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A STUDY OF GLYCOSPHINGOLIPIDS IN AORTA, BLOOD AND SPLEEN AND THE EFFECTS OF ATHEROGENIC DIETS ON THESE LIPIDS IN RABBITS

by

Eric Coles

A Dissertation
Submitted to the
Faculty of the Graduate College
in partial fulfillment
of the
Degree of Doctor of Philosophy

Western Michigan University
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Biochemistry

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LIST OF ABBREVIATIONS

Term	Abbreviation
Bovine	B
Porcine	P
Rabbit	R
Erythrocyte	E or RBC
Plasma	P
Aorta	A
Spleen	S
Leukocyte	L
High-density Lipoprotein	HDL
Low-density Lipoprotein	LDL
Very Low-density Lipoprotein	VLDL
Monoglycosylceramide	GL-1
Diglycosylceramide	GL-2
Triglycosylceramide	GL-3
Tetraglycosylceramide	GL-4
Hematoside	GL-5
Average Chain Length	ACL
Thin-Layer Chromatography	TLC
Gas-Liquid Chromatography	GLC
Trace	T
Not Analyzed	NA

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LIST OF ABBREVIATIONS
(Cont.)

Term	Abbreviation
Not Found	NF
Disintegrations per Minute	dpm

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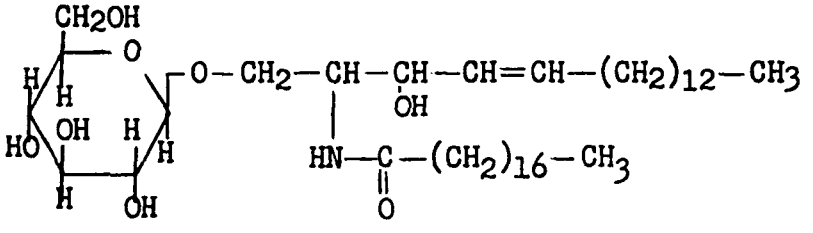
INTRODUCTION

Glycosphingolipids were first isolated by Thudecum in 1884 from brain. Their composition and metabolism in brain have been well studied by numerous investigators (1,2). More recently, these compounds have been obtained from other body tissues (2). Of particular interest in this study was their discovery in aorta (3-5) and blood (6-12).

Research in the area of glycosphingolipids has been primarily directed to analysis and metabolism. This study was designed to determine the detailed structure and possible metabolic interrelationships of this family of lipids in aorta and blood. In order to fully understand the biochemical functions of these compounds, we must first know their distribution, structure and metabolism.

Glycosphingolipids are a family of polar lipids, in which a long chain base is linked, by an amide bond, to a long chain fatty acid and, through a primary hydroxyl group, to a carbohydrate moiety. The carbohydrate may contain from one to five hexose units connected to each other by glycosyl bonds and finally to the hydroxyl group of the long chain base as a β-glycoside. The following structure is an example of a simple glycosphingolipid.



The accepted nomenclature for this compound is . . . N-Stearyl-1-O-D-glucosyl-4-sphingenine (13). This compound is also called a cerebrosde

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or glycosyl ceramide. Other members of the glycosphingolipid family are formed by the addition of one or more hexose moieties to the terminal hexose, through glycosidic bonds. There are many slight variations in the structure of naturally occurring glycosphingolipids. The common name for the base illustrated above is sphingosine. The IUPAC name for sphingosine is 2-amino-4-octadecene-1,3 diol. If the double bond is not present, its common name is dihydrosphingosine and the accepted name is sphinganine (13). Similar dihydroxy amines present in glycosphingolipids contain from fourteen to twenty-six carbons, with the even numbered chain lengths predominating. The hexoses of animal glycosphingolipids are predominantly glucose, galactose and galactosamine. The most pronounced variation in glycosphingolipid structure is found in the fatty acid. The chain length varies from twelve to twenty-eight carbons. Although the even numbered chain lengths between sixteen and twenty-four predominate, all chain lengths are found. The fatty acid may be saturated or contain one or two double bonds. It may also contain a 2-hydroxyl group. A typical fatty acid distribution for a glycosphingolipid contains approximately fifteen fatty acids. Similarities between the fatty acids of two different glycosphingolipids, or the same glycosphingolipid from two different tissues, is evidence for a metabolic relationship between the lipids. That is, two glycosphingolipids may be interconverted by hexose addition or clevage, or a single glycosphingolipid from two tissues may actually be transported from one tissue to the other. This reasoning was employed throughout this study to relate different

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glycosphingolipids found in a particular organ or like glycosphingolipids found in different organs.

The long range objective of this investigation was to determine the origin of glycosphingolipids in aortic tissue. This objective led to a number of short range studies. These were as follows:

1. to study the synthesis of glycosphingolipids in aortic tissue.
2. to determine the quantity and fatty acid distributions of glycosphingolipids in aorta, spleen and various blood fractions for bovine and porcine.
3. to determine the quantity and fatty acid distribution of glycosphingolipids in aorta, plasma and erythrocytes of normal rabbits and rabbits fed various atherogenic diets.

With regard to the overall objective of the research, there are two distinct possible origins for glycosphingolipids in aortic tissue. These lipids may be synthesized in the aortic tissue. This possibility was explored in the first study mentioned above. The second possibility is that the glycosphingolipids in aorta originate in another tissue such as spleen or blood, and are absorbed into the aorta from the blood. If this is the case, the fatty acid distributions of a given lipid from aorta and from the suspected source, should be similar. The second study was designed to explore this possibility. The subjects of this study were bovine and porcine. For the bovine tissues, analyses were performed on aorta, spleen, erythrocytes, leukocytes and three plasma fractions: high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL). In the study with porcine, aorta, spleen, erythrocytes, HDL and LDL were analyzed. Relationships among the various glycosphinglipids in a

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given sample could be made utilizing the results from these studies.

The third study introduced another variable, i.e. the effect of an atherogenic diet on the fatty acid distributions of the glycosphingolipids. If rabbits are fed a diet containing large quantities of fats, they will develop atherosclerosis (14). This is a widely used model system for atherosclerosis and the effects of various dietary factors on the accumulation of lipid in aorta. The glycosphigolipids of aorta, erythrocytes and plasma were analyzed in this study. Again, relationships between a lipid found in two fractions as well as between different glycosphingolipids in the same fraction, were ascertained. Also, the effects on these relationships of the particular diet were studied.

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MATERIALS AND METHODS

All reagents were analytical reagent grade unless specified otherwise. All solvents were distilled before use.

Tissue, Blood and Blood Fractions

Blood, spleen and aortas were obtained at slaughter from bovine and porcine. The animals were from 15 to 18 months old. Spleen and aortas were iced and then stored at -20°C until used for lipid extraction. Aortas used in synthesis experiments, were used within one hour after slaughter. Sodium citrate, 5 mg/liter, was added to blood samples to prevent coagulation.

Whole blood was centrifuged at 3,000Xg for 15 minutes immediately after transport to the laboratory. After centrifugation, plasma was decanted and saved either for analysis or further fractionation. Remaining supernatant was aspirated from leukocytes and erythrocytes. For bovine, the leukocyte layer was carefully removed from the erythrocytes with a spatula. Erythrocytes were then resuspended in a 0.9% NaCl solution and centrifuged at 3,000Xg for 15 minutes. Repeating this process four times allowed complete separation of leukocytes and erythrocytes. Separation was considered complete when the leukocytes retained no red color from the erythrocytes. The final leukocyte preparation was stored at -20°C until used. For porcine, leukocytes were removed from erythrocyte preparations by aspiration and discarded.

Packed erythrocytes obtained by centrifugation of whole blood were

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resuspended twice in an equal volume of 0.9% NaCl solution and centrifuged at 3,000Xg for 15 minutes. Final preparations were stored at -20°C until used for lipid extraction.

To obtain blood from rabbits, they were first injected through the large ear vein, with 5 to 10 mg heparin. After 5 minutes, the hair was removed from the ear with a depilatory. The ear was cut perpendicularly to the ear vein and the ear placed in a partial vacuum apparatus to facilitate bleeding. Figure I is a drawing of this type of apparatus. This procedure yielded 80 to 120 ml of blood per animal. The collected blood was then separated into plasma and packed erythrocytes as described for bovine and porcine blood. Aortas were removed from the bled animals and cleaned of adhering tissue. The rabbits had been divided into four diet groups, six animals in each group. To obtain duplicate analyses, each group was further divided into two groups of three animals each at the time of sacrifice. Blood, as well as aortas within a subgroup were pooled.

Lipoprotein Fractionation

Plasma lipoproteins from bovine were fractionated by a combination of analytical methods (15). Centrifuge tubes were two-thirds filled with whole plasma. A sodium chloride solution, density 1.006 g per ml, was carefully layered onto the plasma to fill the remaining one-third. The tubes were then put into an SW 25.1 rotor and spun at 16,000 rpm in a Beckman Model L preparative ultracentrifuge for one hour. After centrifugation, the lower two-thirds of each tube was removed with hypodermic needle and saved. The remaining one-

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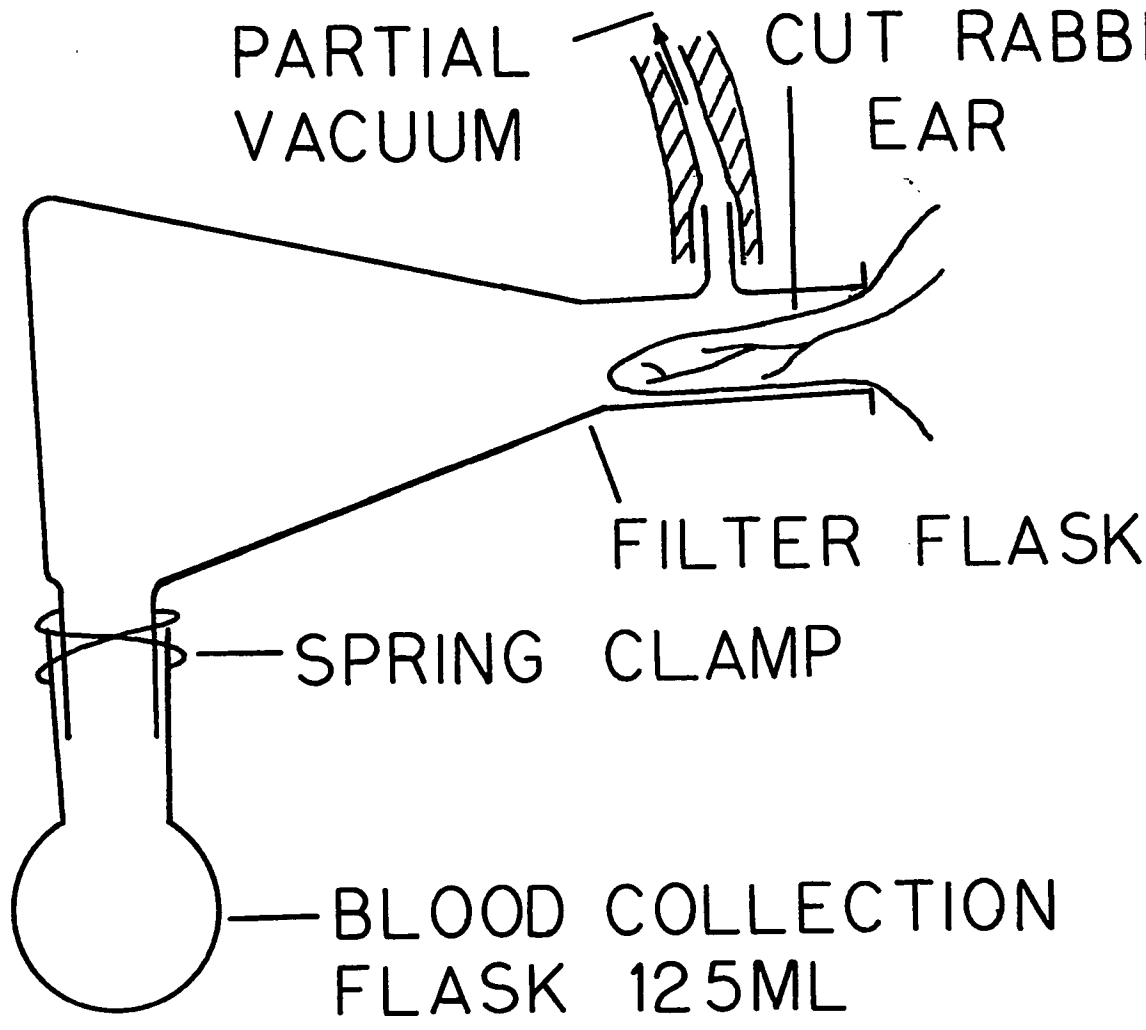
FIGURE I



third, containing chylomicrons, was discarded.

To 30 ml of the centrifuged plasma was added 1.5 ml of a 5% solution of sodium dextran sulfate (grade 2,000, Pharmacia, Uppsala, Sweden), and 1.5 ml of a 22.2% aqueous calcium chloride solution. The solutions were mixed and allowed to stand overnight at 4°C. This procedure precipitated VLDL and LDL. The precipitate was collected by centrifugation of the tubes at 2,000Xg for 20 minutes. The supernatant was saved for HDL analysis. The LDL and VLDL precipitate was dissolved in a minimum of 1.200 g per ml solution, prepared by adding potassium bromide to the 1.006 g per ml solution previously described. The lipoprotein solution was used to fill centrifuge tubes two-thirds full. A sodium chloride solution of density 1.006 g per ml was then layered on top to fill the tubes. The tubes were centrifuged at 37,000 rpm in a SW 39 rotor for 16 hours at room temperature, in a Beckman Model L preparative ultracentrifuge. After centrifugation, the top one-fourth of each tube was removed and saved for VLDL analysis. The middle one-fourth of each tube was removed and used for LDL analysis. Both VLDL and LDL were clearly discernible as yellow bands.

Porcine plasma was treated as bovine plasma to precipitate the light density lipoproteins. This lipoprotein precipitate was not separated into LDL and VLDL as in bovine. Instead, the total lipoprotein precipate was designated LDL. The plasma supernatant from this procedure was again called HDL.

