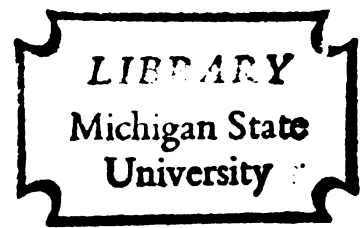


PLASMA CEREBROSIDES IN STROKE AND
MULTIPLE SCLEROSIS

Thesis for the Degree of M. S.
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ABSTRACT

PLASMA CEREBROSIDES IN STROKE AND
MULTIPLE SCLEROSIS

By
Betty Helen Vincent

This investigation was conducted to determine if plasma galactosyl ceramides were elevated in patients with stroke or multiple sclerosis and to determine glycosyl ceramide concentrations in older, normal subjects.

It was hypothesized that central nervous system destruction like that which occurs in stroke or the demyelination characteristic of multiple sclerosis might be reflected by changes in plasma glycosyl ceramides, specifically by an increased percentage of the galactosyl ceramide. If this were shown to be the case, the determination might prove to be useful to monitor the presence or extent of brain destruction or demyelination.

Glycosyl ceramides were analyzed from duplicate 10 ml aliquots of plasma from each of 7 patients with stroke, 5 patients with multiple sclerosis and 5 control subjects. Separation of glycosphingolipids by column and thin-layer chromatography and quantification of glucosyl and galactosyl ceramides by gas-liquid chromatography followed.

There were no significant differences among the three groups. Although the monohexosyl ceramides were elevated in all groups above concentrations reported by other laboratories, average values for controls and multiple sclerosis patients were strikingly similar (28.19 and 28.34 nmoles/ml respectively). The percent of monohexosyl ceramides present as galactosyl ceramide was in the range reported by others (11.05 and 11.40%). Total nanomoles of monohexosyl ceramide in stroke were slightly reduced from those of the other two groups (21.99 nmoles/ml) and percent of galactosyl ceramide was slightly but not significantly elevated (14.32%).

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By
Betty Helen Vincent

A THESIS

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For my husband and children
and
in memory of my father

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INTRODUCTION

Cerebrosides are members of the complex, heterogeneous glycosphingolipid family⁷⁶, and can vary from relatively simple to very complex molecules.

They are known for their importance as structural components in all mammalian tissues and especially in the nervous system. Recent research^{4, 6} has shown them to figure prominently in a group of genetically-determined diseases, the lipidoses, which are characterized by various syndromes involving demyelination, mental retardation, organ and tissue malfunction and shortened life span^{22, 55}. All classes of glycosphingolipids have been implicated in the lipidoses^{4, 6, 61}, and laboratory analyses have shown the presence of cerebrosides and other glycosphingolipids in increased quantities in plasma^{1, 38, 53, 56, 57, 73, 74, 76}, urine¹⁷, cerebrospinal fluid^{57, 70, 71}, and other tissues^{15, 16, 18, 21, 30, 34, 43, 46, 65, 68} depending on the specific enzyme deficiency.

Multiple sclerosis is a disease of the central nervous system of unknown etiology. The occurrence of demyelination in this disease is presumably responsible for the elevation of cerebrospinal fluid cerebrosides. Accumulation of a particular cerebroside, galactosyl ceramide, has been

demonstrated in the cerebrospinal fluid of patients with multiple sclerosis^{11, 71, 72}.

A single study on pooled plasma of patients with multiple sclerosis⁵³ showed no significant differences in cerebrospinal fluid composition or levels from normal controls.

The extent to which the demyelination and associated pathology in stroke causes changes in cerebrospinal fluid and plasma cerebroside has not been documented. The patterns of destructive changes in brain vary in stroke according to the distribution of the occluded arteries. Both gross and microscopic changes occur in gray and white matter with interruption and disintegration of myelin⁵⁵.

The chemical analysis of glycolipids has been dependent upon biopsy or autopsy material and relatively large samples of test material were needed. Furthermore, analytical methods were cumbersome and the sensitivity of these procedures were questionable. Analysis of compounds occurring in the quantities presently found in plasma was impossible and at this stage little progress was made in the understanding of the mechanisms of clinical disorders or the development of diagnostic tests. Analytical methods were needed which could utilize smaller quantities of biological specimens more easily obtained from patients, i.e., blood or urine, and more sophisticated techniques for separating polar compounds of similar physical and chemical

properties in groups of heterogeneous composition such as occur in glycosphingolipids.

In the 1950's, the concomitant development of gas-liquid chromatography and refined techniques in lipid chemistry resulted in a flood of research in this area. The use of these refinements in techniques and instrumentation^{59, 72} now permits the quantification of compounds, formerly reported as "trace" amounts in units as small as a few nanomoles of sample, and on such small amounts of tissue as are found in bone marrow aspirate, urine sediment¹⁷, or in 4 to 10 ml of blood¹⁵.

The result of this progress in the field of neurochemistry has permitted the identification and characterization of many lipidoses from the aspects of tissue constituents, enzyme quantification, and metabolic and synthetic activities. From these kinds of observations, therapeutic methods have evolved⁴⁰ which can potentially benefit patients suffering from certain disorders. Possibly similar detailed studies could clarify some of the etiologic and pathologic mechanisms of other central nervous system diseases, i.e., stroke (CVA) and multiple sclerosis, which have not been as clearly delineated.

Cerebroside levels of concentration have been determined on plasma of normal, healthy individuals usually age 35 or under^{1, 37, 65, 72, 73}. It is not known if plasma cerebroside levels vary with age. Since stroke is generally considered to be a disease of older individuals,

controls were age-matched with those stroke patients. Multiple sclerosis patients generally are younger and plasma values have already been established.

The problem, then, was to assay plasma cerebroside in subjects of apparent good health in the age range of the stroke patients, and compare these levels with values obtained from stroke and multiple sclerosis patients. A demonstration of altered cerebroside content of plasma, especially galactosyl ceramide, in stroke and multiple sclerosis patients could serve these purposes:

- (1) determine if plasma cerebroside reflect damaged brain tissue and ascertain the nature and quantity of the cerebroside;
- (2) serve as a useful clinical tool for the diagnosis and/or monitoring of patients suffering with diseases accompanied by demyelination.

LITERATURE REVIEW

Cerebrosides are a class of neutral glycosphingolipids (see Appendix B) composed of a long chain base, a fatty acid, and one molecule of either glucose or galactose^{61, 76} (Figure 1). The combination of the long chain base with a fatty acid forms a ceramide. Consequently, the cerebro-sides or glycosyl ceramides are usually more specifically called glucosyl or galactosyl ceramide, although the terminology, glucocerebroside and galactocerebroside is often seen.

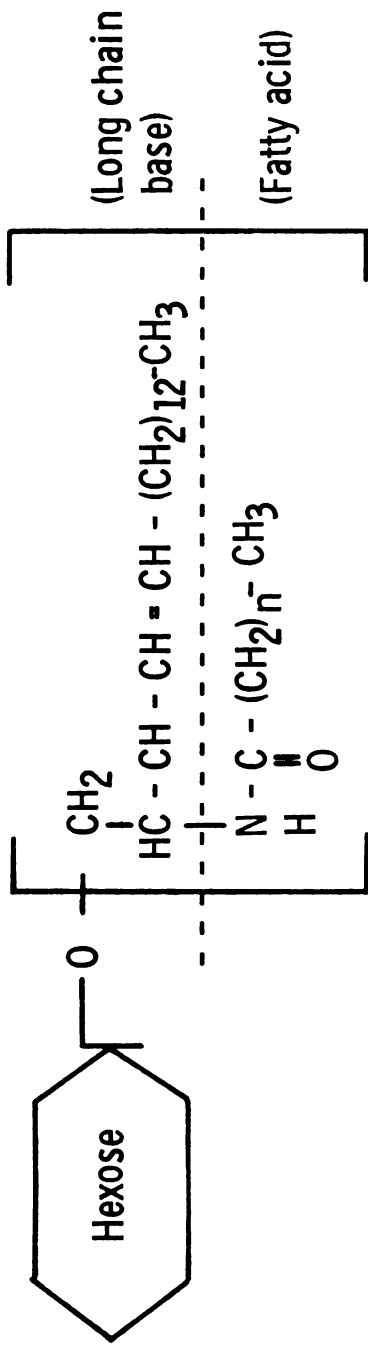
Another compound, closely related as to configuration and location in tissue, especially brain, is sulfatide. This is primarily a sulfated galactosyl ceramide, although a galactosyl-sulfate-glucosyl ceramide has been found⁴¹.

The cerebroside or monohexosyl ceramides are principally structural molecules and have been isolated from a wide variety of mammalian and plant tissues. They play an important role in the structure of biological membranes and myelin.

