A Survey of Glycosphingolipids in the Human Aorta: Their Relationship to Glycosphingolipids of Blood and to Atherosclerosis

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A SURVEY OF GLYCOSPHINGOLIPIDS IN THE HUMAN AORTA: THEIR RELATIONSHIP TO GLYCOSPHINGOLIPIDS OF BLOOD AND TO ATHEROSCLEROSIS

by

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A SURVEY OF GLYCOSPHINGOLIPIDS OF THE HUMAN AORTA: THEIR RELATIONSHIP TO GLYCOSPHINGOLIPIDS OF BLOOD AND TO ATHEROSCLEROSIS

John L. Nappier, Ph.D.

Western Michigan University, 1983

The four major glycosphingolipids, glucosyl ceramide, lactosyl ceramide, galactosyl lactosyl ceramide and globoside were isolated from both diseased and normal tissue from human aorta by solvent extraction followed by solvent partitioning and column chromatography. The levels of each were then determined quantitatively by high pressure liquid chromatography (HPLC) after derivatization. The individual glycosphingolipids were isolated by semi-preparative HPLC and the fatty acid composition of each was determined, after methanolysis, by gas chromatography.

In the diseased tissue, the amount of glycosphingolipid as well as the amount of crude lipid increased relative to the normal tissue. The relative amounts of cerebrosides increased in the diseased tissue whereas the longer carbohydrate chain length glycosphingolipids decreased or remained the same. The fatty acid composition of the glycosphingolipids from the two types of tissues was found to be considerably different. The degree of unsaturation was found to increase in the longer chain length fatty acids in the diseased tissue as compared to the normal tissue. The relative differences in the fatty acid compositions were similar among all four glycosphingolipids.
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CHAPTER I

INTRODUCTION

Glycosphingolipids exist in small amounts in most if not all human and mammalian tissues. They have been studied extensively in neural tissues and blood fractions of humans and animals. Klenk and Lauenstein in 1951, discovered a glycolipid in human erythrocytes which contained galactosamine.\(^1\) Yamakawa and Suzuki found the same compound in human erythrocytes and called it globoside.\(^2\) It was later identified by Yamakawa and coworkers as N-acetylgalactosaminyl-(1-3)-galactosyl-(1-4)-galactosyl-(1-4)-glucosyl ceramide and was found to be the predominant component in a mixture of glycosyl ceramides in human erythrocytes.\(^3\) Further characterization of the remaining glycosyl ceramides resulted in the identification of lactosyl ceramide\(^4\) and a trihexosyl ceramide.\(^5\) The trihexosyl ceramide was later identified as galactosyl-(1-4)-galactosyl-(1-4)-glucosyl ceramide.

Many years prior to the isolation of globoside, cerebroside (glucosyl ceramide) was identified as a component of human serum. In 1958, while developing a quantitative method for the analysis of cerebroside in human plasma, Svennerholm and Svennerholm found evidence to indicate the presence of more complex glycolipids.\(^6\) Further work by them\(^7,8\) and by Vance and Sweeley\(^9\) resulted in the elucidation of the four major glycosphingolipids in human erythrocytes and human plasma: glucosyl ceramide (GL-1),

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galactosyl-(\(\beta_1-4\))-glucosyl ceramide (GL-2), galactosyl-(\(\alpha_1-4\))-galactosyl-(\(\beta_1-4\))-glucosyl ceramide (GL-3) and N-acetylgalactosaminyl-(\(\beta_1-3\))-galactosyl-(\(\alpha_1-4\))-galactosyl-(\(\beta_1-4\))-glucosyl ceramide (GL-4).

Dawson and Sweeley found these four glycosphingolipids in porcine blood in similar concentrations. Coles and Foote also found all four glycosphingolipids in the porcine blood fractions: erythrocytes, plasma, high density lipoproteins (HDL) and low density lipoproteins (LDL). Other investigators have found glycosphingolipids in LDL and to a lesser extent in HDL fractions of human blood plasma. In later work it has been shown that the glycosphingolipids in the LDL and the HDL fractions exchange readily with cell membranes. It has also been shown that the level of HDL and LDL can be correlated with the extent and severity of atherosclerosis. Further, there is evidence to indicate that the LDL fraction is actually retained by human aortic intima. Since the levels of LDL and HDL are associated with the extent of atherosclerosis and it has been shown that some of the components of the HDL and LDL fractions are glycosphingolipids, it becomes apparent that these glycosphingolipids could be involved in the development of atherosclerosis. Glycosphingolipids, as well as gangliosides have been implicated in the loss of contact inhibition, which is a distinct feature in atherosclerosis. Ganglioside levels have been studied in human aorta and the levels have been found to increase in diseased tissue. Furthermore, glycosphingolipids have been known to alter cell growth and promote cellular differentiation. It
is reasonable to assume that they are involved somewhere in the process of atherosclerotic plaque development.

If glycosphingolipids are involved in the development of atherosclerosis in human aorta, then it would be expected that there would be a difference in the levels of glycosphingolipids, or in the characteristics of the glycosphingolipids, or both.

A few studies have been performed on aortas to this end. Foote and Coles determined the levels of cerebroside and its fatty acid distribution in both diseased and normal human aorta. A similar study was performed by Tao and Foote using rabbit and pigeon aortas. Foote and Coles carried out a study using pig aorta in which all four glycosphingolipids were isolated, characterized and quantitated. A comparable study was carried out by Coles and Foote using rabbits in which the levels of the four glycosphingolipids and their fatty acid distributions were compared in the aortas of normal rabbits and rabbits fed a high cholesterol diet.

This present work was an extension of the work done by Foote and coworkers. The levels in normal and diseased aortic tissue of all four of the expected glycosphingolipids (GL-1, GL-2, GL-3, and GL-4) were determined and compared. In addition to determining the levels of the individual glycosphingolipids, fatty acid distribution was determined in each of the glycosphingolipids from both diseased and normal tissue and their levels were compared to see if any obvious or not so obvious differences could be correlated to atherosclerotic plaque development.
CHAPTER II

MATERIALS AND METHOD

The following is a list of reagents and apparatus used in this study:

Reagents

chloroform - freshly distilled in glass before use
methanol - Burdick and Jackson, distilled in glass
methylene chloride - Burdick and Jackson, distilled in glass
hexane - Burdick and Jackson, distilled in glass
acetonitrile - Burdick and Jackson, distilled in glass
ethyl acetate - Burdick and Jackson, distilled in glass
acetone - Burdick and Jackson, distilled in glass
diethyl ether (anhydrous) - Mallinckrodt Inc., analytical reagent
pyridine - Mallinckrodt Inc., freshly distilled from BaO and stored over molecular sieves
hydrogen chloride - Union Carbide, technical grade
argon - Linde
hydrogen - Linde
barium oxide - G. Fredrick Smith Chemical Co., technical grade
sodium hydroxide - Mallinckrodt Inc., reagent grade
sodium methoxide - Matheson, Coleman and Bell
ammonium hydroxide (approx. 58%) - Mallinckrodt Inc.
phosphorus pentoxide - Mallinckrodt Inc., analytical reagent
sodium bicarbonate - Mallinckrodt Inc., analytical reagent
potassium chloride - Mallinckrodt Inc., analytical reagent
p-nitrobenzoyl chloride - Aldrich Chemical Co.
benzoyl chloride - Eastman Kodak, Reagent ACS
3,5-dinitrobenzoyl chloride - Eastman Kodak
benzoic anhydride - Eastman Kodak
bromothymol blue - Eastman Kodak
NHI-F methyl ester standard mixture - Supelco
mannitol - Aldrich Chemical Co.
glucose - Mallinckrodt Inc., analytical reagent
galactose - Aldrich Chemical Co.
galactosamine - Sigma Chemical Co., grade 1
sphingosine - Miles Laboratories
(N-stearoyl)-glucose ceramide - Miles Laboratories
ceramide trihexoside - Supelco, lot no. la 01001
globoside - Supelco, lot no. la 01010
(N-palmitic)-lactosyl ceramide - Miles-Yeda, Ltd.
(N-lignoceric)-lactosyl ceramide - Miles-Yeda, Ltd.
TRI-SIL silylating reagent - Pierce Chemical Co.
Dowex 2-x8 anion exchange resin - Bio Rad Labs.
silica gel ( Unisil ) - Clarkson Chemical Co.