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Rabbit Atherogenic Diets

Twenty-four New Zealand albino male rabbits were divided into four equal groups. One group received plain Wayne rabbit chow, designated as diet I. The three remaining groups received each one of the following diets: II. 1.0 g cholesterol and 2.8 g cottonseed oil per 100 g rabbit chow. III. 2.0 g cholesterol and 2.8 g cottonseed oil per 100 g rabbit chow. IV. 1.0 g cholesterol and 2.8 g lard per 100 g rabbit chow. All animals were fed water and chow ad libitum. The rabbits were maintained on these diets for 180 days. During this period, the weights of the animals were recorded at one week intervals. At the end of the experimental period, blood and various organs were obtained from the animals as previously described.

Extraction of Lipid and Isolation of Glycosphingolipids

Lipid was extracted from packed red blood cells (RBC) as described by Vance and Sweeley (9). The HDL fractions from bovine and porcine plasma and whole plasma from rabbit were first lyophilyzed, and an equal weight of water was added to the dried material. Lipid was extracted from this mixture by the method of Folch et al. (16). Lipid was extracted from spleen, aorta and all light density lipoprotein fractions by the same method. Flasks containing crude lipid were flushed with nitrogen and stored at -20°C.

Glycosphingolipids were isolated from all lipid samples as described by Vance and Sweeley (9). This included silicic acid column chromatography, folled by alkaline methanolysis and preparative silica gel

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thin-layer chromatography (TLC).

Preparation of Fatty Acid Methyl Esters Gas-Liquid Chromatography and Related Assays

Isolated glycosphingolipids were analyzed for fatty acids as previously described (3). This procedure included an acid methanolysis, extraction of the resulting methyl esters with hexane, and the purification by TLC on silica gel of the normal and hydroxy esters and subsequent identification and quantitation by GLC. Methyl esters of the acids 20:0 and 16h:0 were added as internal standards before acid methanolysis. The quantities of glycosphingolipids were calculated using the amounts of methyl esters obtained.

Peaks identified as 24:2 and 26:2 were also characterized by hydrogenation. After hydrogenation, the esters were rechromatographed. The 24:0 and 26:0 peaks were equal to the combined 24:0, 24:1 and 24:2 peaks and the combined 26:0, 26:1 and 26:2 peaks, respectively. The hydroxy acids 24h:2 and 26h:2 were identified by the same procedure. Sugars were analyzed as their trimethylsilyl derivatives by GLC as described by Vance and Sweeley (9). Sialic acid analysis was performed by the method of Warren (17).

GLC - Mass Spectroscopy of Acetoxy Methyl Esters

Acetoxy methyl esters were analyzed by GLC - mass spectroscopy on an LKB - 9,000 gas chromatography - mass spectrometer (LKB Instruments, Inc., Stockholm, Sweden) using a two-foot 1% OV - 1 column. The ionization chamber was usually set at 24 electron volts.

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Standard acetoxy methyl esters run were from the acids 18h:0, 19h:0 and 22h:0 (Applied Science Laboratories, Inc., State College, Pa.).

Synthesis of Cerebrosides in Aortic Tissue

Fresh porcine and bovine aortas were obtained from slaughter and kept in 0.1 M Tris buffer, pH 8.1 at 0°C, while being transported to the laboratory. The aorta was then cut lengthwise and secured to a disecting board. The intima, along with some media was removed. The tissue was then cut into approximately 0.2 cm pieces. For experiments involving unhomogenized tissue either large or cut pieces of intima plus media were used. A crude enzyme preparation was prepared by first grinding the 0.2 cm pieces of intima plus media, using a cold glass homogenizer in a solution of 0.32 M sucrose, 0.014 M mercaptoethanol and 0.001 M EDTA. The suspension was then centrifuged at 8,000Xg for 5 minutes at 0°C, to remove cell debris. The supernatant was removed and used as a crude enzyme preparation.

The incubation mixtures typically contained crude enzyme, buffer (e.g. Tris), detergent (e.g. Triton X-100), Magnesium ions, UTP and a suspected radioactive precursor (e.g. ^{14}C-glucose). Incubations were carried out at 37°C for three hours with gentle agitation. Numerous variations were tried in these experiments. These included: pH, buffers, metal ions, detergents, ^{14}C and ^{3}H precursors of cerebroside, enzyme preparations, coenzymes, energy sources and other activators. After each incubation, the lipid was extracted from the mixture (16). The crude lipid was streaked on a TLC plate (18) along with cerebroside

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standard. The band corresponding to cerebroside was scraped and counted in a Mark I liquid scintillation counter. Incorporation was expressed as disintegrations per minute (dpm) in the cerebroside band. Incorporation into other TLC bands was also noted.

Fatty Acid Distribution Correlations

Correlations between fatty acid distributions for rabbit glycosphingolipids were determined using a PDP-10 computer (Digital Equipment Corporation). Pearson product-moment correlation coefficients were calculated.

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RESULTS AND DISCUSSION

Synthesis of Cerebrosides in Aortic Tissue

For the first experiments unhomogenized tissue was used. Either whole aorta, whole intima plus media, or cut intima plus media, was incubated in Earle's solution (19) along with 10 μc of ^{14}C-glucose. The incubation was carried out at 37° for three hours. Incorporation was determined for crude lipid as well as for cerebrosides as previously described. Results of three typical experiments are presented in Table I.

Table I. Incorporation of ^{14}C-Glucose by Aortic Tissue

Tissue	^{14}C-dpm Crude lipid fraction	^{14}C-dpm Cerebroside band
10 g whole rabbit Aortas	116,000	89
27.7 g porcine intima plus media	552,000	126
15.6 g bovine intima plus media	274,000	278
blank	1,028	27

These experiments showed little evidence for cerebroside synthesis, even though the tissues themselves did synthesize lipids. The procedure to isolate crude lipid carried along some of the ^{14}C-glucose. When put on TLC, this precursor streaked the length of the plate.

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This added to the ^{14}C present in the cerebroside band. In later experiments additional Folch partitions were used (16) to remove the ^{14}C-glucose. These experiments showed even lower levels of ^{14}C in the cerebroside band. The blank illustrated in Table I used these Folch partitions to remove all ^{14}C-glucose before TLC.

Experiments employing tissue homogenates are illustrated by a typical protocol in Table II. These experiments were based on a system utilizing chick brain (20) for glycosphingolipid synthesis.

Table II. Protocol for Cerebroside Synthesis

Incubation mixtures	(^{14}C)-incorporation in the cerebroside band
Complete system[1]	21,20 dpm
plus 0.1 mg ceramide	20
plus 0.1 mg sphingosine	21
minus enzyme preparation	21

1. The complete system contained the following, in a total volume of 1.0 ml: 100 micromoles Tris (pH 8.15); 10 micromoles Magnesium chloride; 10 micromoles UTP; 1 μc (^{14}C)-glucose; 0.6 mg detergent (Cutscum: Triton X-100, 2:1) and crude enzyme (0.55 ml).

Numerous variations in this protocol mixture were tried during the course of the experiments. These variations are presented in Table III.

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Quantities were the same or very similar to the quantities for the corresponding substance listed in Table II.

Table III. Variation of Protocol for Cerebroside Synthesis Experiments

Incubation Ingredients	Variations
Buffers	1. bicarbonate pH. 8.1 2. Tris pH 7.05-8.40 3. Citrate-phosphate pH 6.80-4.40
	4. brucine pH 8.1
Non-labeled Precursors	1. glucose 2. stearic acid 3. ceramide
	4. phycosine 5. sphingosine 6. dihydrosphingosine
Labeled Precursors	1. ^{14}C-glucose 2. ^{3}H-palmitic acid 3. ^{14}C-acetate
	4. ^{14}C-UDP glucose
Cofactors	1. ATP 2. UTP 3. CoA (20 u moles)
Detergents	1. cutscum: triton X-100, 2:1 2. taurocholate
	3. sodium cholate
Metal Ions	1. Mg 2. Ca 3. Mn
Homogenate	1. crude enzyme 2. Mitochondria 3. Microsomes (21)

In all experiments involving tissue homogenates, incorporation into cerebrosides was less than 100 dpm for either ^{14}C or ^{3}H precursors. These experiments did not demonstrate consequential synthesis of cerebrosides in aortic tissue. Although many of the experiments did have incorporation above background, it was decided that further investiga-

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tions of aortic glycosphingolipids should center in the area of analysis and structural determinations. These experiments did not prove conclusively that cerebrosides are not synthesized in aortic tissue, only that systems similar to those used with other tissues did not synthesize measurable quantities of these compounds.

Symbols Used for Glycosphingolipid Fractions

Tissue samples were identified by two letters. The first letter indicated the animal; bovine (B), porcine (P) and rabbit (R) and the second designated the organ. Organ abbreviations were: aorta (A), erythrocytes or RBC (E), plasma (P), spleen (S) and leukocytes (L). Designations for plasma lipoprotein fractions were as previously described. Using this system, bovine aorta samples carry the identifying letters BA. Within each organ, the glycosphingolipids were identified as follows: monoglycosyceramide (GL-1), diglycosyceramide (GL-2), triglycosylceramide (GL-3), and tetraglycosylceramide (GL-4).

Quantities and Fatty Acid Distributions of Glycosphingolipids from Bovine Aorta, Spleen and Blood Fractions

Tables IV and V contain the quantities and fatty acid distributions of glycosphingolipids from bovine aorta, spleen and blood fractions. All bovine samples were obtained from a single animal. Samples BP and BE were averages of duplicate analyses of 75 ml each. The quantities of the remaining tissues were: BL, 3.60 g; BS, 20.0 g; and BA (intima plus media), 14.10 g.

The major glycosphingolipid for all samples except BA, was GL-1.

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Table IV. Percentage Composition of Fatty Acids from Bovine Glycosphingolipids

Fatty Acid	GL-1					GL-2				
	BA	BP	BE	BL	BS	BA	BP	BE	BL	BS
14:0	3.9	1.8	T	T	T	4.3	1.8	T	T	T
14:1	T[1]	T	T	T	T	T	T	T		T
15:0	3.9	T	T	T	T	4.1	T	T	T	T
15:1	T	T	T	T	T		T	T		T
16:0	26.7	27.2	8.1	7.2	17.0	24.9	26.7	20.2	42.7	40.0
16:1	4.3	2.9	2.0	1.3	1.4	5.9	1.8	T	T	
17:0	2.9	1.9	1.3	T	1.1	T	T	T	1.9	1.6
17:1	T	T	T	T		T	T	T		
18:0	20.9	35.6	28.9	26.8	12.3	12.3	21.3	7.4	2.4	3.0
18:1	19.4	22.4	34.9	20.5	12.1	16.9	15.7	4.4	T	1.2
18:2	10.1	5.2	21.6	21.3	6.8	7.8	2.6	1.2	T	T
19:1						T	T			
20:1		T	T		1.2			T		
21:0	T	T	T	T	8.3	T	T	T	T	T

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Table IV. (Cont.)

Fatty Acid	GL-1					GL-2				
	BA	BP	BE	BL	BS	BA	BP	BE	BL	BS
21:1		T	T	1.4	T		2.6			
22:0	3.5	2.9	3.3	17.3	7.9	6.0	5.1	15.1	7.2	7.4
22:1			T	T			T	T	T	1.5
23:0	T	T	T	T	4.5	4.3	10.2	2.3	T	2.1
23:1			T				T		T	T
24:0	4.2	T	T	1.7	19.1	5.7	5.9	31.9	12.9	13.0
24:1		T	T	1.3	5.9	4.2	6.4	10.7	23.9	21.8
25:0		T		1.2	2.5	T		2.7	9.0	8.4
25:1		T				T		T	T	T
26:0	T					3.6		2.0	T	T
26:1						T		2.1	T	T
μ moles	0.44	3.50	7.85	3.95	2.49	0.18	0.29	0.99	2.27	1.27
ACL	17.4	17.3	17.9	18.8	20.1	18.4	19.0	21.4	20.2	20.3

1. Trace (less than 1% of sample)

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Table V. Percentage Composition of Fatty Acids from Bovine Glycosphingolipids

Fatty Acid	GL-3					GL-4					Hem.[1]
	BA	BP	BE	BL	BS	BA	BP	BE	BL	BS	BS
14:0	T	1.5	1.7	1.7	T	6.2	2.5	1.2	2.8	2.1	T
14:1	T	T	T	T	T	T	T	T	T	T	T
15:0	T	T	T	T	T	3.5	T	1.2	T	1.7	T
15:1	1.9	T	T	T	T		T	T	T		T
16:0	15.4	18.8	31.7	17.8	10.9	29.2	24.5	48.6	20.7	23.1	5.4
16:1	1.9	1.7	T	2.8	T	9.9	2.6	T	2.6	T	T
17:0	1.3	T	1.3	T	T	T	T	T	T	T	T
17:1	1.3	T		T		T	T	T	T	T	
18:0	11.9	12.0	13.7	7.7	5.2	13.3	17.2	10.8	11.7	10.1	2.6
18:1	2.2	7.0	4.6	6.9	T	13.9	10.6	14.3	7.8	4.7	T
18:2	T	1.3	1.2	T	T	4.7	1.6	6.6	T	4.2	T
19:1	T		T	T		2.1					
21:0	T	T	T	T	T	T	T	T	T	4.9	T
21:1								T			

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Table V. (Cont.)

Fatty Acid	GL-3					GL-4					Hem.[1]
	BA	BP	BE	BL	BS	BA	BP	BE	BL	BS	BS
22:0	11.0	5.8	10.6	3.9	23.8	6.2	7.5	4.0	15.0	13.8	17.3
22:1				2.4	3.0		4.8	T	T		4.5
23:0	3.6	39.1		47.9	11.3	5.9	5.3	T	3.6	10.4	13.1
23:1	T	T			T		5.8	T	T		T
24:0	23.7	6.4	25.0	4.4	25.8	5.0	9.9	9.7	17.4	15.4	40.2
24:1	16.7	6.4	10.0	4.4	15.6	2.1	5.5	3.5	14.6	7.2	13.3
25:0	5.8			T	4.8				4.0	2.4	3.6
26:0	2.3				T						
26:1	1.0				T						
μ moles	0.66	0.17	0.25	0.36	1.26	0.10	0.13	0.69	0.14	0.17	1.53
ACL	21.3	20.5	20.0	20.7	22.3	17.8	19.3	18.0	20.4	20.1	22.9

1. Hematoside

In BA, GL-3 predominated.