Ceramide: The Long Chain Base

The first component of a ceramide is the long chain base (LCB), which serves as the backbone of the glycosphingolipids. It is the lipophilic portion of the

Figure 1. Diagram of a cerebroside. The portion of the molecule enclosed within brackets is the ceramide portion of the molecule. The long chain base is attached to the hexose by a glycosidic bond. An amide bond links the fatty acid to the long chain base at carbon 2.



molecule and can be any one of a heterogeneous group of aliphatic amino alcohols. Linked to glucose or galactose, at carbon 6 of the hexose by a glycosidic bond it is called psychosine; the fatty acid forms an amide bond at carbon 2 of the LCB.

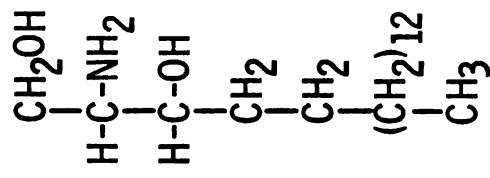
The major long chain base of most mammalian neural and extraneural tissues is the 18 carbon moiety, sphingosine, but homologs to this compound, dihydrosphingosine and phytosphingosine are usually found in lesser quantities (Figure 2). Very small amounts of other amino alcohols, including a 20 carbon compound have been found^{23, 26}. The class of long chain bases in its entirety contains bases with double bonds and branched chains. Sphingosine is an unsaturated dihydroxy base and dihydrosphingosine or sphinganine is a saturated dihydroxy base; phytosphingosine is a trihydroxy base.

Sphingosine was first isolated from brain tissue by Thudicum and further characterized^{32, 48} by techniques involving both synthesis and degradation.

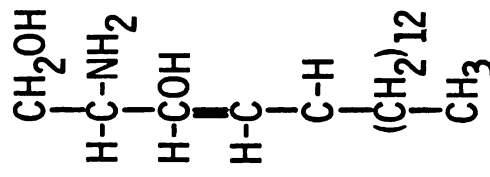
Sphingosine constitutes 80-95% of the total long chain bases in sphingolipids in all mammalian tissues analyzed except kidney²³. Dihydrosphingosine is found in the next largest amount.

Until recently, phytosphingosine had been found only as a plant constituent. Karlsson and Martensson³², however, found that phytosphingosine occurred as approximately half of the long chain base of kidney

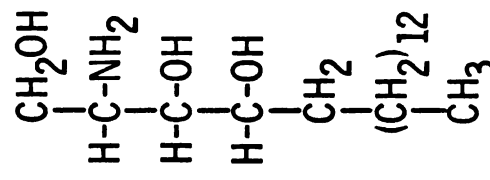
Figure 2. Homologs of sphingosine. Sphingosine is an unsaturated dihydroxy base; dihydroxysphingosine is a saturated dihydroxy base, and phytosphingosine is a trihydroxy base.



dihydrosphingosine



sphingosine



phytosphingosine

cerebrosides. Only glucosyl ceramide was associated with this moiety and its structure and configuration was identical to the plant form. It has also been isolated from small intestine²³ and blood plasma^{69, 72}. None could be found in brain cerebrosides.

The long chain base of human plasma, analyzed by gas-liquid chromatography⁶⁹ showed that 73-82% was sphingosine, 5-12% was dihydrosphingosine, and 10-22% was an unidentified base. Verification of three peaks for sphingosine derivatives was given by Vance and Sweeley⁷² who used trimethylsilylation. Karlsson³³ and Renkonen⁵⁴ found the predominant long chain base of plasma ceramides to be 18:1. Both also found varying amounts of 16:1, 16:0, 17:0, 17:1, 18:0, 18:1, 18:2, and 19:1. The C16:0 and C18:0 forms occurred as trihydroxy bases. Samuelsson^{55, 56} also found 18:1 to be predominant, with 16:1 and 18:2 occurring in substantial quantities. She also identified trace amounts of 17:1, 18:0 and 19:1. Composition of liver long chain base is similar to that of plasma⁵³.

Analysis of the long chain base from glycolipids of central nervous system tissue by more recent methods has shown that 98% of cerebrosides and sulfatides contain 18:1, 2% of 18:0, and traces of lower homologs⁷⁷, one of which is C16²³. Increased relative amounts of C20 are found in brain tissue with increasing age⁷⁷. On the other

hand, higher proportions of C18 dihydrosphingosine (10% of the total) are found in immature human brains²³.

On the basis of the long chain base fraction, it is possible to differentiate glycosphingolipids of kidney, other extraneural tissues, and brain.

Biosynthesis of sphingosine has been studied extensively and has been shown to occur as a condensation of palmitic acid, or an homologous derivative, and serine, with the concurrent decarboxylation of serine. During active myelination, sphingosine is synthesized in situ in the glial cells of the central nervous system, while psychosine, a monohexosyl sphingosine, and other long chain bases are synthesized in the liver. During sphingolipid synthesis, long chain bases from various loci are incorporated into the sphingolipid molecule in this organ⁷⁷.

No diseases have been identified to date in which sphingosine alone is implicated. In demyelinating diseases, however, the total sphingolipid content of nervous tissue is decreased as compared to normal tissue²⁴. In this instance, sphingosine is a part of the total sphingolipid molecule.

Ceramide: Fatty Acids

The second component of a ceramide is a fatty acid which can vary in chain length, hydroxylation, and saturation from one tissue or organ to another. Variation

of these parameters also occurs in developing and aging mammals^{45, 76}. Chains of twelve or fourteen carbons to twenty-six carbons have been reported, and there is some indication that chain length varies from that of normal tissue in certain disease states²⁵. There are varying amounts of alpha-hydroxy and monoenoic acids, but very small quantities of polyunsaturated acids.

Fatty acid composition of brain cerebroside differs in gray matter, white matter, and myelin. Adult brain white matter myelin contains approximately 40% cerebroside¹³, of which galactosyl ceramide contributes nearly the entire amount. Only recently have brain galactosyl ceramide and glucosyl fatty acids been characterized²⁶, and more than 20% were found to be stable alpha-hydroxy acids; polyunsaturates were present in quantities too small for reliable analysis. Svennerholm & Stallberg-Stenhagen⁶⁷ showed brain cerebroside from age 15 years to 79 years to be primarily C24 with large amounts of C25, 26 and 18.

Plasma cerebroside fatty acid analysis⁵⁵ showed the major fatty acid to be C16²³ with smaller quantities of 22:0, 22:1, 24:0, and 24:1 also present. Hydroxy acids were rarely found. Renkonen⁵⁴ reported 23:1 as a major fatty acid component.

Fatty acid patterns in kidney and liver were very similar, but fatty acids in these organs show a higher degree of saturation. Kidney, like brain, contained

hydroxy acids. Twenty to 50 percent of the fatty acids in leukocytes and spleen were C16. Non-hydroxy acids were predominantly aortic material²¹ and fatty acid patterns were similar to those reported for spleen.

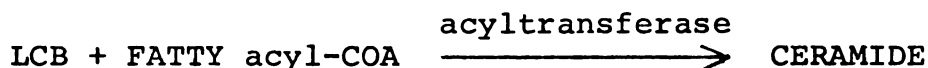
Galactolipids of peripheral nerves showed a pattern intermediate between that of extraneural tissues and brain in that a higher percentage of C16 and C22 acids were present, and there were lesser quantities of C25 and C26⁶⁷.

Cerebroside fatty acids analyzed from cerebrospinal fluid⁵⁷ showed that approximately 50% of the fatty acid present was C24, with 24:1 and 2-hydroxy derivatives also present. Small quantities of C18 (6.4%), C22 (9.3%), C23 (11.7%), C25 (10.0%), and C26 (7.9%) were also reported. This pattern is similar to that of brain.

At birth, the central nervous system content of the longer chain fatty acids, C22 to C26, is relatively low^{46, 66, 77} and the shorter chains (16:0, 18:0 and 18:1)⁴⁵ and non-hydroxy acids predominate. When myelination is complete at about two years of age, adult levels of fatty acids are present. At this time the amounts of longer chain fatty acids have increased to about 90-95% of the total with concomitant increase of monoenes and alpha-hydroxy fatty acids^{62, 77}. Beyond this age level, relatively little change of brain fatty acid patterns occurs. In general, there is a gradual increase in chain length and degree of unsaturation^{45, 66}.

The C18 fatty acids of cerebrosides^{26, 41} are thought to be microsome-bound and formed de novo from palmitic acid via acetyl-COA and malonyl COA, while mitochondria of oligodendrocytes⁷⁷ appear to be responsible for chain elongation⁶³. Desaturation is thought to occur in the acyl-COA derivative. Hydroxy acids were found to be formed from normal fatty acids by decarboxylation²⁵.

Ceramide synthesis occurs in microsomes by the following mechanism:



The long chain base and fatty acyl-COA are converted to ceramide by means of a transferase. Degradation is achieved by hydrolysis to sphingosine in a reversible reaction by enzymes localized in the lysosomes²³.

