ABSTRACT

BIOCHEMISTRY AND METABOLISM OF MAMMALIAN BLOOD GLYCOSPHINGOLIPIDS

By

Robert V. P. Tao

The objective of this research was to chemically characterize the platelet glycosphingolipids and plasma gangliosides as well as to study the metabolic turnover of sphingolipids in a pig induced with reticulocytosis.

Identifications of four neutral glycosphingolipids were made on the basis of sugar molar ratios, studies of permethylation products, and the action of stereospecific glycosidases on these lipids. Lactosylceramide, the most abundant type, accounted for 64% of the total neutral glycolipid mixture. The platelets were rich in a ceramide fraction, representing 1.3% of the total platelet lipids. The neuraminic acid component of hematoside was N-acetylneuraminic acid. Treatment of platelets with trypsin, chymotrypsin or thrombin increased the yield of hematoside as compared with a control, while the level of ceramides was not changed.

In contrast to human platelets, porcine platelets contained trihexosylceramide as the major neutral glycosphingolipid. Sulfatide, hematoside and ceramides were also detected.

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An investigation of the biosynthesis of glycosphingolipids in reticulocyte-rich blood was conducted by incubating [^{14}C]glucose with the whole blood *in vitro* at 37°C for 2 hr. The results suggested that reticulocyte-rich red cells can synthesize glucosylceramide and, to some extent, lactosylceramide as well. Radioactivity was also detected in the hexose moiety of plasma glucosylceramide, suggesting a possible exchange between the plasma and erythrocyte glucosylceramide pools.

A metabolic experiment was conducted to study the turnover of sphingolipids in an anemic pig *in vivo* by injecting [^{14}C]glucose into the pig intravenously as a pulse label, and removing aliquots of blood samples for lipid analyses at frequent intervals throughout a period of 81 days. Analyses were made on plasma, mixed population of red cells, and red cells that had been fractionated into individual groups of cells according to age by density gradient ultracentrifugation.

The results suggested the radioactive glucose was rapidly incorporated into the membrane-bound globoside of the immature erythrocytes in the bone marrow. After being released from the marrow these cells lost a portion of their globoside-containing membrane as they matured in the peripheral circulation. The remodeling of the cells within the circulation continued until it approached the size of a normal adult

cell, after which time the turnover of globoside remained rather constant. The membrane-bound globoside remained with the cell until the time of red cell senescence, and then was released directly into the circulation as a whole unit before cell destruction. It is postulated that this is a major source of all four plasma neutral glycosphingolipids. Erythrocyte glucosylceramide and lactosylceramide did not follow the normal expected red cell survival; instead, they appeared to be in dynamic equilibrium with the plasma glycosphingolipids.

A semilogarithmic plot of specific activity versus time emphasized the biphasic nature of the decay curves of all the sphingolipids studied, suggesting that there were at least two major pools in each of these lipid fractions. Similar half-times of 5.5 and 45.0 days were observed for both erythrocyte GL-3a and GL-4.

When globosides from the fractionated red cell bands were examined, half-times of 0.75, 1.0 and 4.0 days were obtained from the rapid turnover pools of Bands 1, 2, and 3, corresponding to turnover rates of 65.43, 47.04 and 11.50 μmoles/day; whereas 3.3, 5.5, and 9.3 days were obtained from the slow turnover pools of these respective bands which corresponded to turnover rates of 14.78, 11.90 and 6.89 μmoles/day.

Biphasic decay curves were also observed for both plasma GL-1a (t 1/2=0.75, 7.5 days) and G_{M3} (t 1/2=0.9, 1.9 days).

Approximately 93% and 9% of GL-1a and 77% and 36% of G_{M3} were found to be metabolized each day. On the contrary, only approximately 21% was synthesized each day for plasma GL-2a, GL-3a and GL-4.

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FOREWORD

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LIST OF ABBREVIATIONS

Acid Citrate Dextrose	ACD
N-Acetylgalactosamine	GalNAc
N-Acetylglucosamine	GlcNAc
N-Acetylneuraminic Acid	NANA
Adenosine Diphosphate	ADP
Adenosine Triphosphate	ATP
Adenosine Triphosphatase	ATPase
Ceramide containing Hydroxy Fatty Acids	Ceramide-HFA (Cer-HFA)
Ceramide containing Non-hydroxy (normal) Fatty Acids	Ceramide-NFA (Cer-NFA)
Cytosine Monophosphate-NANA	CMP-NANA
Fatty Acid Methyl Ester	FAME
Fucose	Fuc
Galactose	Gal
Gas-liquid Chromatography	GLC
Gas-liquid Chromatography and Mass Spectrometry	GLC-MS
Glucose	Glc
N-Glycolylneuraminic Acid	NGNA
Guanosine Diphosphate-Fucose	GDP-Fuc
Infrared Spectroscopy	IR
Immunoglobulin E	IgE
Mild Alkaline Hydrolysis	MAH
Nuclear Magnetic Resonance	NMR
3'-Phospho-adenosine-5'-phosphosulfate	PAPS
Red Blood Cell	RBC
Uridine Diphosphate-N-acetylgalatosamine	UDP-GalNAc
Uridine Diphosphate-Galactose	UDP-Gal
Uridine Diphosphate-Glucose	UDP-Glc

I. LITERATURE REVIEW

A. Sphingosine

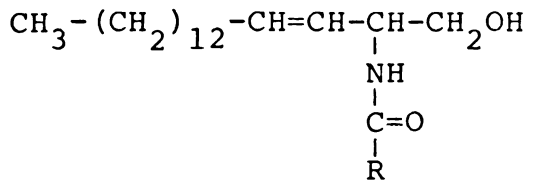
Much of our present knowledge in the field of glycosphingolipids is due to the pioneering work in brain chemistry by Ludwig Wilhelm Thudichum (1829-1901), who discovered spingosine (1) named after sphingein (Gr. "to bind tight"). His work was followed by many distinguished scientists such as Thierfelder, Levene, Rosenheim, and Klenk. Until 1940 the structure of sphingosine was only partly known as an unsaturated dihydroxyamine with a long-chain carbon skeleton, and it was not until 1947 that the correct structure of sphingosine [I] was established by Carter and his collaborators (2). This achievement has paved the way for all the structural studies as well as studies on biosynthesis and metabolism of sphingolipids in the ensuing years. The term "sphingolipid" was also introduced by Carter to designate the lipids derived from the parent base sphingosine.

$$CH_3-(CH_2)_{12}-CH=CH-\underset{OH}{\underset{|}{CH}}-\underset{NH_2}{\underset{|}{CH}}-CH_2OH \qquad [I]$$

B. Ceramide

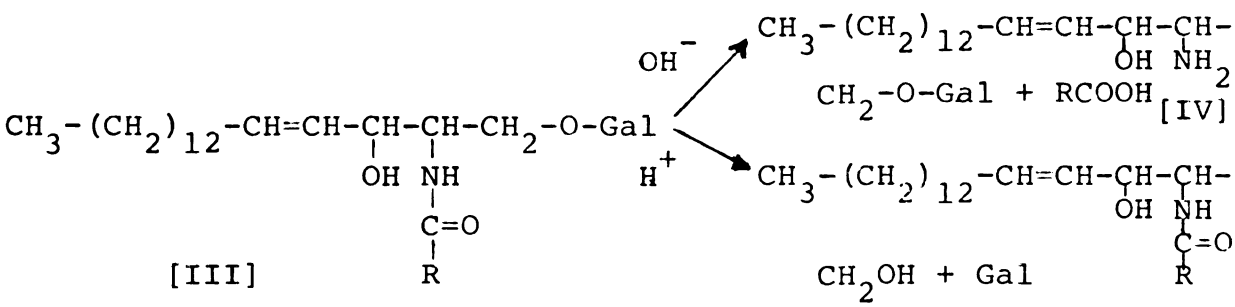
Ceramide [II] is the trivial name for N-acylsphingosine; the name was derived from the waxy texture (cerum) of these

substances (3). They are the only simple derivatives of sphingosines. The fatty acids are bound to the amino group of the long-chain base in amide linkage. Lignocerylsphingosine and cerebronylsphingosine are the outstanding representatives. Thudichum (4) was the first to isolate ceramide and psychosine from cerebroside [III] as degradative products after partial



[II]

hydrolysis under different conditions. Alkaline hydrolysis gave fatty acids and psychosine (galactosylsphingosine)[IV], whereas mild acid treatment cleaved the glycosidic bond with the formation of ceramide.



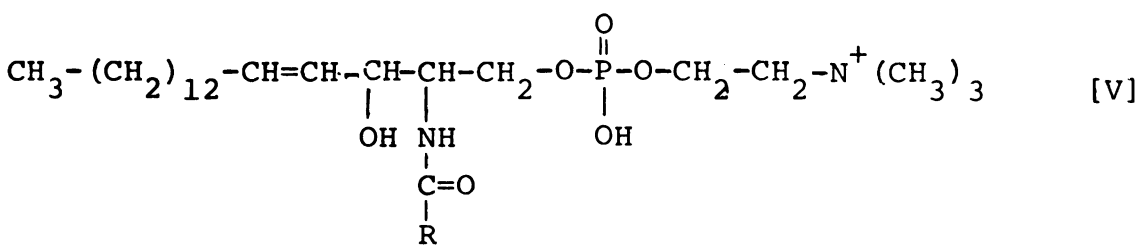
[III]

[IV]

This indicates that the fatty acid is in amide linkage and the hexose in glycosidic bond with sphingosine. A number of investigators have isolated free ceramide from liver (5), spleen (6), and lung (7). Klenk (8) found lignocerylsphingosine in the red cells and Shimojo (9) also demonstrated the

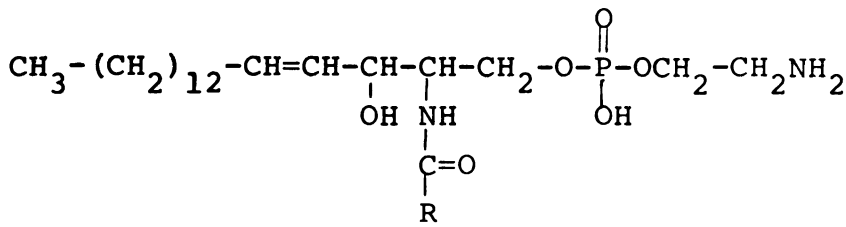
presence of ceramide in pig erythrocytes. Most recently, ceramides were found in human kidney (10), brain (11,12), plasma (13), aorta (14) and pig brain (15). Tannhauser (16) suggested that the presence of N-acylsphingosine in these organs or tissues could serve as a possible precursor in the biosynthesis of cerebroside and sphingomyelin. This view was supported by recent evidence from Kennedy and co-workers (17, 18).

Ceramides have been shown to be part of the molecule in two types of sphingolipids. Sphingophosphatide is characterized by linking ceramide to another polar group via a phosphate diester, such as sphingomyelin [V]. It was discovered in human brain by Thudichum (1). Besides brain and nervous tissues, sphingomyelin was also found to be present in human



[V]

plasma and erythrocytes in rather large amounts. Ceramide phosphorylethanolamine [VI] (another variation of sphingomyelin) and ceramide aminoethyl phosphonates [VII] (ceramide was linked via a phosphonate ester) were found to be present in marine invertebrates (19,20) as well as rumen protozoa (21) and the blowfly Calliphora erythrocephala (22).



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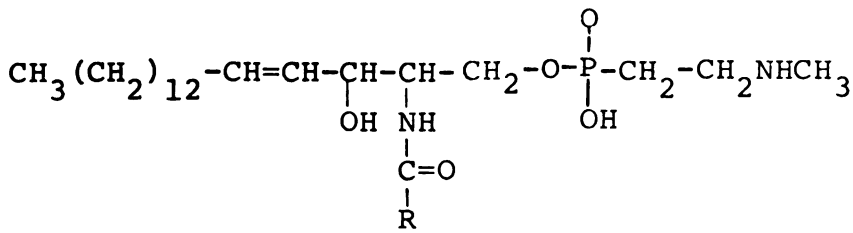


[VII]

The second class of sphingolipids is characterized by the presence of a glycosidic linkage between a carbohydrate unit (mono- or oligosaccharide) and the primary hydroxyl group of the ceramide, ceramide-O-carbohydrate. The carbohydrate chain may contain amino sugars and NANA or NGNA. Various neutral glycosphingolipids, sulfatides, gangliosides and blood group substances are representatives of this group.

C. Neutral Glycosphingolipids

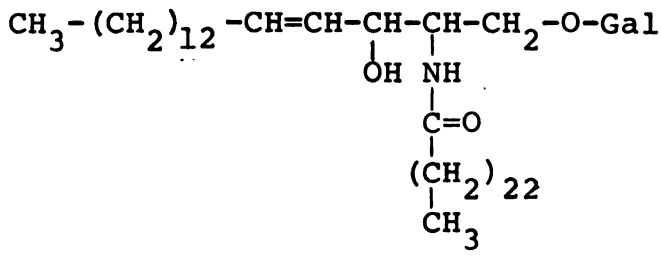
The neutral glycosphingolipids contain one or more glycosyl moieties. They can be divided into monohexosylceramides, dihexosylceramides, trihexosylceramides, tetrahexosylceramides, pentahexosylceramides and polyhexosylceramides. Compounds containing amino-sugars are sometimes called aminoglycolipids. The individual compounds are often named as derivatives of ceramides. For example, a dihexosylceramide containing galactose and glucose in known sequence is called galactosyl-

glucosylceramide. The lipid may be called lactosylceramide if the position and linkages are known, i.e., galactosyl-(β1→4)-glucosyl-(β1→1')-ceramide.

1. Monohexosylceramide

a. Galactosylceramide (Galactocerebroside, GL-1b)

Thudichum (23) was the first to isolate this group of compounds and gave the name cerebrosides to a group of glycosidic substances found in the brain containing 1 molecule each of sphingosine, hexose and fatty acid. The hexose component was identified as galactose by Thierfelder (24). The attachment of sugar to the C-1 position of the sphingosine moiety was demonstrated by Carter and Greenwood (25). Evidence that the glycosidic linkage has the β configuration was presented on the basis of its susceptibility to hydrolysis by β-galactosidase (26) and IR measurements (27). Shapiro and coworkers further confirmed the structural analyses of brain cerebrosides by total synthesis of psychosine (28) and galactocerebroside (29). Brain cerebrosides contain four major fatty acids. The material first isolated by Thudichum was rather impure at that time; however, he was able to distinguish between the two major compounds of this class, cerasine [VIII] and phrenosine [IX], which were later found to contain lignoceric acid, by Levene (30), and α-hydroxylignoceric acid, by Thierfelder (24) and Klenk (31). Besides cerasine and phrenosine, a third cerebroside was obtained from beef brain



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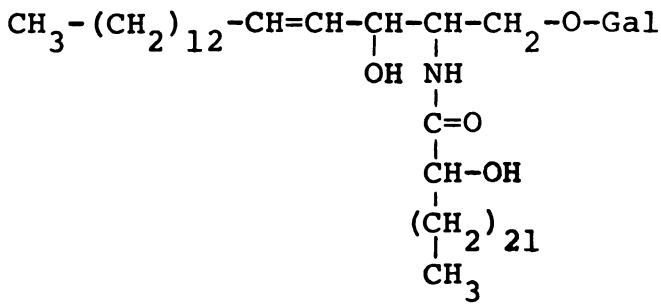
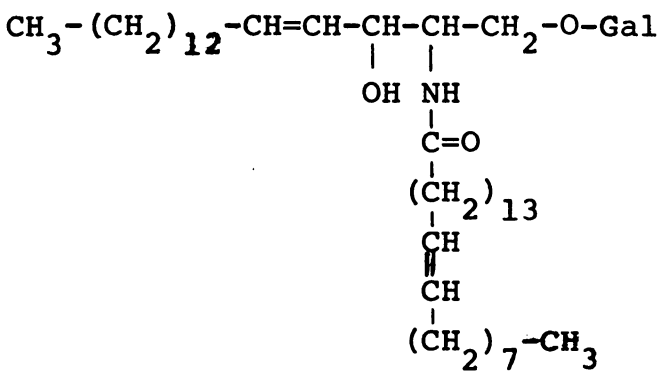
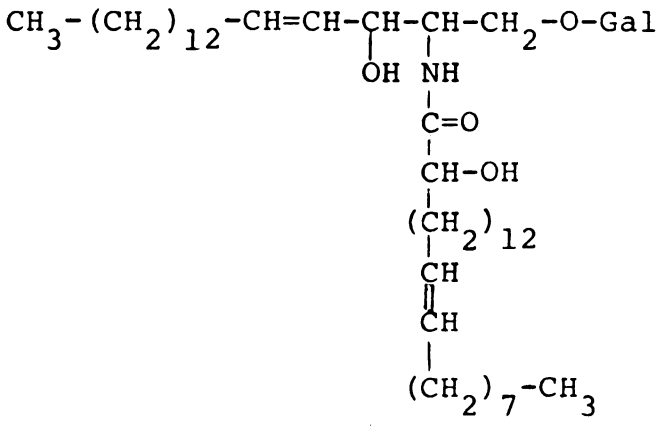


[IX]

and identified by Klenk (32), who named it nervone [X]. The same author also postulated a fourth cerebroside, oxynervone [XI], containing α-hydroxynervonic acid. The sphingosine bases consisted predominantly of C_{18}-sphingosine (98%) and a small quantity of C_{18}-dihydrosphingosine (2%) (33).



[X]



[XI]

Galactosylceramide was found exclusively in the human brain; this lipid accounted for 10-25% of the total lipids from the glial cells in the nervous system (34). It has also been found in extraneural tissues and fluids, such as human kidney (35), intestine (35) and blood. In blood, a small amount of galactosylceramide had been detected in serum (36) (about 10% of the total monohexosylceramides) and a trace amount in human plasma (37). Miras et al. (38) reported that galactosylceramide was the monohexosylceramide present in human leukocytes; however, this was at variance with the findings of Hildebrand et al. (39) and Kampine et al. (40) who found glucosylceramide as the only cerebroside present in leukocytes. This could be explained by the fact that earlier authors based their conclusions on co-chromatography of the isolated material with a galactosylceramide standard in a conventional TLC system which was unsuitable for resolving glucosylceramide from galactosylceramide. Better resolution could be achieved if borate-impregnated silica gel G plates were used (41).

b. Glucosylceramide (Glucocerebroside, Gl-1a)

Glucocerebroside was first isolated in 1940 from the spleen of a patient suffering from Gaucher's disease (26). Glucose instead of galactose was found to be present in this lipid (42). The structure was proposed by Rosenberg and Chargaff (43) as 1-O-(β-D-glucopyranosyl)-N-docosanoyl-D-erythro-sphingosine and confirmed by chemical synthesis (29, 44). Glycosylceramide had been found in a variety of living organisms. In human and pig plasma (37,45), Gl-1a was the major neutral glycosphingolipid present, and accounted for about 48% of the total neutral glycosphingolipid contents. In erythrocytes (37,45), Gl-1a represented 4% (human) and 5% (pig), respectively of the neutral glycosphingolipid fraction. In both human and pig leukocytes (38,45,46), Gl-1a was also found to be a minor component. This lipid was shown to be elevated in both plasma and erythrocytes of patients with Gaucher's disease (47).

2. Dihexosylceramides

a. Digalactosylceramide (Gl-2b)

A digalactosylceramide was found in the kidney of a patient with Fabry's disease by Sweeley and Klionsky (48). The structure was shown to be Gal-(1→4)-Gal-(1→1')-ceramide (49), and recently the anomeric configurations of this lipid were assigned as Gal-(α1→4)-Gal-(β1→1')-ceramide with the aid of stereospecific glycosidases by Li et al. (50). In humans,

GL-2b appears to be present only in kidney (51) and intestines (52), whereas in Fabry's disease abnormal amounts were found in kidney, urinary sediments and pancreas (53-55). GL-2b was never detected in plasma or erythrocytes of these patients. It was reported (38) to be present in human leukocytes; however, this finding could not be confirmed by several other investigators (39,40,52). Hence, the existence of GL-2b in leukocytes is questionable. There was also an early report on this lipid in the brain of a patient with Tay Sachs disease (56).

b. Lactosylceramide (GL-2a)

In 1942, Klenk and Rennkamp (57) isolated a substance from bovine spleen and later from erythrocytes (58), which was found to be composed of sphingosine, fatty acid and two hexose residues (glucose and galactose). The structure of this lipid, isolated from erythrocytes and kidney by Yamakawa and co-workers (59), was shown to bear the following structure Gal-(1→4)-Glc-(1→1)-ceramide. Besides human erythrocytes, Gl-2a has been detected in human plasma (37), serum (60), bone marrow (61) and leukocytes (38-40) as well as porcine plasma (45), erythrocytes (45), and leukocytes (45,46). In human leukocytes (38), Gl-2a accounted for 16% by weight of the total lipids. 4-Sphingenine was the major long-chain base found in Gl-2a of all the blood components, whereas the fatty acid compositions differed considerably. Recently, there

was a report on a patient with lactosylceramidosis, characterized by high levels of Gl-2a accumulation in plasma, erythrocytes, bone marrow cells and other visceral organs (52).

3. Trihexosylceramides

a. Galactosyl-galactosyl-glucosylceramide (GL-3a)

In 1953, Klenk and Lauenstein (58) first suggested the presence of a neutral trihexosylceramide, free of amino sugar, in human erythrocytes. Later, Svennerholm and Svennerholm (60) demonstrated that trihexosylceramide isolated from human serum, spleen and liver contained galactose and glucose in a molar ratio of 2:1. Trihexosylceramide had been identified as the pathologically accumulated glycosphingolipid in Fabry's disease by Sweeley and Klionsky (48). The same lipid was also elevated in the plasma of these patients but not in the red cells (47). Initially, the carbohydrate sequence of GL-3a was deduced as being Gal-Gal-Glc-ceramide upon partial hydrolysis (48). Later, the same lipid was also isolated by Makita (62) and Martensson (51) from normal human kidneys and, on the basis of methylation studies, 1→4 linkages between the sugars were established by Makita and Yamakawa (63). Most recently, the structure of Gal-(1→4)-Gal-(1→4)-Glc-(1→1')-ceramide was confirmed by GLC of the methanolysis products before and after permethylation, periodate oxidation of GL-3a, and mild acid hydrolysis products (49). NMR studies coupled

with stereospecific enzymes (64-66) have established the anomeric configurations of the glycosidic bonds and gave evidence to support the following complete structure-- Gal-(α1→4)-Gal-(β1→4)-Glc-(β1→1')-ceramide. Gl-3a was shown to occur in both the human and pig erythrocytes and also in the plasma fraction. In porcine leukocytes (46), Gl-3a was the observed major neutral glycosphingolipid present.

b. Galactosyl-galactosyl-galactosylceramide (GL-3b)

This lipid has not yet been shown to occur in living organisms. It has been postulated (67), however, that a further addition of one molecule of galactose to the terminal galactose of digalactosylceramide might lead to the formation of this lipid, Gal-(?)-Gal-(α1→4)-Gal-(β1→1')-ceramide. It is possible that this material is present at a very low concentration, which is not able to be detected by the analytical methods employed at present.

c. N-Acetylgalactosaminyl-galactosyl-glucosylceramide

This compound was first demonstrated in the brains of Tay-Sachs patients (56). However, recent studies have shown that the accumulation of this lipid was much more pronounced in Sandhoff's disease than in Tay-Sachs disease.

d. N-Acetylglucosaminyl-galactosyl-glucosylceramide

Recently, this lipid was shown to occur in human and cattle spleen (67) in rather large amounts. The structure was shown to be GlcNAc(β1→3)-Gal-(β1→4)-Glc-(1→1')-ceramide.

4. Tetrahexosylceramides

a. N-Acetylgalactosaminyl-galactosyl-galactosyl-glucosylceramide (GL-4)

In 1951, Klenk and Lauenstein (8) reported the isolation of a glycolipid from the human red blood cells which yielded galactosamine after acid hydrolysis. They found the compound to contain 3 hexose moieties (galactose and glucose in the proportion of 2:1) and 1 galactosamine (40%), fatty acid (29%) and sphingosine (30%). This finding was later confirmed by Yamakawa and Suzuki (68) who showed the molar ratio of fatty acid: sphingosine:neutral hexose:N-acetylgalactosamine was 1:1:3:1. Since the substance formed perfectly round globules (spherocrystals) under the microscope, it was called "Globoside", which is still the commonly used name today.

Globoside is the most abundant neutral glycosphingolipid in human and pig red cell stroma (69). The structure of Gl-4 was first erroneously reported by Yamakawa et al. (70) as GalNAc-(1→6)-Gal-(1→4)-Gal-(1→4)-Glc-(1→1)-ceramide. This error was mainly due to the misinterpretation of a gas chromatographic peak. However, after re-examination Yamakawa et al. (71) proposed the correct structure of globoside, GalNAc-(1→3)-Gal-(1→4)-Gal-(1→4)-Glc-(1→1')-ceramide in 1965. Recently, on the basis of NMR, IR, use of stereospecific glycosidases and permethylation studies, it was concluded that the exact structure of human red cell globoside is GalNAc-(β1→3)-Gal-(α1→4)-Gal-(β1→4)-Glc-(1→1)-ceramide (64).