Apparatus

3 ml Reacti-vials - Pierce Chemical Co.
heating block - Pierce Chemical Co.
silica gel TLC plates - Analtech GF 250µM and 1000µM
homogenizer - Thermovac Macro/micro

GC column packing - Silar 10-C 100/120 mesh on Gas Chrom Q

GC column packing - 3% OV-1 100/120 mesh on Gas Chrom Q

gas chromatograph - Hewlett Packard Model 5730A

integrator - Hewlett Packard Model 3390A

HPLC column - Dupont Zorbax SIL 4.6 mm X 25 cm

HPLC column - Regis Reversible Hi Chrom Nitrile 5 μM, 25 cm x 4.6 mm

HPLC - Varian Model 4200

HPLC - Perkin Elmer Model 601

HPLC detector - Waters Assoc., Model 440

integrator - Autolab System IV

rotary evaporator - Buchi-Rinco Instrument Co.

rotary evaporator - The Upjohn Co. 29

N₂ evaporator - Organomation

Initial Evaluation of Standards

The standard glycosphingolipids were obtained from several sources. The cerebroside standard (GL-1) was a synthetically prepared standard (N-stearoyl)glucose cerebroside obtained from Miles Laboratories. The ceramide trihexoside (GL-3) and globoside (GL-4) were initially purchased from Supelco as 2 mg/ml (lot no. LA 01001) and 5 mg/ml (lot no. LA 01010) solutions in 2:1, CHCl₃:MeOH. The GL-1 standard was prepared by dissolving 10 mg of the solid in 10 ml of 2:1, CHCl₃:MeOH giving a 1 mg/ml solution. The GL-3 and GL-4 standards were diluted to 5 ml with 2:1, CHCl₃:MeOH giving a 0.4 mg/ml solution of GL-3 and a 1 mg/ml solution of GL-4. The three standards
were spotted on a silica gel thin layer chromatography (TLC) plate (Analtech GF 250µ), which had been preheated at least 2 hours prior to spotting and were developed in 24:7:1, CHCl₃:MeOH:H₂O. The TLC tank had been pre-equilibrated prior to the development of the plate for 2 hours with the above solvent system.

Initial Evaluation of HPLC Conditions

The standards were initially benzoylated to increase their UV absorbance for detection by HPLC by a procedure developed by McCluer and Evans.30 Approximately 100 µg of each of the three standards were pipetted into 3 ml Reacti-vials and were evaporated to dryness under a nitrogen stream. A 0.5 ml aliquot of fresh pyridine was pipetted into each vial followed by 50 µl of benzoyl chloride. The vial was capped, shaken and placed in a heating block at 37°C for 16 hours. At the end of 16 hours, the vial was removed from the heating block and placed under a nitrogen stream. The solution was evaporated until no trace of pyridine odor remained. A 3 ml aliquot of hexane was used to transfer the residue from the Reacti-vial to a 15 ml screw cap centrifuge tube. To the centrifuge tube was added 1.8 ml of a saturated solution of Na₂CO₃ in 80:20, MeOH:H₂O. The centrifuge tube was shaken vigorously for 30 seconds. The contents were allowed to settle and the aqueous layer was removed and discarded. The hexane layer was washed three more times in a like manner. A final wash was done with 1.8 ml of 80:20, MeOH:H₂O and then the hexane layer was transferred back to the Reacti-vial and was evaporated to dryness under a nitrogen stream. The residue was
dissolved in 1 ml of CH₂Cl₂ prior to HPLC analysis.

A 20 μl aliquot of the standard solution was injected onto the HPLC under the following conditions:

- **HPLC** - Varian Model 4200
- **COLUMN** - Dupont Zorbax SIL 4.6 mm x 25 cm
- **DETECTOR** - Waters Model 440 UV 254 nm
- **SOLVENT** - gradient 1-8 % CH₃CN in CH₂Cl₂
- **FLOW RATE** - 1.0 ml/ml
- **CHART SPEED** - 0.25 in/min

Isolation of Derivatized Standards

To determine if the standard peaks observed by HPLC corresponded to the correct standards, the standards were isolated by semi-preparative HPLC. The standards prepared in the previous section were combined and concentrated to 50 μl. A 20 μl aliquot was injected onto the HPLC using the conditions indicated above. Each peak was collected after it was eluted through the detector. The collected fractions were subjected to mild alkaline catalyzed methanolysis to remove the benzoyl groups. Each collected fraction was evaporated to dryness under a nitrogen stream. The residue was dissolved in 1 ml of CHCl₃ and 1 ml of 0.6 N methanolic NaOH. The sample was allowed to stand at room temperature with occasional mixing for 1 hour. To the solution was added 1.2 ml of 0.5 N methanolic HCl, 1.7 ml of H₂O and 3.4 ml of CHCl₃. The solution was shaken and then centrifuged to separate the phases. The upper aqueous phase was discarded, the lower organic phase was evaporated.
to dryness under a nitrogen stream and the total extract was spotted on a silica gel TLC plate. The plate was developed in CHCl₃:MeOH:H₂O, 65:25:4 examined under UV light, and then sprayed with bromothymol blue.

**Isolation of Glycosphingolipids From Human Blood**

Due to the inability of obtaining all four pure glycosphingolipid standards from commercial sources, the standards were isolated from human red blood cells. Two pints of packed red blood cells and one pint of whole blood, all of which were outdated, were obtained from the Red Cross and were immediately frozen. The samples were processed according to the procedure of Vance and Sweeley.⁹ The whole blood sample was thawed and then split into two aliquots of 225 ml each, and to each was added 600 ml of MeOH with stirring. Then 1200 ml of CHCl₃ was added to each aliquot. The mixtures were filtered, and the solid residue from both were combined and washed with 200 ml of 2:1, CHCl₃:MeOH. The solid residue was then extracted with 900 ml of 2:1, CHCl₃:MeOH at gentle reflux for 2 hours. The mixture was filtered and the residue was washed with 100 ml of 2:1, CHCl₃:MeOH. This residue was discarded and the extracts, washings and filtrates were combined. The extract was separated into 4-1200 ml fractions in 2 l separatory funnels. To each was added 270 ml of water and they were shaken vigorously. The separatory funnels were allowed to stand overnight. The organic phases from all separatory funnels were combined and evaporated to dryness on a rotary evaporator. The packed red blood cells were processed in a
similar manner.

A silicic acid column was prepared by heating 48 g of silicic acid overnight at 80° C and slurrying it in diethyl ether. The slurry was poured into a 3 cm ID column, and once the packing had settled the column was topped with sand. The excess diethyl ether was allowed to elute through the column and then the column was washed with 180 ml of CHCl₃. The three crude lipid extracts, one from each pint of packed red blood cells and one from the pint of whole blood, were dissolved in a small amount of CHCl₃ and were combined and placed on the column. The CHCl₃ solution was allowed to elute into the column, and the neutral lipids were eluted from the column with 1200 ml of CHCl₃. The pigment was eluted with 120 ml of ethyl acetate, and the glycosphingolipids were eluted with 2400 ml of 9:1, acetone:methanol. Each fraction collected was evaporated to a constant weight in a tared flask on a rotary evaporator. The weights of each fraction were recorded.

The crude glycosphingolipid fraction was dissolved in 12 ml of CHCl₃. A 12 ml aliquot of 0.21 M NaOCH₃ in MeOH was added to the flask. The solution was allowed to stand for 1 hour at room temperature with occasional shaking. After 1 hour, the sample was subdivided into 3 parts of 8 ml each and was placed in 50 ml centrifuge tubes for ease of handling. To each of the tubes was added 4.8 ml MeOH, 6.3 ml of 0.12 N HCl and 10.2 ml of CHCl₃. The samples were shaken vigorously and then centrifuged to separate the phases. The upper phase was discarded, and the lower phase was washed twice more with equal volumes of 1:1, MeOH:H₂O. The organic
layers were combined and evaporated to dryness on a rotary evaporator.

To each of the residues; neutral lipids, pigment fraction and glycosphingolipids, was added 5 ml of 2:1,CHCl$_3$:MeOH. A 10 µl aliquot of each fraction and a 1 mg/ml GL-1 standard solution were spotted on a silica gel TLC plate preheated at 90° C overnight. The plates were developed in CHCl$_3$:MeOH:H$_2$O,100:42:6, which had been equilibrating in the tank for 2 hours. The plates were removed from the solvent after development and were allowed to air dry. After spraying with a bromothymol blue solution, the plates were placed in a tank of NH$_4$OH vapor to enhance the color development.