Ceramides have been isolated from many tissues, where they are probably intermediate forms of other sphingosine-containing lipids, or degradation products of more complex lipids⁸.

Cerebrosides

Cerebrosides are a sub-group of the glycosphingolipids composed^{8, 77} of a ceramide to which glucose (Figure 3) or galactose (Figure 4) is bound by a beta-glycosidic linkage between C6 of the hexose and C1 of the long chain base. They glycosyl ceramides are the compounds of chief concern in this report. They are important

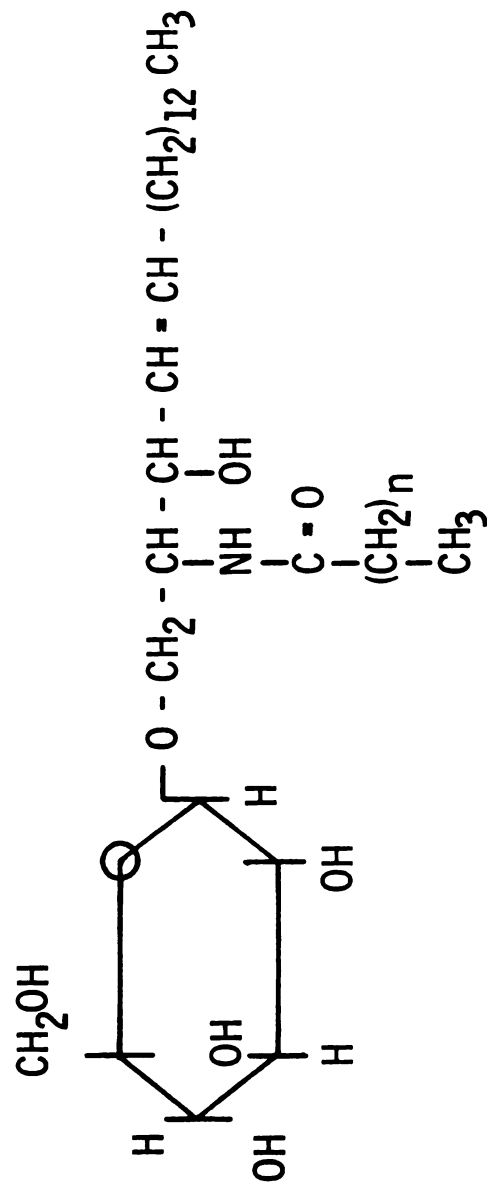


Figure 3. Glucosyl ceramide (glucocerebroside).

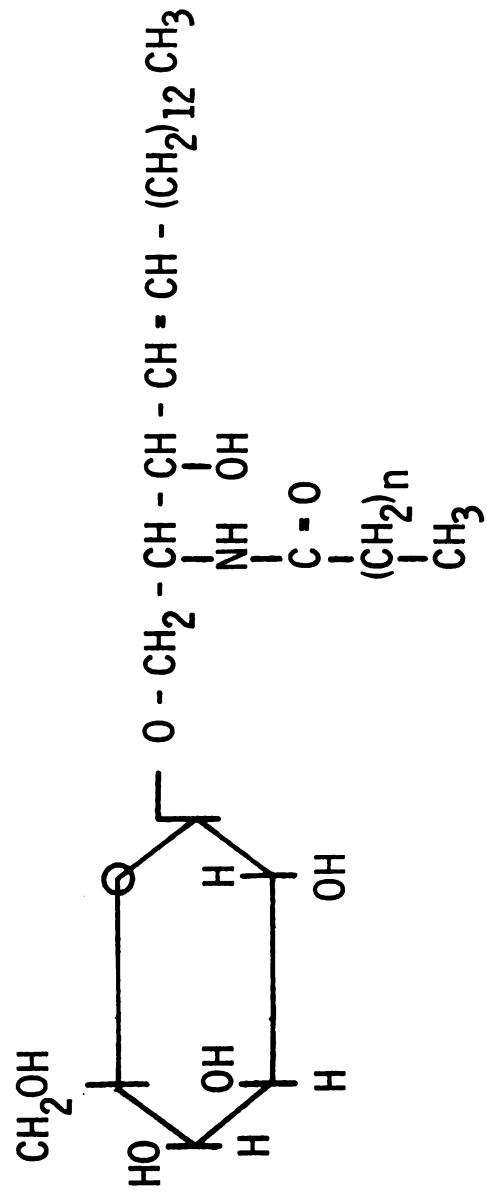


Figure 4. Galactosyl ceramide (galactocerebroside).

structural components of biological membranes, especially myelin, and are closely associated with the sulfatides. Both galactosyl ceramide and sulfatide are present in large quantities in brain white matter, and the sum and ratio of these two compounds function as a sensitive biochemical indicator of the amount of brain myelin present. There is direct sulfation of cerebroside by brain tissue⁶³. Sulfatide catabolism is accomplished via arylsulfatase which degrades it to galactocerebroside^{44, 45, 63}. The sulfate ion can be removed by acid hydrolysis in an analytical procedure, and the galactosyl ceramide can then be quantified⁴¹.

The monohexosyl ceramides may be the precursor to other sphingosine containing lipids, sphingolipids or end stage degradation products⁸.

Extraneural tissues contain only very small amounts of cerebroside when compared to neural tissues, but distinct differences exist from organ to organ between the various classes of sphingolipids⁴¹, and the make-up of individual lipids including cerebroside varies as to hexose moiety, long chain base, and fatty acid.

Cerebroside have been recovered from a wide array of biological sources in both vertebrates and invertebrates⁴¹. Monohexosyl ceramides have been isolated from many biological samples besides brain including plasma^{57, 65, 72, 75}, leukocytes^{30, 31, 34}, erythrocytes¹⁴, liver, kidney, lung, intestine, aorta²¹, placenta, lens^{23, 41}, muscle⁶⁸,

nerve⁶⁸, cerebrospinal fluid^{58, 71}, bone marrow¹⁵, urine¹⁷, and plasma membranes¹⁸.

All mammalian cells and many subcellular organelles are encompassed by a membrane composed of protein and lipid, and myelin is the largest plasma membrane in mammals⁴⁵. Cerebrosides and sulfatides of white matter myelin constitute 20% of the dry weight⁴¹. For many years it was thought that galactosyl ceramide was the only cerebroside present in brain. Recently, however, small quantities of glucosyl ceramide have been reported^{12, 26}.

Dod and Gray¹⁸ found that 7% of the total lipids in rat liver plasma membrane was ceramide monohexoside and none of this was attributed to mitochondrial membranes.

Kidney and erythrocytes^{72, 73} contain largest amounts of the more complex hexosyl ceramides and only small amounts of the monohexosyl ceramides. Of the latter, glucosyl ceramide is found in greater amounts than the galactosyl ceramide. This is also true for placenta⁴¹. On the other hand, monohexosyl ceramides are found in largest quantities in plasma and serum with approximately ten times more glucosyl than galactosyl ceramide present.

Monohexosyl ceramides represent approximately 25% of all neural glycosphingolipids and glucosyl ceramides predominate in intestine, lens, liver, lung, placenta, and spleen²³. Glucosyl ceramides predominate in leukocytes of human blood^{31, 34, 43}, urine sediment¹⁷, and aorta²¹. Human muscle contains only very small amounts of

monohexosyl ceramides, chiefly galactosyl ceramides, but analysis of femoral nerve glycolipids showed an approximate 2000-fold increase in galactosyl ceramides over muscle. Fatty acid patterns of both cerebroside and sulfatides in muscle and nerve were identical, and it was concluded by the authors that these muscle lipids were derived from nerve tissue⁶⁸.

Early investigators described the cerebroside of cerebrospinal fluid as non-phorous containing sphingolipids, while others using comparative mobilities on paper chromatography suggested that lipid patterns of cerebrospinal fluid were similar to those found in plasma. Using gas chromatography-mass spectrometry and borate thin-layer chromatography³⁶, Samuelsson⁵⁸ found no glucosyl ceramide fraction. In subsequent studies⁵⁹ comparing long chain bases, fatty acids, and carbohydrates in human brain and plasma, she found that brain and cerebrospinal fluid had practically identical cerebroside patterns which differed greatly from those of plasma.

The examination of aortic tissue²¹ from diseased and apparently normal vessels shows the cerebroside present to be almost exclusively glycosyl ceramides with values ranging from 0.01 to 0.73% of the total lipid.

Biosynthesis and Degradation of Cerebroside

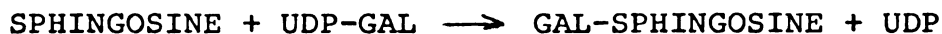
Experimentally, ceramide synthesis has been found to occur in microsomes of the cells in which the lipids are found^{23, 75}. Dawson and Sweeley¹⁴ say that plasma

glycosphingolipids are formed in the liver and that glucosyl ceramides are not synthesized in bone marrow, but do not speculate on location of activity.