In 1956, Matsumoto (72) reported that the chemical composition of the pig erythrocyte glycolipid consisted of galactose, glucose and galactosamine. This was further suggested by Shimojo et al. (9) as being globoside. Later, Miyatake et al. (73) purified the glycolipid from pig red cells and discovered that the structure was actually identical to the human red cell globoside except for the fatty acid composition. The same authors also demonstrated that the glycosidic bond linking N-acetylgalactosamine to galactose was in the β configuration, since the hexosamine was liberated by treatment of the lipid with a β-N-acetylhexosaminidase from pig epididymis tissue. The chemical structure of pig red cell globoside was postulated as being GalNAc-(β1→3)-Gal-(β1→4)-Gal-(β1→4)-Glc-(β1→1)-ceramide. Nevertheless, as a result of a recent study on the anomeric structure of human red cell globoside (64), it is believed that the correct structure for pig red cell globoside will probably be GalNAc-(β1→3)-Gal-(α1→4)-Gal-(β1→4)-Glc-(1→1)-ceramide. Recently, there were several reports (74-76) on Sandhoff's disease (variant of Tay-Sachs disease) which showed that these patients are characterized by extensive accumulation of Gl-4 in the visceral organs, with concomitant deficiency of β-N-acetylhexosaminidase activity.

b. Cytolipin R

In 1967, Rapport et al. (77) isolated a glycosphingolipid from rat lymphosarcoma called cytolipin R. The lipid had the

same carbohydrate composition as Gl-4, but differed from it in immunological activities and R_F values on TLC. It was not until 1972 that the complete structure of cytolipin R, GalNAc-(β1→3)-Gal-(α1→3)-Gal-(β1→4)-Glc-(β1→1')-ceramide, was elucidated by Laine et al. (78), based on the results obtained from the combination of linkage studies with GLC, GLC-MS and the uses of specific glycosidases. The difference in the immunological activities between cytolipin R and globoside were attributed to the difference of the internal linkage, -Gal-(α1→3)-Gal-, between the two lipids.

c. Galactosyl-N-acetylgalactosaminyl-galactosyl-glucosylceramide

This ceramide tetrahexoside was found in patients suffering from G_{M1}-gangliosidosis. This lipid is identical to G_{M1}-ganglioside in structure except that it lacks the neuraminic acid.

d. Galactosyl-N-acetylglucosaminyl-galactosyl-glucosylceramide

Unpublished results (67) have indicated the occurrence of Gal-(β1→4)-GlcNAc-(β1→3)-Gal-(β1→4)-Gal-(1→1)-ceramide in human and cattle spleen.

5. Pentahexosylceramides

a. Forssman hapten

Forssman was the first to report the immunologically active antigens in various organs of the guinea pig (79).

Later in 1939, Brunius (80) established the fact that Forssman hapten contained lipid and hexosamine. This was followed by the isolation of a glycosphingolipid containing hexose and hexosamine from sheep red cells which showed Forssman hapten activity by Papirmeister and Mallette (81). In 1966, Makita et al. (82) isolated Forssman hapten from horse kidney and spleen and showed that it possessed the same carbohydrate sequence (GalNAc-Gal-Gal-Glc-cer in 1:1:1:1 molar ratio) as GL-4 from human red cells and kidney, but differed in chromtographic behavior, optical rotation and blood group activities. The same authors further implicated that the determinant group of this hapten was probably the O-α-N-acetyl-galactosaminyl-(1→3)-galactosyl unit at the non-reducing end, differing from GL-4 in the anomeric configuration of the disaccharide. This conclusion was later confirmed by Yamakawa and co-workers (83) from their studies involving NMR and stereospecific glycosidases, and the structure of GalNAc-(α1→3)-Gal-(β1→4)-Gal-(β1→4)-Glc-(1→1)-ceramide was postulated. However, this was proven wrong recently by Siddiqui and Hakomori (84), who found that Forssman hapten is a ceramide pentahexoside containing 2 moles of N-acetylgalactosamine, 2 moles of galactose, and 1 mole of glucose per ceramide. After hydrolyzing the terminal hexosamine with α-N-acetylgalactosaminidase from pig liver, they were able to obtain a compound identical to globoside in all respects, and which gave a positive precipitin reaction with antigloboside antiserum. With these results and the data

from methylation studies, a revised structure for Forssman hapten was proposed, GalNAc-(α1→3)-GalNAc-(β1→3)-Gal-(α1→4)-Gal-(β1→4)-Glc-(1→1)-ceramide.

b. N-acetylgalactosaminyl-N-acetylgalactosaminyl-galactosyl-galactosyl-glucosylceramide

A very similar pentahexosylceramide was isolated from the dog intestine by Vance et al. (85). Tentatively, the proposed sequence of the carbohydrate chain was GalNAc-GalNAc-Gal-Gal-Glc-ceramide, which was further confirmed by McKibben (86). Details of the linkages between the monosaccharide units and stereochemical configurations of the glycosidic bonds have not yet been established.

c. Galactosyl-galactosyl-N-acetylgalactosaminyl-galactosyl-glucosylceramide

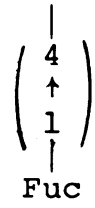
This ceramide pentahexoside was found to be the main component of rabbit erythrocytes and reticulocytes by Eto et al. (87). The structure proved to be Gal-(α1→3)-Gal-(β1→3)-GalNAc-(β1→3)-Gal-(β1→4)-Glc-(β1→1')-ceramide. Since this lipid inhibited the agglutination of human B erythrocytes with its corresponding antibody, the terminal galactose was concluded to be α-glycosidically bound (88).

d. Fucose-containing pentahexosylceramides

(1) Le^a hapten

A third ceramide pentahexoside had been isolated from human adenocarcinoma by Hakomori et al. (89,90). The structure

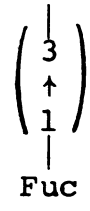
of this lipid was proposed to be Gal-(β1→3)-GlcNAc-(1→3)-Gal-(1→4)-Glc-(1→1)-ceramide



The carbohydrate backbone of this lipid is very similar to the tetrahexoside isolated from human and bovine spleen (see above) with the exception of a 1→3 linkage at the non-reducing end and, of course, the fucose residue which is attached to the N-acetylglucosamine residue in a 1→4 linkage.

(2) X-hapten

Another fucose-containing sphingolipid with the following structure Gal-(1→4)-GlcNAc-(1→3)-Gal-(1→4)-Glc-(1→1)-ceramide



was isolated from erythrocytes and chemically characterized by Yang and Hakomori (91). This lipid contained a novel type of ceramide, which composed of 4-hydroxysphinganine and long-chain 2 hydroxy fatty acids. The carbohydrate moieties were similar to those of the Le^a hapten in composition but there were differences at the terminal galactose (1→4 linked instead of 1→3) and fucose (1→3 linked instead of 1→4). This lipid exhibited no blood group A, B, H or Lewis specificities.

(3) Blood group H substance

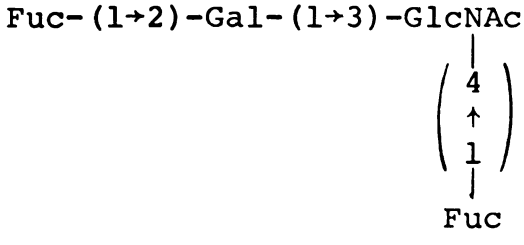
The H substance, isolated from type O blood cells (92), was shown to contain glucose, galactose, fucose and

N-acetylglucosamine. The proposed structure was Fuc-(1→2)-Gal-(1→4)-GlcNAc-(1→3)-Gal-(1→4)-Glc-ceramide. This lipid was active in the inhibition of both H and Le^b hemagglutination.

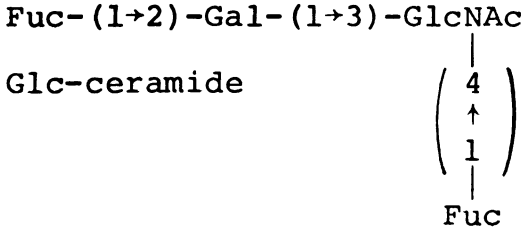
e. Polyhexosylceramides

(1) Le^b Haptens

Two Le^b haptens are known (88). One is a ceramide hexoside with a linear arrangement of oligosaccharides as follows:



Another Le^b active glycosphingolipid has been proposed to be a ceramide octahexoside.



(2) Blood group A substance

Hakomori and Strycharz (92) obtained three different fractions from type A erythrocytes, all of which are immunologically active. All three glycolipids contain the basic carbohydrate contents of galactose, glucose, fucose, N-acetylgalactosamine and N-acetylglucosamine, except one which contains additional sialic acid(s). The postulated sequence

for the A substance is GalNAc-Gal-GlcNAc-Gal-Glc-ceramide.
                                |
                               Fuc

(3) Blood group B substance

Glycosphingolipid with blood group B activity was isolated from type B blood cells. It differs from the A substance by possessing a galactose instead of a N-acetylgalactosamine at the non-reducing end of the carbohydrate chain, Gal-Gal-GlcNAc-Gal-Glc-ceramide. It has been mentioned that
    |
   Fuc
the serological specificities of the determinants from blood group-active glycolipids are very similar to the blood group-active glycoproteins; hence, one could speculate that the linkages between the sugar units would probably be 1→3, 1→3 or 4, 1→3 and 1→4. This certainly needs to be verified.

D. Acidic Glycosphingolipids

1. Sulfatides

a. Ceramide monohexosyl sulphate

Thudichum (1) was again the first to isolate a sulfur-containing lipid from human brain and he named it sulfatide. In 1933, Blix (93) gave more detail descriptions of the sulfatide from brain by showing a composition of sphingosine, amide-bound fatty acid and galactose esterified with sulfuric acid. Initially, the linkage of sulfate to galactose was erroneously reported to be at the C-6 position (94,95);

however, later studies (59,63) clearly demonstrated the sulfate group was located at position 3 of the galactose. This was also confirmed later by chemical synthesis (96,97). The structure was shown to be SO_4-Gal-(1→1)-ceramide.

b. Ceramide dihexosyl sulphate

A sulfatide with both galactose and glucose (lactosyl) has been isolated from human kidney by Mårtensson (98,99). Periodate oxidation and degradation studies indicated that the lactosylceramide was esterified with sulfuric acid at C-3 of the galactose. Methylation studies further confirmed the result and showed that the linkage of galactose to glucose was 1→4, hence the structure is SO_4-Gal-(1→4)-Glc-ceramide.

2. Gangliosides

Gangliosides were first isolated and described by Klenk (100) in 1942. Since these lipids appeared to be characteristic of the ganglion cells, Klenk invented the name "gangliosides". These lipids were found to contain sphingosine, fatty acid, hexose, and a substance which gave a strong purple color with Bial's reagent and was called neuraminic acid. This substance was later shown to be the same compound as sialic acid isolated by Blix (101) from submandibularis mucin. Gangliosides are now defined as acidic glycosphingolipids that contain sialic acid.

In 1956, Svennerholm (102) launched a detailed study of the brain ganglioside fractions and more than a dozen different

gangliosides have been recognized since then. The isolation, differentiation and structural determination of all these compounds were the result of contributions mainly from the laboratories of Klenk, Kuhn and Svennerholm. These works have been discussed in several excellent reviews and will not be discussed in detail here. The major brain gangliosides all contain Gal-GalNAc-Gal-Glc-ceramide as the basic carbohydrate backbone. Some of the representatives are listed in Table 1.

Gangliosides have been detected in many extraneural tissues and fluids, such as spleen (103), liver (104,105), lung (106), kidney (107-110), intestine (111), placenta (112), lens (113,114), adrenal medulla (115), adrenal cortex (116), erythrocytes (117,118) and cerebrospinal fluid (119).

In 1951, Yamakawa and Suzuki (120) obtained a glycosphingolipid, which they called hematoside, from equine erythrocyte stroma. This glycolipid was later confirmed by Klenk and Wolter (121) and re-examined by Klenk and Lauenstein (8). The equine hematoside was shown to contain N-glycolylneuraminic acid attached to the third position of the galactose molecule of the lactosylceramide. Two kinds of hematoside were demonstrated in dog erythrocytes (122), N-acetyl (73%) and N-glycolyl (27%), both of which were linked to the lactosylceramide. Lignoceric acid was the main fatty acid in equine hematoside, while the dog hematoside contained more stearic and nervonic acids. In 1965, the ganglioside

Table 1.--Gangliosides of Mammalian Brain

Symbol	Chemical structure
G_{M3}	NANA-(2→3)-Gal-(β1→4)-Glc-(1→1)-Cer
G_{D3}	NANA-(2→8)-NANA-(2→3)-Gal-(β1→4)-Glc-(1→1)-Cer
G_{M2}	GalNAc-(β1→4)-Gal-(β1→4)-Glc-(1→1)-Cer (3 ↑ 2) NANA
G_{D2}	GalNAc-(β1→4)-Gal-(β1→4)-Glc-(1→1)-Cer (3 ↑ 2) NANA-(8←2)-NANA
G_{M1}	Gal-(β1→3)-GalNAc-(β1→4)-Gal-(β1→4)-Glc-(1→1)-Cer (3 ↑ 2) NANA
G_{D1a}	Gal-(β1→3)-GalNAc-(β1→4)-Gal-(β1→4)-Glc-(1→1)-Cer (3 ↑ 2) NANA-(8←2)-NANA
G_{D1b}	Gal-(β1→3)-GalNAc-(β1→4)-Gal-(β1→4)-Glc-(1→1)-Cer (3 ↑ 2) (3 ↑ 2) NANA NANA
G_{T1}	Gal-(β1→3)-GalNAc-(β1→4)-Gal-(β1→4)-Glc-(1→1)-Cer (3 ↑ 2) (3 ↑ 2) NANA NANA-(8←2)-NANA
G_{Q1}	Gal-(β1→3)-GalNAc-(β1→4)-Gal-(β1→4)-Glc-(1→1)-Cer (3 ↑ 2) (3 ↑ 2) NANA-(8←2)-NANA NANA-(8←2)-NANA

from cat erythrocytes was shown to have one more sialic acid than either the dog or horse (123). It was a disialolactoside with the structure of N-glycolylneuraminyl-(2→8)-N-glycolyl-neuraminyl-(2→3)-galactosyl-(1→4)-glucosyl-ceramide. Lignoceric and nervonic acids were the major fatty acids. Bovine erythrocytes (122) showed a different variation by having N-acetylglucosamine instead of N-acetylgalactosamine in the carbohydrate chain. The proposed structure was Gal-(1→3)-GlcNAc-(1→4)-Gal-(1→4)-Glc-(1→1)-ceramide. Hematoside was the major erythrocyte glycosphingolipid in sea lamprey and it was also shown to be present in calf serum (124). This lipid has also been detected in human erythrocytes (117).

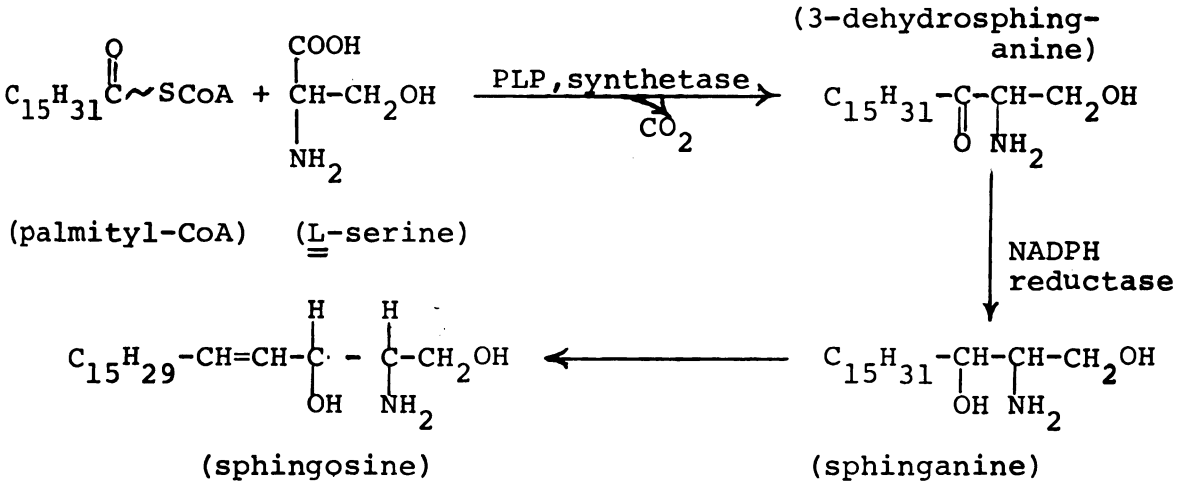
It is interesting to note that variations exist among the major erythrocyte glycosphingolipids of various mammals. The cat, dog and horse contain mainly hematosides, whereas globosides are the major components in the red cells of man, pig, sheep, goat and guinea pig (124). Bovine stroma have both components, whereas the chicken has neither. The precise physiological role of such differences is not understood at present; nevertheless, such differences are important in the selection of a suitable animal for glycosphingolipid metabolic studies.

E. Biosynthesis of Neutral and Acidic Glycosphingolipids

Studies of glycosphingolipid biosynthesis have gained much impetus in recent years; numerous reports have appeared involving *in vivo* and *in vitro* techniques which led to the elucidation of several biosynthetic pathways in the formation of glycosphingolipids. The biosynthesis of complex glycosphingolipids is believed to proceed through the stepwise addition of monosaccharides from sugar nucleotide intermediates (UDP-Gal, UDP-Glc, UDP-GalNAc, GDP-Fuc, etc.) to an appropriate receptor molecule by a multitransferase system (88). It is also believed that different enzymes exist for each step and they are different in substrate and metal requirements, pH optimum, lability to heat and different inhibitors (88). These anabolic enzymes are bound to microsomes and have a pH optimum around 7.0.

1. Sphingosine

In vivo, sphingosine is formed by the condensation of L-serine and palmityl-CoA, with the formation of 3-dehydrosphinganine as the intermediate (125,126). This compound has been synthesized by Mendershausen and Sweeley (127) and by Gaver and Sweeley (128). The reaction requires pyridoxal phosphate. The next step is the catalytic formation of sphinganine by a S-3-dehydrosphinganine:NADPH oxidoreductase (129-131). The final introduction of a double bond gives sphingosine.



2. Ceramide

An enzyme which catalyzes the biosynthesis of ceramides from sphingosine and acyl-CoA derivatives was found in chicken liver, rat and guinea-pig brain by Sribney (132). It was found that either threo- or erythro-sphingosine could be incorporated into ceramide. The specificity of the acylation process of long-chain bases by acyl-CoA esters has been studied extensively by Morell and Radin (133), and was established that ceramide containing nonhydroxy-fatty acids were the precursors of gangliosides and sphingomyelin. The hydroxy-fatty acid containing ceramides were mainly incorporated into galactosylceramide of the cerebron and oxynervon type (133-136).

3. Neutral glycosphingolipids

a. Galactosylceramide (GL-1b)

Two pathways have been postulated for the biosynthesis of cerebrosides on the basis of in vitro studies,

Psychosine Pathway: psychosine + fatty acyl-CoA→cerebroside

Ceramide Pathway: ceramide + UDP-Gal→cerebroside

Psycosine has been synthesized enzymatically from sphingosine and UDP-Gal by a number of investigators (18,137,138) and the formation of cerebroside by acylation of psychosine with acyl-CoA was suggested by Brady (139) in 1962. However, other investigators have proposed that the formation of cerebroside might proceed via the acylation of sphingosine followed by the addition of hexose (136,137,140,141). These two pathways also have supportive evidence from in vivo experiments. Recently, on the basis of mass spectrometric analyses Hammarström presented evidence that the formation of galactosylceramide containing non-hydroxy fatty acid occurred via both pathways. He then found that psychosine can be acylated with acyl-CoA nonenzymatically (142). Whether such dual pathways exist in cerebroside containing hydroxy fatty acids still remains to be investigated.

b. Glucosylceramide (GL-1a)

In 1968, Basu (143) demonstrated the presence of a glycosyl transferase in a particulate fraction from chicken brain which catalyzed the formation of GL-1a from UDP[^{14}C] glucose and ceramide. This finding was recently confirmed by Hammarström using rat brain microsomes.

c. Lactosylceramide (GL-2a)

A rat spleen homogenate was shown by Hauser (140) to incorporate UDP[1-^{3}H]galactose into lactosylceramide and

similar results were obtained by Basu (143) with a particulate fraction from embryonic chicken brain. Kampine et al. (40) obtained radioactive monohexosylceramide and dihexosylceramide when human leukocytes were incubated with [^{14}C]glucose and [^{14}C]galactose. Since human leukocytes contained mainly lactosylceramide, most of the labeling was located in the terminal galactose of the carbohydrate chain. Similar results were obtained irrespective of which sugar was being used as the precursor. This finding seemed to favor a stepwise synthesis of lactosylceramide via GL-1a. When pig bone marrow cells were incubated with [U-^{14}C]glucose, radioactivity was detected in all three of the hexose units of GL-3a (major glycosphingolipid), which seemed to indicate de novo biosynthesis of the hexose moieties by the marrow cells. However, the majority of the label was located in GL-1a and GL-3a with low incorporation detected in GL-2a. This contradicted the theory of stepwise addition of carbohydrate units to a receptor molecule.

d. Digalactosylceramide (GL-2b)

Synthesis of digalactosylceramide was studied in Gray's laboratory (144) using a kidney homogenate. The galactosylation of galactosylceramide by UDP-Gal was demonstrated.

e. Trihexosylceramide (GL-3a)

Hildebrand and Hauser (145) demonstrated that the transfer of a galactosyl moiety from UDP-Gal to lactosylceramide

was catalyzed by an enzyme present in rat spleen homogenate. It was reported that two reactions, catalyzed by different enzymes, occurred in the spleen tissue

glucosylceramide + UDP-Gal → lactosylceramide + UDP

lactosylceramide + UDP-Gal → trihexosylceramide + UDP

Although these two enzymes had the same optimal pH (6.0), they differed from each other in their lability to heat, degree of activation by Mg^{++}, and the extent of inhibition by various sphingolipids.

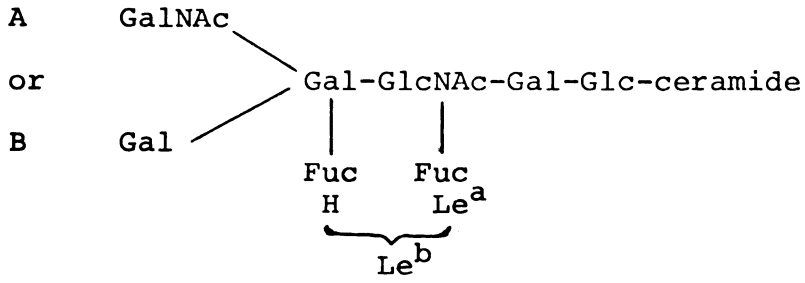
f. Globoside (GL-4)

The biosynthesis of GL-4 *in vitro* has not been fully investigated. It would probably proceed via the N-acetylgalactosylation of GL-3a by UDP-GalNAc, however. Brady (146) was able to obtain incorporation in the glucose, galactose, and N-acetylgalactosamine moieties of globoside after incubating beef bone marrow cells with labeled glucosamine. In a similar type of experiment, Dukes (147) was able to incorporate glucosamine into glycolipids of bone marrow cells *in situ*; the products were tentatively identified as hematosides, gangliosides and globosides.

g. Blood group substances

The blood group-active glycosphingolipids all share the common tetrahexosylceramide backbone, Gal-GlcNAc-Gal-Glc-ceramide. It was shown by Basu *et al.* (148,149) that the

committed step in the biosynthesis of these compounds is the N-acetylglucosylation of lactosylceramide. The transfer of galactose from UDP-Gal to receptor GlcNAc-Gal-Glc-ceramide was demonstrated by the same authors.



It is well established that blood group A and B specificities differ only by a single substituent on the terminal galactose group. The corresponding glycosyl transferases responsible for the formation of these determinants were isolated from the appropriate tissues of blood group A and B individuals respectively (150). Before these transferases can act, the receptor molecule has to be in the right conformation, which is the addition of a fucosyl group at C-2 of the terminal galactosyl moiety. The transferases confer A and B specificity only when this fucosyl group is present.

Marcus and Cass (151) found that Le^a- and Le^b- active glycosphingolipids were associated with the high- and low-density lipoproteins of plasma and were transfered or integrated into the erythrocyte surface membranes. The site of their biosynthesis is not known.