The remaining glycosphingolipid sample was streaked on 3 preparative silica gel TLC plates (1 mm thickness), approximately 1.3 ml each. The plates had been preconditioned as indicated above and were developed in CHCl$_3$:MeOH:H$_2$O,100:42:6. Bromothymol blue was used to visualize the glycosphingolipids. Individual glycosphingolipid areas were scraped with a razor blade and each of the individual silica fractions, from each of the plates, were combined to make a small column. Each of the four columns was eluted with 200 ml of CHCl$_3$:MeOH:H$_2$O,100:50:10. The eluant was collected in separate 250 ml r. b. flasks and evaporated to dryness on a rotary evaporator. Residues were transferred to small vials with 2:1,CHCl$_3$:MeOH. The resulting purified glycosphingolipids were evaporated to a constant weight under a nitrogen stream and the weights were recorded.
Optimization of the Benzoylation Procedure

After the initial benzoylation procedure was determined to be inadequate, several benzoylation procedures were investigated and several derivatizing reagents were tried including benzoic anhydride, \( p \)-nitrobenzoyl chloride and \( 3,5 \)-dinitrobenzoyl chloride.\(^{31,32,33}\) Of these derivatizing reagents the one found to be best was the \( p \)-nitrobenzoyl chloride. The final procedure used was as follows.

The standard or sample compound was pipetted into a 3 ml Reacti-vial and evaporated to dryness under a nitrogen stream. The vial and cap were then placed in a desiccator and dried over \( P_2O_5 \) under vacuum for at least 3 hours, but usually overnight. The vial was removed from the desiccator and approximately 10 mg of \( p \)-nitrobenzoyl chloride was added to the vial. A 1 ml aliquot of pyridine was also added, and the vial was flushed with nitrogen, tightly capped and heated at 60° C for 6 hours. At the end of 6 hours, the vial was removed from the heating block and allowed to cool to room temperature. The pyridine was removed under a nitrogen stream in a water bath at 40° C. The residue was dissolved in 2 ml \( CH_2Cl_2 \) and transferred to a 15 ml screw cap centrifuge tube. The vial was rinsed with 2 ml of 3 % \( NaHCO_3 \) in water and the rinse was added to the centrifuge tubes. The tube was capped and shaken vigorously for 30 seconds and the phases were allowed to separate. The upper aqueous phase was removed and discarded. Two more rinses and washes were done in a similar manner followed by a final 2 ml water rinse and wash. The \( CH_2Cl_2 \) phase was removed and placed back.
in the Reacti-vial and the solvent evaporated under a nitrogen stream. The residue was dissolved in 2 ml of \( \text{CH}_2\text{Cl}_2 \) and was stored at -20° C until analyzed.

**TLC Analysis of Derivatized Standards**

The isolated standards from human blood, the synthetic cerebroside standard, the isolated triglycosyl ceramide from Supelco and two lactosyl ceramide synthetic standards (palmitic and lignoceric) from Miles-Yeda, Ltd. were derivatized with \( p \)-nitrobenzoyl chloride using the above procedure. The derivatives were spotted on silica gel TLC plates and were developed in 5 % \( \text{CH}_3\text{CN} \) in \( \text{CH}_2\text{Cl}_2 \).

**HPLC Analysis of Derivatized Standards**

The initial HPLC conditions were modified to give improved resolution and reproducibility. The above derivatized standards were analyzed using the following HPLC conditions:

- **HPLC** - Perkin Elmer Model 601
- **DETECTOR** - Waters Model 440 Absorbance Detector
- **DETECTOR WAVELENGTH** - 254 nm
- **DETECTOR SENSITIVITY** - 0.1 absorbance units full scale
- **FLOW RATE** - 1.0 ml/min
- **COLUMN** - Regis Reversible Hi Chrom Nitrile 5 µm, 25 cm x 4.6 mm I.D.
- **GRADIENT** - 40-100 % \( \text{CH}_2\text{Cl}_2 \) in hexane over 20 minutes
GRADIENT TYPE - convex
GRADIENT CURVATURE - 999
PRESSURE - 400 psi
INJECTOR - Rheodyne Loop Injector
INJECTION VOLUME - 20 μl loop
CHART SPEED - 0.25 in/min
INTEGRATOR - Autolab System IV
  T1 - 420 sec
  T2 - 960 sec
  T3 - 2200 sec
  PEAK WIDTH - 15 sec
  SLOPE SENS. - 150
  MIN. AREA - 10,000

Preparation of Samples

Human aortas extending from the aortic arch to the bifurcation were obtained at autopsy. All were from embalmed cadavers. The aortas were immediately frozen and were stored in a frozen state until analyzed. Prior to extraction, the aortas were allowed to thaw at room temperature. A general description of the state of the aorta was recorded indicating degree of dryness, extent of diseased tissue and type of diseased tissue. This was done visually. A diseased portion of the aorta was selected and was carefully removed from the aorta by cutting the intima (the inner lining of the aorta) around the diseased portion and lifting out that diseased area. An apparently "normal" area of the aorta was also selected and removed in a similar fashion. Both samples were carried
through the extraction procedure in a parallel manner. The tissues were accurately weighed and were then placed in an appropriately sized homogenization vessel (Thermovac Macro/micro homogenizer) and homogenized for 10 minutes at medium speed using an 18:1 ratio of 2:1, CHCl₃:MeOH to tissue (V:W). The slurry was filtered through a coarse porosity sintered glass funnel under vacuum. The homogenization vessel was rinsed with 2:1,CHCl₃:MeOH (1/6th the original volume), and once more with 1:2,CHCl₃:MeOH (1/6th the original volume). Both rinses were filtered through the residue. An equal amount (1/6th the original volume) of CHCl₃ was added to the filtrate to make the final solvent composition of the extract 2:1,CHCl₃:MeOH. The extract was washed with 1/5 volume 0.1N KCl, and then once again with 0.1N KCl pre-equilibrated with 2:1,CHCl₃:MeOH. During each wash the aqueous layer was discarded. The organic layer was evaporated to dryness on a rotary evaporator at 37°C. The weight of the residue was recorded and represents the crude lipid weight.

Isolation of Glycosphingolipids

A silica gel column was prepared by slurrying 4 g of activated (80°C, overnight) silica gel (Unisil 200-325 mesh) in diethyl ether and pouring it into a 10 mm ID column, which had a plug of glass wool and sand in the bottom. The column was topped with sand and washed with 15 ml of CHCl₃. The crude lipid residue was dissolved in a small amount of CHCl₃, placed on the column and allowed to drain into the column. The neutral lipid fraction was eluted with 100 ml of CHCl₃ and was collected in a 250 ml flat bottom
flask. The glycosphingolipid fraction was eluted with 200 ml of 9:1, acetone:MeOH and was collected in a 250 ml flat bottom flask. Both fractions were evaporated to dryness on a rotary evaporator to a constant weight and transferred to tared screw cap culture tubes. Final weights of both fractions were recorded. The neutral lipid fraction was transferred with CHCl₃ and the glycosphingolipid fraction was transferred with 2:1, CHCl₃:MeOH.

Mild Alkaline Catalyzed Methanolysis

The crude glycosphingolipid residue was suspended in 1 ml of CHCl₃ and 1 ml of 0.21 M NaOCH₃ in methanol was added. The samples were capped and were allowed to stand for 1 hour at room temperature with occasional shaking. At the end of 1 hour, the samples were washed and neutralized with a combination of 1.2 ml MeOH, 1.7 ml of 0.12 M HCl and 3.4 ml CHCl₃. The sample was vigorously shaken and centrifuged for 5 minutes to separate the phases. The upper phase was discarded and the lower phase was washed twice with equal volumes of 1:1, MeOH:H₂O. The aqueous layers were discarded and the organic layer was transferred to a tared screw cap tube, evaporated to dryness under a nitrogen stream and then dried in a desiccator under vacuum overnight. The sample was dissolved in 2:1, CHCl₃:MeOH (5 ml) and was stored at -20°C for later analysis.

Quantitation of Glycosphingolipid Levels in Extracted Aorta Samples

Analysis of the glycosphingolipids from aorta samples was carried out as described in the standard derivatization and HPLC.
section, except for the following changes. A 0.5 mg/ml mannitol standard solution was prepared by dissolving 49.6 mg in 100 ml of methanol. This solution was the internal standard solution, and 20 μl of it was pipetted into the Reacti-vial after the addition of standard or sample (20-400 μl depending upon lipid concentration). The derivatization and HPLC analysis was then performed as indicated previously for the standards.

Isolation of the Glycosphingolipids by Semi-Preparative HPLC

The semi-preparative isolation of the individual glycosphingolipids was performed in a similar fashion as was the quantitative analysis. Samples were derivatized without the addition of mannitol, and a 1 or 2 ml aliquot of the sample was placed in the Reacti-vial prior to evaporation. The extraction after derivatization was carried out as in the quantitative analysis, except that it was necessary to centrifuge the tubes to achieve phase separation. The HPLC conditions were similar, except that a 250 μl injection loop was used instead of a 20 μl loop, and the gradient ran from 30-100 % CH₂Cl₂. The sample was dissolved in a final solution of 60 % CH₂Cl₂ in hexane instead of 100 % CH₂Cl₂.