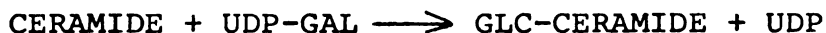
Biosynthesis of galactosyl ceramide has two known pathways^{8, 41}. In the first, the galactose moiety is transferred to ceramide from uridine diphosphate-galactose (UDP-gal):



The second pathway involves the acylation of galactosyl sphingosine:



On the other hand, only one pathway for synthesis of glucosyl ceramides has been elucidated:



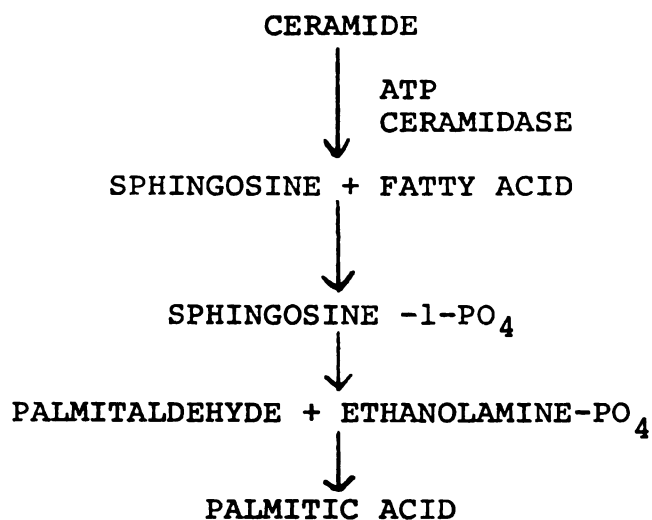
Basu² has found an enzyme in chicken embryo brain which catalyzes this reaction.

This step may be important to synthesis of acidic glycosphingolipids, the gangliosides. Svennerholm⁶⁷ indicates that ganglioside synthesis is accomplished in fetal brain by a step-wise addition of monosaccharide units through the action of a multi-enzyme cloud of glycosyl transferases²³. The formation of glucosyl ceramide in this fashion from glucose and ceramide and, subsequently, to

glucosyl-galactosyl ceramide has been demonstrated in vitro².

Glycolipids undergo complete breakdown when one segment, i.e., the long chain base, fatty acid, or hexose, splits off³⁷.

Glycosyl ceramide is generally thought to be an end product in the degradation of more complex glycosphingolipids⁴¹ with hydrolysis localized in the lysosomes⁷⁴. Burton⁸ shows a series of catabolic reactions utilizing six hydrolases in a system in which there is a step-wise removal of the hexoses, and the end products are glucose and ceramide. Faulty or deficient enzymes specific for cleaving terminal sugars amides are responsible for most of the lipidoses so far encountered^{4, 5, 6, 7, 27, 30, 51}. Ceramide is further degraded²³:



Cerebroside Changes in Development and Aging

The developing brain from an early embryonic stage to maturity undergoes a wide range of biochemical

activities. The earliest stage is characterized by cell proliferation (DNA replication), followed by differentiation (DNA transcription to RNA), then by protein synthesis (RNA translation to protein). During the latter stage, the emerging cell type takes on its own unique morphology and metabolism. With the maturation of specialized cells, i.e., those of the nervous system, myelination begins³.

In man, myelination of the spinal cord tracts begins during the 22nd to the 36th week of fetal life; myelination of the brain tracts follows a short time later. Myelin is not seen in the corpus callosum until 8 weeks post-natally, while most of the myelination of the cerebral hemispheres takes place in the first two years of life²³. This age is taken to be maturity and relatively small amounts of myelin are deposited after this time. The composition of human myelin does not vary to any significant degree between 10 months and 55 years⁴⁷, although data presented by another study⁶⁶ show that fatty acid composition stabilizes at about 25 months and changes only to a small degree between that time and 72 years.

Rapid accumulation of cerebrosides associated with myelination occurs at different ages in different species; as myelin is laid down, the cerebroside content increases proportionally. The lipid patterns, however, are similar.

In the human, neutral glycosphingolipids and sulfatides are present to some degree before the onset of

myelination, and the patterns are very like those of extraneural tissues, especially liver. Glucosyl ceramides predominate with only small amounts of galactosyl ceramides present⁶⁷.

Near full term when myelination begins, the glucosyl ceramide content decreases and the amount of galactosyl ceramide and sulfatide greatly increases⁶⁶. These two compounds continue to increase until myelination is complete. From then on, neither the hexosyl moiety or the sphingosine base changes. The fatty acids change only gradually by chain elongation, hydroxylation, and desaturation^{23, 77}. This seems to be the only effect of aging.

Myelin

Myelin is the most elaborate and extensive of all mammalian membranes. It has been described^{23, 44} as a system of condensed Schwann cell or oligodendrocyte plasma membranes having alternating lamellae consisting of a protein-lipid-protein arrangement. When studied with polarized light, the lipid is seen to be oriented radially to the nerve fiberaxis, the protein, longitudinally⁴⁵.

Myelin is formed from the oligodendrocyte plasma membrane in the central nervous system and from the Schwann cell in the peripheral nervous system. During myelination, the nerve axon is enveloped or ensheathed between nodes of Ranvier by the plasma membrane processes, which are

continuous with the Schwann cell or oligodendrocytes. As many as fifty layers of myelin can be laid down around the axon. Each layer is not fused with the adjacent one, but lies in close proximity to it. By this mechanism, the myelin sheath becomes a multi-layer of the Schwann cell or oligodendrocyte membrane and encircles the axon between nodes of Ranvier²³. There are differences in myelin quantity and composition between infants and adults. Fewer windings of the plasma membrane around the axon are found in infants⁹ and early myelin composition is therefore different from the adult¹³. The myelin of adults comprises 50% of the total dry weight of white matter^{44, 63}.

Myelin is not inert, but, for the most part, is an extremely stable structure. Davison and Dobbing⁹ have classified lipids as to function based on turnover time. Class 1 lipids have a half life of less than one week, function as fuel and energy reserves and for active synthesis and transport. Class 2 components have a $t_{1/2}$ of one to four weeks and function as membrane components. The third class functions as structural components, and the $t_{1/2}$ is greater than four weeks.

Brain galactosyl ceramides show two turnover rates; a half-life of 45-46 days was demonstrated for the first, and the second, a very long half-life, indicating an extremely stable compound. A peak of activity is reached at the time of myelination which then drops to low values⁴¹.

Labelling of fatty acids indicate two pools: incorporation of precursors into C18 rose rapidly and fell rapidly, while C20, C22, and C24 acids increased in activity for two weeks and then slowly declined. Martensson⁴¹ suggests that C18 might represent a non-myelin cerebroside. After 56 days, decay curves gave indications that possibly a third pool existed which was indefinitely stable. Radin and co-workers, also using labelling techniques, reported that sulfatides are formed from cerebroside, but do not undergo breakdown in the normal brain⁴⁹.

In turnover studies on subcellular fractions using ³⁵S-sulfate, a very small fraction of sulfatide was found to undergo rapid turnover exhibiting a half-life of 2 1/2 days. This suggested a very small, very metabolically active center was present in myelin⁴¹, and this has also been considered by others³⁸.

Extraneural cerebroside were found to turn over much more rapidly than does myelin, similar to that of the non-myelin structures of brain.

Onset of myelination is probably triggered by the formation of enzymes during the stage of RNA translation of protein. In instances of inherited genetic defects when a deficiency or lack of essential enzymes occur, hypomyelination or dysmyelination are probably the result⁴⁵.

Many of the lipidoses are accompanied by demyelination attributed to a defective enzyme system, and galactosyl ceramides are known to disappear from myelin⁵⁰.

In addition to its major role as a myelin constituent, another capability has been ascribed to galactocerebroside, that of haptenic activity. Galactosyl ceramide, isolated from brain tissue from several species, has been shown to react with great sensitivity to form immune complexes with anti-brain antibodies²⁹. Rapport and Graf⁵¹ demonstrated that galactocerebroside is antigenic determinants of myelin, and that their structural localization does not exclude their availability for interaction with specific antibody. Immunological activity of glucocerebroside could not be demonstrated.

Other Diseases Accompanied by Demyelination

Other demyelinating diseases occur in which there is no demonstrable mechanism for demyelination. Multiple sclerosis is characterized by demyelination⁴³ in which galactosyl ceramide has been shown to disappear from myelin and elevated quantities appear in the cerebrospinal fluid⁷¹.

Multiple sclerosis has been studied extensively and from many aspects. Reports of gross examination of brain and spinal cord from these patients describe lesions, in general, as small, well circumscribed and widely distributed with areas of apparently intact normal tissue.

Galactosyl ceramides are slightly decreased in brains of patients with multiple sclerosis and deficiencies of lipids also exist in intact white matter and myelin of apparently normal brain tissue²⁴. Gray matter can also be involved, but usually as an extension of a white matter lesion⁵⁵. Plasma cerebroside composition has been determined only on plasma pools from patients with multiple sclerosis⁵⁸; no reports documenting plasma cerebroside values in individual multiple sclerosis patients are available.