4. Acidic glycosphingolipids

a. Sulfatides

In 1960, Goldberg (152) reported the incorporation of [^{35}S]sulfate into rat kidney and liver sulfatides from labeled 3'-phospho-adenosine-5'-phosphosulfate (PAPS). This was later confirmed by McKhann and associates (153) as well as Balasubramanian and Bachawat (154), who demonstrated an enzyme system which could catalyze the transfer of sulfate from PAPS to galactosylceramide acceptor *in vitro*. The sulfate transferase activity was very high in the microsomal fraction of the rat kidney. The major sulfatide of human kidney, sulfo-lactosylceramide, was shown by McKhann and Ho (155) to be the product when lactosylceramide was used as the substrate for the sulfate transferase. Recently, Cumar *et al.* (156) provided additional evidence that exogenous cerebrosides would accept [^{35}S]sulfate from PAPS. In addition, Stoffyn, Stoffyn and Hauser (157) obtained radioactive sulfatide from [^{14}C]galactose-labeled phrenosine and PAPS *in vitro*, using the biosynthetic system described by McKhann and Ho (155). They further showed that the structure of this biosynthetic sulfatide was sulfated at C-3 of the galactose.

b. Gangliosides

The biosynthesis of ganglioside is believed to operate in the same manner as the neutral glycosphingolipids, involving a series of reactions whereby specific sugars are

transferred to the growing glycolipid acceptor by a series of specific glycosyl transferases. The glycosyl transferases were

$$\text{glycolipid-acceptor} + \text{nucleotide sugar} \xrightarrow{Mn^{++},\ \text{transferase}} \text{glycolipid-product} + \text{nucleotide}$$

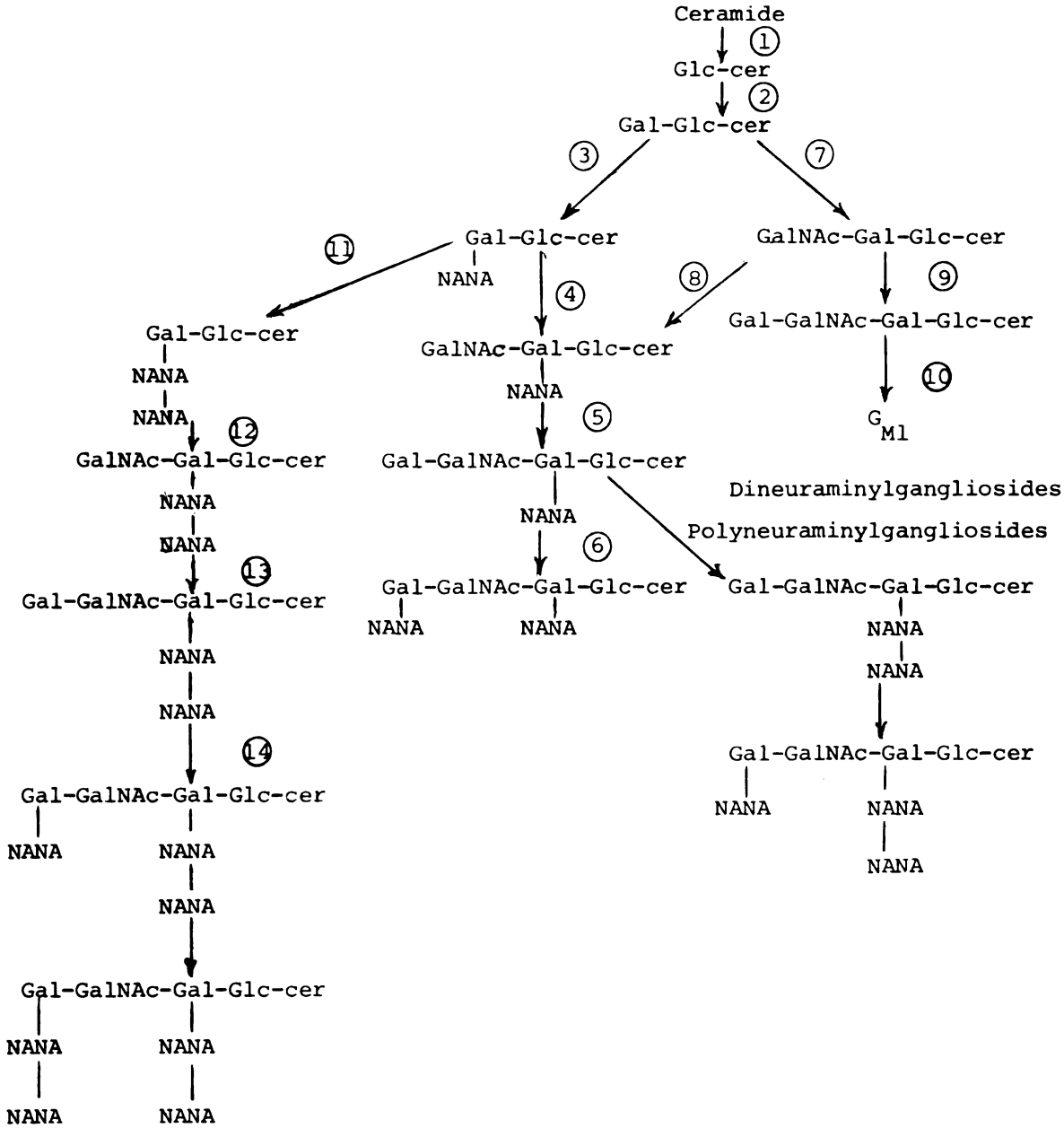
proposed to exist as a membrane-bound multiglycosyltransferase system (158). Different transferases catalyze the transfer of a glycosyl unit to the acceptor, each transferase is specific for its acceptor, and the product of each step is the preferred substrate for the next reaction. In addition to the normal sugar nucleotides mentioned earlier, CMP-NANA is involved in the biosynthesis of gangliosides. The sialyltransferases are a family of enzymes which catalyze the transfer of NANA or NGNA from the corresponding CMP-NANA or CMP-NGNA to the glycolipid receptor. It is believed that a number of different sialyltransferases are involved in ganglioside biosynthesis. Transfer of sialic acid from CMP-NANA to glycosphingolipid was first demonstrated by Kanfer *et al.* (159) *in vitro*. By using a rat kidney homogenate as the enzyme source, and asialoganglioside and tetrahexosylceramide as the acceptors, they were able to obtain a ganglioside fraction identified as G_{M1}. The role of sialyltransferases in the biosynthesis of gangliosides have been studied extensively and systematically by Roseman and associates (158). These workers have described a number of transferases from embryonic chicken brain which catalyze the stepwise synthesis

of gangliosides from ceramide and the appropriate sugar donors (reactions 1-6). The biosynthetic pathway is shown in Scheme I. Experiments from other laboratories suggested that this pathway also occurred in adult frog brain (160,161) as well as young rat brain (162-165). Recent evidence (166,167) also pointed to the existence of a second pathway (reactions 7, 9 and 10) and a third possible pathway (reactions 7, 8 and 5) where the trihexosylceramide and tetrahexosylceramide backbones of gangliosides could be assembled before the addition of sialic acid(s). The galactosyl transferases catalyzing reactions 2 and 5 have been shown to be different by Hildebrand et al. (163), whereas the same galactosyl transferases were involved in the catalysis of reactions 5 and 9 (168). Synthesis of complex gangliosides might also occur via reaction 11 (169); experimental evidences for reactions 7, 8 and 14 had been presented by Cumar et al. (170) and Arce et al. (165).

F. Turnover Studies

Relatively few reports are available about the turnover of glycosphingolipids outside the nervous system. In 1965, Kanfer (171), injected labeled GL-1a into 10-day old rats and recovered most of the activity in the extracted GL-1a and ceramide. Radioactively labeled sulfatides ($^{35}SO_4$) were employed by Davison and Gregson (172) and Pritchard (173) to

Scheme I. Biosynthesis of Gangliosides



study the synthesis and turnover of myelin; most rapid synthesis of sulfatide was found to occur between 20 and 25 days.

Dawson and Sweeley (45) studied the turnover of neutral glycosphingolipids of porcine plasma and erythrocytes in vivo over a 2-month period using [^{14}C]glucose as a pulse label. Their results suggested that red cell GL-2a, GL-3a and GL-4 were synthesized in the bone marrow and released into the plasma during the time of red cell catabolism, whereas GL-1a was not synthesized in the bone marrow and exchanged freely between the plasma and erythrocytes.

Glucose and galactose (174-176), glucosamine (177) and mannosamine (178) are the precursors commonly employed in ganglioside metabolic studies. The radioactivity is incorporated into all portions of the carbohydrate chain of gangliosides and hexosamines are the most efficient precursor of NANA and GalNAc. Radin (174) was the first to demonstrate the incorporation of [1-^{14}C]galactose into cerebrosides and gangliosides of rat brain. This was later confirmed by Burton et al. (177) who showed that all the sugar moieties of gangliosides had the same specific activity. The half-life of the total ganglioside fraction in rat brain was estimated to be about 8-10 days for a glucose or galactose label, and about 24 days for amino sugars (179). This was somewhat different from the data of 20 days obtained by Suzuki (180) using [U-^{14}C]glucose as the precursor. Recently, Suzuki (181) and

Maccioni et al. (182) independently used [1-^{14}C]glucosamine and [6-^{3}H]glucose to study the formation and turnover of rat myelin and brain gangliosides. Suzuki found that the formation and turnover of G_{M1} ganglioside in myelin was different from that of whole brain lipid or whole brain gangliosides. Neither investigator could detect any precursor-product relationship in vivo.

G. Catabolic Degradation of Glycosphingolipids

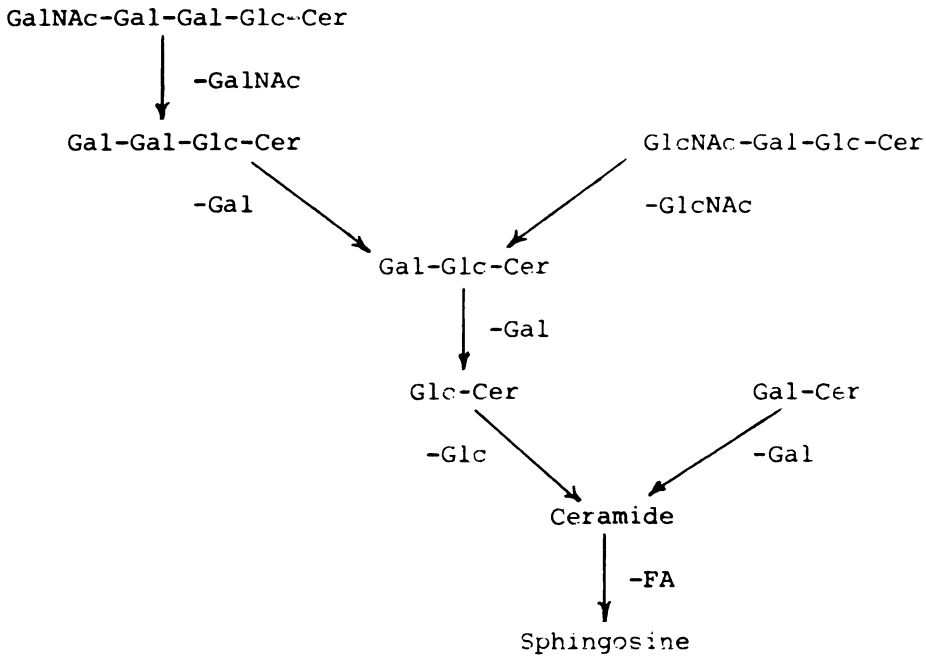
Glycosphingolipids are degraded in a stepwise removal of sugar units by a family of glycosylceramide hydrolases. These enzymes are assured to be located in lysosomes (183) of various organs throughout the body (184). Elucidation of the catabolic pathways of both neutral and acidic glycosphingolipid metabolism gained much success in recent years; this was partly due to the development of enzyme assays which made possible the detection of enzyme deficiencies that occurred in various lipid storage diseases.

In 1964, Sandhoff (185) obtained an enzyme preparation from pig kidney which was able to catabolyze Gal-GalNAc-Gal-Glc-ceramide to ceramide with the formation of trihexosylceramide, GL-2a and GL-1a. Later, Statter and Shapiro (186) demonstrated the recovery of radioactivity in GL-2a and GL-1a from rat after an initial injection of labeled GL-4. An enzyme (hexosaminidase) which could hydrolyze the terminal

N-acetylgalactosamine moiety of globoside was isolated from calf brain by Frohwein and Gatt (187). The same enzyme was equally active towards GalNAc-Gal-Glc-ceramide or GlcNAc-Gal-Glc-ceramide. Recently, two forms of hexosaminidase (A and B form) were identified by Sandhoff (188,189) and by Okada and O'Brien (190-191). In Tay-Sachs disease, the A form was missing while the B form remained normal or elevated (188,190). This correlated well with the finding that in Tay-Sachs disease the main reason for the accumulation of Tay-Sachs ganglioside was due to the absence of an enzyme to hydrolyze it. However, in a variant form of Tay-Sachs disease, characterized by the accumulation of globoside in visceral organs, both A and B forms were missing (188). It was inferred that hexosaminidase A played a physiological role in ganglioside metabolism, whereas hexosaminidase B was involved in globoside degradation (192).

Once the terminal N-acetylgalactosamine was removed from globoside, the remaining trihexosylceramide could be further catabolyzed by an α-galactosidase to lactosylceramide, as shown in Scheme II. In patients with Fabry's disease, the accumulation of an excessive amount of GL-3a was due to the absence of a GL-3a-cleaving enzyme in the plasma (193) and other organs (194) of these patients. On the contrary, enzymatic activity was detected in normal spleen, small intestine, kidney, brain and liver (195).

Scheme II. Biodegradation of Neutral Glycosphingolipids



Digalactosylceramide was shown to possess the same terminal disaccharide as that of GL-3a in linkage and anomeric configuration; however, whether or not its accumulation in Fabry's disease was due to the absence of the same cleaving enzyme is not uncertain. Recent evidence by Mapes and Sweeley (196) indicates that GL-2b is hydrolyzed by a different enzyme than the α-galactosidase involved in GL-3a metabolism.

Lactosylceramide is degraded to GL-1a by a β-galactosidase from rat brain (197,198). The enzyme has been partially purified. An enzyme with similar activity was also demonstrated in a particulate fraction from rat kidney (185). Recently, a new glycosphingolipid storage disease, lactosylceramidosis, was discovered (52). This unusual lipidosis is characterized by a deficiency of lactosylceramide: galactosyl hydrolase activity coupled with excessive accumulation of GL-2a in plasma, erythrocytes, bone marrow, urine sediment and other neural and non-neural tissues.

Further degradation of GL-1a to ceramide has been studied by Brady et al. (199) and by Gatt and Rapport (200). The GL-1a-cleaving enzyme was purified 82-fold from a 100,000 g supernatant fraction of human spleen (201). The enzyme was present in normal but absent in Gaucher spleen (202). GL-1a-cleaving enzyme has also been detected in white blood cells (203). Evidence seems to imply that the major source of accumulated GL-1a in Gaucher's disease might be turnover of

glycosphingolipids from leukocytes (204) or erythrocytes (205).

An enzyme that cleaves galactosylceramide to galactose and ceramide was purified by Hajra et al. (206) from pig brain.

A ceramidase which catalyzes the hydrolysis of ceramides to long-chain base and fatty acids has been purified 200-fold from rat brain by Gatt (207) and Yavin and Gatt (208).

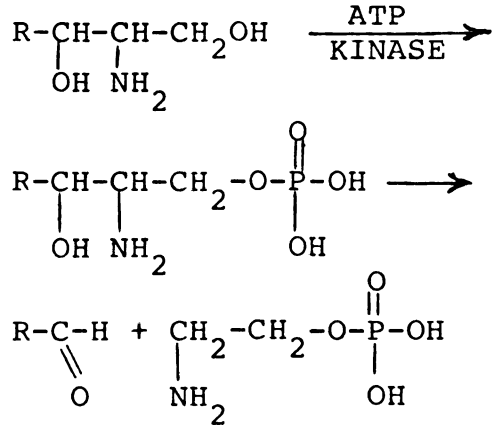
Information about sphingosine degradation has been generated mainly by Stoffel's laboratory, based on in vivo (209-211) and in vitro studies (212-214). Long-chain bases were phosphorylated, then cleaved between C-2 and C-3 to give phosphorylethanolamine and palmitaldehyde, hexadecenal and 2-hydroxypalmitaldehyde from sphinganine, sphingosine and 4-D-hydroxysphinganine, respectively.

SPHINGOSINE

SPHINGANINE

or

4-D-HYDROXYSPHINGANINE

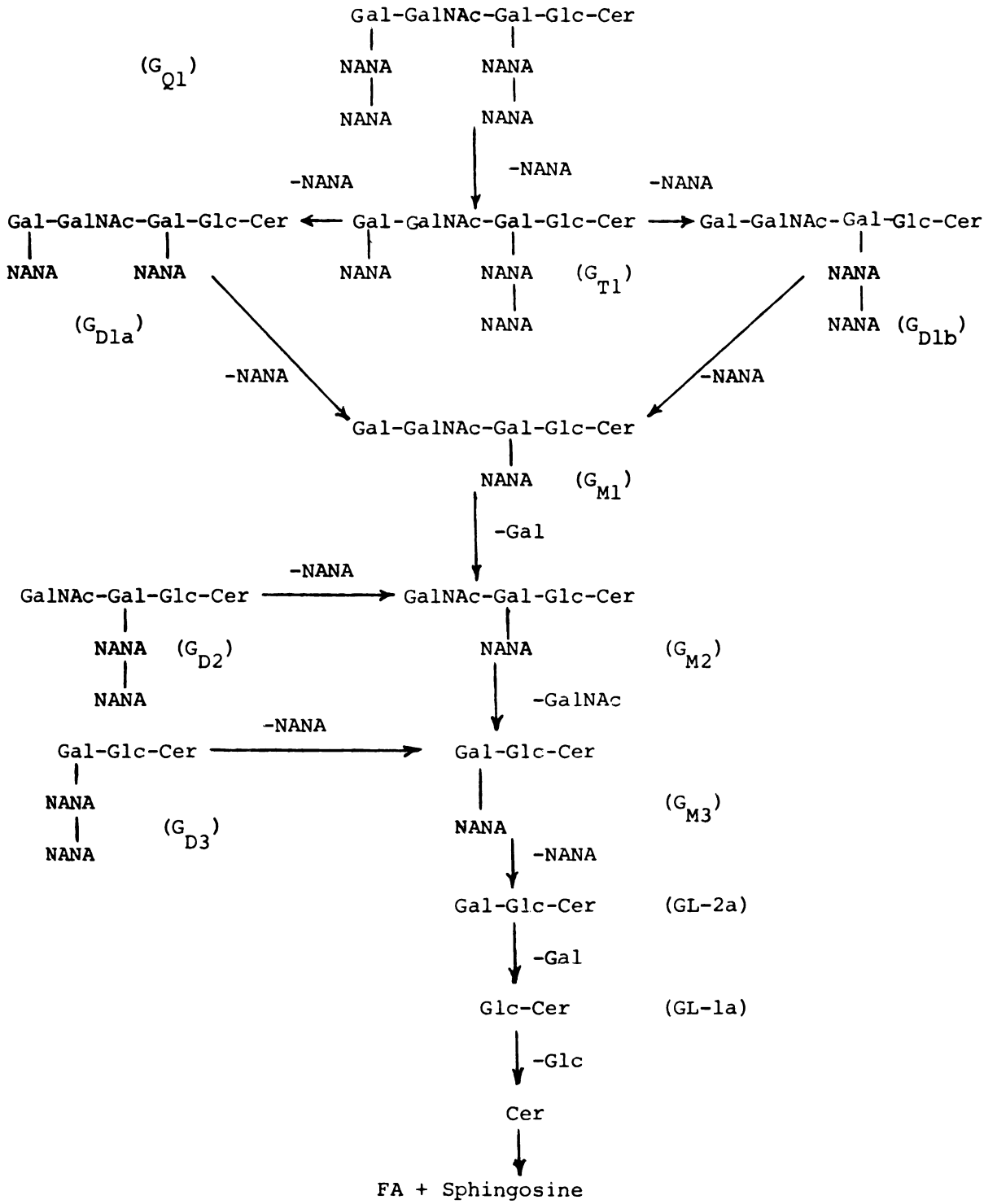


The reaction is initiated by an ATP-dependent kinase (212-215), which has been detected in human erythrocytes (215). Then a pyridoxal-dependent microsomal lyase (214) splits the

phosphorylated base into the C_2-fragment, phosphorylethanolamine, and, in the case of sphingosine, palmitaldehyde.

The biodegradation of gangliosides proceeds via the stepwise removal of monosaccharide units of the carbohydrate backbone by specific hydrolases and sialidases. The scheme for the hydrolysis of G_{T1} to G_{M3} has been proposed (216), and verified by Gatt and associates (217), who isolated and purified several of the glycolipid hydrolases. The degradative pathway is outlined in Scheme III. The degradation of G_{T1} proceeds mainly via G_{D1b} as shown by human brain sialidase (218) as well as those from bacteria origin (219). This finding suggests that the terminal galactose-bound sialic acid is the first to be hydrolyzed, whereas the sialic acid bound to the internal galactose remains intact. It is proposed that the internal sialic acid is more resistant because of steric hindrance caused by the N-acetylgalactosamine bound to C-4 of the internal galactose (220). After N-acetylgalactosamine is liberated by hexosaminidase, the internally bound sialic acid is more easily removed by sialidase (221).

Scheme III. Biodegradation of Brain Gangliosides



II. INTRODUCTION

Glycosphingolipids are important components of mammalian cell membranes. Extensive studies have been made of the composition and metabolism of neutral glycosphingolipids in human erythrocytes (37,222). However, no detailed information is available about the sphingolipid composition and metabolism of human platelets, although several brief reports (223-225) on the existence of sphingolipids in platelets have appeared. Nothing is known about the biochemical structures of the platelet glycosphingolipids but some recent studies have been made of the phospholipid component (226) and glycoproteins (227,228). The lack of information is due, in part, to difficulties in obtaining a sufficient amount of material for adequate biochemical studies.

To complement previous studies on the glycosphingolipids of normal human plasma (37), erythrocytes (37), and leukocytes (38,39) as well as studies of these lipids in patients with storage diseases (47,52,229) and leukemia (39), part of this dissertation was devoted to the determination and characterization of the major sphingolipid constituents of human platelets. It was also interesting to examine the lipids of pig platelets so that a better assessment of the feasibility

of studying platelet glycosphingolipid metabolism *in vivo* could be made.

As part of a general investigation of the blood glycosphingolipids, human plasma gangliosides as well as the major erythrocyte glycosphingolipids in fetal pigs were also studied.

Another area of investigation of this thesis centered on the turnover of various blood sphingolipids in an anemic pig. Previous studies (222) with a normal human and a patient with Fabry's disease demonstrated that the early synthesis (such as liver or other organs) of plasma glycoshpingolipids could only account for 10-20% of the total plasma glycolipids when a stable isotope labeled compound, [6,6-$^{2}H_{2}$]glucose, was used as the precursor, which indicated that the remaining plasma pool had to be derived from some other source, such as the red cells. However, since the incorporation of the label into the red cell glycosphingolipids was not sufficiently high enough to be detected, this hypothesis was not verified until later when Dawson and Sweeley (45) conducted an *in vivo* experiment in a pig, involving a large pulse label of [U-^{14}C]glucose.

The pig was found to be an excellent experimental animal model because it was rather similar to humans in the plasma and erythrocyte glycosphingolipid profiles. Furthermore, the size of the animal permitted withdrawal of 30-40 ml of blood from the animal at various time intervals without affecting

its metabolic status in any way. Dawson and Sweeley (45) concluded from the experiment that GL-1a exchanged freely between plasma and erythrocyte pools, and was not synthesized in the bone marrow, whereas the red cell GL-2a, GL-3a and GL-4 were synthesized in the marrow and were subsequently released into the plasma during the time of erythrocyte catabolism, suggesting that the erythrocyte glycosphingolipids were a major source of the plasma glycolipids. Nevertheless, several questions remained unanswered. One was that the maximum specific activities of erythrocyte GL-2a, GL-3a and GL-4 were reached around 5-7 days after the label was given, and 40-60% of the label was lost from GL-2a and GL-3a, but not from GL-4 during the next few days. This phenomenon might be explained in several ways. One is the possibility of contamination of red cell preparations by other cell components such as leukocytes and platelets. This could result from unsuccessful washing or removal of these components during the centrifugation of the whole blood. The second and more probable explanation may be the presence of some highly metabolically active cells (such as reticulocytes) in the isolated red cell fraction. It is possible that the early loss of label was associated with membrane changes as the reticulocytes mature into normal erythrocytes. If this were true, one would expect to see the specific activity of red cell glycolipids (especially the major erythrocyte glycosphingolipid, globoside) decrease gradually during the entire

life span of the erythrocytes; this was not observed, however. This hypothesis about globoside metabolism in senescent erythrocytes may be verified by conducting a turnover experiment in a pig that has been previously induced with reticulocytosis.

Another puzzling fact was the marked differences observed in the fatty acid composition of the different pig erythrocyte glycosphingolipids. The relative amounts of various fatty acids were similar in GL-3a and GL-4, but distinctively different from GL-1a and GL-2a. For this matter, it is difficult to envisage that GL-2a and GL-3a are the precursors of GL-4. It was the intention of this study in the anemic pig to investigate the turnover of both plasma and red cell ceramides, gangliosides and the neutral glycosphingolipids so that a better perspective could be obtained of the interrelationship between the various plasma and erythrocyte sphingolipid pools. In addition, the experiment was planned to include more time points throughout an eighty day period, since the time points chosen for the earlier study were shown to be inadequate.

As part of this <u>in vivo</u> study, an attempt was made to separate the reticulocytes from the normal cells using gradient ultracentrifugation. If such separation could be achieved, then one could study the turnover of globoside in the young cells versus the old; so that the metabolism of globoside in the red cell aging process could further be assessed.

III. EXPERIMENTAL

A. Materials

1. Non-chemicals

Lipid sources

Human blood	Lansing Regional Blood Center, American Red Cross, Lansing, Mich.
Human platelets	American Red Cross Blood Research Center, Bethesda, Md.
Porcine blood	Meat Laboratory, Dept. of Animal Husbandry, Michigan State Univ., East Lansing, Mich.
Equine blood	Veterinary Clinic, Michigan State Univ., East Lansing, Mich.

In vivo study

Female Yorkshire pigs	Porcine Research Center, Michigan State Univ., East Lansing, Mich.
Pregnant gilts	Maternity Ward, Swine Research Center, Michigan State Univ., East Lansing, Mich.

Miscellaneous

Dialysis tubing (size 20)	Sargent-Welch Scientific Co., Chicago, Ill.
Diazo projection paper (1919-A, size-8½"x11", cat. no. 30012)	B. K. Elliot Co., Pittsburgh, Pa.

Instruments

Gas Chromatograph Model 402	Hewlett-Packard Analytical Instruments, Avondale, Pa.
Sorvall Refrigerated Centrifuge Model RC2-B	Ivan Sorvall Inc., Newton, Mass.
Beckman Model L3-50 Ultracentrifuge	Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.
Beckman LS-150 Liquid Scintillation Counter	Beckman Instruments, Inc., Palo Alto, Calif.
Mass Spectrometer, LKB 9000	LKB, Stockholm, Sweden
Advanced Osmometer, Model 3L	Advanced Instrument, Inc., Needham Heights, Mass.
Gilford Spectrophotometer 2400	Gilford Instrument Laboratories, Inc., Oberlin, Ohio

2. Chemicals

Solvents

Chloroform, methanol, acetone, etc.	J. T. Baker Chemical Co., Philipsburg, N. J. Mallinckrodt Chemical Works, St. Louis, Mo.

Silicic acid chromatography

Unisil (200-325 mesh)	Clarkson Chemical Co., Williamsport, Pa.

Thin-layer chromatography

Precoated silica gel G plates (250 μ)	Quantum Industries, Fairfield, N. J.
Precoated silica gel G plates (250 and 500 μ)	Analtech, Inc., Newark, Del.
Glycosphingolipid standards	Prepared from human, porcine and equine red blood cells.

Thin-layer chromatography (cont'd)

Ceramide (hydroxy fatty acids)	Sigma Chemical Co., St. Louis, Mo.
Ceramide (normal fatty acids)	A gift from Dr. Karin Samuelsson; also from Sigma Chemical Co., St. Louis, Mo.
Ganglioside standards	Supelco, Inc., Bellefonte, Pa.

Acid-catalyzed methanolysis

Hydrogen chloride (lecture bottle)	Matheson Gas Products, East Rutherford, N. J.
Silver carbonate	Mallinckrodt Chemical Works, St. Louis, Mo.
Acetic annydride	J. T. Baker Chemical Co., Philipsburg, N. J.
Hexane (nanograde)	Mallinckrodt Chemical Works, St. Louis, Mo.

Dyes

Bromothymol blue	Matheson Coleman and Bell, Norwood, Ohio
Rhodamine 6 G	Allied Chemicals, Morristown, N. J.
Methyl orange	Fisher Scientific Co., Fair Lawn, N. J.
α-Naphthol	Sigma Chemical Co., St. Louis, Mo.
Resorcinol	Mallinckrodt Chemical Works, St. Louis, Mo.