The major fatty acids of GL-1 were 16:0, 18:0, 18:1 a nd 18:2. Sample BS contained consequential amounts of 21:0, 22:0 and 24:0 and BL of 22:0 in GL-1. The diglycosylceramide of BA and BP contained primarily the same shorter chain fatty acids as GL-1. In BE, BL and BS, GL-2, fatty acids longer than 20 carbons predominated. This difference is reflected in the larger average chain length (ACL) of the fatty acids of BE, BL and BS, GL-2. The fatty acid distributions of GL-3 from bovine had no definite pattern and show little correlation between organs. The major fatty acids were the even numbered between 16 and 24 carbons, plus 23:0. The fatty acids of GL-4 from BA, BP and BE were primarily shorter than 20 carbons, while those from BL and BS were longer than 20 carbons. All samples had 16:0 as a major constituent. Hematoside from BS contained almost exclusively long chain fatty acids and resembled GL-3 from BS.

A comparison of BA fatty acids with the remaining bovine samples, showed BA to be more similar to BP than to BE, BL or BS.

Bovine Plasma Lipoprotein Fractionation

The preparations of BHDL, BLDL and BVLDL yielded only small quantities of glycosphingolipids. The amount of lipid that could be prepared in a sixteen hour centrifuge run was less than 0.1 g. The quantities of glycosphingolipids that were isolated from these samples were less than 20 ug and too small for reliable fatty acid determinations.

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Quantities of Glycosphingolipids from Porcine Blood, Aorta and Spleen

Quantities of glycosphingolipids obtained from porcine are presented in Table VI. The samples were obtained from two animals which were arbitrarily designated I and II. There was reasonably close agreement between corresponding samples from the two animals, with three exceptions. The GL-1 from RBC of animal II was nearly twice that of animal I. Both GL-1 from spleen and GL-3 from RBC, obtained from animal I were twice the corresponding samples obtained from animal II. The data for blood are of the same order of magnitude as the results reported by Sweeley (9); however, some variations were noted. The differences between samples reported here were probably due to individual animal variation, while differences between these data and those of Sweeley are probably also due to animal age and species variation.

In RBC, GL-4 was the major glycosphingolipid. It comprised between 65 and 67 mole percent of the glycosphingolipids. The glycosphingolipids of LDL and HDL were in much lower concentrations than those of RBC. In both plasma fractions GL-1 was found in the highest concentration, but all glycosphingolipid concentrations were of the same order of magnitude. In aorta, GL-1, GL-2 and GL-3 were about equal in concentration, while the amount of GL-4 was approximately half of these.

All of the blood glycosphingolipids appeared to contain 2-hydroxy fatty acids. In RBC, the quantity of hydroxy acids increased with the increasing amount of carbohydrate in the molecule, i.e. from GL-1 to

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Table VI. Glycosphingolipids from Porcine

Glycosphingolipid		GL-1		GL-2		GL-3		GL-4	
Animal		I	II	I	II	I	II	I	II
Tissue		Micromoles[1]							
RBC	normal[2]	2.95	5.12	0.57	0.68	1.75	0.90	6.28	6.52
	hydroxy	T[3]	T	0.19	0.16	0.70	0.26	6.30	6.66
HDL	normal	0.27	0.22	0.12	0.09	0.14	0.15	0.22	0.17
	hydroxy	T	T	T	T	0.01	0.02	0.01	0.04
LDL	normal	0.25	0.19	0.14	0.14	0.18	0.15	0.13	0.12
	hydroxy	0.08	0.14	T	T	0.09	0.08	0.01	0.02
Aorta	normal	0.27	0.24	0.31	0.25	0.27	0.20	0.11	0.08
	hydroxy								
Spleen	normal	2.38	1.54	0.94	1.26	1.06	1.44	0.56	0.55
	hydroxy			0.34	0.20	0.12	0.11		

1. The quantities were obtained from 100 ml of packed erythocytes or plasma and from 20 g spleen, 7.58 g aorta from I and 6.70 g from II.

2. Normal and hydroxy refer to the type of fatty acid in the lipid.

3. Less than 0.01 Micromoles.

GL-4. The cerebrosides contained only a trace of 2-hydroxy fatty acids, while hydroxy acids comprised between 19 and 30 percent of the fatty acids found in GL-2 and GL-3 and about 50 percent in GL-4. From plasma, only GL-1 and GL-3 of LDL contained consequential amounts of hydroxy fatty acids. The remaining plasma glycosphingolipids had barely measurable or just trace amounts of these acids. Spleen glycosphingolipids contained measurable quantities of hydroxy acids only in GL-2 and GL-3. In aorta, no hydroxy fatty acid containing glycosphingolipids were found.

Verification of 2-Hydroxy Fatty Acids in Porcine Glycosphingolipids

Hydroxy acids were identified by their behavior on TLC and GLC as part of the preparative and analytical procedures. However, since these acids have not been reported by others who have studied pig blood glycosphingolipids (10,11), it seemed desirable to further identify these substances as hydroxy acids and show their presence in the glycosphingolipids. GLC - mass spectroscopy was, therefore, done and a carbohydrate - fatty acid ratio determined.

GLC - mass spectroscopy gave the expected molecular ions for 22h:0, 24h:0, 24h:2, 26h:0, and 26h:1 from the RBC GL-4 sample and for 18h:0, 19h:0, and 22h:0 from the standard. The molecular ions were small peaks but were consistently present. The spectra for the standard and sample 22h:0 were identical.

Analyses of GL-4 from whole pig blood gave 3.18 u moles of hexose plus hexosamine and 0.36 u moles normal fatty acid and 0.44 u moles hydroxy fatty acid. Thus, the GL-4 fraction, which chroma-

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tographs as globoside, and would be expected to have a hexose-fatty acid ratio of 4, would have a ratio of 8.8 if only the normal fatty acids are considered. Inclusion of the value obtained for the hydroxy acids gives a ratio of 3.98. This evidence, coupled with the TLC and GLC behavior as well as the mass spectra, seems to clearly indicate that hydroxy acids are present in these lipids.

Normal and 2-Hydroxy Fatty Acid Distributions of Porcine Glycosphingolipids

Normal and 2-hydroxy fatty acid distributions of porcine blood glycosphingolipids are presented in Tables VII and VIII respectively. The distributions for aorta and spleen are presented in Table IX. There was considerable variation from one glycosphingolipid to another as well as from one tissue to another.

The principal normal fatty acids of RBC glycosphingolipids varied from 16:0, 18:0, 18:1 and 18:2 for GL-1 to 22:0, 24:0, and 24:1 for GL-4. The di- and trihexosides from RBC contained more 16:0 than either GL-1 or GL-4 but were otherwise intermediate between GL-1 and GL-4 in the concentrations of C_{18} and C_{24} acids. As the amount of carbohydrate in the molecule increases, there is an increased concentration of all fatty acids longer than 20:0 accompanied by an irregular decrease of fatty acids shorter than 20:0. These observations are reflected in the average chain lengths listed in Table VII.

The principal hydroxy acids of RBC were 24h:0 and 24h:1 which together comprised between 64 and 76 percent of the total hydroxy acids.

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Table VII. Percentage Composition of Normal Fatty Acids in Porcine Blood Glycosphingolipids

Fatty Acid	RBC				HDL				LDL			
	GL-1	GL-2	GL-3	GL-4	GL-1	GL-2	GL-3	GL-4	GL-1	GL-2	GL-3	GL-4
14:0	T	3.6	2.2	T	3.6	3.0	1.8	1.2	3.6	3.4	2.3	3.4
14:1	T	T	T	T	2.0	1.2	1.2	T	T	2.5	T	T
15:0	T	1.8	T	T	1.6	1.2	T	T	1.8	1.6	T	1.6
15:1	T	T	T	T	T	2.2	T	T	T	4.6	T	T
16:0	9.8	41.1	24.5	2.2	24.8	29.4	29.0	13.4	24.4	35.0	35.0	33.1
16:1	1.6	5.0	1.6	T	4.4	3.8	1.8	2.1	4.5	5.7	3.1	5.0
17:0	T	T	T	T	T	1.8	T	T	1.2	T	T	1.0
17:1	T	T	T	T	T	T	T	T	T	1.4	T	T
18:0	24.5	10.1	5.8	1.4	17.9	11.7	7.9	4.6	13.8	8.1	6.0	7.7
18:1	44.6	11.1	3.2	T	14.0	14.6	4.5	3.3	11.0	9.9	2.9	4.6
18:2	17.1	3.8	1.2	T	7.8	5.8	2.4	1.3	7.1	3.8	1.6	1.8
20:1	T	T	T		T	1.3	T	T	T	T	T	T
21:0	T	T	T	T	T	1.4	T	T	T	1.8	T	1.0

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Table VII. (Cont.)

Fatty Acid	RBC				HDL				LDL			
	GL-1	GL-2	GL-3	GL-4	GL-1	GL-2	GL-3	GL-4	GL-1	GL-2	GL-3	GL-4
22:0	2.4	6.6	16.9	22.2	6.9	5.4	6.5	53.2	8.0	4.2	8.5	7.6
22:1	T	T	T	T	1.4	T	11.7	1.6	T	1.0	T	T
23:0	T	1.1		2.2	1.8	2.5	2.2	2.2	3.2	2.2	5.1	2.9
23:1		T	T	T	2.5	2.2	3.4		T	1.5	T	3.4
24:0	T	10.7	28.6	48.8	5.2	4.0	10.1	4.6	10.6	6.1	16.6	10.6
24:1	T	3.6	10.0	13.1	3.9	3.3	10.9	5.4	7.5	3.8	14.6	9.5
24:2	T	1.5	3.0	4.5	2.2	1.6	6.6	3.9	2.3	1.0	4.3	3.4
25:1	T		T	T	T	T		T	1.0	T	T	1.4
26:0		T	1.8	3.4	T	2.2	T	3.2	T	2.4	T	T
26:1		T	1.2	2.2	T				T		T	2.0
ACL	17.8	18.2	20.9	23.1	18.4	18.3	19.9	21.0	18.8	18.0	19.8	19.4

Traces (<1%) of 19:1 were found in RBC GL-1, HDL GL-4 and all of the LDL samples. Traces of 26:2 were found in RBC GL-3 and GL-4. Data are the averages from two samples.

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Table VIII. Percentage Composition of Hydroxy Fatty Acids in Porcine Blood Glycosphingolipids

Fatty Acid	RBC			HDL		IDL		
	GL-2	GL-3	GL-4	GL-3	GL-4	GL-1	GL-3	GL-4
14h:0						4.5	T	
15h:0						3.2	T	
16h:1							30.1	2.0
17h:0	1.0	T	T			6.7	1.2	4.4
17h:1	3.5	1.6				T	T	6.6
18h:0	8.0	3.8	T	28.9	64.2	38.0	6.2	23.7
18h:1				7.4	7.8		3.5	5.7
19h:0				T	2.0	T	1.5	
20h:0	2.4	T	T	T		T	2.0	
20h:1							1.8	
21h:0					T	T	T	1.2
22h:0	13.7	11.7	8.7	1.8	6.6	3.8	8.6	7.3
22h:1		T	T	13.2	4.4	9.8	5.2	7.9
23h:0	2.4	1.8	1.3	15.0	T	8.7	4.8	2.5

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Table VIII. (Cont.)

Fatty Acid	RBC			HDL		IDL		
	GL-2	GL-3	GL-4	GL-3	GL-4	GL-1	GL-3	GL-4
23h:1		T	T				4.6	T
24h:0	47.2	45.5	46.6	19.7	7.2	18.6	11.1	20.3
24h:1	16.7	28.8	29.1	11.2	7.8	4.2	10.4	15.3
24h:2	5.1	6.9	7.2	2.8	T	2.5	6.2	3.1
26h:0	T	T	1.9				2.8	
26h:1			3.0					
26h:2			2.2					
ACL	22.8	23.4	24.0	21.4	19.2	20.1	20.4	21.0

Traces (<1%) of 14h:1 were found in LDL GL-1, 19h:1 in LDL GL-3, 21h:1 in RBC GL-2 and GL-3, 25h:1 in RBC GL-3 and GL-4 and LDL GL-3. Data are the averages from the two samples.

Similar to the normal fatty acids, there was an increase in average chain length from GL-2 to GL-4. Little similarity can be seen between the normal and hydroxy fatty acid distributions except for GL-4. In this glycosphingolipid, average chain lengths were about the same and the 24 carbon acids predominated in both the normal and hydroxy acids. Also, both contain consequential quantities of 22, 23, 24 and 26 carbon saturated acids, as well as some 24 and 26 carbon mono- and diunsaturated acids.

The major normal fatty acids found in HDL glycosphingolipids were 16:0, 18:0 and 18:1 from GL-1 and GL-2; 16:0, 22:1, 24:0, 24:1 from GL-3; and 16:0 and 22:0 from GL-4. The average chain length increased slightly for GL-3 and GL-4 compared to GL-1, but much less than observed for RBC.

The major 2-hydroxy acid present in GL-3 and GL-4 of HDL was 18h:0 which comprised nearly two-thirds of the GL-4 sample. Average chain length was actually less for GL-4 in contrast to other fractions.

The predominant normal fatty acids of LDL were the same as those observed for HDL with somewhat more 24:0. The 2-hydroxy fatty acids of LDL glycosphingolipids were quite variable. Average chain lengths tended to increase as in RBC, but the increases were small and somewhat irregular.

The high concentration of 16h:1 in LDL-3 was surprising. This prompted us to investigate blood from another pig without the use of an internal standard. This was done using whole blood. The GL-3 fraction contained 10.8 percent 16h:1 and 2.3 percent 16h:0. Assuming that

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an equivalent amount of 16h:0 was present in the LDL GL-3 sample reported here, to which internal standard had been added, it can be estimated that the LDL hydroxy GL-3 totals in Table VI should be about 0.01 micromoles higher

Table IX contains the fatty acid distributions from aorta and spleen glycosphingolipids. In aorta, the major fatty acid varied from one glycosphingolipid to another. In GL-1, 16:0, 18:0 and 18:1 predominated while in GL-2 and GL-3, 22:0, 24:0 and 24:1 were the major acids. GL-4 was intermediate between these, containing large amounts of 16:0, 18:0, 22:0 and 24:0. Spleen GL-1 was similar to aorta, but contained more 24:0. Going from GL-1 to GL-4 an increase in the average chain length brought about by increases in 22:0, 24:0 and 24:1 in GL-2, GL-3 and GL-4 is apparent. The hydroxy acids of spleen GL-2 and GL-3 contained primarily the 24 carbon acids. These comprised between 75 and 80 percent of the total acids.

