and hematoside were scraped from TLC plates with a razor blade and eluted from the silica gel with several portions of chloroform-methanol-water (7:7:1 v/v/v). For comparisons of R_f values of GL-5, PGC, and hematoside Permakote TLC plates were used (Applied Science Laboratories, Inc.) These plates have a permanent reusable silica gel adsorbant coating that is much thinner than the adsorbant coating of the preparative plates. This resulted in less spreading of spots and, hence, better resolution was achieved.

TABLE 3

Molar Ratios of Lipid Components

Method	Source	Hexose/ Fatty Acid	<u>Galactose</u> Glucose	<u>Galactose</u> Sphingosine
Sweeley (GL-5)	Whole Blood	3.7	3.1	2.9
	RBC	3.7	3.7	2.7
	Plasma	3.8	3.5	2.8
Yamakawa (PGC)	Whole Blood	3.9	3.5	2.9
	RBC	3.9	3.1	3.0
Svennerholm (Hematoside)	Spleen	1.9	1.5	0.9

TABLE 4

Glycosphingolipid
Fatty Acid Composition

Fatty Acid ¹	GL-5	PGC	Spleen Hematoside
14:0	1.5 ²	1.3	1.1
16:0	10.8	7.3	13.2
18:0	7.7	7.2	22.3
18:1	7.8	7.6	14.3
18:2	4.1	4.0	2.2
20:0			1.2
20:1	T ³	T	4.2
22:0	14.1	16.8	10.4
24:0	9.3	9.3	7.3
24:1	37.2	35.9	13.7
24:2	2.3	2.5	5.2
26:0	3.2	4.1	6.0
ACL ⁴	21.4	21.6	20.3

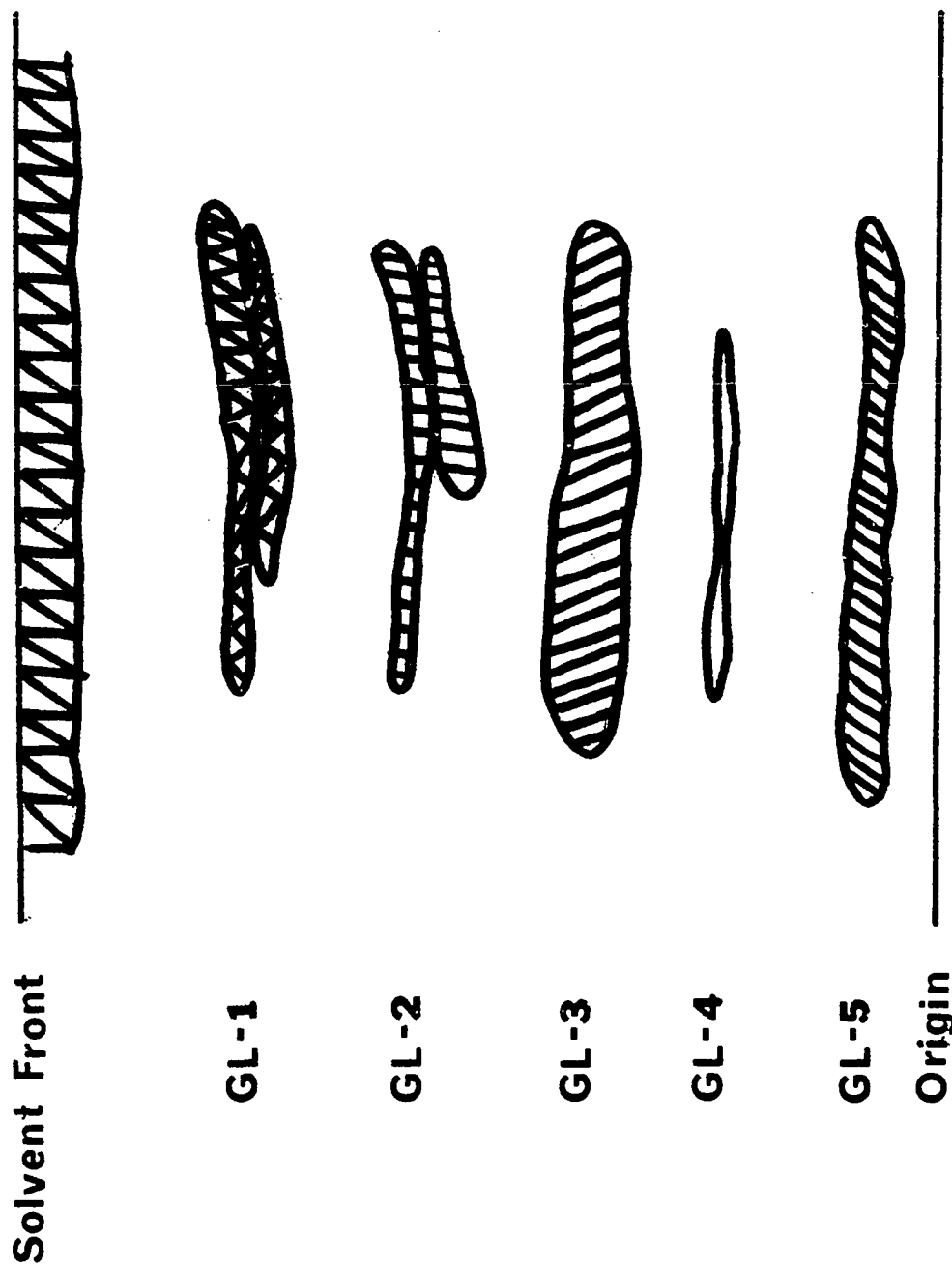
¹Fatty acids are denoted as chain length: number of double bonds.