Gas-liquid chromatography

Hexamethyldisilazane and Trimethylchlorosilane	Applied Science Laboratories, State College, Pa. Anspec Co., Ann Arbor, Mich.
Fatty acid methyl ester standards	Applied Science Laboratories, State College, Pa.

Gas-liquid chromatography (cont'd)

Polysaturated fatty acid methyl esters (PUFA No. 1)	Supelco, Inc., Bellefonte, Pa.
Normal and hydroxy fatty acid methyl esters	Supelco, Inc., Bellefonte, Pa.
Mannitol	Nutritional Biochemical Corp. Cleveland, Ohio
3% EGSS-X on Gas-Chrom Q (100-120 mesh)	Applied Science Laboratories, State College, Pa.
15% ethylene glycol adipate on Chromosorb WHP (80-100 mesh)	Supelco, Inc., Bellefonte, Pa.
16% ethylene glycol succinate on Gas-Chrom P (80-100 mesh)	Supelco, Inc., Bellefonte, Pa.
3% GC-grade SE-30 on Supelcoport (100-120 mesh)	Supelco, Inc., Bellefonte, Pa.
3% ECNSS-M on Supelcoport HD (100-120 mesh)	Supelco, Inc., Bellefonte, Pa.
N-Acetylneuraminyl-lactose	Sigma Chemical Co., St. Louis, Mo.
DL-Sphingosine and Dihydrosphingosine	Miles Laboratories, Inc., Elkhart, Ind.

Assay for sphingosine (Lauter and Trams)

DL-Sphingosine, dihydrospingosine	see GLC
Methyl orange	see Dyes

Assay for ester groups (Rapport and Alonzo)

Hydroxyamine hydrochloride	Mallinckrodt Chemical Works, St. Louis, Mo.

Assay for ester groups (Rapport and Alonzo) (cont'd)

Ferric perchlorate (non-yellow)	G. Frederick Smith Chemical Co., Columbus, Ohio
Tripalmitin	Supelco, Inc., Bellefonte, Pa.

Permethylation study

Sodium hydride (57% oil dispersion)	Alfa Inorganics, Ventron Corp., Beverly, Mass.
Sodium borohydride	Sigma Chemical Co., St. Louis, Mo.

Anomerity study

α-Galactosidase (fig), β-Galactosidase (jack bean), β-Hexosaminidase (jack bean)	Gifts from Dr. Y. T. Li, Tulane Univ., New Orleans, La.
α-Hexosaminidase	Prepared from pig liver by Joseph Sung of this laboratory according to the method of Weissman and Hinrichsen (Biochemistry 8:2034, 1969).
Sodium taurochelate (ox bile)	Sigma Chemical Co., St. Louis, Mo.

Assay for organic phosphorus (Bartlett)

Ammonium molybdate	Fisher Scientific Co., Fair Lawn, N. J.
Sodium bisulfite	Fisher Scientific Co., Fair Lawn, N. J.
1-Amino-2-naphthol-4-sulfonic acid	Mallinckrodt Chemical Works St. Louis, Mo.

Gradient Ultracentrifugation

Albumin, bovine (powder) (Cohn Fraction V; JACS 68:459, 1946)	Sigma Chemical Co., St. Louis, Mo.
Cellulose nitrate tubes (size $1\frac{1}{4}$"x$3\frac{1}{2}$"; 1"x$3\frac{1}{2}$")	Beckman Instruments Inc., Palo Alto, Calif.

Sugars

D-(+)-Galactosamine Hydrochloride	Calbiochem, Los Angeles, Calif.
D-(+)-Glucosamine hydrochloride	Nutritional Biochemical Corp., Cleveland, Ohio
Fucose	Nutritional Biochemical Corp., Cleveland, Ohio
Galactose	Fisher Scientific Co., Fair Lawn, N. J.
Glucose	Mallinckrodt Chemical Works, St. Louis, Mo.
N-Acetylneuraminic acid and N-Glycolylneuraminic acid	Sigma Chemical Co., St. Louis, Mo.

Radioactive isotopes

[U-^{14}C]glucose (S.A. 192 mCi/mM; in ethanol-water 9:1; lot no. 580-031)	New England Nuclear, Boston, Mass.
[U-^{14}C]glucose (S.A. 180 mCi/mM; in 20% ethanol; lot no. 19-119107)	International Chemical and Nuclear Corp., Irvine, Calif.
[1-^{14}C]stearic acid (S.A. 46.08 mCi/mM; in benzene; lot no. 308-211)	New England Nuclear, Boston, Mass.

Miscellaneous

1-Ethyl-3-(3'-dimethyl-aminopropyl)carbodi-imide hydrochloride	Ott Chemical Co., Muskegon, Mich.
Armidexan	Bardley Products, Chicago, Ill.
Heparin	Nutritional Biochemical Corp., Cleveland, Ohio
Experimental drug #744	Parke, Davis and Co., Ann Arbor, Mich.
Aquasol	New England Nuclear, Boston, Mass.

B. Methods

1. Human blood preparation

Human blood (non-outdated or fresh) was obtained from the Regional Blood Center of the American Red Cross in Lansing, Michigan. The separation of plasma from erythrocytes was done according to the method described by Vance and Sweeley (37).

2. Human platelet preparation

Human platelet concentrates were obtained by Dr. Graham A. Jamieson in his laboratory at the American Red Cross Research Center, Bethesda, Maryland. The platelet concentrates were prepared according to a previously established procedure (230) which involved repeated differential centrifugation to remove erythrocytes. The pellet, obtained after centrifuging at 10,000 rpm (18,000 g) at o°C for 5 min, was

used for total lipid extraction. Preliminary studies were done on the total lipid extract from the residues of 73 platelet units after the isolation of platelet membrane glycopeptides by brief trypsin treatment (228). In addition, lipid extracts from non-treated platelets as well as those treated with chymotrypsin and thrombin were prepared for comparison.

3. Porcine platelet preparation

Ten liters of blood were collected from normal healthy pigs at the Meat Laboratory of the Department of Animal Husbandry at Michigan State University, using ACD solution (NIH formula A) as anticoagulant. The pigs were killed by electric shock, then the jugular and carotid vessels were severed. Blood was collected in large round chromatography jars containing 1290 ml of ACD solution (67.5 ml ACD/450 ml blood) with constant agitation. Preparations of platelet concentrates were made according to the method of Cohen and Derksen (231) involving differential centrifugation. After the initial centrifugation, the platelet rich suspensions were transferred to a fine-tip conical centrifuge tube and centrifuged at 1500 g in a desk top clinical centrifuge in order to pack the residual erythrocytes. After each centrifugation, the contents were transferred to another tube without disturbing the erythrocyte pellet. This process was repeated until a faint pinkish ring was observed at the tip of the tube.

The contents were then mixed well and an aliquot was removed for leukocyte, erythrocyte and platelet counts. A smear was also made with the suspension and stained with Wright Stain for microscopic examination. An aliquot was removed and fixed for electron microscopic examination. Finally, the contents were centrifuged once more and the white platelet preparation was freeze-dried in the centrifuge tube leaving the pinkish ring intact. The tube was placed in a lyophilization jar (standing upright) and lyophilized overnight. The dry platelet fraction was removed from the tube in one piece (cone shape), and the tip with the pinkish ring was dissected with a scalpel in order to further minimize red cell contamination.

For electron microscopy, and aliquot of the washed platelet concentrate was fixed with 4% glutaraldehyde in Sorensen's phosphate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in buffer for 1 hour. The specimens were dehydrated rapidly in a graded series of cold alcohols ranging from 70-100% and embedded in Epon. Thick and thin sections were cut on the LKB Ultratome III. The thick sections were stained with toluidine blue and the thin sections were stained with uranyl acetate and lead citrate, and the specimens were examined on the Phillips 300 electron microscope at 600 A°.

4. Extraction of total lipids

Extraction of total lipids from plasma and erythrocytes was performed according to a previously established procedure (37). After the Folch-type partition, total lipids were recovered from the lower phase, while the gangliosides remained in the upper phase.

In the case of human platelets, the trypsinized platelets were extracted with chloroform-methanol 2:1 (v/v) according to the method of Folch, Lees, and Sloane-Stanley (232), using a Waring blender to prepare the chloroform-methanol 2:1 homogenate. The mixture was filtered through a sintered-glass filter, and the extraction was repeated three times with chloroform-methanol 2:1, after which 0.2 volume of 0.75% NaCl was mixed with the combined filtrates (233). The biphasic mixture was allowed to stand overnight. The upper phase was then removed by aspiration, and the lower phase was washed with Folch's "pure solvents upper phase" (chloroform-methanol-water 3:48:47 (v/v)) (234) three times. The combined lower phases were evaporated to dryness in vacuo to yield a fraction of crude total lipids.

Lyophilized porcine platelets were stirred with 400 ml of methanol and 800 ml of chloroform for 30 min at room temperature. The solution was filtered and the residue was washed with 50 ml of chloroform-methanol 2:1 (v/v). The residue was further extracted at a gentle reflux temperature with 250 ml of chloroform-methanol 2:1 (v/v) for 2 hr.

After filtration, the combined extracts were mixed thoroughly with 300 ml of distilled water and allowed to stand in the cold room overnight. The lower phase was washed two more times with Folch's pure solvent upper phase.

5. Isolation of neutral glycosphingolipids (Silicic acid chromatography)

Crude total lipids (lower phase) were fractionated into neutral lipids, glycosphingolipids and phospholipids by silicic acid chromatography as described previously (37). After detecting the presence of free ceramides together with the glycosyl ceramides in the acetone-methanol 9:1 (v/v) fraction from human platelets, a modification was made to elute the ceramides separately with chloroform-methanol 95:5 (v/v) or 98:2 (v/v) before the elution of glycosphingolipids from the Unisil column. The following elution scheme was adopted for all the platelet studies as well as the in vivo pig experiment:

1) freshly distilled pure chloroform without methanol added as preservative
2) freshly distilled chloroform-methanol 95:5 or 98:2 (v/v)
3) acetone-methanol 9:1 (v/v)
4) methanol.

6. Mild alkaline hydrolysis

The crude glycosphingolipid fractions (acetone-methanol 9:1 (v/v) fraction) were treated with base according to the

procedure of Vance and Sweeley (37) and Dawson (52). This procedure destroys phospholipid contaminants which contain labile ester linkages. The hydrolysate is then dialyzed for 48 hours in a large chromatography jar against several changes of distilled water in the cold to remove free glycerol, sugars, salts and other contaminants. The biphasic dialysate was reduced to dryness in vacuo.

7. Thin-layer chromatography

Commercially available pre-coated silica gel G plates were used throughout this study. Both 250 μ and 500 μ plates were employed, depending upon the amount of lipids available for analysis. Plates were usually developed in a paper-lined tank saturated with chloroform-methanol-water 100:42:6 (v/v) or 70:30:5 (v/v) (single development). Samples were suspended in approximately 200 μl of chloroform-methanol 2:1 (v/v) and applied to the plate in one single streak with a Radin-Pelick TLC streaker. This process was repeated two more times with 100 μl of chloroform-methanol 2:1 (v/v). Standard mixtures of glycosphingolipids were always spotted alongside as markers for identification. The TLC plate was air-dried at room temperature after solvent development, and the glycosphingolipid bands were visualized by nonspecific staining with iodine vapor. The individual bands were encircled with a fine hypodermic needle and a record of the TLC plates was made by exposure to diazo projection paper and subsequent development of the paper in a tank saturated with ammonia vapor.

Initial studies, using Quantum plates, indicated that GL-4 and G_{M3} migrated in close proximity and the separation between them was unsatisfactory in the neutral solvent system. Hence, the bands were recovered from the plate in one fraction and the lipids were eluted from the gel as before. The mixture was then separated by TLC using another solvent system: chloroform-methanol-7% NH_4OH 55:40:10 (v/v). This problem was not encountered when Analtech plates were used.

8. Acid-catalyzed methanolysis

After complete sublimation of the iodine vapor, individual bands were scraped with a razor blade, and the neutral glycosphingolipids were eluted from the gel (0.2-0.4 g) with 40-50 ml of chloroform-methanol-water 100:50:10 (v/v) (37) at room temperature. Hematoside was eluted under the same conditions with 40-50 ml of methanol-chloroform-water-pyridine 56:40:12:2 (v/v) (235) and ceramides were eluted with ethyl acetate. The recovered glycosphingolipids were subjected to acid-catalyzed methanolysis. A stock solution of 0.75 N hydrogen chloride in anhydrous methanol was prepared by bubbling dry gaseous HCl into dry methanol at room temperature. The solution could be stored at room temperature for periods up to 2 weeks. In practice, a fresh solution was usually prepared on the day of analysis. In order to make a reliable quantitative estimation of the glycosphingolipids present, an internal standard of mannitol was introduced. The stock solution of mannitol contained 36.4 mg of mannitol in 100 ml of

methanol containing 1% water. A mixture of the glycosphingolipid sample (up to 1 mg), 100-300 µl of mannitol stock solution (0.2-0.6 µmoles) and 3 ml of 0.75 N methanolic hydrogen chloride was heated for 20-24 hr at 75-80°C in a small culture tube with a Teflon-lined screw-cap. The solution was then cooled to ∿25°C and about 200 mg of silver carbonate was added to neutralize the hydrogen chloride. For the amino sugars, 0.2 ml of acetic anhydride was added and the mixture was allowed to stand at ∿25°C for 18 hours in order to convert methyl glycosides of liberated galactosamine and neuraminate to N-acetyl derivatives. The mixture was then centrifuged (1500 g) in a small clinical centrifuge for several minutes, and the supernatant was transferred to another small screw-cap culture tube. The residual silver carbonate was washed 2 more times with a small aliquot of methanol and centrifuged as before. Fatty acid methyl esters were recovered by 3-5 extractions of the combined supernate with equal volumes of hexane, and the methanol solution was evaporated to dryness under a gentle flow of nitrogen.

9. Gas-liquid chromatography

Methyl glycosides were converted to O-trimethylsilyl derivatives by dissolving the residue in 35 µl of a freshly prepared 5:2:1 (v/v) mixture of dry pyridine, hexamethyldisilazane and trimethylchlorosilane (37). After 15 minutes at ∿25°C an aliquot of the cloudy mixture was injected into the gas chromatographic column.

If no hexosamine or sialic acid were present, gas chromatography was normally carried out isothermally at 160° or 170°C on a glass column (6'x$\frac{1}{8}$"i.d.) packed with 3% SE-30 (or 3% OV-1) on 100-200 mesh, acid-washed, silanized diatomaceous earth. Alternatively, the separation could be made by linear temperature programmed analysis on the same column, with an initial temperature of 160°C and a programming rate of 2°C/min to an upper temperature of 230°C. Identifications of fucose, galactose, glucose, galactosamine, glucosamine, sialic acid, and inositol were made by comparison of the observed retention times relative to that of mannitol. The yield of each component was calculated from the total area produced by the various anomeric forms of given sugar, using the area produced by the known amount of mannitol for comparison.

10. Isolation and characterization of gangliosides

The Folch upper phase was dialyzed in the cold for 72 hr against 4 changes of distilled water. The dialysate was lyophilized and subjected to mild alkaline hydrolysis by the method described previously. After dialysis, the hydrolysate was reduced to dryness in vacuo and dissolved in a small amount of solvent for preparative TLC. Alternatively, the Folch upper phase was concentrated to dryness in vacuo and the residue was subjected to mild alkaline hydrolysis as described above. Several solvent systems were utilized in the separation of gangliosides:

TLC plate	Solvent system
Quantum (250 μ)	ascending TLC in a tank saturated with chloroform-methanol-2.5 N NH_4OH (60:40:9) without paper liners; the plate was developed 2 times with adequate drying between each development.
Uniplate (250 μ)	heat activation of plate for 30 minutes at 120°C, one-dimensional ascending TLC in a two-solvent sequential system described by Klibansky et al. (236).

Appropriate bands were detected by iodine vapor and recovered by eluting the gel with methanol-chloroform-water-pyridine 56:40:12:2 (v/v). After acid-catalyzed methanolysis (0.5 N), methyl glycosides and fatty acid methyl esters were recovered and subjected to GLC analysis.

11. Identification of fatty acid methyl esters

Methyl esters of fatty acids were recovered from the acidic methanolysate by hexane extraction. The methyl esters were then purified by preparative TLC on silica gel G with hexane-diethyl ether 85:15 (v/v) as the developing solvent (237), using methyl esters of palmitic and α-hydroxy palmitic acid as markers. Bands were made visible with bromothymol blue, and subsequently scraped from the plate within an hour to avoid extensive losses of short-chain esters by evaporation. A suspension of the silica gel in diethyl ether was packed into a small glass column and the esters were eluted with diethyl ether (40 ml per gm of silica gel) (238). Purified methyl esters were analyzed by GLC at 190°C on a glass column (6 ft by 1/8 in. i.d.) packed with 15% ethylene glycol adipate.

The hydroxy acids were analyzed as their trimethylsilyl methyl ester derivatives (239). The methyl esters were identified by comparing their retention times with those of standards and by co-injection of the unknown with an appropriate standard. Plots of relative retention time versus carbon number were employed for the identification of fatty acids not represented in the standard. Areas were calculated from peak heights and widths at half height and the compositions were expressed as percentages of uncorrected total area.

12. Identification of sphingosine bases

Purified glycosphingolipids were subjected to methanolysis by the method of Gaver and Sweeley (240) using 1 N aqueous methanolic HCl. After methanolysis, the reaction mixture was extracted with hexane 3 to 5 times to remove methyl esters, and the lower phase was neutralized with silver carbonate. The mixture was centrifuged and the supernatant fraction was evaporated to dryness under nitrogen. The residue was dissolved in chloroform and applied to a column containing about one gram of Unisil in chloroform. The column was eluted with 10-15 ml of chloroform (discarded) and the long-chain bases were then recovered with 10-15 ml of methanol. The methanol eluate was evaporated to dryness and bases were N-acetylated with 50 µl of methanol-acetic anhydride 4:1 (v/v) at room temperature overnight. To facilitate the removal of excess acetic anhydride, butanol was added and the mixture co-evaporated under a stream of nitrogen. The acetylated bases

were then converted to O-trimethylsilyl derivatives for GLC at 230°C on 3% SE-30. Reference N-acetyl sphingosines were used as standards.

13. Identification of N-acylneuraminic acid

N-Acylneuraminic acid was liberated from the acidic glycosphingolipid under mild conditions with 0.03 N aqueous hydrochloric acid. After partitioning the hydrolysate with chloroform, the aqueous phase was evaporated to dryness and the product was further purified and identified by column and thin-layer chromatography according to the method of Puro (241). Authentic N-acetyl- and N-glycolylneuraminic acids were used as standards. The N-acylneuraminic acid was also characterized by GLC and GLC-MS as the trimethylsilylated derivative at 220°C on 3% SE-30.

14. Identification of Ceramide

The ceramide fraction, isolated by TLC in the neutral solvent system, was further purified on a 500 μ silica gel G Uniplate (Analtech, Inc.) in chloroform-methanol-glacial acetic acid 192:5:8 (v/v) (10). In other instances, ceramides obtained from silicic acid chromatography (98:2 fraction) were further purified on a 250 μ silica gel G Quantum plate with chloroform-methanol 95:5 (v/v) as the developing solvent (9). Authentic ceramides containing normal and hydroxy fatty acids were used as standards. After exposure to iodine, the band which corresponded to the standard was eluted from the gel

with chloroform-methanol 2:1 (v/v) and ethyl acetate after the iodine had sublimed. The fraction was evaporated to dryness *in vacuo* and the residue was methanolized (37). Fatty acid methyl esters were recovered by hexane extraction and the extract was divided into two equal portions. Ester groups were estimated quantitatively by the method of Rapport and Alonzo (242) on one aliquot and the distribution of fatty acids was analyzed by GLC as described above. The sphingosine content was determined by a modification (243) of the method of Lauter and Trams (244) and the long-chain bases were identified by GLC and GLC-MS analyses of the N-acetyl derivatives as 1,3-di-O-trimethylsilyl ethers.

15. Linkage studies

Neutral glycosphingolipids or gangliosides (1.0 mg) were dissolved in 0.5 ml of dry dimethyl sulfoxide and methylated according to the method of Hakomori (245), using 0.5 ml of the carbanion solution. The contents were sealed in small vials with Teflon caps and sonicated for 30 min, and the reaction was then allowed to proceed for six hours, after which methyl iodide was added (245). After washing the contents with water and chloroform, the chloroform layer was reduced to dryness under a gentle nitrogen flow. The residue was dissolved in 1 ml of 2 N methanolic HCl and methanolysis was carried out at 120°C for 5 hr. Fatty acid methyl esters were extracted into petroleum ether. A small fraction of the

permethylated methyl glycosides in the lower phase was analyzed by GLC on 3% ECNSS-M at 160°C. The remainder of the permethylated methyl glycoside fraction was further hydrolyzed to free sugars with 1 ml of 2 N aqueous HCl at 100°C for 3 hr. The hydrolysate was neutralized, the methylated sugars were reduced with sodium borohydride and alditol acetates were prepared with acetic anhydride-pyridine 1:1 (v/v) according to the method of Pepper and Jamieson (227). The alditol acetates were identified by GLC and GLC-MS (246-248) on 3% ECNSS-M at 175°C. Reference compounds of lactose, N-acetylneuraminyllactose, Fabry kidney trihexosylceramide and globoside (porcine red cells) were employed as standards.

16. Anomerity study

Purified platelet GL-2a, GL-3a, and GL-4 were incubated with α-galactosidase, β-galactosidase, α-N-acetyl-hexosaminidase and β-N-acetyl-hexosaminidase according to the methods described by Hakomori et al. (64) and Laine et al. (78). Approximately 100-200 µg of the purified lipid (except 800 µg of GL-4 was used in the incubation with β-N-acetyl-hexosaminidase) was dissolved in 150-300 µl of 0.05 M sodium citrate buffer (pH 4.3) containing 150-300 µg of sodium taurocholate in a conical centrifuge tube, and the reaction mixture was incubated at 37°C for 20 hr. The reaction mixture was reduced to dryness under a fine stream of nitrogen. One ml of chloroform was added and the contents were sonicated for 3 min

after which it was left at room temperature overnight. After brief centrifugation, the supernatant was removed and the residues were further extracted with chloroform two more times as before. The combined chloroform extracts were evaporated to dryness under a gentle flow of nitrogen and the residues were redissolved in small aliquots of chloroform. Approximately 25% of the lipid was applied to a silica gel G plate together with the authentic glycosphingolipid standards and the purified platelet GL-2a, GL-3a and GL-4 that had not been incubated with the enzymes. The plate was developed in chloroform-methanol-water 100:42:6 (v/v) system. Spots were made visible with α-naphthol and sulfuric acid sprays.

17. Isolation and characterization of platelet Sphingomyelin

A crude mixture of phospholipids (140 mg), eluted from the silicic acid column with methanol, was subjected to mild alkali-catalyzed methanolysis, using 1 ml of 0.6 N methanolic NaOH for each 10 mg of lipid. The solution of lipids was allowed to stand overnight at room temperature. Sphingomyelin was separated from alkali-stable lipids by TLC in chloroform-methanol-water 100:42:6 (v/v) (249). Long-chain bases were liberated from the sphingomyelin (22 mg) by acid-catalyzed methanolysis in 10 ml of the modified aqueous methanolic HCl (240). Selective N-acetylation of the free bases and conversion of the N-acetylated derivatives into 1,3-di-O-trimethylsilyl ethers was accomplished as outlined above, except

that trimethylsilylation was carried out as described by Carter and Gaver (250). The derivatized bases were analyzed by GLC at 230°C on 3% SE-30 and 3% OV-17 columns. Reference samples of N-acetylated sphingosines were employed as standards. Plots of relative retention times versus carbon numbers were used for identifications of bases not represented in the standard. The structures were further confirmed by GLC-MS of the trimethylsilyl derivatives on both polar and non-polar columns, and the aldehydes derived from periodate oxidations of the free long-chain bases (251) were analyzed by GLC and GLC-MS on a 3% EGSS-X column at 130°C.

18. Platelet Phospholipids

Phospholipids, eluted from the silicic acid column (methanol fraction), were concentrated to dryness on a flash evaporator and the residue was dissolved in a known amount of methanol. An aliquot was applied to a 250 μ Quantum plate and developed (ascending) with a two-dimensional technique reported by Klibansky et al. (236). In addition, a single dimensional system was also utilized to separate the individual phospholipids on a preparative basis. Uniplate (250 μ) was activated at 110°C for 90 mins. Sample was spotted on 10-15 tracks and the plate was developed in the appropriate solvent system. Human plasma phospholipids were adopted as standards. Lipids were made visible with iodine vapor. After complete sublimation of the iodine vapor, each individual

group of spots (including the origin) was eluted with methanol from the gel. The methanol eluate was evaporated to dryness under nitrogen flow and the residue was weighed. The residue was resuspended in a known amount of solvent and an aliquot was removed for phosphorus determination by the technique of Bartlett (252). The fatty acid components of the individual phospholipids were also investigated. This was accomplished by separating each group of lipids according to the single-dimensional TLC. The plate was sprayed with 0.005% aqueous solution of Rhodamine 6 G. Each group of individual phospholipid was eluted as before and methanolized with 0.75 H HCl in anhydrous methanol. Fatty acid methyl esters were extracted and identified by GLC at 170°C isothermally on a glass column (6 ft by 1/8 in. i.d.) packed with either 3% EGSS-X or 16% EGS. Peaks of fatty acid methyl esters were resolved by comparing their retention times with the polyunsaturated fatty acid methyl esters derived from cod liver oil and the commercially available standard PUFA No. 1. Co-injection of the unknown with the standard was also used in the process of identification. In addition, fatty acids were also identified by comparing their equivalent chain length values with the established results (253).

19. Platelet neutral lipids

Neutral lipids were fractionated by column chromatography on silicic acid, as described above. Column eluate was concentrated to dryness and resuspended in chloroform. Aliquots

were spotted on multiple tracked TLC plate (Uniplate, 250 μ). The plate was developed with hexane:diethyl ether:glacial acetic acid 90:10:1.5. Individual lipids were located on the developed plate by spraying with a 0.005% solution of Rhodamine 6 G. The lipids were eluted with diethyl ether from the scraped gel. An additional plate was prepared for the identification of cholesterol and cholesterol esters. The plate was sprayed with a mixture of conc. sulfuric acid and acetic acid 1:1 and heated at 90°C for 15 minutes (254). Both cholesterol and cholesterol esters gave red spots against a white background. Fatty acids of triglycerides and cholesterol esters together with the free fatty acids were converted to methyl esters by HCl-methanolysis (2 N). The resulting methyl esters were recovered and checked by GLC as indicated above.