Comparisons of the Fatty Acid Distributions within One Tissue and among Spleen, Aorta and Blood

The glycosphingolipids of RBC contain large quantities of hydroxy acids while those of plasma contain little and those of aorta none. In GL-1 of these three fractions, RBC more closely resembles aorta than does HDL or LDL. This is reflected primarily in The C_{18} acids, although the RBC do contain much larger amounts of 18:1 than aorta. In GL-2 the fatty acid distributions of RBC and both plasma fractions are similar, but all are dissimilar to GL-2 in aorta. GL-3 of aorta again looks more like RBC than those of plasma. In GL-4, aortic fatty acids

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Table IX. Percentage Composition of Fatty Acids in Porcine Aorta and Spleen Glycosphingolipids

Fatty Acid	Aorta-normal[1]				Spleen-normal				Spleen-hydroxy[2]	
	GL-1	GL-2	GL-3	GL-4	GL-1	GL-2	GL-3	GL-4	GL-2	GL-3
14:0	2.4	T	T	6.0	T	T	T	T		
14:1	T	T	T	T	T	T	T	T		
15:0	2.5	T	T	5.0	1.0	T	T	T		
15:1	T	T	T	T	T	T	T	T		
16:0	14.6	13.2	9.5	22.1	12.9	15.0	9.2	5.3		
16:1	2.0	T	T	4.7	T	T	T	T		
17:0	3.9	1.1	1.0	T	1.4	T	T	T	T	1.7
17:1	T	T	T	T	T	T	T			
18:0	34.5	6.8	6.1	9.5	28.8	6.6	4.0	2.8	10.2	4.7
18:1	16.0	T	T	6.3	10.5	T	T	T	2.9	3.6
18:2	3.1	T	T	2.0	4.0	T	T			
19:1	T		T	T	T	T				
20:0									T	1.6
21:0	T	T	1.3	2.0	T	T	T	T		T

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Table IX. (Cont.)

Fatty Acid	Aorta-normal[1]				Spleen-normal				Spleen-hydroxy[2]	
	GL-1	GL-2	GL-3	GL-4	GL-1	GL-2	GL-3	GL-4	GL-2	GL-3
21:1	T		T		T					
22:0	7.6	23.3	22.2	12.2	9.4	29.6	27.0	25.9	4.9	6.5
22:1	5.2	1.4	1.9	5.0	T	T	T	1.3	3.0	3.6
23:0	3.2	2.5	2.8	2.4	3.4	4.3	5.3	6.3	T	T
23:1	T	T	2.9	T	T	T	T	T	1.4	
24:0	2.5	33.7	33.8	12.9	19.8	24.4	32.4	34.8	29.3	41.9
24:1	T	16.4	16.6	5.3	7.3	16.8	18.5	19.9	38.2	29.2
24:2		1.6	1.9	3.2	1.4	3.3	3.7	3.8	9.0	7.3
25:1			T							
26:0	2.5	T	T	1.6	T	T	T	T	1.1	T
26:1					T	T	T		T	T
ACL	18.6	21.9	22.2	19.4	19.9	21.8	22.5	22.8	23.1	23.1

1. Normal fatty acids

2. Hydroxy fatty acids

more closely resemble those in plasma than those in RBC. In conclusion, there is no simple relationship of aortic glycosphingolipids to those of the three blood fractions.

A more striking comparison can be made between aorta and spleen in porcine. The fatty acid distributions of aorta GL-1, GL-2 and GL-3 are similar to those in spleen. They have the same major fatty acids and similar average chain lengths. For GL-4, between these two tissues, the similarity is less pronounced.

HDL and LDL appear to have glycosphingolipids that are related. In each fraction, the different glycosphingolipids have quite similar normal fatty acid distributions. Also, comparison of a given glycosphingolipid between the two fractions shows considerable similarity among the fatty acid distributions. These similarities suggest a probable metabolic interrelationship for these glycosphingolipids. They may well be in equilibrium among the various lipoproteins.

The normal fatty acid distributions of the four glycosphingolipids in porcine RBC are quite dissimilar. From GL-1 to GL-4 the average chain lengths increase, primarily due to increases in 22:0, 24:0 and 24:1. If these lipids are metabolically related by hexose addition, a mechanism involving acyl group selection must be involved. Such a mechanism has been noted for cerebrosides (22). The three hydroxy acid-containing glycosphingolipids from RBC have similar fatty acid compositions. This suggests a closer metabolic relationship among hydroxy fatty acid containing glycosphingolipids, than among those with normal fatty acids.

Both types of fatty acids have very similar distributions in RBC

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globoside. Both globosides may be synthesized from a common pool of fatty acids, some of which are non-selectively hydroxylated either before or after condensing with the amino group of glycosyl sphingosine.

In aorta, GL-2 and GL-3 have quite similar fatty acid distributions. However, both GL-1 and GL-4 are unlike these two lipids and dissimilar to each other as well. Again, any metabolic relationship of these lipids is not evident from their fatty acid distributions.

The normal fatty acids of GL-2, GL-3 and GL-4 in porcine spleen are quite similar. The hydroxy fatty acids of GL-2 and GL-3 are similar and also resemble the normal fatty acids of these lipids. Again, this suggests a metabolic interrelationship of these three lipids, and possibly a hydroxylation of the fatty acids of the glycosphingolipid, as was proposed for RBC.

Comparisons of Normal and 2-Hydroxy Fatty Acid Distributions of Porcine Glycosphingolipids with those of Other Mammals and Tissues

The fatty acids of porcine plasma (HDL and LDL) generally resembled those found in bovine plasma, although no hydroxy acids were found in these bovine samples. In RBC of porcine, GL-1 and GL-2 are similar to their counterparts in bovine RBC. However, the fatty acids of GL-3 and GL-4 from bovine erythrocytes were of much shorter chain length than those of porcine. Again, these bovine glycosphingolipids contained no hydroxy fatty acids as did these lipids in porcine

In a recent paper, Sweeley (12) presented fatty acid distributions from porcine RBC glycosphingolipids. No hydroxy fatty acids were found. The distributions of normal fatty acids of GL-4 reported here and in Sweeley's paper are essentially the same. The normal fatty acids of

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GL-1, GL-2 and GL-3 found by Sweeley, contained the same major fatty acids as those reported here. However, the quantities of each, were consequentially different. These differences could be due to animal variation, species variation or differences in animal age.

The fatty acids found from porcine aorta GL-1 were similar to those found in human aorta (3), where the major acids were 16:0, 18:0 and 18:1. In porcine, however, 18:1 was found in considerably larger concentration than 18:0, in contrast to human aorta. The major fatty acids from the corresponding porcine and bovine aortic glycosphingolipids were generally similar. However, GL-2 and GL-4 of porcine aorta contained more long chain fatty acids than these lipids in bovine.

Spleen glycosphingolipids have been studied in human, horse and bovine (23), and in more detail from bovine in this study. The cerebrosides of porcine spleen had fatty acids similar to those found in bovine spleen. They were also similar to those from equine spleen, but contained less 24 carbon acids. The GL-2 fatty acids of porcine were different than those from bovine. Porcine contained less 16 carbon acids. In GL-2 from porcine, the fatty acids were like those of GL-2 from equine. For GL-3, bovine and porcine had almost identical fatty acid distributions. Data are not available for comparisons of GL-3 in equine and human. Little similarity is seen between bovine and porcine for the GL-4 fatty acids. However, the GL-4 fatty acids from porcine spleen have a high correlation to those of bovine spleen hematosides.

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Rabbit Growth Patterns

Figure II, shows the growth of each rabbit diet group over the course of the experiment. The points are the average weights for each group. Rabbits that did not survive the entire experiment were not counted. Two animals died over the course of the experiment; one each in groups II and III. These animals were the lightest in their respective group for at least ten weeks prior to their death.

It can be seen, from Figure II, that all diet groups grew at approximately the same rate through the first 12 weeks of the experiment. From this point, to the 22nd week, the experimental groups (II, III and IV) lagged behind the control group (I). The experimental animals attained a weight plateau by the 20th week, while the control animals continued to grow to the end of the experiment. It appeared that, at the 23rd week, the control animals had still not attained their weight plateau.

Several observations were made during the dissection of the animals. The experimental animals had fatty livers and spleens, as well as an accumulation of lipid in their aortas. The control animals had normal looking livers, spleens and aortas but had larger amounts of depot fat than the experimental groups.

The gross effects of the atherogenic diets were obvious. The animals which had excess lipid in their diets had accumulated lipids in various organs. This appeared to hamper their full development, since the three experimental groups had lower average weights than the control group.

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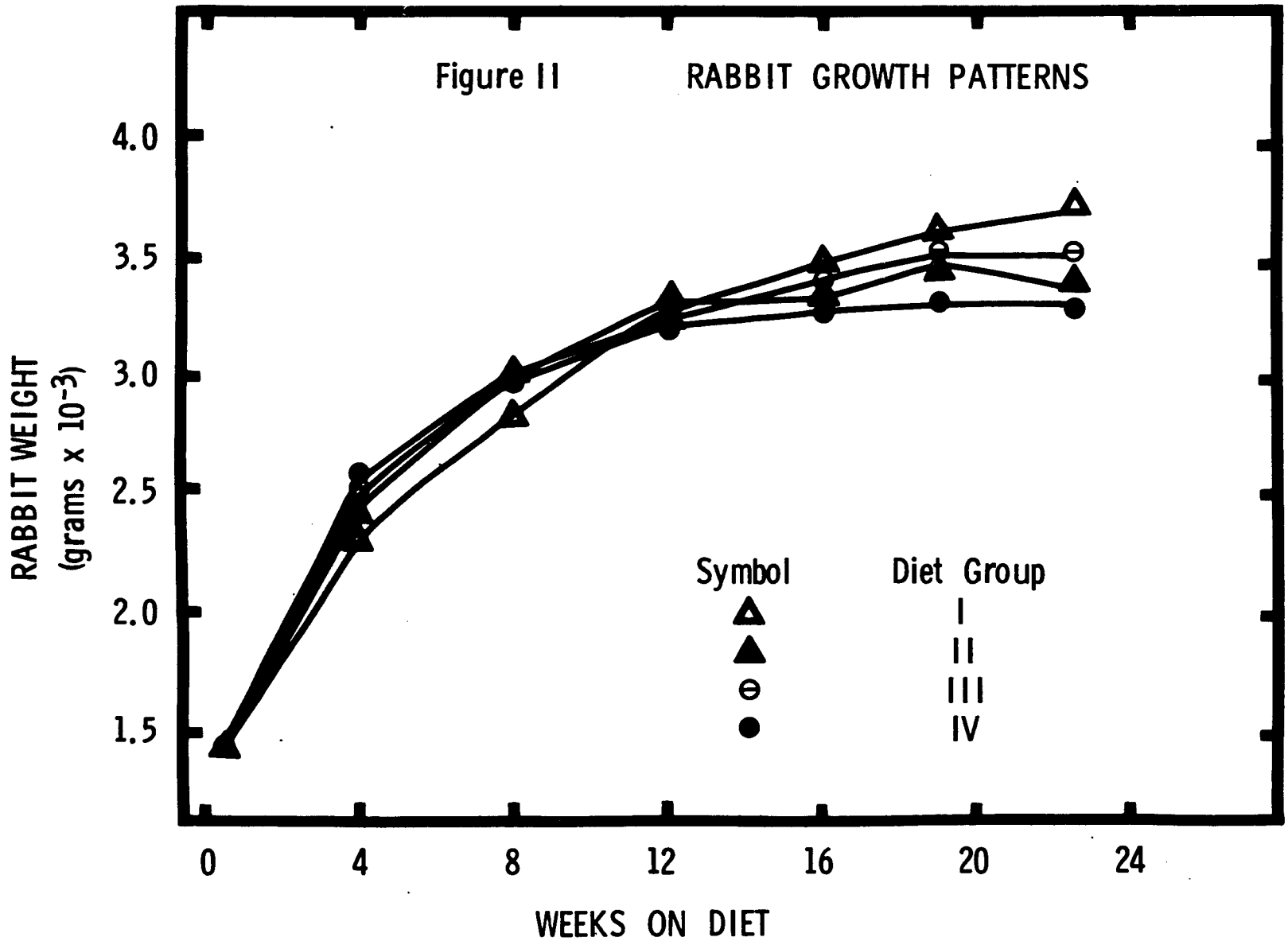

Crude Lipid from Rabbit Blood and Aorta

The quantities of crude lipid obtained from the rabbits are presented in Table X.

Table X. Crude Lipid from Rabbit Blood and Aorta

	Diet Group							
	I		II		III		IV	
Duplicates	1	2	1	2	1	2	1	2
RE	8.5[1]	7.0	7.9	8.8	12.4	12.0	12.3	11.1
RP	1.6	1.5	20.1	7.5	34.0	25.5	28.8	36.0
RA	126[2]	113	116	125	113	92	110	150

1. mg lipid per ml sample, for RE and RP
2. mg lipid per g tissue, for RA

The amount of lipid per g aortic tissue did not change consequentially from control to experimental groups. However, the average weight of experimental animal aortas was 1.8 times that of the control animal aortas. This indicated that additional non-lipid material had developed in the aorta, along with the accumulation of lipid during the diet period.

There was more lipid per ml in RBC in the experimental animals than in the controls. This was slight for group II and about 50 percent greater in groups III and IV. Differences were also noted in the percent RBC of total blood volume. The percent RBC of total blood volume was 33$\pm$ 2 percent for group I; 24$\pm$ 2 percent for group II;

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17± 2 percent for group III and 21± 1 percent for group IV. The RBC of the experimental animals thus occupied consequentially less of the blood volume than in the control group. Conversely, of course, the plasma of the experimental animals occupied a larger percentage of the blood volume than in the control group. The most notable effect of the high lipid diets was seen in the plasma. In all cases, the mg lipid per ml for plasma was at least ten times as great for experimental animals as compared to control animals. It may be that the relative increase in plasma volume is simply a reflection of the increased quantities of lipid that need to be accommodated in the blood.