²Amounts are given as a percentage of total area on gas chromatograms.

³Trace (less than 1% of sample).

⁴Average chain length.

Figure 3
Thin Layer Chromatography



Thin layer chromatography of GL-5, PGC, or hematoside was performed using 4 different solvent systems. The results are shown in Table 5. GL-5 from whole blood, RBC, or plasma co-chromatographed with PGC of whole blood or RBC in all 4 solvent systems. Hematoside co-chromatographed with GL-5 or PGC only in the 70% aqueous propanol solvent system.

Determination of Hexosamine

In the colorimetric determination of hexosamine the relationship between absorbancy and hexosamine concentration was linear over the range of 0-25 ug/sample (see Figure 2). Concentrations above 25 ug/sample showed a negative deviation from a straight line. Results of the hexosamine determination on GL-5, PGC, and hematoside are presented in Table 6. Yamakawa found 12.4% hexosamine in PGC. Yamakawa's theoretical value was 15.3% hexosamine.

TABLE 5

Thin Layer Chromatography of GL-5, PGC and Hematoside

<u>Method</u>	<u>Source</u>	<u>Solvent System</u>			
		<u>C:M:W</u> 100:42:6	<u>C:M:W</u> 60:35:8	<u>70% Aqueous</u> <u>Propanol</u>	<u>C:M:7% NH₃</u> 55:40:10
		<u>R_f</u>			
Sweeley (GL-5)	Whole Blood	.11	.57	.65	.72
	RBC	.11	.57	.65	.72
	Plasma	.11	.57	.65	.72
Yamakawa (PGC)	Whole Blood	.11	.57	.65	.72
	RBC	.11	.57	.65	.72
Svennerholm (Hematoside)	Spleen	.16	.65	.65	.85

TABLE 6

Analysis of Hexosamine in GL-5, PGC, and Hematoside

Glycosphingolipid	Source	Percent Hexosamine by wt.	Standard Deviation
(Sweeley) GL-5	RBC	15.3	(0.6)
	Plasma	12.7	(0.3)
	Whole Blood	14.1	(0.5)
(Yamakawa) PGC	Whole Blood	14.2	(0.5)
(Svennerholm) Hematoside	Spleen	0	(0.1)

DISCUSSION

From the TLC data (Table 5) it can be concluded that hematoside and GL-5 are two different compounds. Coles observed similar chromatographic properties of these two compounds in the chloroform:methanol:water (100:42:6 v/v/v) and the 70% aqueous propanol solvent systems. As can be seen from the data in Table 5, it is possible for two different compounds to have similar migrating properties in one particular solvent system. However for two different compounds to co-chromatograph in two entirely different solvent systems is very unlikely. All parts of the molecule must contribute to the adsorption-desorption process on the TLC plate. Perhaps in certain solvent systems the carbohydrate moiety is more influential than the fatty acid or sphingosine moieties. In considering compounds such as these in which the fatty acid composition may vary one could suppose that two glycosphingolipids with different carbohydrate moieties may possibly co-chromatograph in two different solvent systems due to their fatty acid moieties being a predominant factor in the adsorption-desorption process. In the long run though, the property of a compound involved in TLC will be influenced by all parts each to a greater or lesser extent. The proper solvent system would in the end bring these differences out.