Millar⁴³ has suggested that multiple sclerosis could involve a defect of the blood-brain-barrier and has outlined a sequence of events which possibly accompanies demyelination. This sequence is similar to that described for vascular disease. Whether or not this sequence is correct is less important than the likelihood that the blood-brain-barrier may be altered in this disorder which probably involves an autoimmunity to myelin basic protein.

Loss of myelin has been reported in stroke following massive destruction of brain tissue which may occur following cerebrovascular accident. Stroke is a general term often used synonymously with cerebrovascular accident (CVA) to indicate infarction or hemorrhage in the brain resulting from vascular disease. Generally, infarctions are secondary to thrombosis or embolism, while hemorrhages follow rupture of microaneurysms on vessels altered by hypertension. Appendix A lists the terms used to describe diagnoses of patients used in this study. Clinically, it

is preferable to designate the site of occlusion or damage as specifically as possible. The terms should include the site and location of injury as well as the vascular distribution and the exact vessel involved, if that is possible. A transient ischemic attack (TIA) is symptomatic of occlusive disease since tissue hypoxia or anoxia caused by ischemia during incomplete or intermittent blockage gives transient neurological symptoms.

Vascular disease is an on-going process which can begin at a fairly early age and progress for a normal lifespan without clinical manifestations. Those subjects who have occult vascular disease could be considered to be normal. In these individuals, the build-up of fatty streaks and plaques upon the intima of the vasculature, which increases with age, is unaccompanied by significant symptoms. In others, infarction and brain destruction follows thrombosis or formation of emboli⁵⁵. Gross examination of the brain following stroke has shown the myelin to be interrupted.

Hausheer and Bernhard²⁷, in comparing atherosclerotic aortas with normal aortas, found that total lipids as well as cerebrosides increase as atherosclerosis increases; both glucosyl and galactosyl ceramides are present. Foote and Coles²¹ in their study suggest that cerebrosides found in the human aorta, especially in fatty streaks, originate in plasma. Studies documenting plasma cerebroside changes in cerebrovascular disease are not available.

MATERIALS AND METHODS

Blood samples were obtained from hospitalized patients. In most instances, specimens from stroke patients were obtained within 2 weeks following onset of symptoms (see Appendix A).

Fifty milliliters of blood were drawn into heparin, the samples centrifuged, the plasma separated from the cells, and frozen until analyzed.

The controls used were five apparently healthy individuals age-matched with stroke patients. Fifty milliliters of blood from each subject were drawn into heparin. Following centrifugation, the plasma was separated from the cells, and analysis immediately initiated, without freezing the plasma. Duplicate 10 milliliter samples of plasma (measured to the nearest 0.05 ml) were analyzed according to the method of Vance and Sweeley⁷². No hemolyzed samples were used.

Redistilled solvents were used throughout. Unisil (200-325 mesh) was obtained from Clarkson Chemical Company (Williamsport, Pa.). Commercial thin layer plates coated with silica gel G (Quantum Industries, Fairfield, N. J.) were pre-run in diethyl ether to remove contaminants and reduce the amount of plasticizer. The silylating reagent,

Regisil, was obtained from the Regis Chemical Company (Chicago, Ill.). Supelco Inc. (Bellefonte, Pa.) supplied the Supelcoport column packing (3% SE 30).

Folch Extraction and Column Chromatography

To the serum samples were added 120 milliliters of chloroform and 60 milliliters of methanol (Figure 5). The mixture was stirred with a magnetic stirring bar for 15 minutes at room temperature, and then filtered. The precipitate on the filter paper was washed with 20 ml of chloroform-methanol (2:1, v/v). Forty milliliters of distilled water (from glass) were mixed with the filtrates and allowed to stand overnight in the cold.

The lower phase was removed from this biphasic system and evaporated to dryness on a rotary evaporator with gentle heating (50°C). This total lipid portion was taken up in 0.5 ml of chloroform and applied to a 2 gm Unisil column. The neutral lipids were removed from the column with 50 ml of chloroform and discarded; the glycosphingolipids, with 100 ml of acetone-methanol (9:1, v/v).

Alkali-Catalyzed Methanolysis

Solvents were removed from the glycosphingolipids, the residues transferred to screw-capped vials with chloroform-methanol (2:1, v/v) and dried under a thin stream of nitrogen. Mild alkali-catalyzed methanolysis was performed at this point to remove phospholipid contaminants¹⁹.

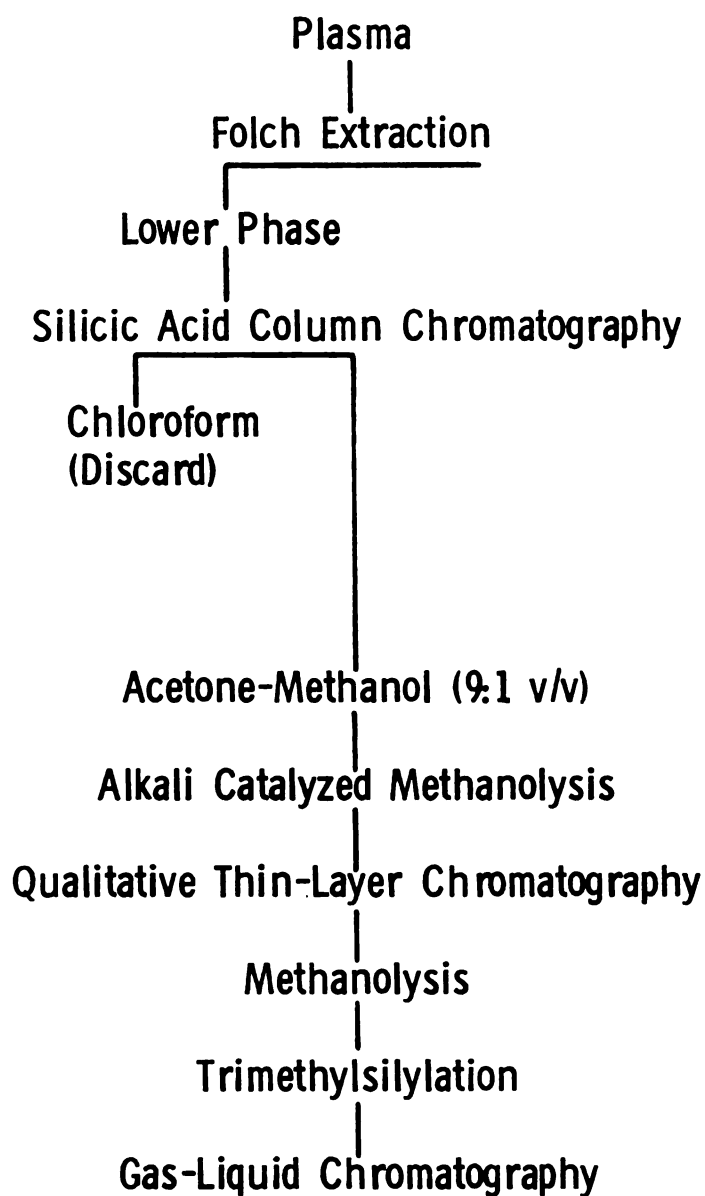


Figure 5. A summary of cerebroside isolation.

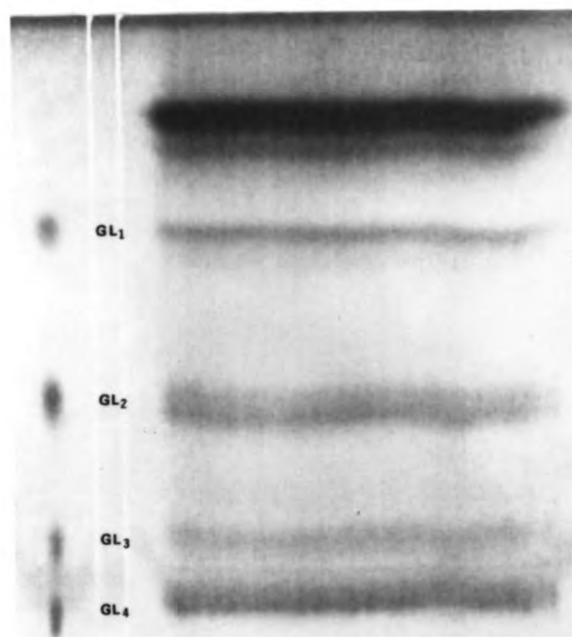


Figure 6. Thin-layer chromatogram of glycosyl ceramides from human plasma. The bands which were visualized after exposure to iodine vapor are monohexosyl ceramide (GL₁), dihexosyl ceramide (GL₂), trihexosyl ceramide (GL₃), and globoside (GL₄).