Identification of Glycosphingolipids from Rabbit Blood

Analyses were performed on glycosphingolipids isolated from whole blood of mature rabbits, purchased from Pel - Freez Biologicals, Inc. (Rogers, Arkansas). The five glycosphingolipids were isolated as previously described.

The isolated lipids were run on TLC (9) along with the four glycosphingolipids isolated from porcine blood. A representation of the TLC plate is presented in Figure III.

The Rf values for GL-1, GL-2 and GL-3 from rabbit corresponded to the faster moving component of the double bands found for those compounds from porcine blood. Most of the GL-3 from porcine was in the slower moving band, but the faster moving band was discernible. The GL-4 band from rabbit was also faint but discernible and corres-

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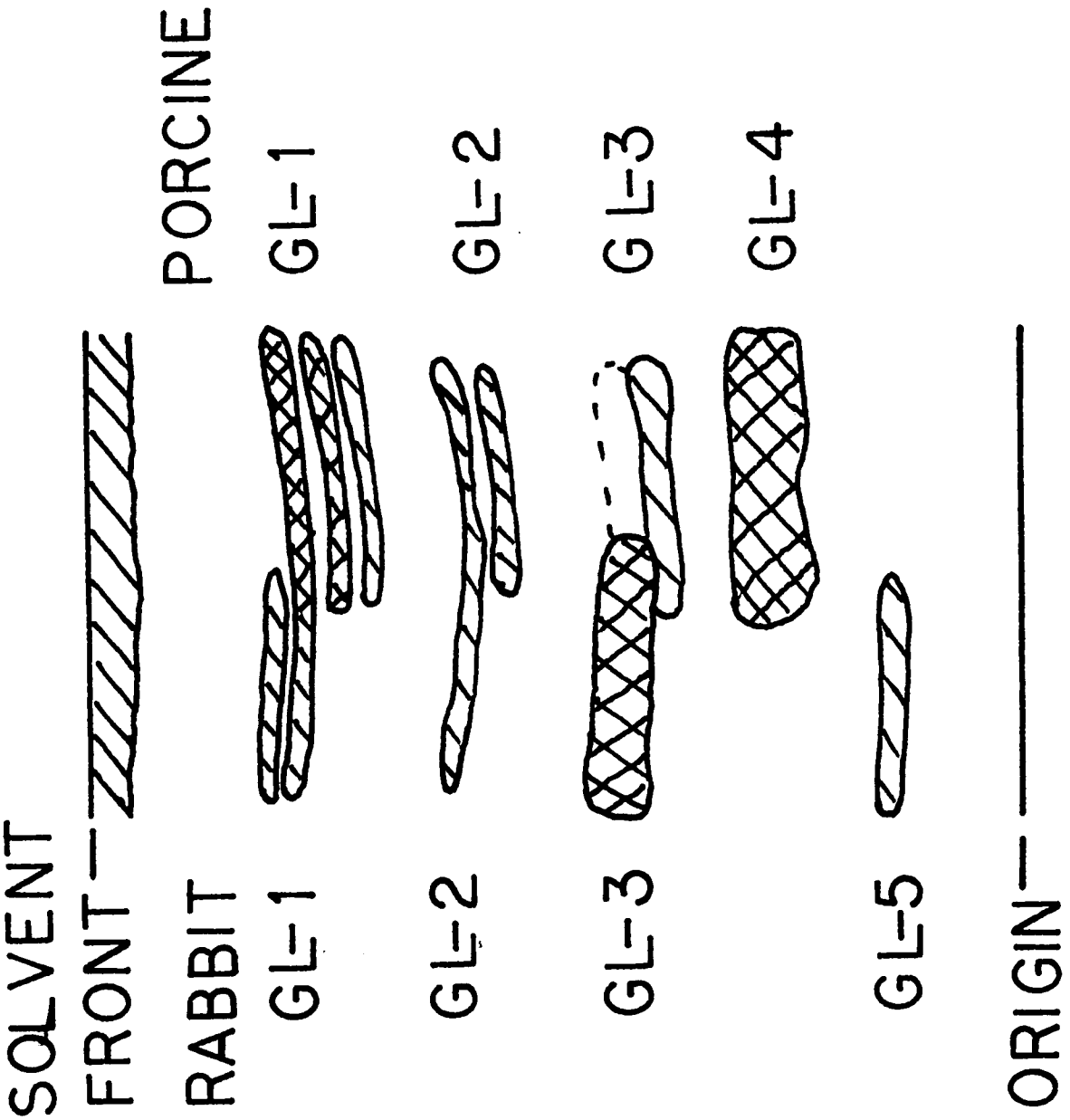
FIGURE III



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ponded to the GL-4 band for porcine. The GL-5 band from rabbit blood did not correspond to any lipid present in the porcine sample. This band (GL-5) was suspected to be either a pentaglycosylceramide (22) or hematosides which are found in human spleen (23). Two TLC systems were used (to aid in the identification). The first system was chloroform: methanol: water (100:42:6) (9) and the second was 70 percent aqueous 1-propanol (23). The first TLC system gave an Rf for hematosides of 0.16 and the second, an Rf of 0.65. In both systems, GL-5 co-chromatographed with human spleen hematosides.

Further identification was done by subjecting the rabbit glycosphingolipids to acid methanolysis, which releases methyl esters and methyl glycosides. Quantities of glucose, galactose and fatty acid were then determined for each lipid and ratios of hexose/fatty acid and galactose/glucose calculated. The quantity of GL-4 was not sufficient to perform this experiment. The results of these analyses are presented in Table XI.

From the data in Table XI and Figure III, GL-1, GL-2 and GL-3 appear to be the same lipids reported by Sweeley (12) for porcine. In GL-1, the sugar is predominantly glucose, although some galactose cerebroside was found. The ratio galactose/glucose for GL-2 indicates that this is a mixture of diglycosylceramides, in which the compound containing one each of glucose and galactose probably predominates. The ratios for GL-3 are consistent with a triglycosylceramide having two galactose moieties and one glucose. In addition, partial methanolysis experiments were performed on GL-3. Acid methanolysis was

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Table XI. Hexose/Fatty Acid and Galactose/Glucose Ratios for Rabbit Blood Glycosphingolipids

Glycosphingolipid	moles hexose/moles fatty acid	moles galactose/moles glucose
GL-1	0.98	1/4.7
	1.03	1/6.6
GL-2	1.90	1/1.3
	1.94	1/1.2
		1/1.2
GL-3	3.08	1.9/1
	3.02	2.0/1
		1.9/1
GL-5	2.03	2.0/1
	1.90	2.0/1

performed for several time periods from 10 minutes to 180 minutes, after which the amounts of galactose and glucose released were determined. The results of these experiments are presented in Figure IV.

It can be seen from Figure IV, that the points from 5 to 30 minutes lie on a straight line. This line extrapolated to zero methanolysis time, gives a ratio of galactose/glucose of 7.9. This figure should represent the ratio of these sugars in the terminal position of the saccharide portion of GL-3.

The synthesis of glycosphingolipid is believed to take place by the addition of hexose units to pre-existing glycosphingolipid precursors. Therefore, GL-2 would be formed by the addition of a hexose to GL-1. From Table XI, it can be seen that glucose is the predominent hexose in cerebroside. However, GL-2 has approximately one

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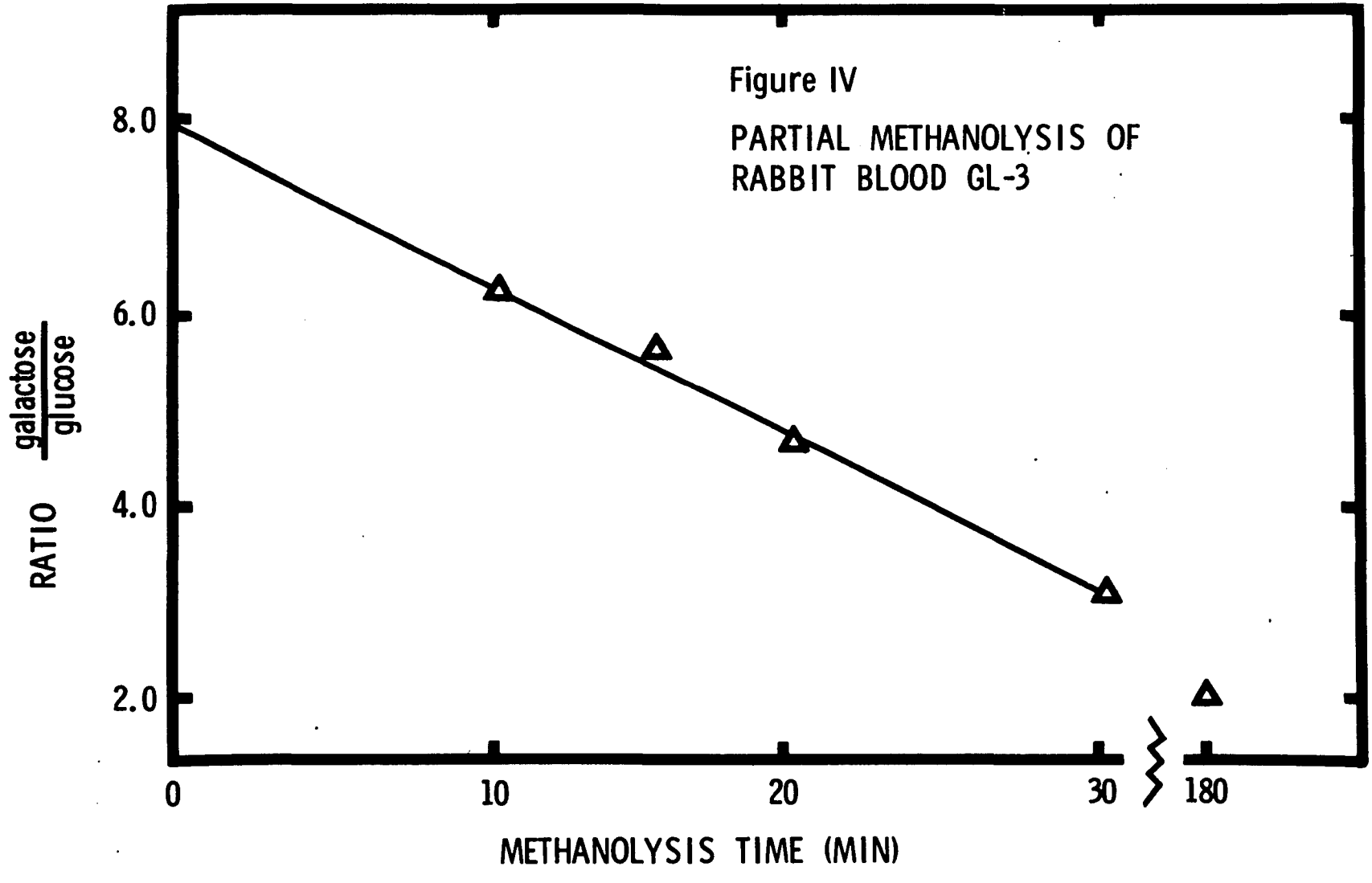
Figure IV

PARTIAL METHANOLYSIS OF RABBIT BLOOD GL-3



glucose for each galactose in the molecule. Therefore, assuming that GL-1 is a precursor for GL-2, the predominant sequence of sugars in GL-2 is glucose-galactose, counting from the ceramide portion of the molecule. This reasoning can also be employed for GL-3. Since the ratio galactose/glucose is approximately two, the predominant sequence of sugars in GL-3 is glucose-galactose-galactose. The fact that the ratios galactose/glucose are not whole numbers, indicates that other sequences of sugars do exist.

The results obtained for GL-5 in Table XI were not conclusive. Although the results from TLC and the sugar/fatty acid ratios appear to correspond to those for hematosides, the ratio galactose/glucose, which should be one, was high. Additional data were obtained by determining the quantity of sialic acid for GL-5. This experiment gave a hexose/sialic acid ratio of 2.0:0.85, which contrasts to the theoretical ratio of 2:1. It may be concluded that GL-5 is a mixture of lipids containing a high percentage of hematoside.

Yamakawa et al. (22) reported a pentaglycosylceramide to be the major glycosphingolipid in rabbit RBC. This lipid had a ratio galactose/glucose of three and also contained N-acetyl-glucosamine. The lipid did not migrate with hematoside on TLC but did migrate more slowly than GL-4. The pentaglycosylceramide was not found in this study, although it may have been present to a small extent in GL-5, since its TLC properties are similar to those of hematosides. Unfortunately, in the study reported here, the only RBC sample analyzed for GL-5 was from diet group IV. In that sample GL-5 amounted to only

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10 mole percent of the glycosphingolipids. In Yamakawa's study, the pentaglycosylceramide was more than 50 mole percent of the glycosphingolipids. At present this problem is unresolved.

Quantities of Glycosphingolipids in Rabbit Blood and Aorta

The quantities of glycosphingolipids found in rabbit aorta, plasma and erythrocytes are presented in Tables XII and XIII. Of the fifty-four pairs of like samples, forty-three had deviations from the mean of less than twenty percent. Eight of the remaining pairs had deviations of from twenty to thirty percent. The three remaining pairs, RP - II, GL-1; RP - II, GL-5; and RP - IV, GL-4 had deviations of 36, 42 and 35 percent, respectively. These deviations are based on the data in Table XII. The reliability of the data in Table XIII is about the same.

Sweeley examined the precision of this glycosphingolipid assay in a study on human blood (9). In triplicate analyses of the quantities of the four lipids from a single donor, the average deviation from the mean was 12 percent for the four glycosphingolipids. The assays were performed on both plasma and erythrocytes. The average deviation of the assays between two donors was 16 percent for these lipids in plasma and 15 percent for erythrocytes. The average deviation from the mean of the fifty-four pairs reported here, in Table XII, is 13 percent. Although greater precision in duplicate assays is generally desirable, the precision obtained in this study with rabbits does compare favorably with the error study done by Sweeley.