20. Preparation of radioactive glucocerebroside

a. Hydrolysis of GL-1 by barium hydroxide

The method of hydrolysis employed here was a modification of the procedure described by Carter and Fujino (255), adapted for small samples. To 20 mg of GL-1 (Gaucher spleen) 1 ml of dioxane was added and warmed slightly to dissolve all material. One ml of 10% $Ba(OH)_2$ was then added drop-wise and the mixture was refluxed for 12 hr at 90°C in a pear-shaped flask fitted with a small distillation unit. The reaction mixture was transferred to a conical centrifuge tube containing

3.25 ml of water. The reaction flask was further rinsed with dioxane-$Ba(OH)_2$ 1:1 (v/v) and water to ensure removal of all the contents. The combined reaction mixture was allowed to stand at room temperature overnight. The contents were centrifuged and the residue was extracted twice with hot ethanol and once with hot chloroform-methanol 1:1 (v/v). The combined extracts were concentrated in vacuo to give crude glucosylsphingosine which was purified by silicic acid chromatography in order to remove any unhydrolyzed Gl-1. The crude sample was applied to a column packed with 0.9 gm of Unisil in chloroform. The materials were eluted subsequently with 30 ml each of chloroform-methanol 8.5:1.5 and methanol. Both fractions were further purified by TLC using two different solvent systems, chloroform-methanol-2.5 N NH_4OH 60:40:9 and chloroform-methanol-water-conc. NH_4OH 48:14:1:1. Standard glycosphingolipid and psychosine were used as markers. Glucosylsphingosine migrated slightly ahead of galactosylsphingosine (psychosine) in both systems, and it was eluted from the gel with methanol.

b. Coupling of glucosylsphingosine with [1-^{14}C] stearic acid

The coupling reaction was done according to a previous report (256) on the synthesis of radioactive ceramides with a slight modification. Five mg of [1-^{14}C]stearic acid and 5.1 mg of glucosylsphingosine were dissolved in 3 ml of CH_2Cl_2-methanol 1:1 in a large heavy-walled conical centrifuge

tube containing 10 mg of carbodiimide. The tube was stoppered and left standing in a closed incubator at 40°C overnight. To the centrifuge tube 25 ml of chloroform and 25 ml of water were added, mixed and centrifuged. The lower layer was washed twice with 1% $NaHCO_3$, twice with 0.1 N HCl and then twice with water. After removal of water from the last washing, some ethanol was added to the lower phase and the contents were concentrated to dryness in vacuo. The material was purified by silicic acid chromatography and TLC as described above. The band that corresponded to the GL-1a standard was scraped and eluted from the gel with chloroform-methanol-water 100:50:10 (v/v). The recovered material was dissolved in a small amount of solvent and an aliquot was removed for radioactivity counting. Acid-catalyzed methanolysis was also carried out on a small aliquot of the sample in order to analyze fatty acids, sphingosine and sugar derived from this lipid.

21. Experiment with fetal pigs

Two Hampshire sows of 1 and 1 1/2 year old were selected from the experimental station herd. Both were bred to Hampshire boar; one was in her second gestation while the other was her first. Using the absence of further estrus as an indication of conception, the pregnant sows were chosen at specific periods during gestation for Caesarean section. The operations were performed by Dr. David J. Ellis of the College

of Veterinary Medicine, Michigan State University, at intervals of 45 and 90 days after the first breeding.

The gilts were anesthetized by I. M. injection of an experimental drug #744 in a dosage of 1 mg/lb of body weight.

a. 45-day fetuses

The fetuses were removed from the uterus and a hemostat clamped on the umbilical cord to prevent loss of blood. The litter contained 11 fetuses in total. Each fetus was weighed and blood was removed by direct heart puncture. Difficulties were encountered in sampling the blood; attempts to remove blood from the umbilical veins and arteries were also unsuccessful. Out of the 11 fetuses, only 1 ml of blood was collected in a heparinized tube and the majority of this sample was contaminated by the mother's blood.

b. 90-day fetuses

A total of 11 fetuses were also obtained from this litter. Blood samples were obtained from the anterior vena cava as described by Carle and Dewhirst (257). An average of 3 ml of blood was obtained from each fetus.

c. Analysis of blood samples

Red cells were separated from the samples as described previously and followed with lipid extractions. Globoside was isolated and quantitated. In addition, a sample of the blood from the gilt was also analyzed which served as a control.

22. Red Cell fractionation technique

Separation of young and mature erythrocytes was by ultracentrifugation over a discontinuous density gradient of isotonic albumin solution according to the method described by Winterbourn and Batt (258), which was a modification of the procedure originally described by Piomelli et al. (259), adapted for use on a larger scale.

a. Albumin solutions

Albumin was dissolved in water to give a 40% solution. This was done in the cold room with stirring at low speed to avoid foaming. The osmolality, density and albumin concentration of the solution were measured, and the osmolality adjusted to 290 milliosmoles by adding solid NaCl. Osmolality was recorded on an Advanced Osmometer Model 3L in Dr. Anthony Bowdler's laboratory. Albumin concentration was measured by reading the optical density of a series of diluted solutions of unknown concentration at 280 nm in a Gilford Model 2400 recording spectrophotometer. Density was measured with a hydrometer ranging 1.000-1.2000 in scales or calculated directly from the albumin concentrations (259).

The stock albumin solution was diluted with 0.92% saline solution to give six solutions with albumin concentration ranging between 30-40% (densities ranging between 1.075 and 1.100).

b. Preparation of gradients

Gradients were prepared at 4°C in cellulose nitrate centrifuge rubes. The size of tubes varied with the amount

of albumin solutions and red cell suspensions employed in the experiment. Albumin solutions of decreasing specific gravity were layered on each other with the aid of a peristaltic pump. The pump was fitted with 2 ft of Tygon tubing attached with a fine capillary tip at both ends. One end was placed in the tube containing the albumin solution, and the other end was placed inside the cellulose nitrate tube with the capillary tip leaning against the inside wall. By operating the pump at low to moderate speed, the albumin solutions were layered without creating any air bubbles. Interfaces between layers were clearly visible.

c. Ultracentrifugation

Red cell suspensions (hematocrit approximately 75%) were carefully layered over the albumin with a Pasteur pipette. Tubes were balanced using a small amount of isotonic saline. The tubes were centrifuged at 4°C for 30 min at 25,000 rpm in a Beckman Model LS-50 Ultracentrifuge with a swinging bucket, 25.1, 25.2 or 41 depending on the size of the centrifuge tubes used.

After centrifugation, the different red cell bands were collected in another centrifuge tube using the peristaltic pump in a similar manner as described previously, only by switching the two ends of the Tygon tubing around so that the materials were removed instead of being delivered.

The red cells were separated from albumin by mixing with an equal volume of isotonic saline and centrifuged for 10 min

at 4000 rpm in a Sorvall refrigerated centrifuge. After washing 3-5 times, the cells were ready for further study.

23. Separation of young and mature red cells from a normal and anemic dog

To check the fractionation technique, normal and anemic canine blood was used since samples were readily available. Three tubes of gradients were prepared, as described above, in cellulose nitrate tubes (1"x 3 1/2"0 and 1.2 ml each of red cell suspensions from old normal human blood, normal dog and anemic dog (hemolytic anemia, etiology unknown; reticulocyte count 15-20%) were layered over the albumin solutions and ultracentrifugation was done in a SW 25.1 rotor. After washing the cells thoroughly with isotonic saline, the amount of red cells was estimated visually in a calibrated centrifuge tube. The cells were resuspended in saline and blood smears were prepared, air-dried and stained for microscopic examination.

24. Separation of young and mature erythrocytes from a normal and an anemic pig

Layers of 1.9 ml each of albumin solutions of decreasing specific gravity were carefully layered on each other as before in four cellulose nitrate tubes of 9/16" in diameter by 3 1/2" in length. Interfaces were clearly visible. Aliquots of 0.6 ml of red cell suspensions from normal and anemic dog, normal and anemic pig (20% reticulocytes) were layered on the top of the gradient. The tubes were centrifuged in a SW 41

rotor and the hemoglobin contents of the recovered cell layers were measured and expressed as the percentage of the total.

25. Checking hemolysis of the erythrocytes in albumin solutions

To six conical centrifuge tubes containing 1 ml of 30, 32, 34, 36, 38, and 40% albumin solutions, respectively, were added 0.1 ml of packed pig erythrocytes; the contents were mixed well on a Vortex mixer, and incubated at 4°C for 30 min. Then 1 ml of isotonic saline was added to each tube, mixed, and centrifuged at 4000 rpm for 10 min. The supernatant solution was examined for the Soret band and hemoglobin content at 416 nm and 540 nm, respectively, in a Gilford spectrophotometer.

26. Red cell size distribution

This was obtained in a Celloscope Model 112 red cell counter with a setting of 75 μ for the opening. Cells were diluted in phosphate buffered saline (pH 7.4). The Celloscope was linked to a multi-channel analyzer and by a teletype print-out. The relative number of cells in successive channels was recorded. The mean channel number was calculated by the following equation,

$$\text{mean channel number} = \frac{\Sigma i.Ci}{\Sigma\ Ci}$$

where i is equal to the channel number and Ci is the number of cells in a given channel i.

27. In vivo pig experiment

a. Induction of anemia (reticulocytosis)

Generation of reticulocytosis in animals can be accomplished with chemicals (such as phenylhydrazine) or bleeding. Since the effects of chemicals on blood glycosphingolipid metabolism in the pig are not known, it was decided to induce anemia in the pig by bleeding.

Bleeding was performed by Dr. Elwyn Miller with daily removal of blood from the anterior vena cava according to the method of Carle and Dewhirst (257). Standard hematological techniques were employed in monitoring the degree of anemia. Microhematocrits were obtained in duplicate with capillary tubes and centrifuged for 5 min in an International Model MB centrifuge. Hemoglobin content was determined at 540 nm using the cyanomethehemoglobin method (260). Air-dried blood smears in sextuplicate were stained with new methylene blue for the supravital staining of reticulocytes. Routinely, 1000 red cells were examined on 3-4 blood smears and the average of number of cells containing reticulum was expressed as percentage of the total number of erythrocytes.

Preliminary bleeding of several pigs, ranging in size between 10-13 kg, demonstrated that removal of 50 ml blood daily gave a rather slow anemic response judging by the hematological criteria. Hence, it was decided to bleed the pig on a 100 ml basis in the following in vivo experiment.

b. Induction of anemia in Pig 123-6

A female Yorkshire pig (123-6), weighing 10 kg, was put on an iron-deficient ration described in Table 2.

Table 2.--Rations Pan-fed to Pig 123-6

Ingredients	Percent
Ground yellow corn (shelled)	79.00
Soybean meal, 49% C.P.	18.00
Dicalcium phosphate	1.00
Ground limestone	1.00
Iodized salt	0.50
Vitamin B mix*	0.20
Vitamin D_2 premix (9000 I.U./g)	0.02
Vitamin A palmitate (30,000 I.U./g)	0.04
Vitamin E premix (125,000 I.U./lb)	0.25
Vitamin B_{12} premix (60 mg/lb)	0.03
Zinc sulfate (36% Zn)	0.02

*Contained riboflavin, 2 g/lb; pantothenic acid, 4g/lb; niacin, 9 g/lb; choline chloride, 90 g/lb.

The special ration was prepared by Dr. Elwyn Miller, and the calculated analyses of the ration indicated the composition of 16% protein, 0.65% calcium, 0.54% phosphorus and 30 ppm of iron (which is one-half the standard requirement for this size pig). The pig was pan-fed with 400 gm of this ration per day (two times/day).

During this time daily removal of 100 ml blood had already begun and anemia was expected to develop. On day 9 (and subsequently on day 15 and 24), 200-300 mg of Armidexan (elementary iron as ferric hydroxide in complex with a low molecular weight dextran form) was given to the pig I.M. in order to ensure the availability of an adequate iron pool for red cell production. At the same time, the ration was changed to the normal growth diet containing 60 ppm of iron; however, the ration was still pan-fed in order to limit the growth rate of the pig. Between day 15 and the actual day of radioisotope administration, 100-150 ml of blood was taken daily in order to maintain a steady level of anemia in the pig. Achievement of a steady level (state) was judged on the basis of leveling-off of both the packed cell volume and hemoglobin content, and also the oscillation of the reticulocyte counts within a fairly narrow range.

c. Radioisotope administration

Once the anemic condition was maintained at a steady level, 10 mCi of [U-^{14}C]glucose (192 mCi/mM, in ethanol-water 9:1 solution) was given to the pig I.V. This was done by restraining the pig in a tray developed by Dr. Elwyn Miller (261). Two syringes (one filled with the isotope while the other was empty) were connected to a 3-way valve which was in turn attached to 10 cm rubber tubing fitted with an 18 gauge needle. This set-up prevented any possibility of needle displacement from the site of injection in case the pig moved.

All parts in contact with blood were pre-rinsed with heparinized saline. Once the needle was in the vein, approximately 5-10 ml of blood was drawn into the empty syringe; the valve was turned to the second syringe containing the isotope, which was subsequently administered. Immediately following that, the valve was turned back to the first syringe whereby the previously drawn blood was re-injected back into the pig to flush out any remaining isotope trapped in the assembly line. The pig was individually confined.

d. Treatment of blood samples

Blood samples were withdrawn from the pig according to the following schedule. Approximately 100-130 ml of blood was removed daily between Day 0 and Day 10, while 40 ml of

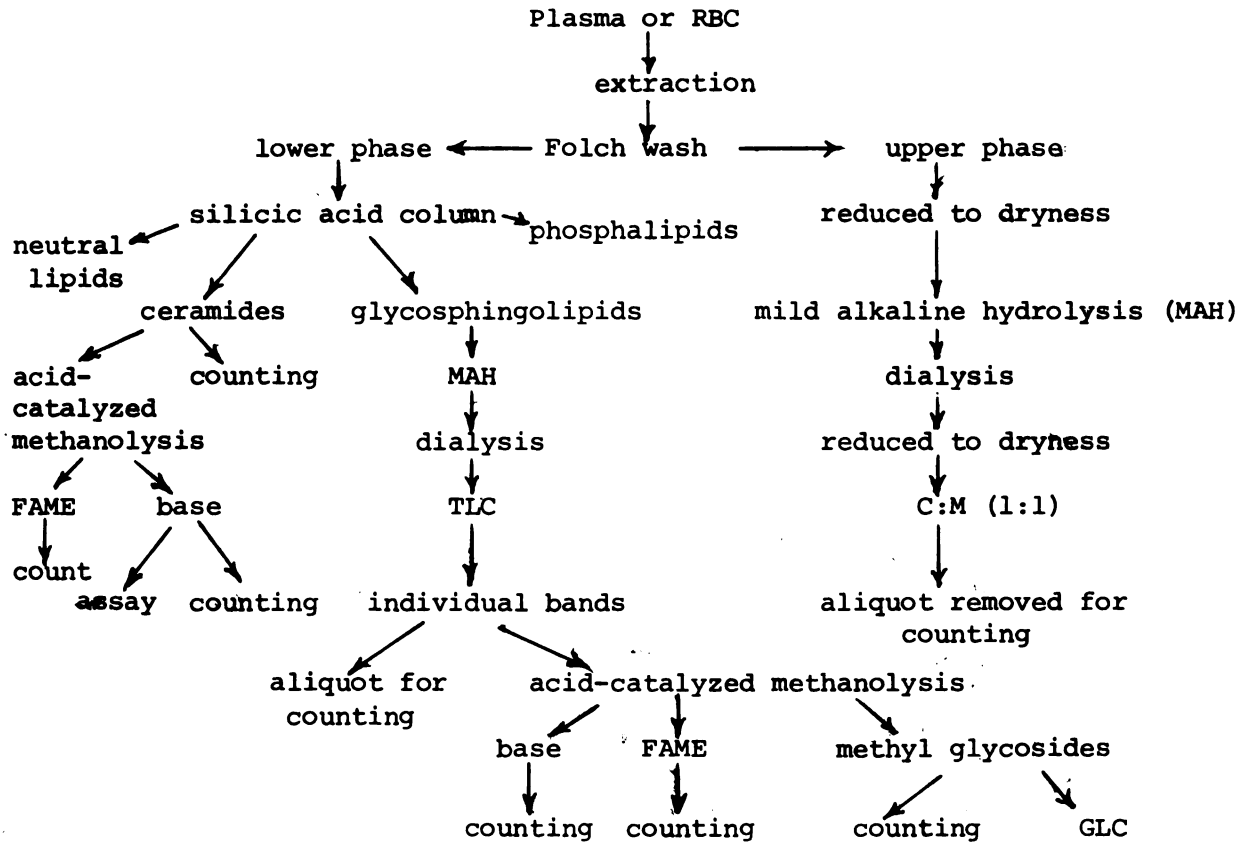
6 hr	Day 7	Day 35	Day 63
12 hr	8	41	66
Day 1	9	46	70
2	10	50	75
3	14	53	81
4	20	55	
5	25	57	
6	30	60	

blood was removed on the other days. The weight of the pig was maintained constant for the first ten days by restricting the food intake as before. After the initial 10 day period, the pig was allowed to grow normally by feeding from a self-feeder ad. lib.

Erythrocytes were separated from plasma by centrifugation and washed extensively with saline as before. Lipids were extracted from all of the plasma and erythrocyte samples.

In addition, on days 2, 3, 4, 6, 7, 8, 10, 14, 20, 30, 41, 50, 60, 70 and 81, aliquots of the packed red cells were subjected to gradient fractionation by the ultracentrifugation technique. Extraction of total lipids and isolation of glycosphingolipids were carried out according to the procedure described above in the Methods, and presented schematically below.

Scheme IV. Analysis of Blood Glycosphingolipids.



The only apparent difference in the procedure cited above was the counting of radioactivity in the glycolipid and/or its components derived from these molecules. Radioactivity was monitored in two ways--the purified sphingolipid was

dissolved in an appropriate solvent and a known amount of the lipid was transferred to a scintillation vial. The contents were concentrated to dryness under a stream of nitrogen and 10 ml of Aquasol was added for counting. Specific activity was expressed as counts per minute per ml of plasma or erythrocyte. Alternatively, in the case of neutral glycosphingolipids, individual components of fatty acid hexose and sphingosine were separated according to their different solubility properties after acid-catalyzed methanolysis (45). The hexose moieties were divided into two equal halves and one was used for the quantitation of methyl glycosides by GLC, while the other was used for radioactivity determination. Specific activity was expressed as counts per minute per μmole. Counting was done in a Beckman Model LS-150 liquid scintallation counter.

Plasma and erythrocyte ceramides were purified by TLC and recovered by solvent extraction. An aliquot of the lipid was counted in Aquasol. In addition, erythrocyte ceramides containing normal and hydroxy fatty acids were further subjected to acid-catalyzed methanolysis after which the fatty acid methyl esters were recovered by hexane extraction. The hexane extracts were reduced to dryness in a scintillation vial and 10 ml of scintillation fluid was added. Long-chain bases were obtained by reducing methanol fractions to dryness under a flow of nitrogen and redissolving the residues in a known amount of ethyl acetate. Aliquots were removed in

duplicate for sphingosine assay and the remainder was counted for radioactivity.

Upper phases from both the plasma and erythrocyte samples were concentrated to dryness *in vacuo*. The residues were subjected to mild alkaline hydrolysis at room temperature for 1 hr, after which the hydrolysate was extensively dialyzed. The dialysate was again concentrated to dryness *in vacuo* and the residues were dissolved in a small amount of chloroform-methanol 1:1 and an aliquot was transferred to a scintillation vial for radioactivity determination. Since specific activities were not similarly expressed in all of the sphingolipids studied, the following table summarizes how results were presented.

Specific Activity (cpm/μmole)	Specific Activity (cpm/ml RBC or plasma)
plasma Gl-la, GL-2a, GL-3a, GL-4 and G_{M3}	erythrocyte GL-la, GL-2a, GL-3a, GL-4 and G_{M3}
erythrocyte ceramides	erythrocyte gangliosides
erthrocyte GL-4 (8 time points from first 10 days)	erythrocyte ceramides
	plasma ceramides
	plasma gangliosides

e. Red cell fractionation

Cell fractionations were done on red cell samples obtained at 2, 3, 4, 6, 7, 8, 10, 14, 20, 30, 41, 50, 60, 70 and 81 days. Layers of 9 ml each of albumin solutions of decreasing specific gravity were carefully layered on each other in

three cellulose nitrate tubes of 1 1/2 inches in diameter by 3 1/2 inches in length. Aliquots of 2.5 ml of red cell suspensions (hematocrit approx. 75%) were layered on top of the albumin solution. The tubes were centrifuged at 4°C for 30 min at 25,000 rpm with a SW 25.2 swinging bucket. At several time points (beginning and end) throughout the 80 day period photographs were taken of the gradient tubes after centrifugation so that a permanent record could be kept.

Individual red cell bands were recovered as before, pooled, and the hemoglobin contents were measured. The cells were then washed with isotonic saline extensively to remove all traces of albumin. The volume of the packed cells was recorded and the lipids were extracted from the cells as before. Glycosphingolipids were isolated by silicic acid chromatography and further purified by TLC. Globoside was eluted from the gel and subsequently degraded by acid-catalyzed methanolysis. Fatty acid methyl esters were removed by hexane extraction and the hexane layers reduced to dryness in a scintillation vial and counted. The methyl glycosides and sphingosine bases were partitioned with chloroform-methanol 2:1 and water. Half of the methyl glycoside fraction was quantitated by GLC while the other half and the sphingosine fraction were concentrated to dryness in scintillation vials and radioactivity determined after adding 10 ml of Aquasol. Specific activity was expressed as counts per minute per μmole.

27. In vitro study

During the generation of reticulocytosis in pig 123-6 (before administration of the radioisotope), 100 ml of blood (reticulocyte count 40%) was removed from the pig and incubated with 30 μCi of [U-^{14}C]glucose in an Erlenmeyer flask at 37°C (Dubnoff shaker) for 2 hr. The flask was saturated with 100% O_2 and stoppered. The pH of the blood was 7.25. After incubation, the plasma was removed by centrifugation at 600 g and the cells were washed three times with 0.92% of isotonic saline.

Three tubes containing 9 ml each of the albumin solutions were prepared as before and 6 ml of the red cell suspension (hematocrit approx. 75%) was layered on albumin in each tube. The tubes were centrifuged at 4°C for 30 min at 25,000 rpm in a SW 25.2 rotor. After ultracentrifugation the tubes seemed overloaded with cells; hence, the top layer which contained most of the cells was removed from each tube and pooled. The cells were washed extensively with isotonic saline and glycosphingolipids were isolated. The lipids were subjected to acid-catalyzed methanolysis and radioactivity of the individual components was determined. The methyl glycosides derived from each glycosphingolipid were also quantitated by GLC. Specific activity was expressed as counts per minute per μmole.

IV. RESULTS

A. Isolation, Purification and Characterization of Human Plasma G_{M3} Ganglioside

1. Discovery of Human Plasma G_{M3} Ganglioside

The presence in plasma of a ganglioside was discovered as a result of changing from one brand of TLC plates to another. In this laboratory commercially available pre-coated silica gel G plates have been employed routinely in the isolation of glycosphingolipids. In the past, Brinkman plates were always employed and the separation of various glycosphingolipids was very satisfactory in the neutral solvent system; globoside usually gave a single band. However, delivery of these plates from the manufacturer was slow and resulted in work delays. Hence, I tested another brand of pre-coated plates (Quantum Industries) that had just appeared on the market. The separation and yield of glycosphingolipids from these plates were equally satisfactory as the Brinkman plates. Furthermore, scraping gel from these plates was more easily accomplished because they contained less binder.

When using Quantum plates in the purification of human plasma glycosphingolipids, it was observed that the GL-4

fraction was resolved into two closely migrating bands, at times apparently depending on the daily fluctuation of room temperature and humidity. It was originally believed that the second band was a fraction of GL-4 with a different fatty acid composition, but further investigation ruled out this possibility. Gas-liquid chromatography of the methyl glycosides derived from the material after acid-catalyzed methanolysis gave a peak which corresponded in retention time to the methyl ester methyl ketal of trimethylsilylated sialic acid on a programmed analysis. The presence of a sialic acid-containing glycosphingolipid was therefore suspected.

2. Search for A Solvent System

Initially, an attempt was made to develop the TLC plates in the neutral solvent system (ascending) two times in the same direction. This was a useful modification since the two bands separated a little better than with one development but was not sufficient for complete purification. A search was then made for a different solvent system to resolve the mixture. Efforts were mainly concentrated on published ganglioside solvent systems using various combinations or proportions of chloroform, methanol, water and ammonia. Standard GL-4 (isolated from erythrocytes by Mr. Paul Snyder of this laboratory) and gangliosides (isolated from horse erythrocytes by the author) were employed as markers. It was discovered

that chloroform-methanol-7% NH_4OH (55:40:10, v/v/v) gave the best separation between the two lipids.

3. Separation of Sialic Acid-Containing Glycosphingolipid and Globoside from Human Plasma

Glycosphingolipids derived from 50 ml of human plasma were first purified by TLC (Quantum plate) in the chloroform-methanol-water 100:42:6 (v/v) system. The two bands (in the region of the standard GL-4) were removed from the plate in one fraction and eluted from the gel with chloroform-methanol-water mixture. The lipids were then separated by a second TLC (Quantum plate) using chloroform-methanol-7% NH_4OH 55:40:10 (v/v) as solvent. As shown in Figure 1, the unknown lipid (Band B, R_F = 0.51-0.54) and globoside (Band C, R_F = 0.38-0.42) were well separated from each other and from another compound (Band A) that has not been identified.

4. Identification of Lipid B by Gas-liquid Chromatography

Gas-liquid chromatography enables one to obtain detailed information about the nature and relative amounts of the sugar constituents in a given glycosphingolipid. An identification of fucose, galactose, glucose, galactosamine, glucosamine and sialic acid can be made by comparison of the observed retention times relative to that of mannitol with those of standards as shown in Figure 2 and Table 3.