HCl Methanolysis

A stock solution of 2 μ moles/ml of mannitol standard was prepared in methanol (see Appendix C). Thirty microliters (60 nanomoles) of mannitol and 3 ml of 0.5N methanolic HCl were added to the cerebroside residue, and incubated at 80°C for 22-24 hours. After cooling, methyl esters of fatty acids were extracted by 3 treatments of equal volumes of hexane. The remaining portion of the methanolysis mixture containing methyl glycosides and sphingolipid bases were neutralized with silver carbonate, centrifuged, and transferred to screw-capped vials. Fatty acid methyl esters and methyl glycosides were reduced to dryness under nitrogen.

Trimethylsilylation

Trimethylsilylation was accomplished by treating both products of methanolysis with 25 μ l of bis(trimethylsilyl) fluoroacetamide plus 1% trimethylchlorosilane (Regisil) and 25 μ l of dimethylformamide for 15 minutes prior to injection into the gas-liquid chromatography apparatus.

The TMSi derivatives of methyl glucosides, methyl galactosides, and the mannitol standard separated readily (Figure 7) on the 3% SE 30 column at 60°C.

Calculations were made according to Vance and Sweeley⁷³ and converted to nanomoles/ml.

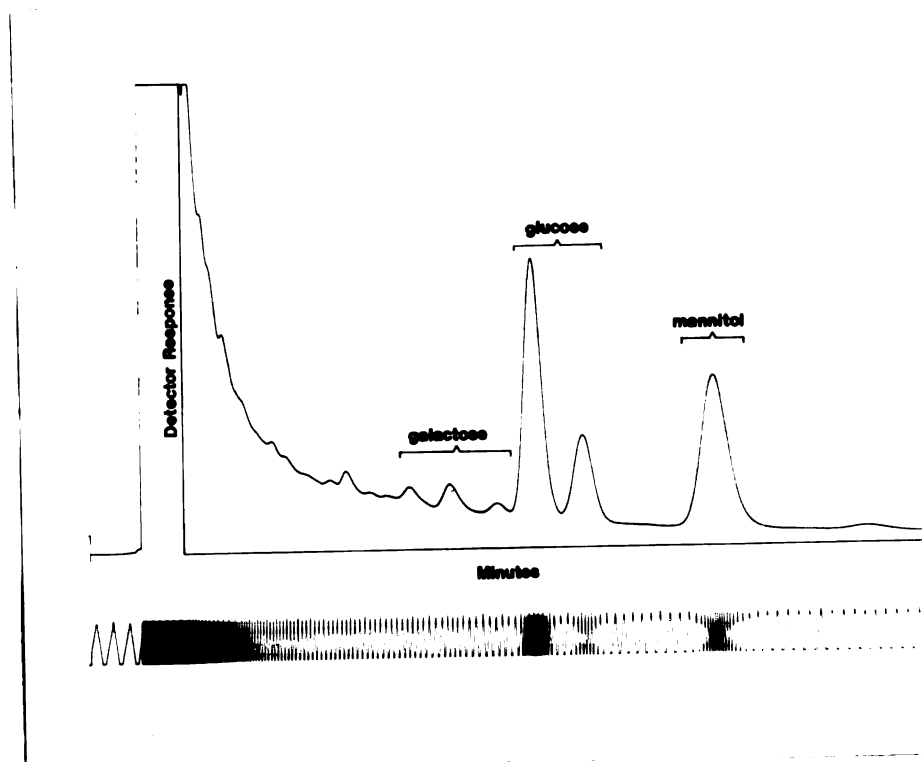


Figure 7. Gas-liquid chromatogram of TMSi methyl glycosides of D-glucose and D-galactose with mannitol added as an internal standard. Run on a 2mm x 3mm column of 3% SE-30 on 100/120 Supelcoport at 150°C. A Hewlett-Packard F & M model 402 gas chromatogram was used with a flash heater at 250°C and a nitrogen carrier gas flow rate of 35 ml/minute.

RESULTS

The values given in Table 1 indicate the levels of total monohexosyl ceramides (glycosyl cerebroside), galactosyl ceramide, and percent galactosyl ceramide of the individuals constituting the control group. The mean galactosyl ceramide was 3.07 nanomoles/ml (nM); the total cerebroside was 28.18 nM/ml; percent galactosyl ceramide was 11.05; nM/ml of glucosyl ceramide was 25.02.

Table 2 shows the average of paired samples of the group of multiple sclerosis patients. Total cerebroside (28.34 nM/ml), galactosyl ceramides (3.19 nM/ml), percent galactosyl ceramides (11.40), and glucosyl ceramides (25.20 nM/ml) show a striking similarity to those values determined for the control group. The range of percent galactosyl ceramides of the control group was 9-14%, for the multiple sclerosis group, 8-15%.

The concentration of cerebroside in plasma of individuals suffering from stroke is presented in Table 3. Total cerebroside (21.99 nM/ml), glycosyl ceramides (17.75 nM/ml), and galactosyl ceramide (2.80 nM/ml) were decidedly lower than those values of the control group, while the percent galactosyl ceramides (14.32) was higher. Even so, no significant difference ($p > 0.05$) at the 5%

Table 1. Concentration of glycosyl ceramides*. Average of paired samples (nanomoles/ml) of controls.

Patient	Age-Sex	Controls		Total GL ₁	%GAL
		GAL	GLC		
V.W.	50m	5.11	38.62	43.74	11.76
J.D.	61m	2.89	28.87	31.76	9.08
B.G.	47f	2.79	24.92	27.71	10.14
F.D.	50m	2.10	17.51	19.61	10.71
C.H.	66m	2.46	15.16	18.11	13.55
$\bar{X}$		3.07	25.02	28.19	11.05

*Abbreviations: GAL indicates galactosyl ceramide; GLC, glucosyl ceramide; and GL₁, glycosyl ceramide.

Table 2. Concentration of glycosyl ceramides in plasma.*
Average of paired samples (nanomoles/ml) of
multiple sclerosis patients.

Patient	Age-Sex	Multiple Sclerosis		Total GL ₁	%GAL
		GAL	GLC		
D.P.	24f	1.94	11.35	13.34	14.95
D.W.	44f	2.88	31.64	34.52	8.34
E.M.	46m	2.79	22.00	24.80	10.51
C.V.	28m	4.81	35.77	40.53	11.86
L.V.	37f	3.54	25.25	28.49	11.37
$\bar{X}$		3.19	25.20	28.34	11.40

*Abbreviations: GAL indicates galactosyl ceramide; GLC, glucosyl ceramide; and GL₁, glycosyl ceramides.

Table 3. Concentration of glycosyl ceramides in plasma.*
Average of paired samples (nanomoles/ml) of
stroke patients.

Patient	Age-Sex	Stroke		Total GL ₁	%GAL
		GAL	GLC		
A.K.	65m	1.56	11.20	12.76	12.21
E.S.	84f	4.57	14.90	19.47	25.66
B.M.	76m	4.69	25.57	30.36	14.52
G.M.	58m	2.28	22.65	24.93	9.26
P.S.	55m	1.20	8.55	9.75	12.32
C.D.	56m	1.96	14.49	16.45	11.94
D.S.	76m	3.36	36.89	40.23	8.36
$\bar{X}$		2.80	17.75	21.99	14.32

*Abbreviations: GAL indicates galactosyl ceramide; GLC, glycosyl ceramide; and GL₁, glycosyl ceramide.

level was observed between the total cerebroside of the stroke and multiple sclerosis groups when compared with the control group (Table 4). Table 5 gives a comparison of nanomoles/ml and percent of galactosyl ceramides of the three groups. Using analysis of variance, there was no significant difference at the 5% level ($p > 0.05$).

Patient E.S. showed 25.66% of galactosyl ceramide (mean = 14.32%). The range of percent for the other patients in the group was 8-14%. The diagnosis of this patient was cerebral thrombosis (stroke), and she had been a known diabetic for fifty years. Her case history (see Appendix A) gives no indication, however, of any diabetic neuropathy. The question of the effect of age upon the galactosphingolipid content of plasma certainly arises in this instance.

Analysis of the glycosyl ceramides and percent galactosyl ceramides, excluding patient E.S., showed (Table 6) the total cerebroside (22.41 nanomoles/ml) and percent galactosyl ceramides (11.44) to be more closely related to the control group. The range of the percent galactosyl ceramides between the individuals constituting the stroke group is the same (8-15%) as for the multiple sclerosis group (Table 7). Galactosyl ceramides are decreased to 2.51 nanomoles/ml. There was no significant difference ($p > 0.05$) between these two groups as determined by nested analysis of variance.

Table 4. Total glycosyl ceramides, galactosyl ceramides, and glucosyl ceramides of all groups (nanomoles/ml).

	GL ₁	GAL	GLC
Controls	28.19	3.07	25.02
Multiple Sclerosis	28.34	3.19	25.20
Stroke	21.99	2.80	17.75
p=>0.05			

Table 5. Galactosyl ceramides in plasma (nanomoles/ml).