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Table XII. Glycosphingolipids from Rabbit

Glycosphingolipid		GL-1	GL-2	GL-3	GL-4	GL-5
Diet Group						
RA-I[1]	1	0.87[2]	NF[3]	0.91	0.43	NF
	2	1.40	NF	1.03	0.39	NF
RA-II	1	1.23	0.36	0.59	0.27	0.59
	2	1.33	0.49	0.96	0.40	0.64
RA-III	1	1.25	0.51	0.61	0.31	0.51
	2	2.32	0.75	0.75	0.40	0.98
RA-IV	1	1.47	0.60	0.66	0.43	1.03
	2	1.27	0.57	0.89	0.44	0.89
RP-I	1	0.54[4]	0.34	0.30	NF	0.84
	2	0.53	0.45	0.35	NF	0.81
RP-II	1	0.96	0.34	1.22	0.16	0.25
	2	0.45	0.40	0.99	0.11	0.10
RP-III	1	2.07	1.36	2.50	0.76	0.18
	2	1.39	1.37	1.41	0.80	0.10
RP-IV	1	1.13	0.27	0.90	0.27	0.12
	2	1.05	0.40	0.92	0.13	0.14

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Table XII. (Cont.)

Glycosphingolipid		GL-1	GL-2	GL-3	GL-4	GL-5
Diet Group						
RE-I	1	2.05	0.59	2.93	0.54	NA[3]
	2	1.19	0.51	3.13	0.39	NA
RE-II	1	0.62	0.48	3.02	0.31	NA
	2	0.67	0.65	4.75	0.31	NA
RE-III	1	2.05	0.76	5.10	0.53	NA
	2	2.02	0.71	3.98	0.73	NA
RE-IV	1	1.98	0.82	4.20	0.42	0.88
	2	2.91	0.59	3.96	0.50	0.82

1. RA (rabbit aorta), RP (rabbit plasma), RE (rabbit erythrocytes), I through IV (diet groups)

2. Units are, micromoles lipid per 10 g aortic tissue for RA

3. NF (not found); NA (not analyzed)

4. Units are micromoles lipid per 100 ml sample for RP and RE

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Table XIII. Glycosphingolipids from Rabbit

Glycosphingolipid		GL-1	GL-2	GL-3	GL-4	GL-5
Diet Group						
RA-I	1	0.69[1]	NF	0.72	0.34	NF
	2	1.24	NF	0.90	0.33	NF
RA-II	1	0.98	0.48	0.47	0.30	0.47
	2	1.15	0.81	0.82	0.37	0.55
RA-III	1	1.11	0.46	0.55	0.29	0.62
	2	2.52	0.84	0.82	0.43	0.71
RA-IV	1	1.33	0.54	0.60	0.38	1.02
	2	0.85	0.38	0.59	0.30	0.59
RP-I	1	4.74	2.88	2.56	NF	0.72
	2	3.46	2.93	2.30	NF	0.53
RP-II	1	0.43	0.15	0.55	0.07	0.11
	2	0.59	0.39	1.23	0.14	0.14
RP-III	1	0.54	0.35	0.64	0.19	0.04
	2	0.54	0.54	0.57	0.31	0.04

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Table XIII. (Cont.)

Glycosphingolipid		GL-1	GL-2	GL-3	GL-4	GL-5
Diet Group						
RP-IV	1	0.39	0.95	0.31	0.09	0.04
	2	0.29	1.08	0.26	0.04	0.04
RE-I	1	2.42	0.69	3.31	0.64	NA
	2	1.70	0.72	4.45	0.55	NA
RE-II	1	0.78	0.62	3.80	0.38	NA
	2	0.76	0.74	5.40	0.36	NA
RE-III	1	1.61	0.62	4.13	0.43	NA
	2	1.68	0.59	3.44	0.61	NA
RE-IV	1	1.61	0.66	3.40	0.34	0.72
	2	2.62	0.53	3.58	0.45	0.74

1. micromoles lipid per g of crude lipid (for RA, RP and RE).

As can be seen in Table XII, GL-1 was the major glycosphingolipid found in rabbit aorta. The concentration of the remaining lipids found were less than that of GL-1, but were of one order of magnitude. GL-2 and GL-5 were not found in the control animal aortas. The lower limit of the assay is approximately 0.05 micromoles of isolated glycosphingolipid. The pooled aortas from group I animals weighed 3.5 g. Therefore, using the units used in Table XII (micromoles per 10 g tissue), NF represents less than 0.14 micromoles in this case. GL-2 and GL-5 were both found in appreciable quantities in all three groups of experimental animals. The concentrations of GL-1, GL-3 and GL-4 from rabbits were approximately the same as those found for porcine (Table VI). However, GL-2 was also found in appreciable quantities from porcine aorta.

The control group of RP did not contain measurable quantities of GL-4. The remaining glycosphingolipid concentrations in the control animals were all of the same order of magnitude. Trends can be seen when comparing the control and experimental animal glycosphingolipids from RP. The concentration of GL-1 was greater in the experimental animals than in the control animals. Diet group III had the largest concentration of GL-1. A similar trend can be seen in GL-1 from RA. Here, GL-1 averages slightly higher in the experimental animals and diet III animals contain the largest quantity of this lipid. The RP concentration of GL-2 was elevated, compared to the control animals in diet group III only. This amounted to a three to four fold increase, while groups II and IV were essentially the same as the control group.

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This pattern was reflected in the aorta concentrations to a lesser degree. GL-4 was not found in the control group plasma and in only small amounts in experimental animals II and IV. Group III again had a much higher concentration of this lipid. The previous trends noted for the glycosphingolipids are reversed for GL-5, which had its highest concentration in diet group I in RP samples.

Unfortunately, GL-5 was not recognized as an important component in RE samples in the first three diet groups. This lipid was analyzed only in animals from group IV. The effects of the atherogenic diets on the concentration from RE were small. the concentration of GL-1 was lower in diet group II animals than from RE of the remaining three groups. Little change was noted in the concentrations of GL-2, GL-3 and GL-4 from RE, when comparing the control animals to the experimental animals. The major glycosphingolipid in RE was GL-3, which comprised over fifty mole percent of the glycosphingolipids GL-1 through GL-4.

Fatty Acids of Rabbit Aorta Glycosphingolipids

Tables XIV and XV contain fatty acids distributions for rabbit aorta glycosphingolipids. The major fatty acids for cerebrosides in the control diet group (I) were 16:0, 18:0 and 18:1. The distribution is very similar to that reported earlier from this laboratory (24). From the experimental animals (II, III and IV) the major fatty acids for this lipid were 16:0, 22:0, 24:0 and 24:1. These

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Table XIV. Percentage Composition of Fatty Acids from Rabbit Aortic Glycosphingolipids

Glycosphingolipid	RA-GL-1				RA-GL-2				RA-GL-3			
Diet Group	I	II	III	IV	I[1]	II	III	IV	I	II	III	IV
Fatty Acid												
14:0	16.6	1.2	T	T	2.1	1.9	2.3	3.0	2.1	1.4	1.3	
14:1	T	T	T	T	3.0	2.3	T	T	T	T	T	
15:0	T	T	T	T	2.0	1.7	1.5	T	T	T	T	
15:1	T	T	T	T	T	T	T	T	T	T	T	
16:0	26.7	18.4	18.7	16.5	21.0	19.8	26.6	16.9	14.2	14.2	14.3	
16:1	5.8	T	T	T	T	2.4	1.9	3.8	T	2.8	1.7	
17:0	T	T	T	T	T	T	T	T	T	T	T	
17:1	T	T	T	T	T	T	T	T	T	T	T	
18:0	14.5	8.8	5.9	7.2	7.3	6.4	12.9	10.7	7.1	6.6	6.5	
18:1	16.1	5.9	3.9	10.0	4.4	4.1	4.0	5.5	2.0	3.4	2.5	
18:2	4.8	5.0	2.6	3.4	2.3	T	T	T	T	T	T	
20:1				T			T				T	

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Table XIV. (Cont.)

Glycosphingolipid	RA-GL-1				RA-GL-2				RA-GL-3			
Diet Group	I	II	III	IV	I	II	III	IV	I	II	III	IV
Fatty Acid												
21:0	T	1.6	1.6	1.8	2.5	2.6	1.9	1.9	2.7	2.9	2.9	
21:1	T	T	T	T	T	T	T		T	T	T	
22:0	3.7	12.8	13.8	12.8	12.3	13.1	10.6	21.9	18.2	16.3	16.1	
22:1	2.1	2.3	1.2	1.8	T	T	T	T	T	T	T	
23:0	T	5.4	5.7	4.4	3.8	4.5	3.0	1.8	5.9	5.0	4.4	
23:1		T	T	T	T	T	T				T	
24:0	9.6	13.1	12.9	11.4	14.2	12.2	10.5	26.3	22.1	20.0	17.6	
24:1	T	16.5	22.5	24.5	11.3	17.4	17.6	8.2	12.4	12.9	19.4	
24:2		5.7	8.8	3.5	4.3	6.2	2.2	T	4.9	5.0	3.0	
26:0		3.3	2.3	2.6	9.4	5.5	5.0		8.6	9.5	10.4	
ACL	17.5	20.9	21.4	21.1	20.6	20.8	20.2	20.6	21.8	21.6	21.8	

1. Not found

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Table XV. Percentage Composition of Fatty Acids from Rabbit Aortic Glycosphingolipids

Glycosphingolipid	RA-GL-4				RA-GL-5			
Diet Group	I	II	III	IV	I[1]	II	III	IV
Fatty Acid								
14:0	5.5	4.2	3.3	2.2		1.7	1.3	T
14:1	T	T	T	T		T	T	T
15:0	T	1.9	2.3	1.8		T	T	T
15:1	T	1.7	2.1	2.0		T	T	T
16:0	31.6	20.2	18.9	20.6		9.3	9.2	9.2
16:1	7.2	3.3	6.0	4.3		T	2.0	T
17:0	T	T	T	T		T	T	T
17:1	T	T	T	T		T	T	T
18:0	14.6	10.7	9.2	9.3		4.8	4.2	4.1
18:1	7.3	6.8	7.6	6.1		2.3	2.6	1.0
18:2	T	2.5	2.1	2.4		T	T	T
20:1								T
21:0	T	3.6	4.2	4.8		1.5	1.5	1.4

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Table XV. (Cont.)

Glycosphingolipid	RA-GL-4				RA-GL-5			
Diet Group	I	II	III	IV	I	II	III	IV
Fatty Acid								
21:1		T	T	T		T	T	T
22:0	11.0	10.5	10.9	10.6		15.6	12.9	14.0
22:1	T	T	T	T		1.3	1.4	1.8
23:0	12.0	3.0	2.6	2.2		5.6	4.7	4.3
23:1		T	T	T		T	T	T
24:0	8.1	13.6	12.4	13.8		18.6	14.6	14.5
24:1	2.6	4.7	7.0	7.0		24.8	29.3	39.6
24:2	T	1.8	2.6	2.9		8.7	11.1	6.2
26:0		11.2	9.0	10.2		5.7	5.2	3.9
ACL	18.6	20.0	20.0	20.2		22.3	22.3	22.6

1. Not found

changes are reflected in the larger ACL. The earlier report (24) did not show these changes. However, those experiments extended over a three-month period while experiments reported here were for a six month period. These data indicate that changes in the cerebroside fatty acids occurred between the third and the sixth month of the experiment. The fatty acid distribution for cerebrosides in the control diet is also similar to that reported for normal human aorta (3). However, the fatty acid distribution for cerebrosides in atherogenic tissue from human is quite unlike that reported here for the experimental animals. In human tissue, the atherogenesis produced no increase in long chain fatty acids. The fatty acid distribution for BA-GL-1 reported here, is similar to RA-GL-1 in the control animals, but unlike that of the experimental animals. The same fatty acids predominate in PA-GL-1 as in RA-GL-1. However, the relative percentages of these acids are quite different.

Diglycosylceramide was not found in the control rabbit aortas. From the experimental groups, the major fatty acids for this lipid were 16:0, 22:0, 24:0 and 24:1, the same as for GL-1 from the experimental groups. The higher molecular weight glycosphingolipids (GL-2 through GL-5) have not been previously reported for rabbit aortic tissue. The fatty acids of BA-GL-2 were of shorter chain length than those of RA-GL-2 from the experimental rabbits. The same acids predominated in RA-GL-2 and PA-GL-2 as in RA-GL-1. However, the ACL of the PA-GL-2 was greater than RA-GL-2.

The major fatty acids of RA-GL-3 were the same as those of RA-GL-2. However, in GL-3 the longer chain fatty acid were in higher

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concentration. For GL-3, the experimental diets caused a decrease in 16:0, 18:0 and 22:0 along with increases primarily of 24:1, 24:2 and 26:0, when compared to the control group. While these changes were not as pronounced as those for GL-1 from aorta, there was an increase in ACL for the experimental groups. The major fatty acids of RA-GL-3 are the same as those of BA-GL-3 and PA-GL-3.

In GL-4 of RA, the major fatty acids were 16:0, 18:0, 22:0 and 23:0 for the control animals and 16:0, 18:0, 22:0, 24:0 and 26:0 for the experimental. These fatty acid distributions, however, contain more of the shorter acids than found in GL-3 from aorta. The effects of the atherogenic diets are again seen as a decrease in shorter chain fatty acids with an increase in long chain acids. As in the other lipids from RA, the fatty acid distribution of RA-GL-4 of the control diet is similar to BA-GL-4 and PA-GL-4. GL-5 was not found in the control group from RA. The fatty acids of the experimental groups for GL-5 in RA all contained 16:0, 22:0, 24:0 and 24:1 as their major fatty acids. The ACL was longer than that found for any of the other glycosphingolipids from RA. GL-5 was not found in either BA or PA samples. However, the fatty acids of hematosides from BS are similar to those found from RA-GL-5, with the concentrations of 24:0 and 24:1 approximately reversed.

The effects of the atherogenic diets on the fatty acid distributions are further illustrated in Table XVI. A comparison of animals on diet I with those on diets II, III and IV shows two trends. The ratio 24:1/24:0 is greater for animals on the experimental diets than on the control diets, except for GL-4 where they are equal for animals

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Table XVI. Percentage of 24:2 and 24:1/24:0 Ratios for Rabbit Glycosphingolipid Fatty Acid Distributions

	RA											
	GL-1				GL-2				GL-3			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
24:1/24:0	0.1	1.3	1.7	2.4		0.8	1.4	1.7	0.3	0.6	0.6	1.1
24:2[2]	NF	5.7	8.8	3.5		4.3	6.2	2.2	T	4.9	5.0	3.0

	GL-4				GL-5			
	I	II	III	IV	I	II	III	IV
24:1/24:0	0.3	0.3	0.6	0.5		1.4	2.0	2.7
24:2	T	1.8	2.6	2.9		8.7	11.1	6.2

	RE											
	GL-1				GL-2				GL-3			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
24:1/24:0	1.9	1.3	2.2	2.7	6.8	3.6	4.0	6.0	12.2	8.5	6.9	8.6
24:2	3.0	4.6	4.8	T	12.1	13.3	9.8	5.2	18.4	24.0	17.3	6.6

	GL-4				GL-5[1]			
	I	II	III	IV	I	II	III	IV
24:1/24:0	7.5	4.7	5.2	6.4				7.0
24:2	16.0	11.9	10.4	6.1				6.0

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Table XVI. (Cont.)