A daily initial run was done with a standard mixture of the glycosphingolipids to determine the exact retention time of the individual glycosphingolipids. Once the retention times were established, the samples were injected onto the column and fractions were collected after the eluant had passed through the UV detector.
If the retention times varied slightly, fractions were collected at the beginning through the end of the sample peak rather than exactly at the retention time of the standards.

Determination of The Fatty Acids of The Isolated Glycosphingolipids

The isolated glycosphingolipids from the semi-preparative HPLC separation were evaporated to dryness under a nitrogen stream in a water bath at 40° C. The samples were analyzed by a modified method of Foote and Coles. To the samples was added 2 ml of 5% dry HCl in methanol. The tubes were flushed with nitrogen, capped and heated at 75° C for 15 hours. After 15 hours, the tubes were allowed to cool and the resultant methyl esters were extracted with four 2 ml portions of hexane. The hexane extracts were combined and evaporated to dryness. The residue was dissolved in 100 μl of hexane and 2-10 μl of the sample was injected on a gas chromatograph under the following conditions:

GAS CHROMATOGRAPH - Hewlett Packard Model 5730A
COLUMN - 6ft., 3% Silar-10C 100/120 mesh on Gas Chrom Q
INJECTION PORT TEMPERATURE - 200° C
FID TEMPERATURE - 250° C
CARRIER GAS - Argon
CARRIER GAS FLOW - 40 ml/min
PROGRAM (TEMPERATURE) - 100-230° C @ 4° C/min
INTEGRATOR - Hewlett Packard Model 3390A
TIME - 30 min
ATTENUATION - -1
THRESHOLD - 0
PEAK WIDTH - 32

The standard used to determine the retention time of the fatty acid methyl esters was NHI-F (Supelco). A 50 mg/ml solution was prepared in hexane and was diluted to 0.5 mg/ml in hexane. A 2 μl injection of the standard solution was made onto the column.
CHAPTER III

RESULTS

TLC and HPLC Evaluation of Standards

The initial TLC evaluation of the three standards that were available at that time, showed that only the synthetically prepared glucose cerebroside (GL-1) was pure. The standards obtained from Supelco, triglycosyl ceramide (GL-3) and globoside (GL-4), were both found to give two spots on TLC analysis. The HPLC analysis of these standards gave double peaks for all three. The triglycosyl ceramide and globoside peaks were interlaced. To verify whether or not the peaks observed on the HPLC corresponded to the actual compounds, the fractions were collected as they came off the HPLC. The collected fractions were subjected to mild alkaline catalyzed methanolysis to remove the benzoyl groups. The isolated samples were then spotted on a TLC plate and compared to standards. The results are shown in Table 1. The GL-1 peak appears to be pure and has an $R_f$ similar to that of the standard, GL-1. The first GL-3 peak did not have a corresponding spot, probably indicating that there was insufficient compound isolated to be seen by TLC. The first GL-4 peak gave TLC spots with $R_f$'s which correspond to GL-3 and GL-4. It appears that peak 1 corresponds to GL-1, peak 4 and probably peak 2 correspond to GL-3 and peak 3 and peak 5 correspond to GL-4. The TLC analysis of the peaks corresponding to GL-4 appear to show that the
Table 1
Evaluation of Glycosphingolipid Standards by HPLC and TLC

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>HPLC Peak</th>
<th>TLC R&lt;sub&gt;f&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GL-1</td>
<td>0.67</td>
</tr>
<tr>
<td>2</td>
<td>GL-3, peak 1</td>
<td>-&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>GL-4, peak 1</td>
<td>0.22, 0.32</td>
</tr>
<tr>
<td>4</td>
<td>GL-3, peak 2</td>
<td>0.30</td>
</tr>
<tr>
<td>5</td>
<td>GL-4, peak 2</td>
<td>0.21, 0.35</td>
</tr>
<tr>
<td>GL-1 std&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>0.68</td>
</tr>
<tr>
<td>GL-3 std&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>0.29, 0.32</td>
</tr>
<tr>
<td>GL-4 std&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>0.21, 0.25</td>
</tr>
</tbody>
</table>

a - number of the fraction sampled from HPLC.
b - peak comparison to fraction number; standards for GL-3 and GL-4 produced two peaks for each.
c - TLC analysis of HPLC fractions after mild alkaline catalyzed methanolysis.
d - no spot observed.
e - standards spotted on to TLC plate; not isolated by HPLC.

The latter was contaminated by GL-3. Since the GL-3 peak was not contaminated with GL-4, the separation by HPLC appears to be successful and the contamination of the GL-3 peaks with GL-4 must have occurred after HPLC isolation of the peaks, possibly during the collection of the HPLC effluent. It could also be that the methanolysis performed to remove the benzoyl groups was a bit too severe and caused the degradation of some of GL-4 to GL-3 or a compound with an R<sub>f</sub> similar to that of GL-3. The second GL-3 peak
gave a spot with an $R_f$ similar to that of the GL-3 standard. The second GL-4 peak also gave TLC spots with $R_f$'s which correspond to GL-3 and GL-4. Isolation of these standards from human red blood cells was pursued for two reasons. One was the need for more standards, and the second was because the standards initially available gave double peaks by HPLC and double spots by TLC. The cause of the double peak is discussed later, but may be from incomplete derivatization and/or the presence of hydroxy fatty acids. Human red blood cell glycosphingolipids are known to be free of hydroxy fatty acids, and because of that they were used as a source of the glycosphingolipid standards.

All four glycosphingolipids were isolated from human red blood cells in the following amounts: GL-1 8.4 mg, GL-2 15.4 mg, GL-3 16.4 mg and GL-4 58.5 mg. These standards were evaluated for purity along with the initial cerebrosides and some more recently purchased synthetic lactosyl ceramide (GL-2). The isolated standards and the commercial standards were all found to give a single spot by TLC. While the isolation of the standards was continuing, several different benzoylation procedures and different HPLC conditions were investigated. Upon completion of the isolation of the standards, the benzoylation procedure and HPLC procedure had been optimized. The standards were derivatized and analyzed by the new procedures and found to give single peaks for all four of the glycosphingolipids. It was not known whether or not the isolation of the standards free of hydroxy fatty acids, or the change in derivatization reagents eliminated the double peaks. The GL-2 peak
appeared to be a combined double peak, but was sufficiently separated from the other peaks to be useful. The reason for the double peak is unknown. An example of a HPLC chromatogram of the standards is shown in Figure 1.

Isolation of the Crude Glycosphingolipids

Eight aortas were carried through the extraction procedure. From six of the aortas, both plaque and normal tissue was taken. From one, only plaque tissue could be found. A plaque sample and a calcified sample, but not a normal sample, were taken from another aorta. The samples were processed according to the extraction procedure previously described. The results are shown in Table 2. Table 3 gives weights as a per cent of tissue. The crude lipid weight, expressed as a per cent of tissue weight, shows that in every case the level of crude lipid increases in the diseased tissue as compared to the normal tissue. The glycosphingolipid weight, expressed as a per cent of tissue weight, shows that the amount of glycosphingolipid also increases in the diseased tissue relative to the normal tissue. In general, it appears that the glycosphingolipid increases by approximately the same relative amount as the crude lipid increases. Again in Table 3, by comparing the weight of the glycosphingolipid as a per cent of crude lipid, it can be seen that there is very little difference between the levels in diseased tissue as compared to normal tissue.
Figure 1. An HPLC Chromatogram of the Four Glycosphingolipid Standards.
Table 2

Summary of Glycosphingolipid Extraction

<table>
<thead>
<tr>
<th>Crude Sample Number</th>
<th>Neutral Tissue Weight (g)</th>
<th>HPLC Lipid Weight (g)</th>
<th>HPLC Lipid Weight (g)</th>
<th>GSL Weight (mg)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-N(^b)</td>
<td>2.396</td>
<td>0.18497</td>
<td>0.15118</td>
<td>0.757</td>
</tr>
<tr>
<td>25-N</td>
<td>2.911</td>
<td>0.15119</td>
<td>0.10709</td>
<td>1.186</td>
</tr>
<tr>
<td>25-P</td>
<td>1.335</td>
<td>0.10544</td>
<td>0.08385</td>
<td>1.046</td>
</tr>
<tr>
<td>31-N</td>
<td>0.460</td>
<td>0.03308</td>
<td>0.01876</td>
<td>0.198</td>
</tr>
<tr>
<td>31-P</td>
<td>1.646</td>
<td>0.24008</td>
<td>0.17507</td>
<td>1.255</td>
</tr>
<tr>
<td>37-N</td>
<td>4.877</td>
<td>0.19539</td>
<td>0.13979</td>
<td>1.157</td>
</tr>
<tr>
<td>37-P</td>
<td>3.161</td>
<td>1.37401</td>
<td>0.84522</td>
<td>5.811</td>
</tr>
<tr>
<td>43-P</td>
<td>4.286</td>
<td>0.42812</td>
<td>0.24478</td>
<td>4.731</td>
</tr>
<tr>
<td>43-C</td>
<td>6.053</td>
<td>0.42381</td>
<td>0.23583</td>
<td>2.872</td>
</tr>
<tr>
<td>49-N</td>
<td>1.564</td>
<td>0.10436</td>
<td>0.07067</td>
<td>0.677</td>
</tr>
<tr>
<td>49-P</td>
<td>7.062</td>
<td>0.76570</td>
<td>0.60234</td>
<td>6.075</td>
</tr>
<tr>
<td>55-N</td>
<td>1.743</td>
<td>0.13200</td>
<td>0.09530</td>
<td>0.958</td>
</tr>
<tr>
<td>55-P</td>
<td>7.010</td>
<td>0.81779</td>
<td>0.66514</td>
<td>6.300</td>
</tr>
<tr>
<td>61-N</td>
<td>0.818</td>
<td>0.05906</td>
<td>0.03814</td>
<td>0.542</td>
</tr>
<tr>
<td>61-P</td>
<td>4.395</td>
<td>0.47572</td>
<td>0.35870</td>
<td>4.614</td>
</tr>
</tbody>
</table>