	GAL	%GAL
Controls	3.07	11.05
Multiple Sclerosis	3.19	11.40
Stroke	2.80	14.32
p=>0.05		

Table 6. Concentration of glycosyl ceramides, galactosyl ceramides, and percent galactosyl ceramide* of the stroke group. Patient E.S. is excluded. Average of paired samples (nanomoles/ml).

Patient	Age-Sex	Stroke GAL	Total GL ₁	%GAL
A.K.	65m	1.56	12.76	12.21
B.M.	76m	4.69	30.36	14.52
G.M.	58m	2.28	24.93	9.26
P.S.	55m	1.20	9.75	12.32
C.D.	56m	1.96	16.45	11.94
D.S.	76m	3.36	40.23	8.36
$\bar{X}$		2.51	22.41	11.44

*Abbreviations: GAL indicates galactosyl ceramide and GL₁, glycosyl ceramide.

Table 7. Concentration of galactosyl ceramides and glycosyl ceramides (nanomoles/ml) and percent galactosyl ceramides of the stroke, multiple sclerosis, and control groups when patient E.S. is excluded from the stroke group.

	GAL	%GAL	GL ₁
Control	3.07	11.06	28.19
Multiple Sclerosis	3.19	11.40	28.34
Stroke	2.51	11.44	22.42
p=>0.05			

DISCUSSION

The values for controls in this report are higher than previously given for normals, but the individuals who compose this group were older than control groups previously studied. The percent of galactosyl ceramides is slightly lower (9-14%) than reported by Rathke and Jones⁵³. The control group was age-matched with stroke patients. The range of percent galactosyl ceramides found in multiple sclerosis patients was also slightly lower (8-15%), but the mean percent galactosyl ceramides of these patients (11.40) compared very closely with that of the controls (11.05). The values for galactosyl ceramides (2.80) and total cerebrosides (21.99) were lower than the controls, but were not significantly lower; percent galactosyl ceramides were higher (14.32), but still within the range found in the controls, and not significantly different.

Patient E.S. had 25.66% galactosyl ceramides. This patient was an 84 year old female, and a known diabetic for fifty years, although her case history gave no indication of any diabetic neuropathy (see Appendix A). The question of the effect of age on glycosphingolipid content of plasma certainly arises in this patient. In fact this

patient could probably be included in three different categories: aging, stroke, and other neurological disorders. For this reason, it was decided to re-evaluate the data excluding this patient. The galactosyl ceramide mean dropped from 2.80 nM/ml to 2.51 nM/ml; total cerebroside rose only a small amount, to 22.41 nM/ml as compared to 21.99 nM/ml in the original calculation. The mean percent galactosyl ceramides was found to be 11.44%. This was very similar to the mean percent galactosyl ceramides found in the control group (11.05%) and the group of multiple sclerosis patients (11.40%).

There are several points in the analytical procedure where errors could have been incorporated.

Gangliosides, that is, glycosphingolipids containing neuraminic acid, and sphingolipids having five or more molecules of carbohydrate, are soluble in both chloroform-methanol mixtures and in water; cerebroside having one to four carbohydrate moieties are not¹⁹. This is the basis for the Folch extraction procedure²⁰. Gangliosides, moreover, are strongly influenced in biphasic systems⁷⁷ by K^+ and Ca^{++} . Some workers have used KCl in the aqueous phase to facilitate quantitative separation of gangliosides from cerebroside. The work described in this paper was performed using distilled water. It is conceivable that some gangliosides remained with the cerebroside. This problem should have been resolved, however, by the thin-layer chromatographic procedure. GL_1 bands co-migrated

with known standards; bands were well separated and there was no overlap. Products of the methanolysis procedure were identified as methyl glycosides of hexoses by gas-liquid chromatography.

Any work done with plasma bears the risk of some contamination from erythrocytes despite careful centrifugation and removal of plasma from the red cell layer. Glycosyl ceramides occur in small quantities in erythrocyte stromata^{72, 73} and in leukocytes³⁴. The possibility does exist that some very small quantity of glucosyl ceramides from blood cells was co-analyzed. There was, however, no visible hemolysis in any sample, and any red or white cells escaping visual detection would have contributed an insignificant amount of cerebroside.

Tourtellotte and Haerer⁷¹ conducted an exhaustive study on lipids in cerebrospinal fluid in 156 patients diagnosed as having multiple sclerosis or retrobulbar neuritis. Patients were categorized according to number of exacerbations, stage of remission, age, length of time the patient had had the disease, amount of disability, levels of cerebrospinal protein, and acute or chronic stage of the disease. Cerebroside were elevated in all patients, and more so in patients showing the greatest elevations in cerebrospinal fluid protein and those patients over 40 years of age. The assumption is that patients over 40 have had multiple sclerosis for longer periods of time and have greater areas of demyelination

resulting in breakdown of the blood-brain-barrier with an increase in CSF protein. Except for patient E.M. who is described as having had multiple sclerosis for 14 years, case histories of patients do not contain this information. There are no data available as to whether the patient is undergoing an initial bout of the disease or an exacerbation. Neither is any information given regarding the number of exacerbations the patient has had, nor the possible extent of his disease. This kind of data might help to explain the variation between patients in this study, and the experiment should have been designed so that the information was included. The higher amounts of galactosyl ceramides in the plasma of patients C.V. and L.V. may indicate more extensive demyelination.

On the other hand, even though an increase in cerebrospinal fluid galactosyl ceramide has been reported in multiple sclerosis patients, the total body quantity of cerebrospinal fluid is small compared to that of plasma. Consequently, assuming that products of demyelination do appear in plasma, these products would suffer a several-fold dilution which may not be sufficient to elevate the concentration of galactosyl ceramide above a rather wide normal range.

This assumes that the blood-brain-barrier permits entrance of substances from the central nervous system into plasma. Communication with the brain via the blood has been shown to be highly selective, and the

possibility exists that unless this barrier is damaged directly in the process of demyelination, galactosyl ceramides cannot gain access to plasma.

The mean values of glycosyl and galactosyl ceramides for stroke patients are reduced from those of control and multiple sclerosis subjects, but not to a significant degree. The percent galactosyl ceramides was found to be higher. Although considerable variation of glycosyl and galactosyl ceramides was observed, this variation was also present in the other two groups.

As in the case of the multiple sclerosis patients, more complete case histories might have proved helpful. For example, more specific documentation of the onset of symptoms, the stage in the course of the disease when plasma was withdrawn from the patient, and an estimation of the severity of the disease might have proved helpful in determining if a time interval elapsed before products of destroyed brain were dumped into plasma.

Massive destruction of brain is known to disrupt the blood-brain-barrier, and the prediction that galactosyl ceramides would appear in blood plasma is a natural one. This conclusion, however, is not consistent with the data. The variation in concentration of galactosyl ceramide encompassed a wide range. Two explanations are possible:

- 1) The time of sampling could be very significant; a time interval may be required before molecules from destroyed brain tissue appear in plasma, and earlier values

may be normal or subnormal. It may be that cerebroside are removed from plasma and deposited upon the intima prior to stroke and contribute to thrombus formation. Following stroke, cerebroside from damaged brain are returned to plasma, and 2) the extent of the lesion may be a contributing factor. Smaller lesions contribute smaller amounts and, as postulated for cerebrospinal fluid, dilution of these products of brain destruction, may not produce a significant increase above the fairly wide range of normal values.

The source of plasma cerebroside has not been established although Dawson and Sweeley¹⁴ have postulated that glycosphingolipids from senescent erythrocytes establish an equilibrium with plasma, and Kattlove et al. present formulae for calculating quantities of cerebroside catabolyzed daily from leukocytes. Except for blood cells, the equilibrium of the various extraneural organs with plasma in health and disease has not been verified.

Plasma is known to be a carrier of and a depot for other metabolic products from these organs. While long chain bases and fatty acids are known to be organ specific, whether these molecules form a composite in plasma following degradation is uncertain.

It is of interest to note that the fatty acids found in plasma were predominantly of the shorter chain lengths, especially palmitic acid (C16), and that it is this particular fatty acid which is thought to be the basic

fatty acid molecule passing from blood to brain and ultimately undergoing elongation, hydroxylation, and desaturation⁷⁵ in the formation of brain glycosphingolipids.

Communication between plasma and brain constituents is a highly selective process. Elevation of glycosphingolipid levels in both plasma and urine have been found in some lipid storage diseases, even those accompanied by demyelination. These lipids have been attributed to underdestruction, not overproduction, due to enzyme defects and are generally not considered to be products of demyelination.

Studies on the mechanism of aging have brought to light few biochemical changes occurring in the aging process in normal subjects. No information is available to describe if, or at what point, organ structures such as kidney or liver begin to degenerate on a small scale throwing off structural molecules, such as cerebroside, into plasma. This could explain the elevation of plasma cerebroside found in the older control subjects used in this study.