	RP											
	GL-1				GL-2				GL-3			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
24:1/24:0	0.8	1.1	1.5	2.2	0.9	1.1	1.3	2.3	1.2	1.2	1.9	2.5
24:2	0.0	7.1	9.2	5.0	3.5	3.2	4.9	4.2	2.7	9.5	13.1	5.6

	GL-4				GL-5			
	I	II	III	IV	I	II	III	IV
24:1/24:0		1.0	1.5	2.4	1.2	1.2	1.2	2.0
24:2		4.2	4.8	3.4	1.2	1.8	4.3	3.5

1. In diet groups I, II and III of RE, GL-5 was not analyzed. Remaining blanks indicate that particular lipid was not found in the sample.

2. Percentage of this acid in each sample.

on I and II. This equality in the ratio is due mainly to an increase in the concentration of 24:1. The second trend is the greater concentration of 24:2 in the experimental animals than in the controls.

Although the fatty acid distributions from the experimental animals were similar, there were consistent differences between groups II, III and IV. From Table XVI it can be seen that the ratio 24:1/24:0 increases so that diet group I $\leq$ II $\leq$ III < IV. An exception is the lower ratio for group IV in GL-4. The equality between II and III in GL-3 is the result of rounding off. The value for III as measured was 0.08 larger than for II. While there are some equalities in these ratios, the overriding trend is for an increase with increasing diet number. The 24:2 concentrations also show some differences among the various atherogenic diets. In this instance the order is I < IV < II < III, with the only exception being again in GL-4, where the concentration of 24:2 in group IV is the highest.

These several changes, brought about presumably by the atherogenic diets, will be discussed more later, along with changes in plasma and RBC.

Fatty Acids of Rabbit Plasma Glycosphingolipids

Tables XVII and XVIII contain the percentage fatty acid distributions for rabbit plasma. The fatty acid distributions of GL-1 were markedly altered by the atherogenic diets. In the control group, the major fatty acids were 16:0, 18:0, 18:1 and 18:2. In the three remaining diet groups the amounts of 18 carbon acids

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Table XVII. Percentage Composition of Fatty Acids from Rabbit Plasma Glycosphingolipids

Glycosphingolipid	RP-GL-1				RP-GL-2				RP-GL-3			
Diet Group	I	II	III	IV	I	II	III	IV	I	II	III	IV
Fatty Acid												
14:0	1.5	2.5	T	T	1.5	4.2	T	5.1	1.7	T	T	T
14:1	T	T	T	T	T	3.2	2.1	3.9	T	T	T	T
15:0	T	T	T	T	T	7.9	4.0	3.8	T	T	T	T
15:1	T	T	T	T	T	3.2	2.1	2.9	T	T	T	T
16:0	23.9	16.0	13.9	13.5	8.1	22.5	29.8	18.1	15.8	9.0	8.5	10.0
16:1	6.2	T	T	T	1.0	2.5	1.4	T	3.3	T	T	T
17:0	T	T	T	T	T	T	T	T	T	T	T	T
17:1	T	T	T	T	T	T			T		T	
18:0	15.8	6.1	5.6	5.4	5.5	11.1	8.1	5.5	9.0	3.9	5.2	5.6
18:1	19.5	3.2	T	T	2.7	6.7	4.0	2.1	8.6	T	T	1.0
18:2	13.1	1.3	T	T	1.7	4.6	2.2	T	2.0	T	T	1.0
20:1	T											T

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Table XVII. (Cont.)

Glycosphingolipid	RP-GL-1				RP-GL-2				RP-GL-3			
Diet Group	I	II	III	IV	I	II	III	IV	I	II	III	IV
Fatty Acid												
21:0	T	1.8	1.7	1.8	1.7	1.4	T	T	1.8	1.9	1.5	1.8
22:0	5.0	17.5	18.4	17.6	16.1	7.5	9.7	11.0	15.4	19.5	15.3	17.6
22:1	T	T	T	T	T	1.6	3.8	T	1.0	T	3.6	T
23:0	5.5	11.4	11.1	11.3	17.5	5.1	6.1	6.8	12.3	13.7	10.0	11.4
23:1	T	T	T	T	T		T	T	T	T	T	T
24:0	3.9	16.0	15.7	14.0	19.8	6.9	9.4	11.2	11.1	19.6	14.7	13.3
24:1	3.0	16.9	24.3	31.4	18.7	7.5	12.3	25.5	13.6	22.9	28.1	32.7
24:2		7.1	9.2	5.0	3.5	3.2	4.9	4.2	2.7	9.5	13.1	5.6
25:1	T	T				T		T	T	T		T
26:0	2.5	T	T	T	2.0	1.2	T	T	1.7	T	T	T
26:1		T		T		T						
ACL	18.6	21.2	22.0	22.1	22.0	18.6	19.5	20.4	20.6	22.4	22.4	22.2

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Table XVIII. Percentage Composition of Fatty Acids from Rabbit Plasma Glycosphingolipids

Glycosphingolipid	RP-GL-4				RP-GL-5			
Diet Group	I[1]	II	III	IV	I	II	III	IV
Fatty Acid								
14:0		4.8	2.8	2.2	4.6	9.2	8.6	2.9
14:1		2.4	T	T	T	T	T	2.1
15:0		1.7	T	T	1.6	T	T	T
15:1		T	T	T	T	1.4	3.3	T
16:0		20.0	22.2	26.7	15.6	15.6	21.1	19.3
16:1		6.2	1.3	2.2	5.1	4.1	5.0	3.4
17:0		T	T	T	1.1	T	T	T
17:1		T	T		T	T		
18:0		9.4	17.5	11.4	13.4	9.6	9.8	7.9
18:1		6.1	4.6	5.5	9.3	7.6	9.7	4.4
18:2		3.3	5.7	2.7	3.6	6.1	4.4	2.0
21:0		1.5	1.3	1.6	3.1	3.2	1.6	1.7

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Table XVIII. (Cont.)

Glycosphingolipid	RP-GL-4				RP-GL-5			
Diet Group	I	II	III	IV	I	II	III	IV
Fatty Acid								
21:1						T		
22:0		11.1	12.1	11.2	7.8	11.3	7.2	12.7
22:1		1.7	T	T	5.1	4.5	T	T
23:0		6.9	6.6	8.5	7.9	8.0	6.2	7.3
23:1				T				T
24:0		8.2	7.6	7.2	4.7	4.2	6.9	10.0
24:1		8.2	11.7	17.3	5.8	4.9	8.1	19.8
24:2		4.2	4.8	3.4	1.2	1.8	4.3	3.5
26:0		4.3	1.7	T	10.1	8.4	3.8	3.1
26:1		T	T				T	
ACL		19.6	19.9	19.9	19.8	19.6	19.2	20.5

1. Not found

decreased and the predominant fatty acids were 16:0, 22:0, 23:0, 24:0 and 24:1. These changes are reflected in the larger ACL for the experimental groups as compared to that of the controls. The changes in rabbit plasma caused by the atherogenic diets were similar to the effects of the diets observed for GL-1 from rabbit aorta. The fatty acid distribution of RP-GL-1 is similar to that of both bovine and porcine plasma GL-1.

The effects of the atherogenic diets on the diglycosylceramide from rabbit plasma were opposite to those observed for the monoglycosylceramide. In RP-GL-2 of the control group, the long chain fatty acids 22:0, 23:0, 24:0 and 24:1 predominated. However, in the three experimental groups, the shorter chain fatty acids were increased, leading to a smaller ACL for these fatty acid distributions. A comparison of GL-2 from rabbit plasma with GL-2 from bovine and porcine plasma shows that the fatty acids of the latter samples are more similar to those of the experimental rabbits than to those of the control rabbits.

The effects of the atherogenic diets on RP-GL-3 fatty acids was less pronounced than noted for GL-2 and GL-1. The major fatty acids for all RP-GL-3 samples were 16:0, 22:0, 23:0, 24:0 and 24:1. In this glycosphingolipid the experimental diets increased the concentration of fatty acids longer than 21 carbons, resulting in a larger ACL. The fatty acids of BP-GL-3 and PHDL and PLDL-GL-3 are not similar to RP-GL-3 in either the control or the experimental animals.

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RP-GL-4 was not found in the control animals. The major fatty acids for this lipid from the experimental groups, were 16:0, 18:0, 22:0 and 24:1. These fatty acid distributions are again unlike those found for this lipid from porcine and bovine plasma. The fatty acid distributions of RP-GL-5 samples were relatively unaffected by the atherogenic diets. The even numbered carbon acids from 14 through 26 were found in consequential quantities in all samples. The ACL of these lipids had a range of only 19.2 to 20.5.

Additional effects of the atherogenic diets on fatty acids from rabbit plasma glycosphingolipids can be seen in Table XVI. There is an increase in the ratio 24:1/24:0 from group I through group IV except in GL-3 where the group I and II samples have equal ratios. Again in all but one instance (GL-2 groups I and II) there is a general order of 24:2 concentration for the various diet groups so that I IV, II III. Both of the above trends in 24 carbon acids were noted in fatty acid distributions for rabbit aorta glycosphingolipids.

Fatty Acids of Rabbit Erythrocyte Glycosphingolipids

Tables XIX and XX contain the fatty acid distributions for rabbit RBC glycosphingolipids. The changes caused by the experimental diets in RE-GL-1 are less pronounced than those observed in aorta. All cerebroside samples from RE had 16:0, 18:0, 18:1, 18:2, 22:0 and 24:1 as the major fatty acids. The experimental rabbits showed an increase in 18:0 and decreases in 18:1 and 18:2 when compared to the controls. Also, slight increases in fatty acids longer than 22

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Table XIX. Percentage Composition of Fatty Acids from Rabbit Erythrocyte Glycosphingolipids

Glycosphingolipid	RE-GL-1				RE-GL-2				RE-GL-3			
Diet Group	I	II	III	IV	I	II	III	IV	I	II	III	IV
Fatty Acid												
14:0	T	T	1.3	T	1.7	1.6	3.2	2.9	T		T	T
14:1	T		T	T	1.8	3.3	1.5	1.2	T		T	T
15:0	4.1	T	T	T	2.0	1.5	1.0	T	2.0		T	T
15:1	T		T	T	T	2.7	T	T	T		T	T
16:0	16.5	21.4	17.6	18.2	11.6	12.6	17.7	16.0	4.4	4.3	5.9	7.1
16:1	2.1	T	1.6	T	T	T	3.6	2.5	T	T	T	T
17:0	T	T	T	1.4	T	T	T	T	T	T	T	T
17:1	T		T	T								
18:0	14.7	23.2	25.5	30.8	3.0	6.9	6.6	6.4	1.4	1.3	2.1	1.7
18:1	14.5	8.2	9.8	12.8	1.7	2.4	5.6	5.7	T	T	1.7	T
18:2	19.4	6.8	5.4	5.5	T	1.3	T	1.5	T	T	T	T
19:1	T				T			T	T			
20:1	1.0	1.0	T	1.9					T			

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Table XIX. (Cont.)

Glycosphingolipid	RE-GL-1				RE-GL-2				RE-GL-3			
Diet Group	I	II	III	IV	I	II	III	IV	I	II	III	IV
Fatty Acid												
21:0	1.0	T	1.1	T	T	1.2	T	T	T	T	T	T
21:1		1.0	T	T					T		T	T
22:0	8.8	9.9	7.9	7.0	5.9	8.1	6.3	7.6	5.2	6.8	7.1	7.3
22:1	2.5	2.0	2.1	3.9	14.1	3.1	1.7	3.0	2.2	1.5	3.3	2.0
23:0	2.4	4.3	3.6	2.3	3.4	1.9	2.3	4.3	3.3	3.1	3.2	2.6
23:1	T	T	T	T	T	3.9	4.6	T	T	T	T	T
24:0	2.6	7.6	6.2	4.0	5.1	7.4	6.3	5.9	4.6	5.8	7.3	7.3
24:1	5.1	9.8	13.0	10.9	34.8	26.7	25.1	35.5	56.2	49.2	50.4	63.1
24:2	3.0	4.6	4.8	T	12.1	13.3	9.8	5.2	18.4	24.0	17.3	6.6
25:1	T				T	T	T		T	T	T	T
26:0	2.2	T	T	1.3	2.8	1.9	4.7	2.3	1.4	2.6	1.7	2.1
26:1					T	T	T		1.0	1.4	T	
ACL	19.0	19.6	19.6	19.2	21.8	21.1	20.8	21.0	23.2	23.4	23.1	23.1

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Table XX. Percentage Composition of Fatty Acids from Rabbit Erythrocyte Glycosphingolipids

Glycosphingolipid	RE-GL-4				RE-GL-5			
Diet Group	I	II	III	IV	I[1]	II[1]	III[1]	IV
Fatty Acid								
14:0	T	1.9	3.8	T				T
14:1	T	T	T	T				T
15:0	3.7	T	1.2	T				T
15:1	T	2.7	T	2.8				T
16:0	10.6	14.8	18.9	15.3				6.1
16:1	T	2.5	5.8	2.5				T
17:0	T	T	T	T				T
17:1	T							T
18:0	3.4	7.4	5.8	5.0				4.0
18:1	2.5	5.3	7.3	5.7				3.3
18:2	T	T	T	T				T
19:1	T							
21:0	T	1.6	T	T				T

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Table XX. (Cont.)

Glycosphingolipid	RE-GL-4				RE-GL-5			
Diet Group	I	II	III	IV	I	II	III	IV
Fatty Acid								
22:0	5.3	7.3	5.3	6.6				7.7
22:1	2.6	4.6	1.2	4.8				3.0
23:0	3.6	1.9	T	1.1				4.0
23:1	T	3.1	3.6	5.6				T
24:0	5.8	5.0	5.1	5.3				7.8
24:1	43.6	23.7	26.5	33.9				54.5
24:2	16.0	11.9	10.4	6.1				6.0
25:1	T	T	T	T				T
26:0	3.0	6.2	5.1	5.3				3.7
26:1	T	T	T					
ACL	22.3	21.2	20.6	21.4				22.8

1. Not analyzed

carbons were noted. The fatty acids of both BE-GL-1 and PE-GL-1 contained larger amounts of the 18 carbon acids and less 16:0 than those found for RE-GL-1.