Figure 1. Thin-layer chromatography of hematoside from human plasma.

Thin layer chromatographic separation of an unknown lipid (Band A), hematoside (Band B) and globoside (Band C) from human plasma. The plate was developed in chloroform-methanol-7% NH_4OH (55:40:10, v/v/v).

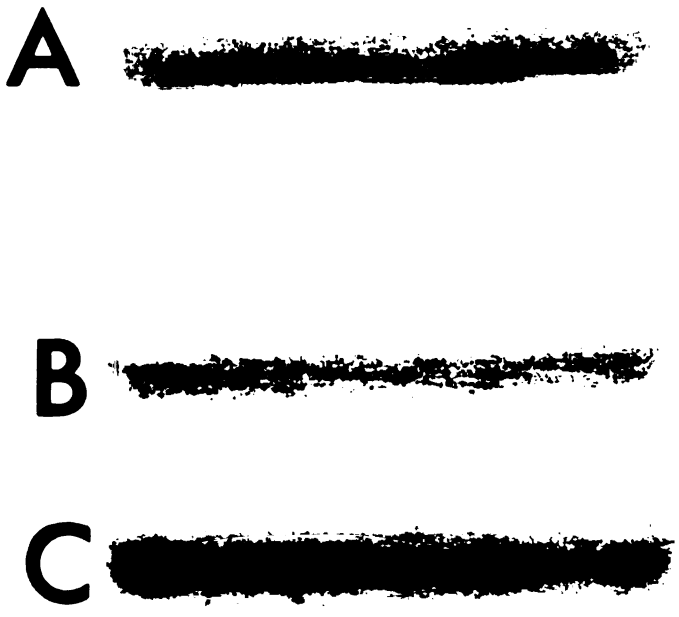


Figure 1

Figure 2. Gas-liquid chromatography of trimethylsilyl methyl glycosides.

Analysis of O-trimethylsilyl derivatives of methyl glycosides on 3% SE-30, programmed from 160° to 230°C at 2°C/min with a carrier gas flow rate of 10 ml/min (nitrogen). Peaks correspond to the following sugars: fucose (1,2,3); galactose (4,5,6); glucose (7,8); mannitol (9); N-acetylgalactosamine (10,12); N-acetylglucosamine (11,13,14); and N-acetylneuraminic acid (15).

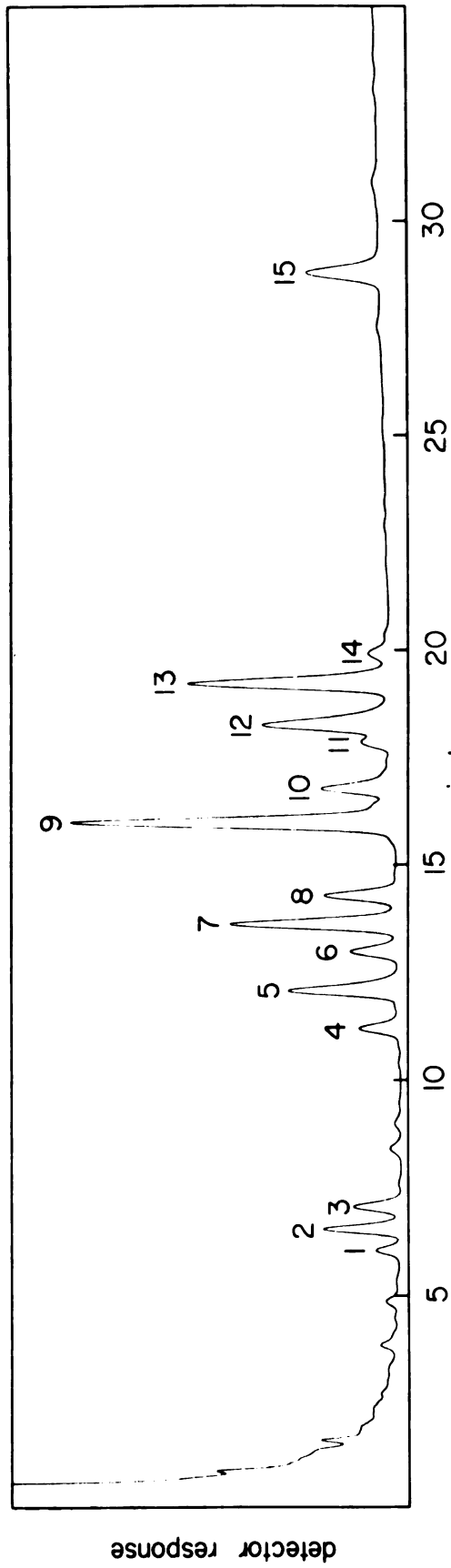

detector response
minutes
5
10
15
20
25
30
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 2

Table 3. Relative Retention Behavior of Trimethylsilyl Methyl Glycosides

Compound	Retention Time[a]	Correction Factor[b]
Fucose	0.17, 0.19, 0.21	1.56
Galactose	0.47, 0.53, 0.62	1.25
Glucose	0.69, 0.77	1.25
Mannitol	1.00	1.00
N-Acetylgalactosamine	1.15, 1.43	1.36
N-Acetylglucosamine	1.34, 1.66, 1.87	1.36
Methyl N-acetylneuraminate	not determined	0.98

[a]The retention times are relative to the time for hexa-O-trimethylsilylmannitol, which was 15±1 min on 3% SE-30 at 160°C (isothermal) with 48 ml per min nitrogen flow rate.

[b]These factors are used to correct observed areas in a gas-liquid chromatograph with flame ionization detector; they are based on molecular weight ratios.

The lipid recovered from Band B was subjected to acid-catalyzed methanolysis and the mixture was reacted with acetic anhydride to convert the neuraminate to an N-acetyl derivative. Methyl glycosides were identified by gas-liquid chromatography as O-trimethylsilyl derivatives on a 6 ft. U-shaped glass column packed with 3% SE-30. After injection of the sample the column temperature was increased linearly from 160°C to 230°C at a rate of 2°/min. Peaks of galactose, glucose and neuraminic acid derivatives were identified by their

retention behavior and comparison of their mass spectra with those of authentic compounds. A typical gas-liquid chromatogram is shown in Figure 3. Based on the areas of peaks on the gas-liquid chromatograms, the calculated average molar ratios of galactose and neuraminate to glucose were 0.99 and 0.63 for the plasma ganglioside, as shown in Table 4.

5. Identification of Sialic Acid Derived from Lipid B

The lipid was reacted with aqueous acid under mild conditions to liberate intact N-acetylneuraminic acid (115). After extraction with chloroform the aqueous phase was evaporated to dryness and the product was purified by column chromatography and TLC according to the method of Puro (241). Thin-layer chromatography (Figure 4) showed that the R_F value (0.41) of the sialic acid from the lipid was identical with that of authentic N-acetylneuraminic acid, whereas N-glycolylneuraminic acid, liberated from hematoside of equine erythrocytes, had an R_F value of 0.28.

The lipid recovered after mild acid hydrolysis was subjected to acid-catalyzed methanolysis and the trimethylsilyl methyl glycosides were analyzed by GLC. The only components found were galactose and glucose in a molar ratio of 1:1 as shown in Figure 3. It was therefore concluded that human plasma lipid B was an N-acetylhematoside (G_{M3} ganglioside).

Figure 3. Retention times of trimethylsilyl methyl glycosides from ganglioside of normal human plasma.

Methyl glycosides were identified as O-trimethylsilyl derivatives on a 6 ft. U-shaped glass column packed with 3% SE-30 in a Hewlett-Packard 402 Gas Chromatograph. After in injection of the sample the column temperature was increased linearly from 160°C to 230°C at a rate of 2°/min. Top tracing, plasma ganglioside before mild acid hydrolysis, peaks correspond to the following sugars: galactose (1,2,3); glucose (4,5); and N-acetylneuraminic acid (6). Bottom tracing, plasma ganglioside after mild acid hydrolysis.

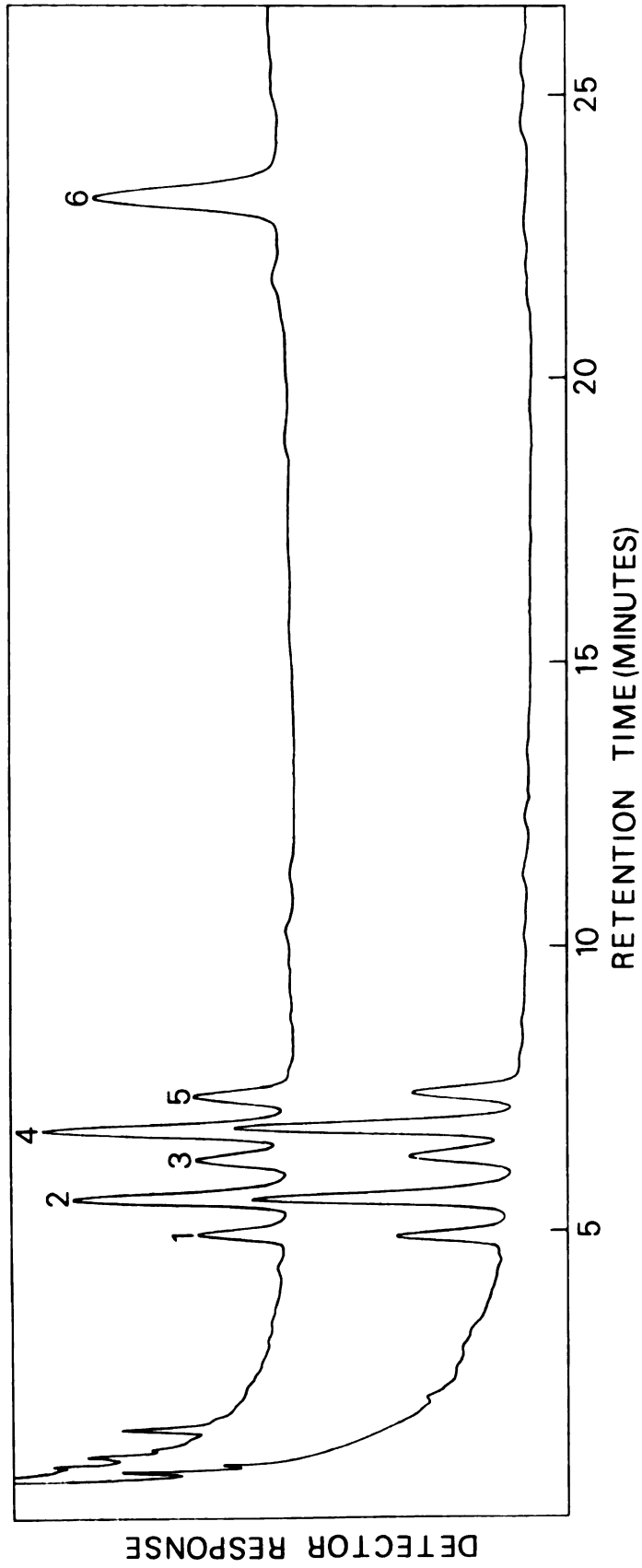

DETECTOR RESPONSE
RETENTION TIME(MINUTES)
5
10
15
20
25
1
2
3
4
5
6

Figure 3

Table 4. Concentration of G_{M3} Ganglioside and Globoside in Human Plasma

Sample	G_{M3} Ganglioside[a]			Globoside		
	Gal/Glc	NANA[b]/Glc	nmoles/ml	Gal/Glc	GalNAc/Glc	nmoles/ml
1	1.01	0.68	3.1	1.77	0.71	2.5
2	0.99	0.59	3.3	1.83	0.90	2.6
3	0.96	0.62	3.6	1.86	0.75	2.7
Mean	0.99	0.63	3.3	1.82	0.80	2.6

[a]The values are for total recovered G_{M3} ganglioside in upper and lower phases.

[b]N-Acetylneuraminic acid.

Figure 4. Thin-layer chromatography of neuraminic acid derived from human plasma ganglioside.

Human plasma ganglioside was dissolved in 0.5 ml of 0.03 N HCl (aqueous) and heated at 85°C for 2 hr. The products of hydrolysis were partitioned with chloroform and water, and the aqueous phase was applied to a small column containing 1 g Dowex-I in the acetate form. The mixture was first eluted with 10-20 ml of water and then with 10-20 ml of 1 M formic acid. The recovered neuraminic acid residue was identified by TLC in n-propanol-1 N NH_4OH-water (6:2:1) solvent system. The plate was activated at 120°C for 15 min.

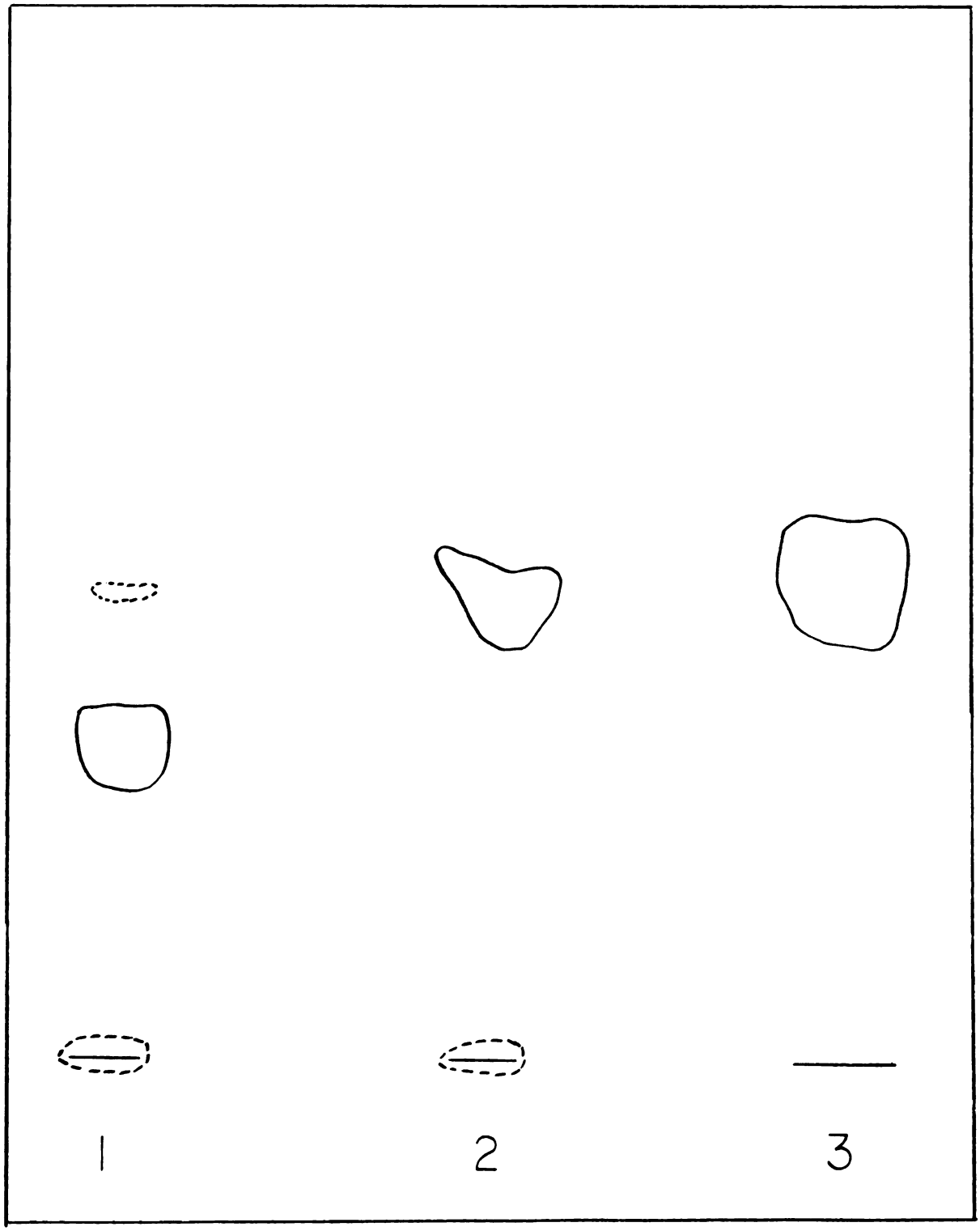


Figure 4

6. Linkage Studies of Human Plasma Hematoside

Methylation studies were carried out, and gas-liquid chromatographic analyses were made of both the permethylated methyl glycosides and alditol acetates on a 6 ft. glass column packed with 3% ECNSS-M on 100-120 mesh Supelcoport. The gas-liquid chromatographic peaks of the permethylated methyl glycosides were not resolved as clearly as the alditol acetates. However, since the permethylation study involves a rather long procedure and there is ample material available, it is a good practice and worthwhile undertaking to inject a small aliquot of the permethylated methyl glycoside into a gas-liquid chromatograph prior to the hydrolysis step, so that an additional piece of information about the retention times of various sugars can be obtained. This gives an added insurance in case the latter part of the procedure does not work for one reason or another. The technique of using the combination of GLC and GLC-MS analyses of the partially methylated alditol acetates is definitely by far a more accurate and superior method in determining the linkages between the monosaccharide residues of glycosphingolipids.

Permethylation of human plasma G_{M3} led to the production of methyl-2,3,6-tri-O-methyl-α,β-glucoside and methyl-2,3,6-tri-O-methyl-α,β-galactoside, as shown in Figure 5. Conditions for permethylation were checked with lactose and N-acetylneuraminyllactose standards. The retention times of the methyl ethers are presented in Table 5. Identification of

Figure 5. Gas-liquid chromatography of permethylated methyl glycosides from lactose, N-acetylneuraminyl-lactose, and human plasma ganglioside.

Peak 1 corresponds to methyl-2,3,4,6-tetra-O-methyl-α and β-galactoside; peak 3 to methyl-2,3,6-tri-O-methyl-β-glucoside; peak 4 to methyl-2,4,6-tri-O-methyl-β-galactoside; and peak 5 to α anomers of 3 and 4. Analysis was made on a 6 ft. glass column packed with 3% ECNSS-M on 100-120 mesh Supelcoport maintained isothermally at 160°C using helium as carrier gas.

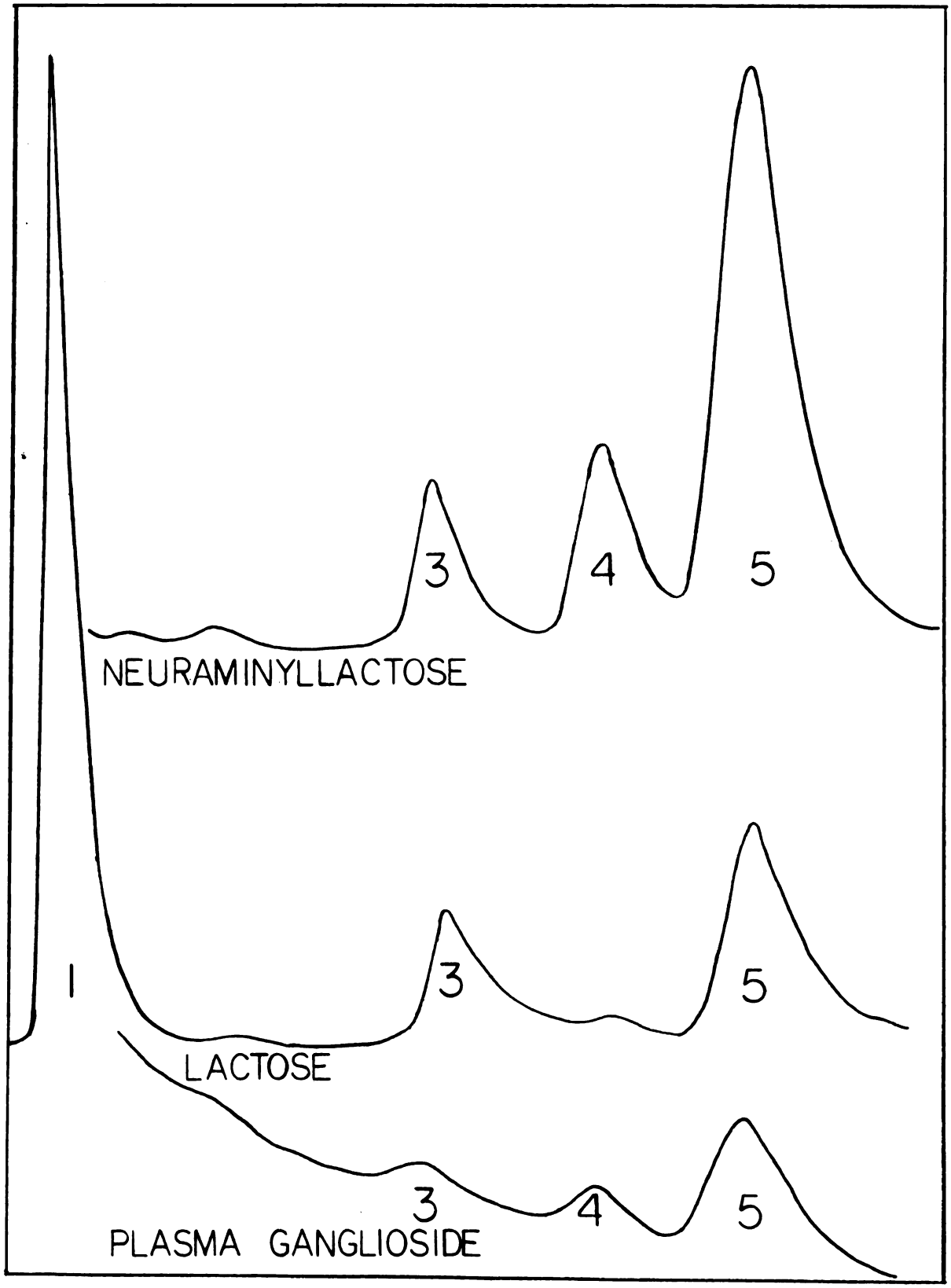

3
4
5
NEURAMINYLLACTOSE
1
3
5
LACTOSE
3
4
5
PLASMA GANGLIOSIDE

Figure 5

Table 5. Retention Times of Permethylated Methyl Glycosides from Human Plasma Ganglioside[a]

Compounds	Retention Time (Min)			
	1	3	4	5
Lactose	2.8	5.3	---	7.3
N-Acetylneuraminyllactose	---	5.4	6.5	7.4
Human plasma ganglioside	---	5.4	6.5	7.4

[a]Peaks of permethylated methyl glycosides are denoted as 1, methyl-2,3,4,6-tetra-O-methyl-α,β-galactoside; 3, methyl-2,3,6-tri-O-methyl-β-glucoside; 4, methyl-2,4,6-tri-O-methyl-β-galactoside; 5, α anomers of 3 and 4.

Table 6. Retention Times of Partially Methylated Alditol Acetates from Human Plasma Ganglioside[a]

Compounds	Retention Time (Min)	
	(b)	(d)
N-Acetylneuraminyllactoce	30.1	33.3
Human plasma ganglioside	30.0	33.0

[a]Peaks of partially methylated alditol acetates are represented as (b), 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol; and (d), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol.

methylated sugars were made according to published results (262).

Gas-liquid chromatography of the partially methylated alditol acetates from G_{M3} (Figure 6) revealed two peaks which corresponded to the retention times of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol. The identification of all alditol acetates was confirmed by GLC-MS; their fragmentation patterns were identical with the published results (241,247, 248).

These data led to the conclusion that the N-acetyl-neuraminic acid of human plasma G_{M3} is attached to C-3 of the external galactose unit, which is, in turn, linked at C-4 of the glucose residue. Thus, the structure of plasma G_{M3} is NANA-(2→3)-Gal-(1→4)-Glc-(1→1)-ceramide.

7. Hematoside from Folch Upper Phase

After it was apparent that plasma lipid B was G_{M3}, the upper layer from an equilibration of total lipid extracts with water by the procedure of Folch et al. (232) was examined for gangliosides. G_{M3} was the major constituent of this fraction. The partition coefficient of G_{M3} between the upper and lower phases was 0.4; that is, 71% of the total plasma G_{M3} was recovered in the lower chloroform layer. In addition, the presence of some hexosamine-containing gangliosides were also detected and will be discussed later.

Figure 6. Gas-liquid chromatography of partially methylated alditol acetates from N-acetylneuraminyllactose and human plasma ganglioside.

Peak (b) corresponds to 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol; and peak (d) to 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol. Analysis was made on a 6 ft. glass column packed with 3% ECNSS-M on 100-120 mesh Supelcoport maintained isothermally at 175°C using helium as carrier gas.