It was observed in this investigation that when GL-5 and hematoside were put on preparative TLC plates side by side and eluted with chloroform-methanol-water (100:42:6 v/v/v) as was done

many times in order to pool these compounds, the R_f values were essentially the same. The relatively thick coating of silica gel (approximately 0.6 mm) seemed to cause the bands to spread thus making measurement of R_f less accurate. In most cases GL-5 and hematoside appeared to have the same migration on these preparative plates.

On Permakote plates, the much thinner coat of silica gel (approximately 0.25 mm) resulted in less spreading of spots. The increased resolution resulted in observed differences in R_f values of GL-5 and hematoside. TLC results also left little doubt that GL-5 and the pentaglycosylceramide obtained using Yamakawa's method were in fact the same compound.

Since PGC has five sugars in its carbohydrate moiety it is a very polar lipid. Defining it as a lipid requires some qualification. The general definition of a lipid is a biological compound that is soluble in fat solvents, i.e. chloroform, methanol, benzene, and acetone²⁰. PGC was insoluble in acetone. In Yamakawa's method, acetone was used to precipitate PGC, and in Sweeley's method the solute of the F-2 fraction from the silicic acid column (acetone-methanol 9:1 v/v) would occasionally precipitate out.

The low recovery of PGC using Sweeley's method (Table 1) compared to that using Yamakawa's method (Table 3) may be due to a large loss during the 'Folch Wash' of the chloroform-methanol (2:1 v/v) extract. Most of the PGC may have gone into the aqueous methanolic layer along with the gangliosides and other more polar lipids. In an attempt to make GL-5 less soluble in the upper layer,

the upper layer was made 0.1 M in KCl. The addition of salt did not increase the amount of PGC isolated from the lower layer, but did reduce the amount of pigmented contaminants in the F-2 and GL-5 fractions. This made it advantageous to use the KCl upper layer in Sweeley's method throughout the investigation.

Sweeley's method was initially devised to get glycosphingolipids from human blood containing up to 4 sugar moieties. The occurrence of the more polar GL-5 in Coles' study was not expected. Although Sweeley's method yielded a greater amount of total glycosphingolipids, Yamakawa's method was approximately 2.5 times more efficient for isolating PGC from rabbit blood. Yamakawa's method is also faster. Not only is the overnight wait for equilibration of the aqueous partition eliminated, the need for preparative TLC is eliminated as well. In Sweeley's method, the RBC and whole blood fractions were contaminated with pigment after the silicic acid chromatography step, and could not be separated during TLC. In Yamakawa's method this problem seemed to be eliminated by the acetone precipitation of the crude glycolipid fraction.

The galactose/glucose ratios found by GLC were somewhat higher than expected for PGC if Yamakawa's structure is accepted. It would be expected that equal amounts of these sugars would give equal peak areas since they are stereoisomers; however, experiments using equal amounts of glucose and galactose, and experiments using hydrolyzed lactose gave a galactose/glucose ratio larger than one. This problem has been documented in the literature²¹. One possible cause for the high ratio may be due to the GLC detector being more

sensitive to galactose.

Another possible cause for the high galactose/glucose ratio could have been incomplete acid methanolysis. If the methanolysis was carried out in a HCl concentration less than 1 N or if it was carried out for less than 20 hours, then the galactose/glucose ratios were constantly high. Since glucose is attached to sphingosine and is presumably the last hexose cleaved, the glucose values may be low. Hence, the ratios would be high.

In the determination of hexosamine, the average percentage of hexosamine was 14.1% for all samples. This represents one mole hexosamine per 1560 molecular weight units. The theoretical molecular weight of the pentaglycosylceramide described by Yamakawa is 1427 with a 20 carbon saturated fatty acid.

SUMMARY

From the GLC and TLC results obtained in this study, it is concluded that the unknown compound denoted GL-5 and first described by Coles in his original report is indeed the pentaglycosylceramide reported by Yamakawa. It is further concluded that GL-5 contains 3 moieties galactose, 1 moiety glucose, 1 moiety N-acetylglucosamine, 1 moiety fatty acid and 1 moiety sphingosine base.

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