The major fatty acids of RE-GL-2 were again the same for all diet groups. These were 16:0, 24:1 and 24:2. The ACL of this lipid were, hence, greater than for GL-1. The effects of the experimental diets on these distributions were small, and no consequential trends were noted. These distributions are similar to those found for BE-GL-2, but unlike those of PE-GL-2, which contained primarily the shorter carbon acids as well as hydroxy fatty acids.

The triglycosylceramides for RE contained primarily 24 carbon acids. As in GL-2, the experimental diets had little effect on the fatty acid distributions. A comparison of RE-GL-3 to that lipid from bovine and porcine erythrocytes shows little similarity among their fatty acids. PE-GL-3 also contained hydroxy acids, in contrast to RE-GL-3.

Although the ACL for RE-GL-4 is smaller than for RE-GL-3, the same fatty acids predominated. Notable differences were the larger quantity of 16:0 in RE-GL-4 as compared to RE-GL-3 and the decrease in 24:1 for GL-4. Again, the effects of the diets were subtle. The fatty acids for GL-4 were quite unlike those of this lipid in PE. The major fatty acids of RE-GL-4 are the same as those of PE-GL-4, although the percentages for each acid are different. Also, porcine globosides contained large quantities of hydroxy acids unlike globosides from rabbit erythrocytes.

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It was unfortunate that GL-5 was not analyzed from the first three diet groups. However, at the time, it was not one of the compounds of interest. Later it was recognized to be a consequential glycosphingolipid and its fatty acid distribution was determined. The major fatty acids of GL-5 in RE were the 24 carbon acids. The distribution was similar, primarily to GL-3. In both cases, 24:1 comprised between 50 and 60 percent of total acids.

Some effects of the experimental diets on the ratio 24:1/24:0 and the percent 24:2 can be seen in Table XVI. There is a general decrease in the ratio 24:1/24:0 from the control to the experimental groups for GL-2, GL-3 and GL-4.

Within the experimental groups for all glycosphingolipids, except GL-5, the order of the ratio is II III IV except in GL-3 where the value for III is low.

The concentration of 24:2 shows no consistent trend, as it did in aorta, except that it was always lowest in GL-4.

Alterations of Fatty Acid Synthesis due to Atherogenic Diets

It has been reported that pigeons, which spontaneously develop atherosclerosis, have an increased rate of fatty acid synthesis in aortic tissue (25). A similar result was obtained with rabbits in which atherosclerosis had been induced by a high cholesterol diet (26). In both studies, the increased synthesis was localized in the mitochondria, and was primarily due to chain elongation of the acids 16:0, 18:0, and 18:2. The data obtained in the present study

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are consistent with increased fatty acid synthesis by chain elongation in as much as the atherosclerotic animals had larger chain length fatty acids in their aortic glycosphingolipids. Thus, in atherosclerotic animals, it would be expected that the sixteen and eighteen carbon acids would be diminished and acids with 22, 24, and 26 carbons would be in greater concentration. This was generally the case for all of the glycosphingolipids.

The added dietary fats had different fatty acid distributions. These distributions were, for cottonseed oil 16:0-27.3%, 18:0-2.0%, 18:2-50.5% and for lard 16:0-26.2%, 16:1-4.0%, 18:0-13.5%, 18:1-42.9%, 18:2-9.0% (27). These differences have probably affected the glycosphingolipid fatty acid distributions directly to some extent. However, it seems obvious that the trends discussed cannot be simply a reflection of the different fatty acid intake. Thus, diet III generally caused a greater change than diet II with the same fatty acids in the diet.

Correlations of the Fatty Acid Distributions of Glycosphingolipids from Rabbit Aorta, Plasma and Erythrocytes

It can be seen that a fatty acid distributions are a complex description of a particular glycosphingolipid. Previously, investigators have relied on a subjective analysis when comparing one fatty acid distribution to another, in order to determine relationships between two lipids. It is obvious that this type of analysis has limited value. In this study, an attempt was made to more accurately determine similarities between various distributions.

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In each case the average Pearson correlation was determined as described in the methods section. For this study, fatty acids found as traces were arbitrarily given percentages of 0.4 and acids not found were given percentages of 0.0.

Table XXI presents the correlations among the fatty acid distributions of a particular glycosphingolipid from a particular tissue. These correlations indicate changes in the fatty acid brought about by the various atherogenic diets.

The effects of the atherogenic diets on the fatty acids of GL-1 were pronounced. For both aorta and plasma, there is a very low correlation between the fatty acids of this lipid from group I and the remaining three diet groups. This again illustrates the large changes in GL-1 brought about by the experimental diets. The experimental diets do not have so great an effect on the erythrocyte GL-1. This is reflected by a higher correlation between group I and groups II, III and IV than was seen for aorta and plasma. The correlations among the experimental diets in GL-1 were all high (.90). In all three tissues, one pattern was noted for GL-1. Group II had a higher correlation with III than with IV, and the correlation between diets III and IV was always greater than between diets II and IV. This observed pattern seems reasonable from the make-up of the diets. Diet II contained one percent cholesterol and 2.8 percent cottonseed oil. In diet III the amount of cholesterol was raised to two percent. In diet IV there was only one percent cholesterol but 2.8 percent lard, instead of cottonseed oil. From this it can be seen that the change in the percent of

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Table XXI. Correlations Among Glycosphingolipid Fatty Acid Distributions in the Four Diets

GL-1

	Aorta				Erythrocytes				Plasma			
Diet	I	II	III	IV	I	II	III	IV	I	II	III	IV
I	1[1]	.55	.40	.44	1	.80	.76	.76	1	.40	.26	.23
II		1	.97	.95		1	.98	.93		1	.97	.92
III			1	.96			1	.96			1	.98
IV				1				1				1

GL-2

Diet	I	II	III	IV	I	II	III	IV	I	II	III	IV
I	1	0[2]	0	0	1	.92	.86	.91	1	.48	.58	.79
II		1	.95	.93		1	.95	.93		1	.93	.72
III			1	.94			1	.95			1	.81
IV				1				1				1

GL-3

Diet	I	II	III	IV	I	II	III	IV	I	II	III	IV
I	1	.92	.90	.83	1	.99	1.0	.97	1	.84	.76	.81
II		1	.99	.95		1	.99	.93		1	.96	.94
III			1	.97			1	.97			1	.96
IV				1				1				1

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Table XXI. (Cont.)

GL-4

Diet	Aorta I	Aorta II	Aorta III	Aorta IV	Erythrocytes I	Erythrocytes II	Erythrocytes III	Erythrocytes IV	Plasma I	Plasma II	Plasma III	Plasma IV
I	1	.82	.82	.80	1	.90	.88	.93	1	0	0	0
II		1	.98	.99		1	.96	.95		1	.74	.75
III			1	.99			1	.94			1	.95
IV				1				1				1

GL-5

Diet	I	II	III	IV	I	II	III	IV	I	II	III	IV
I	1	0	0	0	NA[3]	NA	NA	NA	1	.93	.86	.70
II		1	.98	.94		NA	NA	NA		1	.88	.71
III			1	.97			NA	NA			1	.82
IV				1				1				1

1. Diagonal rows of 1, indicate any distributions correlated with itself would have a perfect correlation.

2. Zeros indicate one of the lipid samples was not found.

3. (NA) Not analyzed.

cholesterol from diet II to III changed the fatty acids of GL-1 less (a higher correlation) than a change from cottonseed oil to lard, as from diet II to IV.

Correlations among GL-2 fatty acid distributions were similar to those for GL-1, although GL-2 was not found in group I aortas. In plasma, there was a large change in the fatty acid distributions of GL-2 from the control to the experimental groups. This is reflected in a low correlation between group I and groups II, III and IV. Again, as in GL-1, the atherogenic diets had a much smaller effect on the fatty acids of GL-2 from erythrocytes than was observed for plasma. In all three tissues the pattern observed among the experimental groups for GL-2 was the same as observed for GL-1. In GL-2, group II correlated higher with group III than with IV, and the correlation between groups III and IV was higher than that between groups II and IV.

The atherogenic diets had a much smaller effect on GL-3 fatty acids, than on either GL-2 or GL-1 fatty acids. This is reflected in high correlations between the control group and the experimental group in all three organs. However, the same general trend was observed here as was observed for GL-2 and GL-1. That is, the atherogenic diet affected the fatty acids of aorta and plasma more than those of erythrocytes. Correlations among the three experimental diet groups follow the same trend that was observed for GL-2 and GL-1.

Correlations among GL-4 acids follow the same pattern as those of GL-3, although GL-4 was not found in group I from the plasma fraction. The correlations for GL-5 are unfortunately incomplete,

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since GL-5 was not analyzed in the first three groups from erythrocytes and was not found in group I from aorta. From results for plasma, the correlations indicate that the diets changed this lipid very little.

These correlations again point out that the atherogenic diets have their greatest effect on plasma and aorta, and a lesser effect on erythrocytes. This is consistent with the previously discussed observations. It was noted that the amount of crude lipid from plasma of rabbits fed atherogenic diets was ten times that of plasma from control animals. Although the amount of crude lipid per g tissue was not changed for aorta, the amount of tissue per aorta was increased when comparing control and experimental animals. It was also noted that experimental animals had aortas that were covered with fatty plaques and were hence grossly changed from normal rabbit aortas.

Table XXII presents the correlations between the glycosphingolipid fatty acid distributions of aorta and plasma along with aorta and erythrocytes, within a given group. For lipids GL-1 through GL-4, all correlations between RA and RP are higher than between RA and RE. Data from BL-5 are incomplete, in contrast to those for the other four lipids. The correlation of RA and RP is lower than that of RA and RE. These data indicate that the fatty acids of aorta glycosphingolipids more closely resemble those of plasma than erythrocytes. As discussed previously, significant glycosphingolipid synthesis could not be demonstrated; therefore, these

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compounds are probably transported from the blood. The correlations presented in Table XXII are evidence for the transport of glycosphingolipids from plasma to aorta rather than from erythrocytes to aorta.

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Table XXII. Correlations Between Glycosphingolipid Fatty Acid Distributions of Aorta, Erythrocytes and Plasma, within a Diet Group

Diet	I	II	III	IV
		GL-1		
RA to RP[1]	.83	.93	.96	.94
RA to RE	.65	.82	.68	.49
		GL-2		
RA to RP	0[2]	.75	.89	.87
RA to RE	0	.50	.88	.78
		GL-3		
RA to RP	.81	.86	.77	.86
RA to RE	.18	.42	.48	.65
		GL-4		
RA to RP	0	.74	.77	.81
RA to RE	.16	.54	.64	.51
		GL-5		
RA to RP	0	.43	.50	.83
RA to RE	NA	NA	NA	.91

1. Correlation of rabbit aorta to rabbit plasma.

2. Zeros indicate one of the lipids was not found.

SUMMARY

Experiments were performed to determine the level of cerebroside synthesis in bovine aortic tissue. Although there was incorporation into crude lipid in many experiments, no consequential incorporation into cerebrosides was noted. These experiments did not prove conclusively that cerebrosides are not synthesized in aortic tissue, only that systems similar to those used with other tissues did not synthesize measurable quantities of these compounds.

Glycosphingolipids were isolated from bovine and porcine aorta, spleen and various blood fractions. The quantity and fatty acid distribution of each lipid was determined. The predominant lipid in all tissue, except aorta, was GL-1. In aorta, GL-3 predominated. The fatty acids of these lipids were quite variable. It was noted that the fatty acid distributions of BA were more like those of BP than the remaining bovine samples. The attempt to fractionate the various lipoproteins in BP did not yield reproducible results.

The major glycosphingolipid in porcine erythrocytes was GL-4. In PE samples, the ACL of the fatty acid distributions increased with increasing hexose content of the lipid. Hydroxy acids were also identified to be major components in these lipids, especially in GL-4. The quantities of the four glycosphingolipids in PHDL, PLDL and PA samples were all of the same order of magnitude. The fatty acids of these lipids were quite variable. In PS, GL-1 was again the predominant glycosphingolipid. In these lipids the longer chain

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fatty acids predominated.

Five glycosphingolipids were isolated from rabbits that were fed normal or one of three atherogenic diets for a period of six months. These lipids, identified as GL-1 through GL-4, were identical to those found in human blood. GL-5 was characterized as probably hematoside with possibly some pentaglycosylceramide. The major glycosphingolipid in RE samples was GL-3. In aorta and plasma either GL-1 or GL-3 were the predominating glycosphingolipids. The quantities of most of the glycosphingolipids were greater in the experimental animals than in the control animals. Generally, diet III animals had the greatest quantities.

The primary effects of the atherogenic diets on the fatty acids of rabbit glycosphingolipids are typified by RA-GL-1. The experimental animals had higher concentration of the longer chain fatty acids than the controls. This was reflected by a larger ACL for the experimental groups. The primary increases were in the 24 carbon acids. Further changes were also seen in the 24 carbon acids. The experimental animals had an increased 24:1/24:0 ratio and an increased percentage of 24:2. Plasma and aorta showed larger effects of the atherogenic diets than did the erythrocytes, although the general effects in all three tissues were the same. It was postulated that these changes were due to increased fatty acid synthesis, primarily chain elongation, induced by the atherosclerosis brought on by the experimental diets.

The correlations of the fatty acid distributions also indicated

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that the diets had their greatest effects on plasma and aorta glycosphingolipids. The correlations between aorta and plasma were consequentially higher than the correlations between aorta and erythrocytes. This, and the aforementioned fatty acid changes, indicate a probable transport of glycosphingolipids from plasma to aorta.

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VITA

The author was born February 1, 1944 in New York City. He later moved to Union, Washington, where he received his education through high school. After two years at a junior college, he transferred to Western Washington College where he received his BA in chemistry in 1966. The following Fall he enrolled at Western Michigan University and completed an MA in chemistry in Spring 1968. His studies have continued at this institution, and this thesis represents the final requirement for a Ph.D.

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