\(^a\) - determined by summation of individual glycosphingolipid HPLC analyses.

\(^b\) - N indicates normal tissue, P indicates plaque tissue and C indicates calcified tissue.
Table 3
Comparison of Crude Lipid and Glycosphingolipid Weights

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Crude Lipid Weight, % Tissue Weight</th>
<th>GSL Weight, % Tissue Weight</th>
<th>GSL Weight, % Crude Lipid Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-N</td>
<td>7.72</td>
<td>0.0316</td>
<td>0.409</td>
</tr>
<tr>
<td>25-N</td>
<td>5.19</td>
<td>0.0408</td>
<td>0.785</td>
</tr>
<tr>
<td>25-P</td>
<td>7.90</td>
<td>0.0784</td>
<td>0.992</td>
</tr>
<tr>
<td>31-N</td>
<td>7.19</td>
<td>0.0431</td>
<td>0.599</td>
</tr>
<tr>
<td>31-P</td>
<td>14.58</td>
<td>0.0762</td>
<td>0.523</td>
</tr>
<tr>
<td>37-N</td>
<td>4.01</td>
<td>0.0237</td>
<td>0.592</td>
</tr>
<tr>
<td>37-P</td>
<td>43.47</td>
<td>0.1840</td>
<td>0.422</td>
</tr>
<tr>
<td>43-P</td>
<td>9.99</td>
<td>0.1100</td>
<td>1.105</td>
</tr>
<tr>
<td>43-C</td>
<td>7.00</td>
<td>0.0474</td>
<td>0.678</td>
</tr>
<tr>
<td>49-N</td>
<td>6.67</td>
<td>0.0433</td>
<td>0.649</td>
</tr>
<tr>
<td>49-P</td>
<td>10.84</td>
<td>0.0860</td>
<td>0.793</td>
</tr>
<tr>
<td>55-N</td>
<td>7.57</td>
<td>0.0550</td>
<td>0.726</td>
</tr>
<tr>
<td>55-P</td>
<td>11.67</td>
<td>0.0900</td>
<td>0.771</td>
</tr>
<tr>
<td>61-N</td>
<td>7.22</td>
<td>0.0660</td>
<td>0.918</td>
</tr>
<tr>
<td>61-P</td>
<td>10.82</td>
<td>0.1050</td>
<td>0.970</td>
</tr>
</tbody>
</table>

a - determined by HPLC analysis.
b - N indicates normal tissue, P indicates plaque tissue and C indicates calcified tissue.
Quantitation of the Individual Glycosphingolipids by HPLC

The calculation of the amounts of the individual glycosphingolipids from the HPLC analysis was done by assuming that the absorbances of the benzoyl groups solely account for the detector response. A similar assumption was made by Ullman and McCluer in their work on the quantitation of glycosphingolipids by HPLC. The amount of glycosphingolipid is related directly to the detector response relative to the response of the internal standard, mannitol, after derivatization. Mannitol has 6 hydroxyl groups which can be benzoylated. The glycosphingolipids not only have hydroxyl groups which can be benzoylated, but also have amine groups which can be benzoylated. The individual glycosphingolipids, their number of hydroxyl and amine groups, and the total number of benzoyl groups attached is given in Table 4. The assumption is that the detector response is directly related to the number of benzoyl groups attached to the derivatized molecule. By comparing the detector response of the internal standard at a known concentration to that of the unknown concentrations of the glycosphingolipids, the unknown concentration can be determined. To test the validity of this assumption, glycosphingolipid standards of known concentrations were derivatized and analyzed by HPLC using an integrator to determine the area of each peak. Knowing the concentrations of the solutions allows the determination of the amount of glycosphingolipid injected on to the column, and the calculation in picomoles of glycosphingolipid which corresponds to the obtained peak area. By dividing the peak area by
Table 4
Theoretical Number of Glycosphingolipid Benzoyl Groups

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of OH$^a$</th>
<th>No. of NH$^b$</th>
<th>Total Benzoyl Groups$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mannitol</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>GL-1</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>GL-2</td>
<td>8</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>GL-3</td>
<td>11</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>GL-4</td>
<td>13</td>
<td>2</td>
<td>15</td>
</tr>
</tbody>
</table>

$^a$ number of hydroxyl groups available for benzoylation.
$^b$ number of amino- or amido- groups available for benzoylation.
$^c$ total number of groups which can theoretically be benzoylated.

the picomoles of glycosphingolipid present, a detector response/picomole is obtained. By dividing the response/picomole by the number of benzoylated groups in the derivatized glycosphingolipid molecule a normalized response, i.e. a response/benzoyl group, is obtained. If the initial assumption is valid and the standards are pure, the normalized response should be the same for all four glycosphingolipid standards. The results of the evaluation are shown in Table 5. As can be seen from the table, with the exception of GL-3, the standard responses compare quite well with each other, the average being within 90-97% of the mannitol standard. Also from Table 5 it can be seen that the reproducibility from injection to injection is good, having standard deviations ranging from 1.3-5.3%.
Table 5
Evaluation of Glycosphingolipid Standard Detector Responses

<table>
<thead>
<tr>
<th>Run No.</th>
<th>NR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>%man&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>%man&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>%man&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>%man&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>92.0</td>
<td>32.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>95.0</td>
<td>11.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.7</td>
<td>28.85&lt;sup&gt;c&lt;/sup&gt;</td>
<td>85.2</td>
</tr>
<tr>
<td>2</td>
<td>61.19</td>
<td>91.4</td>
<td>65.03</td>
<td>97.1</td>
<td>19.88</td>
<td>29.7</td>
<td>57.40</td>
<td>85.7</td>
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<tr>
<td>3</td>
<td>63.35</td>
<td>91.2</td>
<td>67.94</td>
<td>97.8</td>
<td>20.74</td>
<td>29.8</td>
<td>66.20</td>
<td>95.3</td>
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<tr>
<td>4</td>
<td>53.42</td>
<td>91.7</td>
<td>57.69</td>
<td>99.1</td>
<td>17.41</td>
<td>29.9</td>
<td>54.24</td>
<td>93.1</td>
</tr>
<tr>
<td>5</td>
<td>49.59</td>
<td>83.8</td>
<td>57.34</td>
<td>97.0</td>
<td>16.95</td>
<td>28.7</td>
<td>56.88</td>
<td>96.2</td>
</tr>
<tr>
<td>6</td>
<td>54.71</td>
<td>90.3</td>
<td>58.92</td>
<td>97.2</td>
<td>17.38</td>
<td>28.7</td>
<td>58.83</td>
<td>97.1</td>
</tr>
<tr>
<td>avg</td>
<td>90.1</td>
<td>97.2</td>
<td>30.1</td>
<td>92.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td>3.1</td>
<td>1.3</td>
<td>1.8</td>
<td>5.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> - normalized response based on detector response and number of benzoyl groups.

<sup>b</sup> - expressed as a per cent of the internal standard, mannitol.

<sup>c</sup> - values are lower because a different HPLC procedure was used.

The GL-3 standard was found to have a considerably lower response than the rest, and this is believed to be a reflection of either its purity or the accuracy of its concentration rather than of its response. If this is true, it will in no way affect the calculation of the amount of GL-3 in the unknown samples, as that calculation is based on the theoretical number of benzoyl groups on the derivatized GL-3 molecule relative to the response of the internal standard. The actual concentration of the GL-3 standard does not enter into the calculation of the concentration of GL-3 in the unknown samples.
The calculations for the samples are done by taking the peak area of the internal standard, which is present at a known concentration, and determining the response/picomole as was done for the standards. The normalized response is then determined by dividing the response/picomole by 6, the number of benzoyl groups present in the derivatized mannitol molecule. Once the normalized response has been determined, it is multiplied by the number of benzoyl groups on the derivatized individual glycosphingolipid molecules to determine the response/picomole for each of the four glycosphingolipids. The response/picomole value is then divided into the peak area for the peak associated with that glycosphingolipid, giving the amount in picomoles of that particular glycosphingolipid.