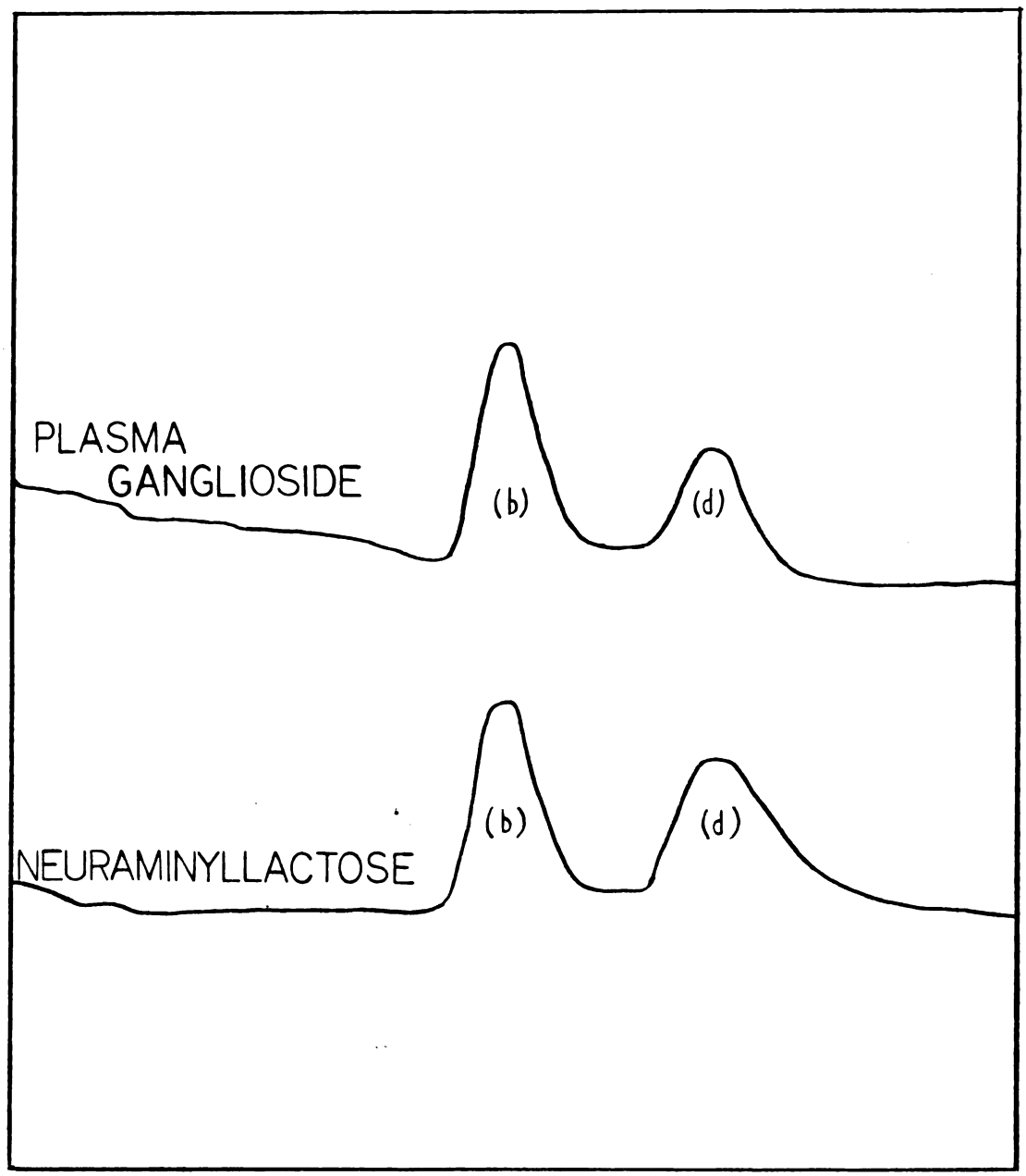


Figure 6

8. Fatty Acid Composition of Human Plasma Hematoside

Methyl esters of fatty acids were recovered after acid-catalyzed methanolysis and were analyzed by gas chromatography at 190°C on 15% ethylene glycol adipate polyester. The fraction of G_{M3} recovered from lower phase after Folch-type partition contained significant proportions of palmitic, stearic, behenic, tricosanoic, lignoceric and nervonic acids, of which palmitic and stearic acids comprised 22% of the total mixture (Table 7). The longer-chain fatty acids from C_{20} to C_{24} represented 68% of the total mixture. There were no detectable α-hydroxy fatty acids in the mixture.

The fraction of G_{M3} recovered from the upper phase had a different fatty acid composition in which palmitic, stearic and oleic acids accounted for 80% of the total mixture (Table 7).

9. Quantitative Estimation of the Sugars by Gas-liquid Chromatography

To quantitatively estimate the sugars present in a given glycosphingolipid, mannitol was added to the purified glycolipid as an internal standard prior to acid-catalyzed methanolysis. The yield of each component was calculated from the total area produced by the various anomeric forms of a given sugar, using the area produced by the known amount of mannitol for comparison. Factors to correct for differences in molecular weights of the trimethylsilyl derivatives were

Table 7. Fatty Acid Composition of Plasma G_{M3} Ganglioside

Fatty acids are denoted as chain length:number of double bonds. The amounts found are given as a percentage of total area on gas chromatograms.

Component	Lower Phase %	Upper Phase %
14:0	0.7	1.6
15:0	0.8	1.4
15:1	0.3	tr
16:0	10:7	38.6
16:1	1.0	tr
17:0	1.4	1.5
17:1	0.6	tr
18:0	10.9	29.7
18:1	3.5	10.7
19:0	1.9	1.7
20:0	5.3	5.7
21:0	1.6	---
21:1	0.6	---
22:0	23.0	8.6
23:0	9.9	tr
24:0	18.9	tr
24:1	9.0	tr

given in Table 3. Using the ratio of 1.25, observed for the area of trimethylsilyl mannitol to that of trimethylsilyl methyl glycosides with equal amounts (mass) of glucose and mannitol, the yield of glucose from gas-liquid chromatographic data was calculated by the equation:

$$\mu\text{moles of glucose} = \frac{\text{area of glucose peaks}}{\text{area of mannitol peak}} \times \frac{}{1.25 \times \mu\text{moles of mannitol added}}$$

The concentration of G_{M3} in human plasma is shown in Table 4. The mean level of this ganglioside in three plasma samples was 0.33 μmole/100 ml; the individual analyses of 50 ml aliquots of the same plasma gave 0.14, 0.17, and 0.17 μmole.

10. Other Human Plasma Gangliosides from Upper Phase

Preliminary studies of the G_{M3} ganglioside level in Folch-type upper phase indicated the presence of some hexosamine-containing sialo-glycosphingolipids. They were present as minor components and 150 ml of plasma was therefore extracted for gangliosides according to the procedure listed in Methods. Thin-layer chromatography revealed a number of slow-migrating bands (Figure 7, lane 3, Bands II and III) in addition to G_{M3}, the fast-moving major ganglioside (Band I). Both N-acetyl-galactosamine and N-acetylglucosamine were observed in the lipids obtained from several of these bands by gas-liquid chromatography on a programmed run. This was further confirmed

Figure 7. Thin-layer chromatography of Folch upper phase gangliosides from normal human plasma and platelets.

The Folch upper phases from human platelets (73 units) and plasma (150 ml) were dialyzed in the cold for 72 hr. The dialysate was subjected to mild alkaline hydrolysis and dialyzed again as before. The gangliosides were purified by TLC, using 250μ Uniplate heat activated at 120°C for 30 min. The plate was developed sequentially in chloroform-methanol-1.25 N NH_4OH (60:40:9) and n-propanol-water (70:30) solvent systems. In lane 1, reference standard ganglioside mixture containing mono-, di-, and trisialogangliosides. In lane 2, platelet gangliosides: hematoside (A), hematoside containing different fatty acid composition (B), hexosamine-containing ganglioside (C). In lane 3, plasma gangliosides: hematoside (I), hexosamine-containing gangliosides (II, III).

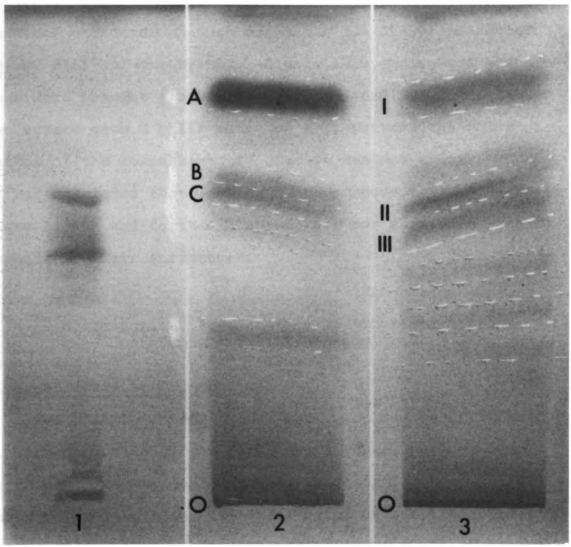


Figure 7

by comparing the retention times of the hexosamines with authentic N-acetylgalactosamine, N-acetylglucosamine and mannitol standards on both non-polar (3% SE-30) and polar (3% OV-225) columns by GLC. Sialic acid, galactose and glucose were present along with these hexosamines. The latter two sugars gave a molar ratio of 1:1, whereas the molar ratios of the hexosamines to glucose was not unity. Thus, it was concluded that more than one component was present in these lipids and further studies were not conducted with respect to their structures.

B. Isolation, Purification and Characterization of Human Platelet Sphingolipids

1. Human Platelet Concentrates

The platelet concentrates were prepared by Dr. Graham A. Jamieson using differential centrifugation to remove erythrocytes. Residual erythrocyte contamination was estimated to be less than 0.2% (v/v) based on spectrophotometric assays (540 nm) of the hemoglobin present in the supernatant solution.

2. Lipid Composition of Human Platelets

The yield of total lipids from 73 platelet units was 1.32 g. The relative proportions (weight %) of neutral lipids, glycosphingolipids plus ceramide, and phospholipids from the total lipid extract of trypsinized human platelets are given Table 8. Neutral lipids constituted 25% of the total lipid

Table 8. Total Lipid Composition of Human Platelets

Sample	Total mg	Neutral Lipids		Glycosphingolipids[a]		Phospholipids	
		Weight mg	% of TL[b]	Weight mg	% of TL[b]	Weight mg	% of TL[b]
1	372	88	24	31	8	244	66
2	279	74	26	22	8	180	65
Mean			25		8		66

[a]Includes ceramide and G_{M3}. The yield is for crude glycoshpingolipids before mild alkali-catalyzed methanolysis; phospholipid accounted for 50-60% of the weight.

[b]Total lipid.

fraction, whereas the shpingolipids and phospholipids accounted for 8% and 65%, respectively.

3. Identification of Platelet Glycosphingolipids by Thin-layer Chromatography

Commercially available pre-coated plates of silica gel G were used for the separation of glycosyl ceramides. Previous experience with Quantum plates had demonstrated that the separation of globoside and hematoside could be achieved by means of two solvent systems. The use of two plates for this procedure is not economical when there are many samples to be analyzed, such as in a turnover study. Hence, an attempt was made to resolve these components with TLC in a single development. This was achieved with Uniplates from Analtech. An example of the TLC of glycosphingolipids from 50 ml of plasma is shown in Figure 8. Plasma globoside (Band E) and hematoside (Band F) were separated very well. In addition, ceramide (Band A) could also be identified, but with lipids derived from erythrocytes and platelets further purification of ceramides was required.

Uniplates also gave good separation of platelet globoside (R_F = 0.16) and hematoside (R_F = 0.07), as shown in Figure 9. After development in chloroform-methanol-water 70:30:5 (v/v), four neutral glycosphingolipids (Figure 9--I, II, III, and IV) were located in the same areas as glucosylceramide, lactosylceramide, Fabry trihexosylceramide and porcine globoside,

Figure 8. Thin-layer chromatography of sphingolipids from normal human plasma.

Thin-layer chromatography (on 500μ pre-coated silica gel G plates from Analtech, Inc.) of sphingolipids from normal human plasma. In lane 1 reference standard ceramide. In lane 2, reference standards from top to bottom are glucosylceramide, lactosylceramide, Fabry trihexosylceramide and porcine globoside. In lane 3, ceramide (A), major neutral glycosphingolipids (B-E) and hematoside (E) are separated from crude total glycosphingolipid fraction of plasma. Unlabeled zone at top contains primarily methyl esters that were released from contaminating phospholipid by mild alkali-catalyzed methanolysis. The solvent system used was chloroform-methanol-water (70:30:5, v/v/v).

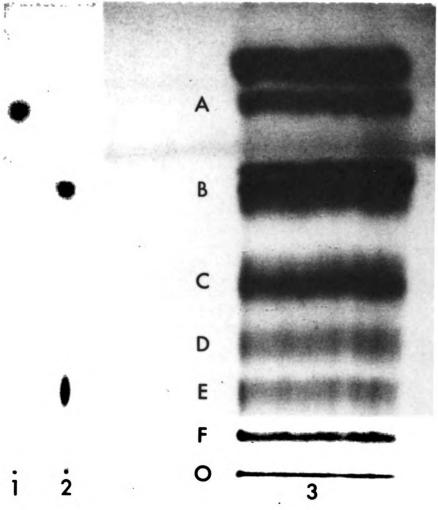


Figure 8

Figure 9. Thin-layer chromatography of glycosphingolipid fraction from washed normal human platelets treated with trypsin.

Thin-layer chromatography (500μ pre-coated silica gel G plates from Analtech, Inc.) of glycosphingolipid fraction from washed normal human platelets treated with trypsin. In lane 1, reference standards from top to bottom are glucosylceramide, lactosylceramide, Fabry trihexosylceramide and porcine globoside. In lane 2, major neutral glycosphingolipids (I-IV) and acidic glycosphingolipid (V) are separated from crude total glycosphingolipid fraction of platelets. Unlabeled zone at top contains primarily ceramides along with methyl esters that were released from contaminating phospholipid by mild alkali-catalyzed methanolysis. The solvent system used was chloroform-methanol-water (70:30:5, v/v/v).

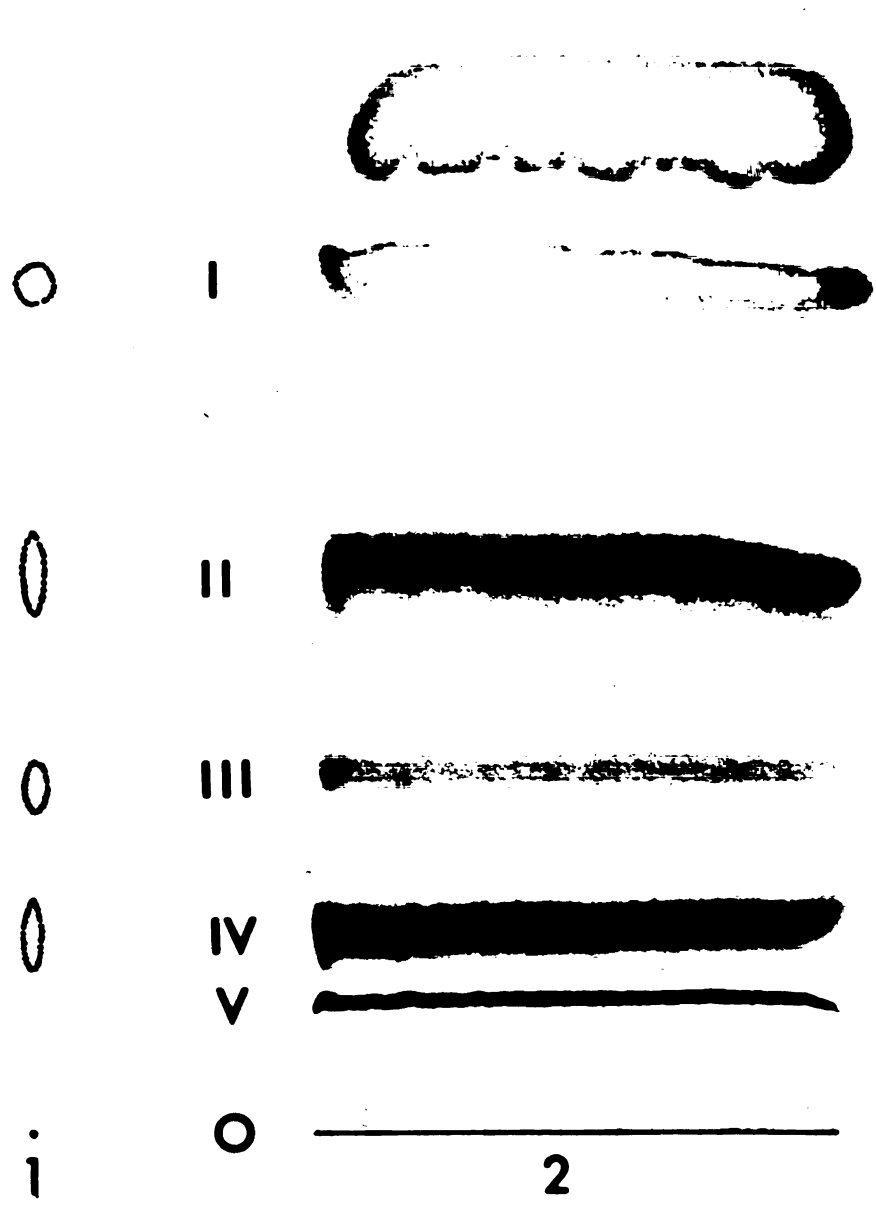


Figure 9

respectively, and an additional band (V) which migrated between the origin and globoside in this solvent system was shown to contain sialic acid by resorcinol spray.

4. Identification of Platelet Glycosphingolipids by GLC

Representative gas-liquid chromatograms of the O-trimethylsilylated methyl glycosides of the neutral glycosphingolipids are shown in Figure 10. The calculated average molar ratios of galactose to glucose were 1.1, 2.2, and 2.1 for lipids II, III, and IV, respectively (Table 9). Glucose was the only sugar component in lipid I. N-Acetylgalactosamine was also present in IV, and the molar ratio of N-acetylgalactosamine to glucose was 0.8. The sialic acid-containing lipid (V) had a galactose to glucose ratio of 1.2 and a molar ratio of N-acylneuraminic acid to glucose of 0.54, as shown in Figure 11. These results agree with the assignment of lipids I-V as monohexoside, dihexoside, trihexoside, tetrahexoside and sialo-lactoside.

5. Identification of Sialic Acid from Platelet Lipid V

After splitting the neuraminic acid by mild acid hydrolysis, thin-layer chromatography revealed the presence of N-acetylneuraminic acid (R_F = 0.40) when compared with the authentic N-acetylneuraminic acid standard. This was further confirmed by GLC of the liberated neuraminate as the trimethylsilylated derivative.

Figure 10. Gas-liquid chromatography of trimethylsilyl methyl glycosides from major neutral glycosphingolipids of normal human platelets treated with trypsin.

Analysis of O-trimethylsilyl derivatives of methyl glycosides with a Hewlett-Packard 402 gas chromatograph equipped with a 6 foot 3% SE-30 column maintained at 165°C. Peaks correspond to the following sugars: galactose (1,2,3); glucose (4,5); mannitol (6); and N-acetylgalactosamine (7,8). From top to bottom: GL-4 (IV), GalNAc-(1→3)-Gal-(1→4)-Gal-(1→4)-Glc-(1→1)-ceramide containing Gal:Glc:GalNAc (2.1:1.0:0.8); GL-3 (III), Gal-(1→4)-Gal-(1→4)-Glc-(1→1)-ceramide containing Gal:Glc (2.2:1.0); GL-2 (II), Gal-(1→4)-Glc-(1→1)-ceramide containing Gal:Glc (1.1:1.0); and GL-1 (I), Glc-(1→1)-ceramide containing only glucose.

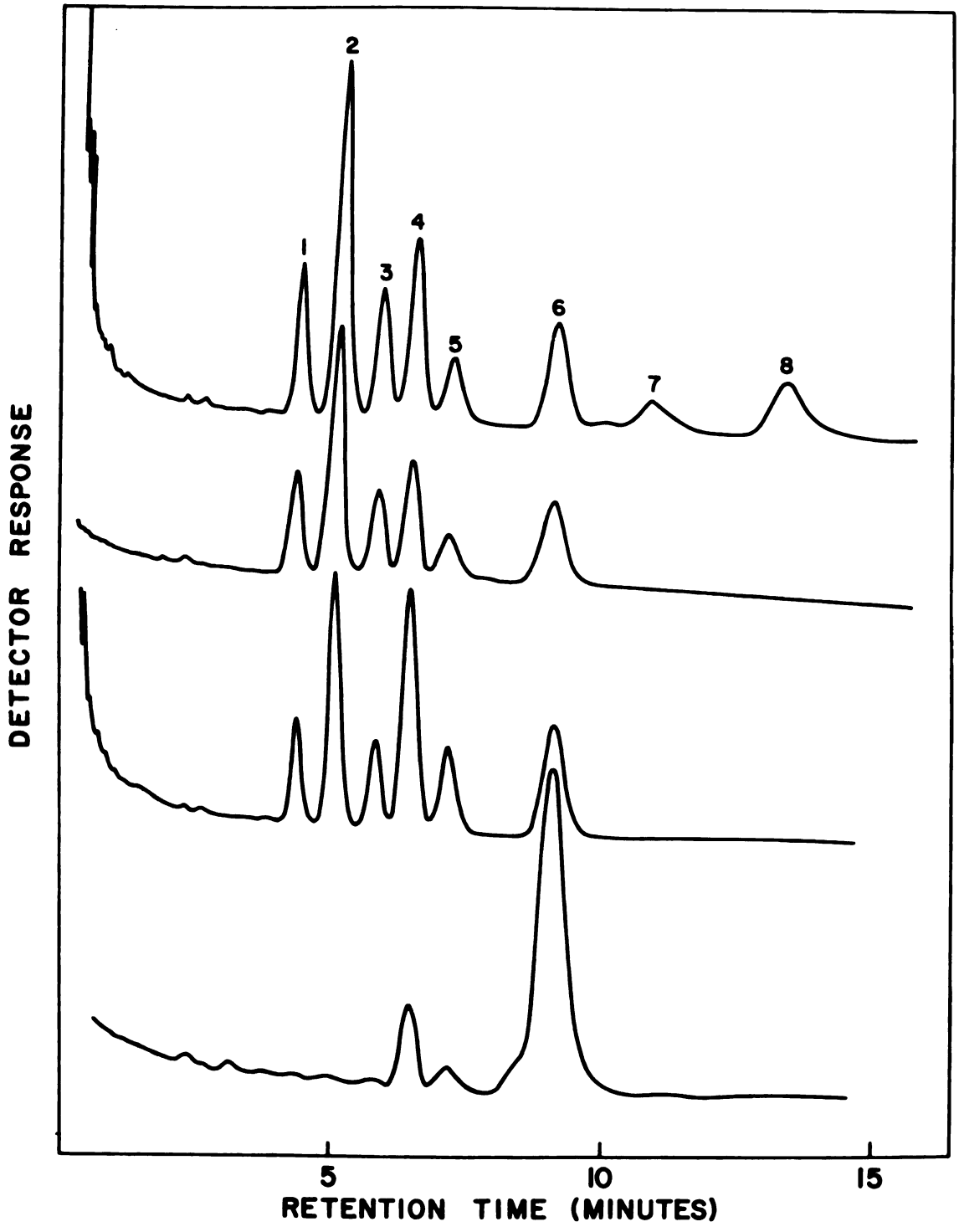


Figure 10

Table 9. Concentration of Glycosphingolipids in Human Platelets

Sample[a]	Lower Phase											
	GL-1	GL-2		GL-3		GL-4				G_{M3}	
	µmoles g TL	Gal[b] Glc	µmoles g TL	Gal Glc	µmoles g TL	Gal Glc	GalNAc Glc	µmoles g TL	Gal Glc	NANA Glc	µmoles g TL
1	0.22	1.1	4.81	2.1	1.31	2.1	0.85	1.17	1.1	0.55	1.32
2	0.21	1.1	5.03	2.2	1.33	2.1	0.80	1.31	1.2	0.53	1.40
Mean	0.22	1.1	4.92	2.2	1.32	2.1	0.83	1.24	1.2	0.54	1.36
3	0.35	1.0	4.29	1.9	1.70	1.9	0.60	1.48	1.0	0.52	0.38

[a]Samples 1 and 2 are different weights and were analyzed at different times (about two months apart); they represent the lipid extract from trypsin-treated platelet residues. Sample 3 is the lipid extract from washed human platelets which were not pre-treated with trypsin.

[b]Molar ratios of sugars as determined by GLC.

Figure 11. Gas-liquid chromatogram of trimethylsilyl methyl glycosides from G_{M3} ganglioside of normal human platelets treated with trypsin.

Gas-liquid chromatogram of O-trimethylsilyl derivatives of methyl glycosides from G_{M3} (lower phase), NANA-(2-3)-Gal-(1→4)-Glc-(1→1)-ceramide containing NANA:Gal:Glc (0.6:1.1:1.0) of washed normal human platelets treated with trypsin. Peaks correspond to the following sugars: galactose (1,2,3); glucose (4,5); mannitol (6); and N-acetylneuraminic acid (9). Analysis was made on 3% SE-30, programmed from 165° to 230°C at 2°C/min with a carrier gas flow rate of 30 ml/min (nitrogen).

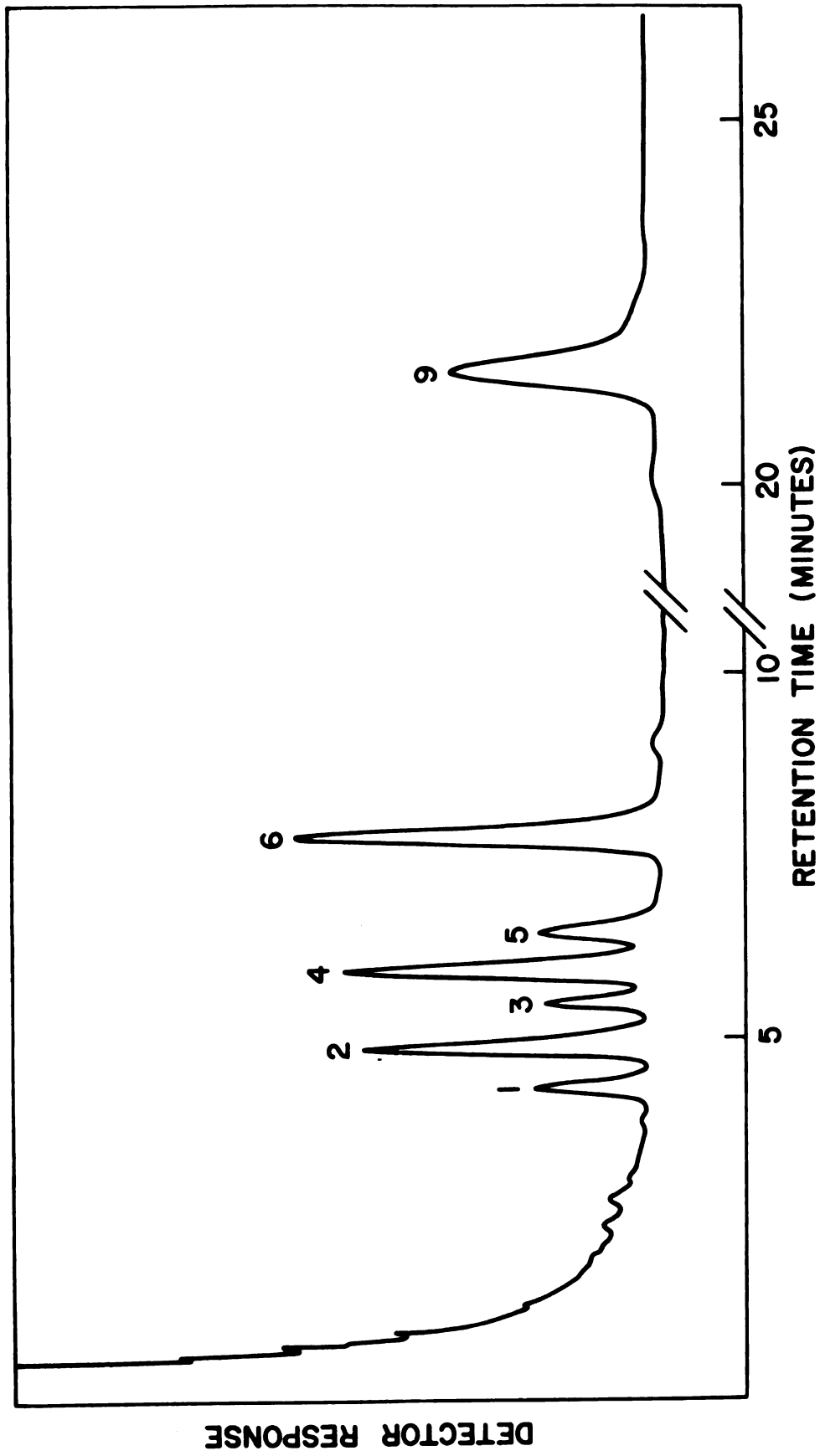

DETECTOR RESPONSE
RETENTION TIME (MINUTES)
5
10
20
25
1
2
3
4
5
6
9

Figure 11

6. Linkage Studies of Platelet Glycosphingolipids

a. Lipid I (ceramide monohexoside)

It is well established that the hexose is linked to ceramide through a glycosidic linkage; limited amounts of material prevented permethylation studies. Analysis of the trimethylsilyl derivatives showed that only methyl glucoside was present in this lipid.

b. Lipid II (ceramide dihexoside)

The methylated products of the dihexoside contained α and β methyl-2,3,4,6-tetra-O-methyl-galactoside and α and β methyl-2,3,6-tri-O-methyl-glucoside (Figure 12 and Table 10). Gas-liquid chromatography of the partially methylated alditol acetates revealed two peaks for lipid II with the same retention times (Figure 13 and Table 11) as 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol. The structures of the methylated monosaccharides were further confirmed by GLC-MS; their fragmentation patterns were identical to those reported for 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-hexitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-hexitol (241,247,248) and agreed with those obtained with reference standards (Table 12). The results were consistent with the structure of the ceramide dihexoside from human platelets being Gal-(1→4)-Glc-(1→1)-ceramide.