SUMMARY

Duplicate plasma samples from seven stroke patients, five multiple sclerosis patients, and five control subjects age-matched with stroke patients were analyzed to determine the levels of concentration of cerebroside. The analysis involved the use of the Folch extraction procedure to separate the total lipids, silica gel column and thin-layer chromatography to separate monohexosyl ceramides, HCl methanolysis to rupture the glycosidic and amide bonds of the cerebroside molecule, and quantification of the hexose moiety by gas-liquid chromatography.

While the normal values in this study were elevated above those previously reported, the percent of galactosyl ceramides was similar to that determined on younger patients. The concentration of plasma cerebroside in control subjects and multiple sclerosis patients was strikingly similar. Cerebroside values for the stroke patients were lower and the percent galactosyl ceramides were higher than for the control subjects. No values, however, varied to a significant degree ($p > 0.05$).

Recalculation of data from the stroke group following elimination of the patient who appeared to be afflicted with more than one disease entity and who showed an

approximate two-fold elevation of galactosyl ceramides above the other patients in the stroke group gave the following results: the total cerebroside values were slightly elevated, galactosyl ceramides decreased considerably, but not to a significant degree, and the percent galactosyl ceramides was in very close agreement with the other two groups.

The pathology of stroke and multiple sclerosis is described and possible reasons for the wide variation in patient cerebroside values given. The influence of aging on plasma cerebroside values is discussed.

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APPENDICES

APPENDIX A

APPENDIX A

Patient Information

The data listed below gives the patient's identification, age and sex, the date the plasma sample was obtained from the patient, the date of onset of symptoms, the date admitted to the hospital, and the diagnosis:

<u>PATIENT</u>	<u>AGE-SEX</u>	<u>DATE SAMPLED</u>	<u>ONSET SYMPTOMS</u>	<u>ADMISSION</u>	<u>DIAGNOSIS</u>
D.W.	44f	11-14-72	10-30-72	11-8-72	multiple sclerosis
D.P.	24f	11-14-72	6- -72	11-9-72	multiple sclerosis
E.M.	46m	11-16-72	14 years ago	11-14-72	multiple sclerosis
L.V.	37f	- - - -	8-22-72	8-30-72	multiple sclerosis or disseminated encephalomyelitis
C.V.	28m	9-19-72	3 weeks	9-17-72	multiple sclerosis or disseminated encephalomyelitis

Appendix A (Cont'd)

<u>PATIENT</u>	<u>AGE-SEX</u>	<u>DATE SAMPLED</u>	<u>ONSET SYMPTOMS</u>	<u>ADMISSION</u>	<u>DIAGNOSIS</u>
G.M.	58m	9-26-72	1 1/2 years	9-17-73	transient cerebral ischemia
P.S.	55m	12-6-72	12-1-72	12-4-72	stroke
E.S.	84f	9-26-72	9-18-72	9-18-72	diabetic (50 years) stroke
D.S.	76m	- - -	7-22-72	7-22-72	stroke
C.D.	56m	9-6-72	7-24-72	9-2-72	cerebral thrombosis
A.K.	65m	8-15-72	8-k-72	8-11-72	embolus
B.M.	76m	9-21-72	9-7-72	9-9-72	stroke

APPENDIX B

APPENDIX B

Nomenclature

Sphingolipids are defined as non-glycerol lipids which are grouped by the presence of a long-chain aliphatic base, sphingosine or its homolog. Glycosphingolipids, ceramides, and sphingomyelins are classes of compounds found in this group:

glycosphingolipid: ceramide derivative linked to
1 or more moles of carbohydrate

ceramide: N-acyl fatty acid derivative of
sphingosine

sphingomyelin: ceramide-1-phosphorylcholine

The glycosphingolipids are further classified as cerebrosides, ceramide oligosaccharides, and gangliosides.

Cerebrosides or glycosyl ceramides are monohexosyl ceramides having either glucose or galactose as the sugar moiety; some workers use the designation of GL₁. The individual sugar compounds are termed glucosyl (glc-cer) or galactosyl (gal-cer) ceramides; glucocerebrosides and galactocerebrosides are terms which are also used. Ceramide oligosaccharides can be expressed by indicating the hexoses in sequence, such as galactosyl-galactosyl-glucosyl ceramide. Another classification is listed below:

Appendix B (Cont'd)

GL ₂	dihexosyl ceramide
GL ₃	trihexosyl ceramide
GL ₄	globoside

Sulfatide is either galactosyl-SO₄-ceramide, or galactosyl-SO₄-glucosyl ceramide.

The gangliosides consist of a ceramide to which is attached several moles of hexose and carbohydrate derivatives including hexoseamines and sialic acid. Sialic acids include N-acylneuraminic acids, their esters, and derivatives of alcoholic hydroxyl groups. The two most frequently encountered hexosamines are glucosamine and galactosamine.

APPENDIX C

Preparation of Glassware

All glassware, including separatory funnels, chromatography columns and tanks are first washed and dried using prescribed techniques for the washing of laboratory glassware. This is followed by further rinsing with distilled water, acetone, and chloroform methanol (2:1) in that order.

Preparation of Thin-Layer Chromatography Plates

Commercial plates are chromatographed in diethyl ether and permitted to dry prior to sample application to remove contaminants and plasticizer. Plates are scribed with a pencil using a ruler or template; an 8 to 10 cm area is marked off with double lines for the test area and parallel to this, a 2 cm strip again bounded by double lines for spotting standards. A band 2 cm from the top is used for sample identification.

Preparation of Chromatography Tanks

Tanks and covers are washed according to the procedure given above under preparation of glassware. They are then washed with a few ml of the solvent system to be used. A strip of filter paper 7 to 8 inches wide is fitted inside

Appendix C (Cont'd)

the tank so that it will not touch the sides of the plates. One hundred seventy-five to two hundred ml of the solvents (chloroform-methanol-water, 100:42:6) are placed in the tank. The cover is weighted. Tanks should be equilibrated for at least two hours prior to use, kept out of direct sunlight, drafts and heat, and must be used within three days or before significant evaporation of any solvent.

Reagents and Solutions

A. Solvents are redistilled from glass before use.

The use of plastic containers and stop-cock grease should be avoided.

B. Water is glass distilled; plastic containers contribute interferring plasticizers.

C. Methanolic NaOH (0.6N)

1. Dissolve 2.4 gm of sodium hydroxide pellets in 100 ml of methanol.
2. Gently warm at 50°C or use a sonicator until dissolved.
3. Replace every 3 weeks.

D. Methanolic HCl (0.5N)

1. Bubble 1.86 gm of gaseous HCl into 100 ml of methanol. This is a hazardous procedure and must be performed in a hood. CAUTION!

Appendix C (Cont'd)

2. The shelf-life is 3 weeks.

E. Mannitol standard (2 μ l/ml)

1. Dissolve 36.4 mg of mannitol in 1 ml of distilled water with gentle heating at 40-50°C.
2. Add 99 ml of methanol. Stopper tightly
3. Check frequently for contamination by GLC after addition of silylating reagent and methanolic HCl, warming 2 hours at 80° and trimethylsilylation.

Mini-Columns for Cerebroside Extraction

Disposable Pasteur pipets are plugged with glass wool and rinsed with chloroform-methanol-water (100:50:10 by vol). Each pipet is fitted with a cardboard collar made from an inch square of medium weight cardboard which has been centrally punctured. The tip of the pipet is inserted into a test tube; and the collar holds it upright.

The desired GL band is scraped from the thin-layer chromatography plate onto glassine paper and carefully introduced into the mini-column. GL₁ cerebrosides are extracted with approximately 10 ml of chloroform-methanol-water (100:50:10).

Iodine Vapor Tank

Approximately 25 gm of crystalline iodine is sprinkled on the bottom of a thin-layer chromatography tank

Appendix C (Cont'd)

and covered. Chromatograms are allowed to stand upright in the tank until the bands are seen as yellow, horizontal lines. The color fades upon removal from the vapor tank and may need to be re-exposed.

Blueprints

As soon as GL bands have been visualized by exposure to iodine vapor, the thin-layer chromatography plate is placed face up on an x-ray view box. A sheet of 8 1/2 x 11 blueprint paper (maximum speed, ammonia-develop, standard weight paper, grain co., Scott Graphics, Holyoke, Mass. 0804) is placed face down on the plate and left in position until the background is white and the GL bands are yellow. Experimentation is required to determine the optimum exposure time. When the bands on the plate fade, the plate may be re-exposed to the iodine.

Ammonium Hydroxide Tank

Into the bottom of a 2000 ml glass beaker or other suitable container which has been fitted with a cover, is placed a few ml of concentrated NH_4OH . The blueprint is slightly rolled to fit inside the container. Exposure is completed when the cerebroside bands appear blue on a white background. The NH_4OH should be replaced periodically.

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