The results of the HPLC analysis of the individual glycosphingolipids are given in Table 6. The results are expressed in nanomoles of the individual glycosphingolipids per mg of total glycosphingolipid found by HPLC. From the table it appears that the level of GL-1 increases more in the diseased tissue as compared to the normal tissue than did GL-2, GL-3 and GL-4. Table 7 shows the comparisons better by expressing each glycosphingolipid as a ratio of its concentration in the diseased tissue compared to that in the normal tissue. As can be seen from the ratios, GL-1 concentration was greater in all but one sample from diseased areas, while GL-4 was lower from all samples from diseased areas. GL-2 and GL-3 were mixed, but in general, GL-2 was relatively greater from diseased aorta than was GL-3. This suggests that the shorter carbohydrate chain length glycosphingolipids are more predominant in diseased areas from aorta.
Table 6
Level of Glycosphingolipids Found

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>GL-1</th>
<th>GL-2</th>
<th>GL-3</th>
<th>GL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-N</td>
<td>515.0</td>
<td>280.3</td>
<td>305.1</td>
<td>97.8</td>
</tr>
<tr>
<td>25-N</td>
<td>308.8</td>
<td>264.5</td>
<td>348.8</td>
<td>141.1</td>
</tr>
<tr>
<td>25-P</td>
<td>539.7</td>
<td>306.5</td>
<td>223.7</td>
<td>78.9</td>
</tr>
<tr>
<td>31-N</td>
<td>820.3</td>
<td>271.0</td>
<td>106.0</td>
<td>41.6</td>
</tr>
<tr>
<td>31-P</td>
<td>631.4</td>
<td>426.2</td>
<td>141.4</td>
<td>&lt;18.6</td>
</tr>
<tr>
<td>37-N</td>
<td>408.9</td>
<td>465.8</td>
<td>257.5</td>
<td>&lt;43.4</td>
</tr>
<tr>
<td>37-P</td>
<td>580.8</td>
<td>425.9</td>
<td>166.0</td>
<td>8.1</td>
</tr>
<tr>
<td>43-P</td>
<td>669.6</td>
<td>392.4</td>
<td>149.3</td>
<td>33.7</td>
</tr>
<tr>
<td>43-C</td>
<td>685.6</td>
<td>325.9</td>
<td>145.1</td>
<td>43.4</td>
</tr>
<tr>
<td>49-N</td>
<td>191.9</td>
<td>503.5</td>
<td>276.2</td>
<td>83.4</td>
</tr>
<tr>
<td>49-P</td>
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<td>319.4</td>
<td>432.2</td>
<td>287.1</td>
<td>55.7</td>
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a - nanomoles of the individual glycosphingolipid found by HPLC per mg of total glycosphingolipid found by HPLC.
## Table 7

Comparison of Glycosphingolipid Ratios$^a$

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</table>

$^a$ - a ratio found by dividing the nanomoles/mg found in diseased tissue by the nanomoles/mg found in normal tissue. A value above 1 indicates an increase, a value below 1 indicates a decrease.

---

### Analysis of the Fatty Acids From the Isolated Glycosphingolipids

The individual glycosphingolipids were isolated by semi-preparative HPLC as previously described and were methanolized releasing the fatty acids as methyl esters, sphingosine and sugar moieties. The fatty acid methyl esters were extracted into hexane and analyzed by gas chromatography. Since the individual glycosphingolipid concentrations were determined by HPLC, only the relative amounts of the fatty acids were of concern. The samples obtained were small and approached the sensitivity of the flame ionization detector (FID) on the gas chromatograph. To obtain reasonably reliable data the individual samples were run at least in
duplicate and in most cases were run in quadruplicate. During the analysis, a peak was observed which appeared to correspond to the fatty acid, 20:1. As the analysis continued this peak was found to increase in size over time up to the point where it corresponded to over half of the fatty acid present. It was concluded that this peak was an artifact of either the derivatization process or the clean-up procedure and was eliminated in any further calculations.

As mentioned before, a standard was used to determine the retention times of the fatty acid methyl esters. This was NHI-F mixture of methyl esters. Analysis of the standard solution of known relative concentrations over a period of time gives a determination of the accuracy and precision of the assay method. The results of the analysis of 14 standard injections are given in table 8. An

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<th>Mean(%)^</th>
<th>Std Dev</th>
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^ an average of 14 values.
example of a chromatogram is shown in figure 2. As can be seen in Table 8, the reproducibility is relatively good when the level of fatty acid is 7% or above. Below that level the reproducibility gave coefficients of variation of 10-15%.

Figure 2. A GC Chromatogram of the NHI-F Methyl Ester Standards.
The results of the fatty acid analyses of the individual glycosphingolipids is given in Table 9. The values reported are means of 2 to 5 individual values.

### Table 9

**Per Cent Distribution of Fatty Acids**

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<tr>
<td>GL-3</td>
<td>26.2</td>
<td>5.7</td>
<td>6.2</td>
<td>6.1</td>
<td>-</td>
<td>13.1</td>
<td>15.1</td>
<td>13.9</td>
<td>15.7</td>
</tr>
<tr>
<td>GL-4</td>
<td>20.9</td>
<td>7.4</td>
<td>7.0</td>
<td>8.4</td>
<td>6.3</td>
<td>15.3</td>
<td>15.1</td>
<td>16.2</td>
<td>14.0</td>
</tr>
<tr>
<td>61-N GL-1</td>
<td>19.4</td>
<td>7.8</td>
<td>7.2</td>
<td>9.0</td>
<td>-</td>
<td>7.8</td>
<td>15.8</td>
<td>11.9</td>
<td>14.1</td>
</tr>
<tr>
<td>GL-2</td>
<td>17.4</td>
<td>9.8</td>
<td>7.5</td>
<td>9.2</td>
<td>-</td>
<td>8.0</td>
<td>21.1</td>
<td>9.6</td>
<td>11.7</td>
</tr>
<tr>
<td>GL-3</td>
<td>17.5</td>
<td>9.0</td>
<td>8.9</td>
<td>10.4</td>
<td>4.7</td>
<td>7.3</td>
<td>19.0</td>
<td>7.7</td>
<td>6.7</td>
</tr>
<tr>
<td>GL-4</td>
<td>17.4</td>
<td>11.8</td>
<td>8.9</td>
<td>12.1</td>
<td>-</td>
<td>10.0</td>
<td>19.6</td>
<td>15.8</td>
<td>-</td>
</tr>
</tbody>
</table>

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Table 9
Per Cent Distribution of Fatty Acids (Continued)

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>16:0</th>
<th>CN17</th>
<th>18:0</th>
<th>18:1</th>
<th>20:0</th>
<th>22:0</th>
<th>23:0</th>
<th>24:0</th>
<th>24:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>61-P GL-1</td>
<td>16.4</td>
<td>2.4</td>
<td>6.2</td>
<td>1.8</td>
<td>2.3</td>
<td>11.8</td>
<td>8.0</td>
<td>17.0</td>
<td>29.8</td>
</tr>
<tr>
<td>GL-2</td>
<td>17.4</td>
<td>-</td>
<td>14.5</td>
<td>10.6</td>
<td>-</td>
<td>11.6</td>
<td>8.1</td>
<td>-</td>
<td>37.8</td>
</tr>
<tr>
<td>GL-3</td>
<td>16.6</td>
<td>7.8</td>
<td>9.8</td>
<td>11.6</td>
<td>8.2</td>
<td>10.9</td>
<td>12.0</td>
<td>12.7</td>
<td>15.1</td>
</tr>
<tr>
<td>GL-4</td>
<td>25.4</td>
<td>5.4</td>
<td>11.1</td>
<td>8.8</td>
<td>-</td>
<td>8.3</td>
<td>10.5</td>
<td>11.1</td>
<td>13.0</td>
</tr>
</tbody>
</table>

a - an average of between 2 and 5 analyses.
b - CN17 is a peak which had a retention time to long for 17:1. It had a carbon number of 17.6 compared to 17.3 for 17:1.

Identification of fatty acids not included in the known standards was achieved by a plot of carbon number against the retention time for the standards. The unsaturated fatty acids such as 18:1, had retention times corresponding to carbon numbers of 18.3, or 0.3 past the corresponding whole carbon number. All substantial peaks had either whole carbon numbers or were 0.3 past the whole number, except a peak having a carbon number of 17.6. The identity of this peak was not pursued further.

As can be seen from the data, there is a high degree of variation from sample to sample. However, if the normal tissue results are averaged, the plaqued tissue results are averaged, and the two averages compared, a pattern begins to emerge which can be seen in Table 10. From this table it is apparent that there is a
Table 10
Averages of Per Cent Fatty Acid Distribution$^a$

<table>
<thead>
<tr>
<th>Sample</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>20:0</th>
<th>22:0</th>
<th>23:0</th>
<th>24:0</th>
<th>24:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL-1n</td>
<td>14.6</td>
<td>6.2</td>
<td>6.7</td>
<td>7.7</td>
<td>5.8</td>
<td>13.0</td>
<td>17.5</td>
<td>19.3</td>
</tr>
<tr>
<td>GL-1p</td>
<td>18.7</td>
<td>2.9</td>
<td>8.3</td>
<td>6.4</td>
<td>3.2</td>
<td>12.0</td>
<td>10.2</td>
<td>15.3</td>
</tr>
<tr>
<td>GL-2n</td>
<td>16.4</td>
<td>7.3</td>
<td>7.8</td>
<td>10.2</td>
<td>6.8</td>
<td>11.4</td>
<td>18.0</td>
<td>22.6</td>
</tr>
<tr>
<td>GL-2p</td>
<td>18.9</td>
<td>6.0</td>
<td>7.9</td>
<td>8.5</td>
<td>6.3</td>
<td>11.8</td>
<td>18.5</td>
<td>16.8</td>
</tr>
<tr>
<td>GL-3n</td>
<td>17.5</td>
<td>9.1</td>
<td>8.3</td>
<td>11.6</td>
<td>5.9</td>
<td>9.6</td>
<td>21.2</td>
<td>16.7</td>
</tr>
<tr>
<td>GL-3p</td>
<td>20.6</td>
<td>7.3</td>
<td>8.7</td>
<td>10.0</td>
<td>6.7</td>
<td>10.7</td>
<td>17.9</td>
<td>13.9</td>
</tr>
<tr>
<td>GL-4n</td>
<td>16.4</td>
<td>9.2</td>
<td>9.1</td>
<td>10.9</td>
<td>5.8</td>
<td>9.8</td>
<td>21.8</td>
<td>17.4</td>
</tr>
<tr>
<td>GL-4p</td>
<td>19.5</td>
<td>7.2</td>
<td>8.8</td>
<td>10.4</td>
<td>4.0</td>
<td>10.3</td>
<td>17.6</td>
<td>11.7</td>
</tr>
</tbody>
</table>

$^a$ - each number is the average of all values obtained for normal(n) or plaque(p) tissue.

marked difference in the levels of fatty acids in the glycosphingolipid samples taken from diseased tissue and normal tissue. This becomes even more apparent when the differences between the normal tissue and diseased tissue are tabulated as they are in Table 11. From these differences it can be seen that the levels of 16:0 and 24:1 increase, and the levels of 23:0 and 24:0 decrease in diseased tissue relative to normal tissue. The levels of 18:0, 18:1, 20:0 and 22:0 are approximately the same in both diseased and normal tissue.
Table 11
Summary of Differences in Per Cent Between Diseased and Normal Tissue

<table>
<thead>
<tr>
<th>Sample</th>
<th>16:0</th>
<th>CN17</th>
<th>18:0</th>
<th>18:1</th>
<th>20:0</th>
<th>22:0</th>
<th>23:0</th>
<th>24:0</th>
<th>24:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL-1</td>
<td>+4.1</td>
<td>-3.3</td>
<td>+1.6</td>
<td>-0.6</td>
<td>-2.6</td>
<td>-1.0</td>
<td>-7.3</td>
<td>-4.0</td>
<td>+9.1</td>
</tr>
<tr>
<td>GL-2</td>
<td>+2.5</td>
<td>-1.3</td>
<td>+0.1</td>
<td>-1.7</td>
<td>-0.5</td>
<td>+0.4</td>
<td>+0.5</td>
<td>-5.8</td>
<td>+7.4</td>
</tr>
<tr>
<td>GL-3</td>
<td>+3.1</td>
<td>-1.8</td>
<td>+0.4</td>
<td>-1.6</td>
<td>+0.8</td>
<td>+1.1</td>
<td>-3.3</td>
<td>-2.8</td>
<td>+5.0</td>
</tr>
<tr>
<td>GL-4</td>
<td>+3.1</td>
<td>-2.0</td>
<td>-0.3</td>
<td>-0.5</td>
<td>-1.8</td>
<td>+0.5</td>
<td>-4.2</td>
<td>-5.7</td>
<td>-</td>
</tr>
</tbody>
</table>

a - values are differences between normal tissue and plaque tissue from table 10, + indicates more in the plaque tissue and - indicates less in the plaque tissue.

Analysis of the Hexoses From the Glycosphingolipids

The results of the analysis of the semi-preparative isolates of the individual glycosphingolipids for the sugar residues was inconclusive. Although the presence of galactose and glucose was verified, quantitation of the relative amounts was not successful. The standards were easily quantitated through the assay procedure, but the assay of the samples lacked reproducibility. This was thought to be due to the small sample size. The semi-preparative isolation was performed twice on selected samples and the duplicate samples were combined in an attempt to increase the sample size. Analysis of these samples was also unsuccessful. It was felt that insufficient sample was present for a quantitative analysis of the sugar residues in the samples.
It is apparent from the results that either the initial derivatization procedure was not yielding single components, or the presence of the hydroxy fatty acids resulted in two peaks. The benzoylation procedure used for the initial work was the one reported by Ulman and McCluer using benzoyl chloride. Unless the reaction conditions are mild enough this reaction with glycosphingolipids can result in the formation of two products: the expected O-benzoylated derivative, where all the available hydroxyls are benzoylated, and the N-benzoylated derivative where in addition to the hydroxyls the amide linking the fatty acid to the sphingosine is derivatized. If the reaction was to go to completion, there would be only one product consisting of complete O-benzoylation and N-benzoylation. It appears that in the data presented, the N-benzoylation reaction was not going to completion resulting in two products and consequently two peaks occurring on the HPLC.

In an attempt to eliminate the extra peaks, different derivatizing reagents were used. McCluer and Evans reported the use of benzoic anhydride, which did not react at all with the amide functionality on the glycosphingolipid molecule under the conditions used. Initial attempts using this derivatizing reagent had only limited success. Yamazaki and coworkers used p-nitrobenzoyl chloride to react only with the amide functionality after acetylation of the
hydroxyl groups. Their primary reason for acetylation was to aid in the initial separation of the crude glycosphingolipid. Since in this instance that was not necessary, derivatization was performed with p-nitrobenzoyl chloride directly, without prior acetylation. This gave single peaks for pure standards indicating that both the O-benzoylation and the N-benzoylation reactions had gone to completion.

The level of total glycosphingolipid determined as a per cent of tissue weight was remarkably constant from aorta to aorta. Considering only the normal areas of the aorta, the values obtained ranged from 0.0237 to 0.0660 % with most values being relatively close to the mean of 0.0430 %. This is a good indication that the extraction procedure and HPLC analysis were reproducible. The values obtained for the diseased tissue, or more specifically for the plaque tissue were also remarkably constant, falling within a range of 0.0762 to 0.184 %. This also shows a marked increase in the level of glycosphingolipid in the diseased tissue over that observed for normal tissue. This corresponds well with the noted increase in the amount of crude lipid expressed as a per cent of tissue weight. The findings in Table 3 show that the level of glycosphingolipids in the diseased tissue relative to the normal tissue did not change when expressed as a per cent of crude lipid, indicating that as the level of crude lipid increased the level of glycosphingolipid increased in a corresponding manner. The one sample of calcified tissue 43-C, showed levels of glycosphingolipid similar to those of normal tissue and lower than those observed in plaque tissue.
The levels of the individual glycosphingolipids as determined by HPLC showed quite a bit of variability between aortas. For instance, the level of GL-1 in normal tissue expressed as a per cent of total glycosphingolipid on a molar basis (Table 6) ranged from 18 to 66%. The diseased tissues also showed similar variabilities. The variability in the other three glycosphingolipids was not as large, but it still was apparent. Even with this large variability between aortas, certain patterns between diseased tissue and normal tissue can be observed. In almost every case, the per cent of GL-1 increased markedly in diseased tissue as compared with normal tissue. The levels of GL-2 appeared to remain approximately the same, and the level of GL-3 showed a slight decrease in diseased tissue. The glycosphingolipid, GL-4, showed even more of a decrease than did GL-3. In general, it appears that the relative level of the longer carbohydrate chain length glycosphingolipids decreased in the diseased tissue whereas the shorter carbohydrate chain length glycosphingolipids increased.

The level of cerebroside found in human aorta is similar to that found earlier by Foote and Coles. They found 0.01 to 0.73% expressed as a per cent of crude lipid, whereas in the present work the values observed ranged from 0.09 to 0.51%. They also did not find a particularly noticeable increase in the level of cerebroside as a per cent of crude lipid, but did notice a marked increase in the level of crude lipid as a per cent of tissue as was the case here.

No other studies have been done on glycosphingolipid levels in human aorta, but Foote and Coles did a study using pig aorta.
Their findings of the relative per cent of the individual glycosphingolipids agree reasonably well with those observed here, except that in the present study the value of GL-1 was somewhat higher. This is not surprising, as in the present study it was observed that the level of GL-1 increased with an increase in the degree of atherosclerosis, and the human aortas had a much higher degree of atherosclerosis than did the pig aortas which were free of disease. Likewise, in a study using rabbits, the level of GL-1 increased in the aortas exposed to the high cholesterol diets.\textsuperscript{28}

The analysis of the individual glycosphingolipids for their fatty acid composition revealed that the most striking characteristic is the high degree of variability between samples of different aortas. This high degree of variability between aortas has been reported elsewhere.\textsuperscript{25} The primary fatty acids found were 16:0, 18:0, 18:1, 20:0, 22:0, 23:0, 24:0 and 24:1. The level of 16:0 in normal tissue varied from 7.2 to 20.8\%. The level of 24:0 varied from 10.8 to 42.3\%. The remaining fatty acids varied but to a lesser degree.

Though the analysis of only eight aortas makes it difficult to ascertain exact statistically significant differences, by averaging the normal tissue results and the plaque tissue results significant trends become apparent. Even though large differences do occur between different aortas, comparison of individual values with those obtained by averaging, reveals that the trends reflected in the averaged results are representative of the individual values.

From these results (Table 10 and 11) it can be seen that the level of the fatty acid 16:0 increased by 2-4\% and that of 24:1
increased by 5-9%. The level of 23:0 decreased by 3-7% and that of 24:0 by 3-6%. It is interesting to note that the increases and decreases in the fatty acid composition of each of the glycosphingolipids appear to parallel one another with no major differences occurring between any of the glycosphingolipids.

Foote and Coles observed a decrease in the fatty acid ratio of 18:0/18:1 in cerebrosides from diseased human aorta as compared to that from normal aorta cerebrosides. A similar decrease is seen for the current data when looking at the fatty acid ratio 24:0/24:1. This latter ratio modification is consistent with that found from rabbit aortas where the ratio was lower for the glycosphingolipids from rabbits that had been maintained on an atherosclerotic diet. The twenty-four carbon and eighteen carbon acids are related by chain elongation and beta-oxidation. The normal 24:0/24:1 ratio found in pig aorta ran from 1 to 4. In the present and earlier studies in human aorta, the ratios from normal tissue ranged from 1.02 to 2.36, close to the values from pig aorta. The ratios for the plaque tissue in human aorta show a marked decline, ranging from 0.65 to 1.02. This compares well with the values found using rabbits. These results indicate that as the atherosclerotic plaque develops the degree of unsaturation increases, especially in the longer chain length fatty acids.

The amount of individual glycosphingolipids had values which on a percentage basis compare quite well with values obtained for human whole serum by Dawson and Sweeley and for human whole serum and for various lipoprotein fractions, including VLDL, LDL, HDL2 and HDL3, by
Dawson, Kruski and Scanu. Foote and Coles work using pig aortas found levels of GL-3 and GL-4 which were somewhat higher than what was found in this study, but their values agree reasonably well with the values obtained for pig blood serum by Dawson and Sweeley. The differences may represent species variations. These findings lend support to the hypothesis that the glycosphingolipids present in the aorta are deposited there from serum, more specifically from the lipoprotein fractions of serum. The levels of the individual glycosphingolipids have been determined in the human heart. They were found to be much higher in GL-3 and GL-4 than the levels found in serum, suggesting that these glycosphingolipids probably were not deposited from serum, but were probably synthesized in situ.

Comparing the levels of individual glycosphingolipids in the diseased tissue with those found in normal tissue indicates that the levels of GL-1 are higher in diseased tissue, but as the length of the carbohydrate chain increases beyond that of GL-1 the level of glycosphingolipid decreases in the diseased tissue. It has been suggested that GL-1 and possibly GL-2 freely exchange between plasma lipoproteins and erythrocytes and probably also between different lipoprotein fractions. If this is true, then it might be expected that GL-1 and GL-2 would exchange between tissue components in the intima of the aorta, and that in the progression of the atherosclerotic disease, more of the GL-1 and GL-2 would be deposited in the aorta and therefore show an increase in concentration. Other investigators have suggested that though the exchange between lipoproteins and tissue cells takes place, it is not a simple free
exchange and more complex mechanisms may be involved. They also suggest, as have still others, that there are two pools of glycosphingolipids, one in the erythrocytes and one in the plasma, and with the exception of GL-1 no exchange takes place. It is further suggested that any transfer of glycosphingolipids that does occur between plasma and erythrocytes is not significant. This however does not preclude the transfer of glycosphingolipids between plasma lipoprotein fractions and the intima of cell membranes. It has been shown that transfer of glycosphingolipids does occur between LDL and HDL and that HDL can stimulate endogenous synthesis of GL-2 in leukocytes and GL-3 in fibroblasts. This stimulation may be due to the ability of HDL to provide GL-1 for the synthesis of GL-2 and GL-3 in these instances.

Since the levels of the glycosphingolipids tend to indicate that the glycosphingolipids may be absorbed from the plasma, it was hoped that the fatty acid composition of these lipids, or at least of GL-1, would show similarities to that of lipids found in human serum. However, the fatty acid composition found in the aorta did not correspond with that found in plasma or lipoprotein fractions or in erythrocytes of pigs. One thing that was noted was that the fatty acid composition of GL-1 in erythrocytes and in plasma lipoprotein fractions was significantly different from the fatty acid composition of GL-2, GL-3 and GL-4 from the same blood components. This supports the hypothesis that GL-1 is freely exchangeable between blood components, whereas GL-2, GL-3 and GL-4 are probably not and are derived from the same pool within the individual blood components.
The fatty acid compositions of all four glycosphingolipids from aorta were found to be quite similar, and considerably different from the values reported in the literature for GL-1 from erythrocytes or from other blood components.\textsuperscript{9,35,36} It would appear that the glycosphingolipids in the aorta either all come from the same pool, because of their similar fatty acid composition, or they are synthesized \textit{in situ} possibly by carbohydrate elongation or by the degradation of GL-4. It would appear from the fatty acid composition that GL-1 is not absorbed from the blood unless this occurs by selective absorption or by transport based on the fatty acid composition of the molecule.

In diseased tissue, there is a decrease in the levels of glycosphingolipids with longer carbohydrate chain length and an increase in the levels of glycosphingolipids with shorter carbohydrate chain length. This suggests that there may be a change in the rate of glycosphingolipid metabolism in diseased tissue. Synthesis by addition of carbohydrate may be inhibited, or the degradation of GL-4 may be enhanced by the development of the atherosclerotic plaque.

The relative changes in the fatty acid composition of the glycosphingolipids between the diseased and the normal tissue are uniform and would suggest that such changes are due either to changes in the initial synthesis of GL-1 in the aorta, or due to changes in the selective absorption or transport of GL-1 into the aorta from the plasma based on its fatty acid composition. Selective metabolism based on fatty acid composition is also a possibility. Investigators
who have done work with gangliosides in human aorta have suggested that the increase in the concentration of these gangliosides was due to an increase in the synthesis rather than an increase in deposition from blood components. 21

In summary, the level of crude lipid was found to increase in diseased tissue as compared to normal tissue. Correspondingly, the level of glycosphingolipid was found to increase in diseased tissue, but only to the same extent as the increase in crude lipid. Of the four glycosphingolipids found in aorta, GL-1 increased in diseased tissue and GL-3 and GL-4 decreased, at least in their relative amounts. The relative level of GL-2 appeared to remain constant. Analysis of the fatty acid composition of the individual glycosphingolipids revealed that the primary fatty acids were 16:0, 18:0, 18:1, 20:0, 22:0, 23:0, 24:0 and 24:1. Comparison of the fatty acid distribution between normal and diseased tissue showed that the fatty acids 16:0 and 24:1 increased while 23:0 and 24:0 decreased. The degree of unsaturation increased in the longer chain length fatty acids in the diseased tissue as compared to the normal tissue. The relative increases and decreases in the fatty acid distribution in the glycosphingolipids were similar among all four glycosphingolipids isolated.
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