Figure 12. Gas-liquid chromatography of permethylated methyl glycosides derived from lactose, Fabry trihexosylceramide (CTH), porcine erythrocyte globoside (GL-4), neuraminyllactose, and platelet glycosphingolipids.

Peak 1 corresponds to methyl-2,3,4,6-tetra-O-methyl-α and β-galactoside; peak 2 to methyl-2,3,6-tri-O-methyl-β-galactoside; peak 3 to methyl-2,3,6-tri-O-methyl-β-glucoside; peak 4 to methyl-2,4,6-tri-O-methyl-β-galactoside; and peak 5 to α anomers of 2,3, and 4. Peaks 2 and 3 are partially resolved, not quantitative with respect to peak areas. Analysis was made on a 6 ft. glass column packed with 3% ECNSS-M on 100-120 mesh Supelcoport maintained isothermally at 160°C using helium as carrier gas. Peak identifications were made by comparing with published results (262).

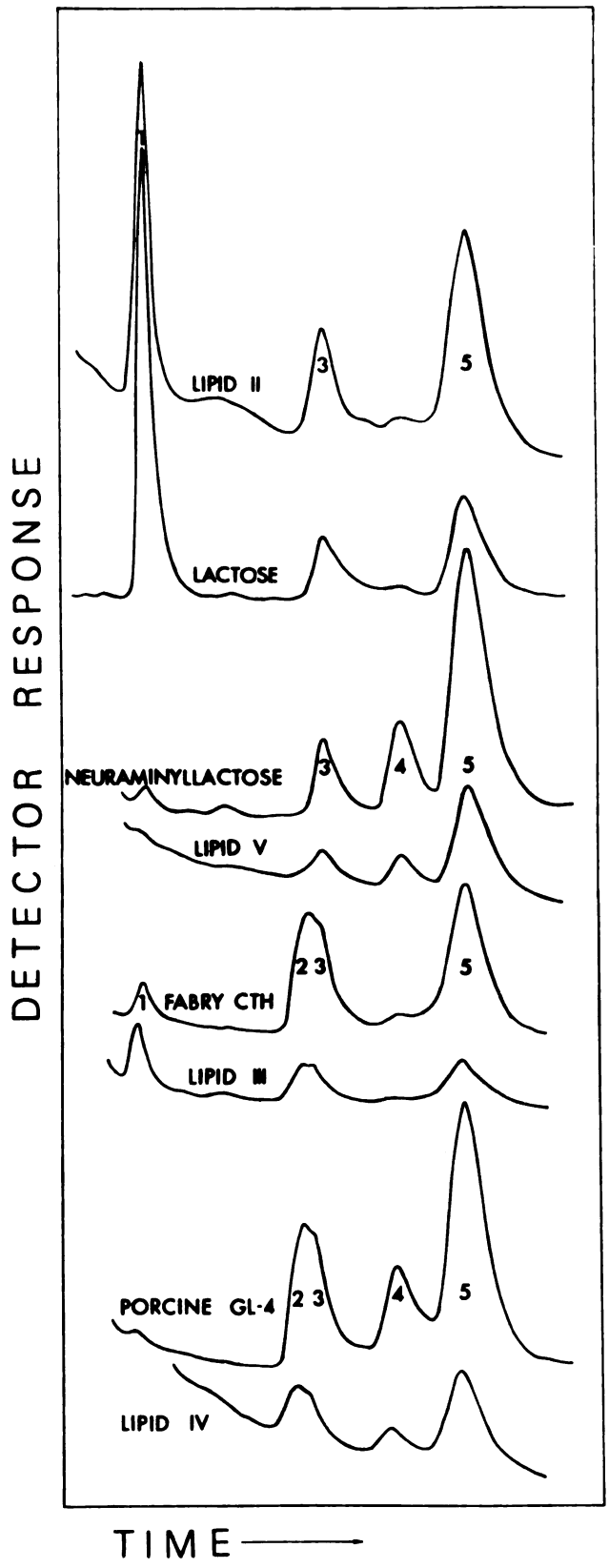

DETECTOR RESPONSE
LIPID II
3
5
LACTOSE
NEURAMINYLLACTOSE
3
4
5
LIPID V
1
FABRY CTH
2 3
5
LIPID III
PORCINE GL-4
2 3
4
5
LIPID IV
TIME

Figure 12

Table 10. Retention Times of Permethylated Methyl Glycosides from Platelet Glycosphingolipids[a]

	1	2[b]	3[b]	4	5
Lactose	2.8	----	5.3	----	7.3
Lipid II	2.9	----	5.4	----	7.4
Fabry kidney GL-3	2.9	5.2	5.3	----	7.4
Lipid III	2.9	5.2	5.4	----	7.5
Porcine erythrocyte GL-4	----	5.2	5.3	6.5	7.4
Lipid IV	----	----	5.4	6.5	7.4
N-acetylneuraminyllactose	----	----	5.4	6.5	7.4
Lipid V	----	----	5.4	6.5	7.4

[a]Peaks of permethylated methyl glycosides are denoted as 1, methyl-2,3,4,6-tetra-O-methyl-α and β-galactoside; 2, methyl-2,3,6-tri-O-methyl-β-galactoside; 3, methyl 2,3,6-tri-O-methyl-β-glucoside; 4, methyl-2,4,6-tri-O-methyl-β-galactoside; 5, α anomers of 2, 3 and 4.

[b]Partially resolved by GLC, not quantitative with respect to peak areas.

Figure 13. Gas-liquid chromatography of partially methylated alditol acetates derived from lactose, neuraminyl-lactose, Fabry trihexosylceramide (CTH), porcine erythrocyte globoside (GL-4), and platelet glycosphingolipids.

Peak (a) corresponds to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol; peak (b) to 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl galactitol; and peaks (c) and (d) to 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl galactitol and -glucitol, respectively. Analysis was made on a 6 ft. glass column of 3% ECNSS-M on 100-120 mesh Supelcoport maintained isothermally at 175°C using helium as carrier gas.

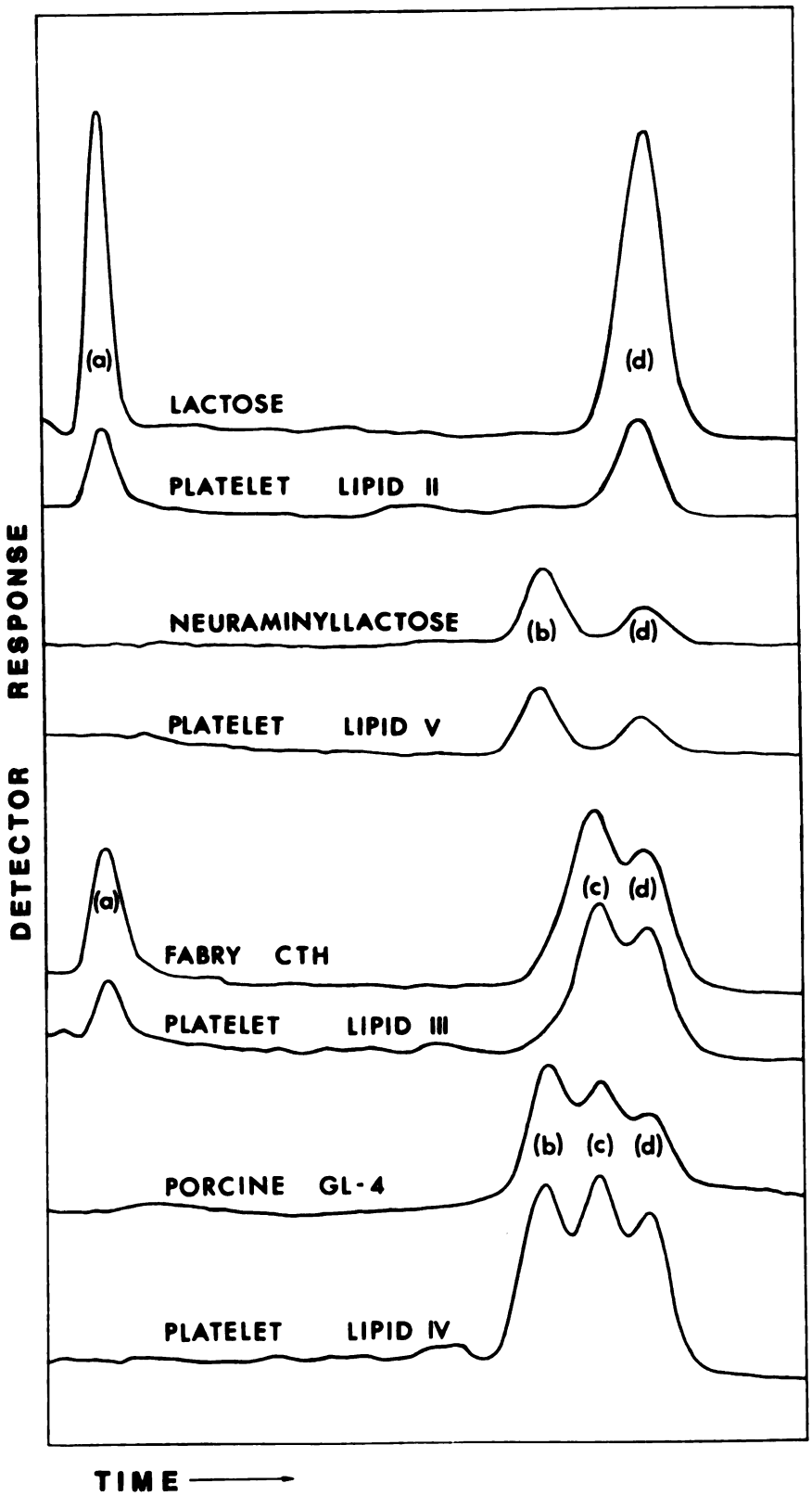

DETECTOR RESPONSE
(a)
(d)
LACTOSE
PLATELET LIPID II
NEURAMINYLLACTOSE
(b)
(d)
PLATELET LIPID V
(a)
(c) (d)
FABRY CTH
PLATELET LIPID III
(b) (c) (d)
PORCINE GL-4
PLATELET LIPID IV
TIME

Figure 13

Table 11. Retention Times of Partially Methylated Alditol Acetates from Platelet Glycosphingolipids[a]

	GLC Peaks			
	(a)	(b)	(c)	(d)
Lactose	15.9	---	---	32.9
Lipid II	16.3	---	---	32.7
Fabry kidney trihexosylceramide	16.3	---	31.7	33.2
Lipid III	16.3	---	31.7	32.9
Porcine globoside	---	29.9	31.5	33.0
Lipid IV	---	30.1	31.8	33.8
N-acetylneuraminyllactose	---	30.1	---	33.3
Lipid V	---	29.7	---	32.9

[a]Peaks of partially methylated alditol acetates are represented as (a), 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol; (b), 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol; (c), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol; and (d), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol.

Table 12. Mass Spectrometric Identification of Methylated Alditol Acetates from Platelet Glycosphingolipids. Numbers are expressed as percentage of peak intensity to mass number 43 according to Bjorndal et al. (246-248).

Intensity m/e	Lactose		Platelet GL-2[a]		Fabry GL-3A			Platelet GL-3[a]			Porcine RBC Gl-4			Platelet GL-4			N-Acetyl-neuraminyl-lactose		Platelet G_{M3} (lower phase)	
	(a)	(d)	(a)	(d)	(a)	(c)	(d)	(a)	(c)	(d)	(b)	(c)	(d)	(b)	(c)	(d)	(b)	(d)	(b)	(d)
43	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
45	36	18	32	16	36	18	17	34	17	17	27	20	18	24	18	14	27	18	24	17
71	11	6	8	5	8	6	5	7	6	4	7	6	5	6	5	6	8	8	7	5
75	5	3	5	3	4	3	4	4	3	3	4	4	4	2	4	3	-	3	-	3
87	14	14	14	16	13	16	14	14	14	15	10	15	16	9	14	13	8	17	7	16
99	-	12	-	9	-	10	11	-	12	12	7	12	14	5	9	10	6	15	3	12
101	42	16	30	13	33	16	15	32	15	16	19	16	17	15	16	13	19	17	17	17
113	5	20	6	21	6	20	18	5	20	19	-	22	20	-	21	20	-	22	-	19
117	32	41	27	41	28	38	39	29	38	42	51	42	41	46	40	45	49	41	52	42
127	-	-	-	-	-	-	-	-	-	-	5	-	-	5	-	-	6	-	4	-
129	25	7	20	6	23	9	9	22	7	6	30	7	6	26	8	4	29	6	31	5
145	25	-	16	-	18	-	-	17	-	-	-	-	-	-	-	-	-	-	-	-
161	18	3	14	4	15	4	4	15	4	4	15	4	3	14	4	3	15	2	15	2
205	10	-	7	-	8	-	-	8	-	-	-	-	-	-	-	-	-	-	-	-
233	-	11	-	11	-	9	10	-	9	12	6	10	12	5	9	12	6	13	8	15

Peak (a), 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol
Peak (b), 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol
Peak (c), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol
Peak (d), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol

c. Lipid III (ceramide trihexoside)

Analysis of the methylated derivatives of the platelet trihexoside disclosed three peaks with retention times identical with α and β methyl-2,3,4,6-tetra-O-methyl-galactoside, α and β methyl-2,3,6-tri-O-methyl-galactoside and α and β methyl-2,3,6-tri-O-methyl-glucoside derived from the corresponding standards (Figure 12 and Table 10). Partially methylated alditol acetates gave three peaks with retention times identical with those of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol by GLC (Figure 13 and Table 11). With the reasonable assumption, based on previous reports (51,106,263), that the glucose is linked 1→1 to ceramide the gas chromatographic evidence is thus consistent with the characterization of the trihexoside as Gal-(1→4)-Gal-(1→4)-Glc-(1→1)-ceramide.

d. Lipid IV (ceramide tetrahexoside)

After methylation and acid hydrolysis of the neutral glycosphingolipid IV, GLC of partially methylated hexoses revealed three peaks (Figure 12 and Table 10) corresponding to the retention times of α and β methyl-2,3,6- and -2,4,6-tri-O-methyl-galactosides and α and β methyl-2,3,6-tri-O-methyl-glucoside, respectively. After reduction and acetylation, three peaks having the same retention times as 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol were obtained from the ceramide tetrahexoside

(Figure 13 and Table 11). These results indicate that the two galactose residues are linked to the glucose unit in a similar fashion as that of the platelet GL-3a, with the exception of an additional N-acetylgalactosamine which is attached to C-3 of the external galactose moiety. Hence, the structure of this tetrahexoside is GalNAc-(1→3)-Gal-(1→4)-Gal-(1→4)-Glc-(1→1)-ceramide.

e. Lipid V (sialo-dihexosylceramide)

GLC of partially methylated monosaccharides after methylation and hydrolysis of intact platelet acidic glycosphingolipid V disclosed the presence of α and β methyl-2,3,6-tri-O-methyl-glucoside and α and β methyl-2,4,6-tri-O-methyl-galactoside (Figure 12 and Table 10). GLC of the partially methylated alditol acetates gave two peaks which corresponded to the retention times of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol (Figure 13 and Table 11). These experiments revealed that the structure of the carbohydrate chain of this ganglioside was identical with the N-acetylneuraminyllactose standard. Thus, the structure of this ganglioside is NANA-(2→3)-Gal-(1→4)-Glc-(1→1)-ceramide.

7. Enzymatic Hydrolysis of Platelet Neutral Glycosphingolipids

A TLC separation of products resulting from incubation of platelet neutral glycosphingolipids with specific glycosidases is shown in Figure 14.

Figure 14. Thin-layer chromatography of platelet glycosphingolipids and hydrolysis products by various glycosidases.

Lane 1, reference standard glycosphingolipids from top to bottom are glucosylceramide (GL-1a), lactosylceramide (GL-2a), Fabry trihexosylceramide (GL-3a) and porcine globoside (GL-4). Lane 2, hydrolysate of platelet GL-2a with fig α-galactosidase, showing no reaction and recovered original GL-2a. Lane 3, hydrolysate of platelet GL-2a with jack bean β-galactosidase, showing GL-1a and a small amount of unhydrolyzed GL-2a. Lane 4, hydrolysate of platelet GL-3a with fig α-galactosidase, showing GL-2a as the hydrolyzed product. Lane 5, hydrolysate of platelet GL-3a with jack bean β-galactosidase, showing no reaction and recovered the original GL-3a. Lane 6, hydrolysate of platelet GL-3a with fig α-galactosidase plus jack bean β-galactosidase, containing GL-1a only. Lane 7, hydrolysate of platelet GL-4 with α-N-acetylhexosaminidase, no reaction occurred and recovered the original GL-4. Lane 8, hydrolysate of platelet GL-4 with β-N-acetylhexosaminidase showing a product of GL-3a and a α-galactosidase contaminant. Lane 9, original platelet GL-2a without enzyme treatment. Lane 10, original platelet GL-3a without enzyme treatment. Lane 11, original platelet GL-4 without enzyme treatment. Lane 12, reference standard glycosphingolipids same as in lane 1. A 250μ pre-coated silica gel G plate (Analtech, Inc.) was used with a solvent system of chloroform-methanol-water (100:42:6).

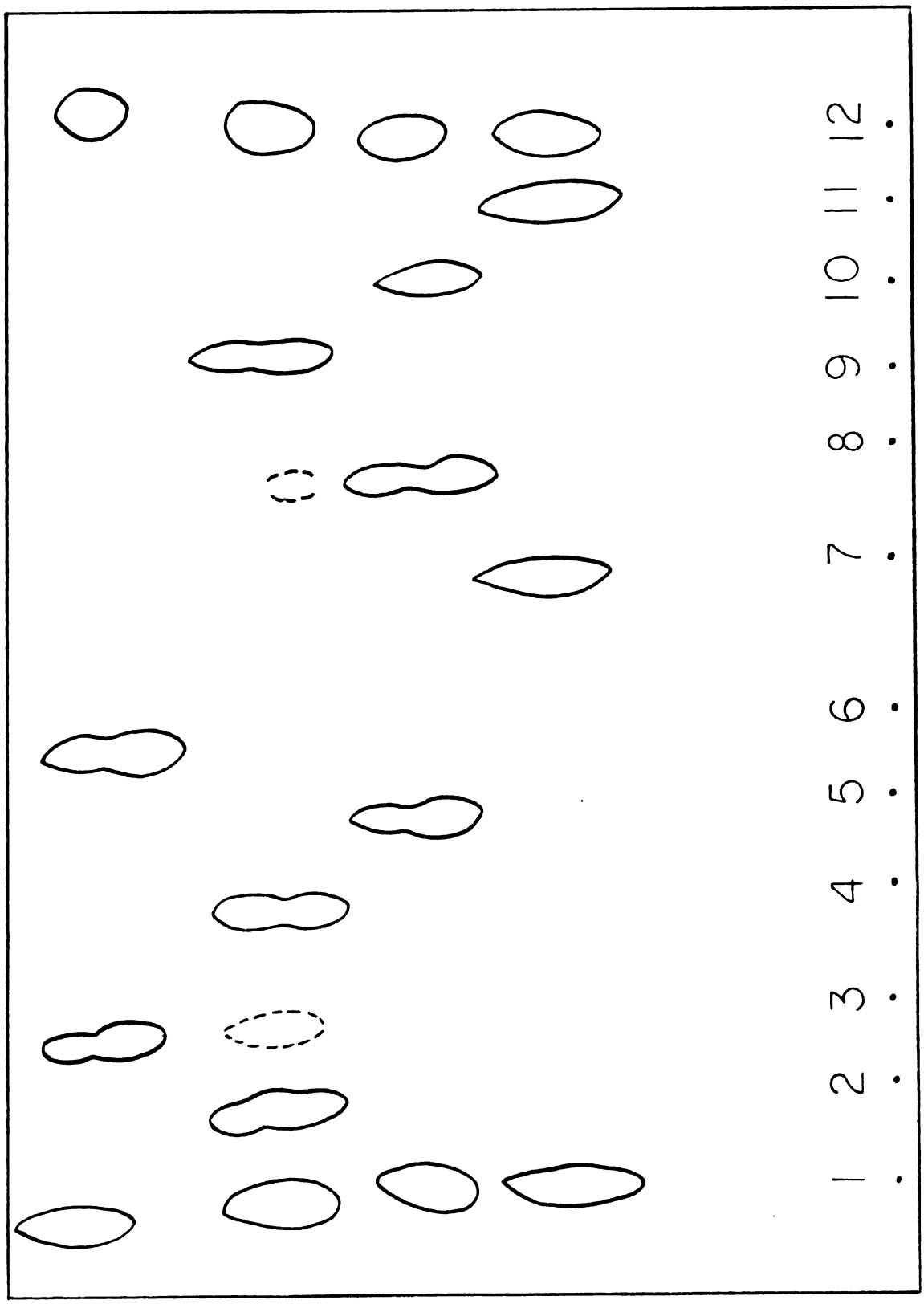


Figure 14

a. Platelet GL-4

When pig liver α-N-acetylhexosaminidase was incubated with GL-4, no reaction occurred. Jack bean β-N-acetylhexosaminidase, however, hydrolyzed the lipid to a product with TLC behavior similar to Fabry GL-3a and platelet GL-3a.

b. Platelet GL-3a

Degradation of platelet GL-3a to ceramide dihexoside took place with fig α-galactosidase but not with jack bean β-galactosidase. Combined reaction of fig α-galactosidase and jack bean β-galactosidase with GL-3a produced one component with a TLC migration rate similar to that of GL-1a.

c. Platelet GL-2a

When fig α-galactosidase was incubated with platelet GL-2a, no reaction occurred; jack bean β-galactosidase cleaved the lipid to a product with TLC properties similar to those of GL-1a. The yield of the reaction was limited because of lower activity of the enzyme and the excessive amount of substrate present in the reaction mixture.

These results showed that the stereochemical configuration between the galactose and glucose was β in GL-2a, and the anomeric linkage between the two galactose residues in GL-3a had an α-configuration while the internal galactose was β-glycosidically linked to the glucose. In GL-4, the anomeric linkage between the N-acetylgalactosamine and galactose had a β-configuration. Due to the limited supply of the galactosidases the hydrolysis product of GL-4 was not

degraded further by these enzymes. Nevertheless, in view of the similar TLC behavior of the hydrolyzed product with that of the authentic platelet GL-3a standard, it is likely that the anomeric configurations in the product are identical with those of platelet GL-3a. Therefore, the complete structures of platelet glycosphingolipids are tentatively assigned as Glc-(1→1)-ceramide (GL-1a), Gal-(β1→4)-Glc-(1→1)-ceramide (GL-2a), Gal-(α1→4)-Gal-(β1→4)-Glc-(1→1)-ceramide (GL-3a) and GalNAc-(β1→3)-Gal-(α1→4)-Gal-(β1→4)-Glc-(1→1)-ceramide (GL-4), respectively. The stereochemical configurations of the glycosidic linkages in GL-1a and G_{M3} ganglioside were not determined.

8. Fatty Acid Composition of Platelet Glycosphingolipids

The fatty acid compositions of platelet glycosphingolipids are listed in Table 13. All of the lipids contained primarily 20:0, 22:0, 24:0 and 24:1, although 16:0 and 18:0 were also present. 2-Hydroxy acids were not found. Long-chain fatty acids ($\geq$20:0) constituted 73-77% of the total normal fatty acids in GL-2a, GL-3a, GL-4 and G_{M3}, whereas 9-14% of normal acids were dominated by 16:0 and 18:0. GL-1a showed a high percentage of 18:0 (64.0%) and 18:1 (21.9%) with less of 16:0 (3.0%) and the long-chain acids.

Table 13. Fatty Acid Composition of Human Plasma, Erythrocyte and Platelet Glycosphingolipids[a]

	Ceramide	GL-1		GL-2		GL-3		GL-4		G_{M3} (lower phase)		
Component	Platelet	Platelet	RBC	Platelet	RBC	Platelet	RBC	Platelet	RBC	Platelet	RBC	Plasma
						% of total fatty acids						
14:0	0.5	0.6	-	1.3	-	0.8	-	1.0	-	tr.	-	0.7
15:0	tr.	1.0	-	0.6	-	0.8	-	1.1	-	tr.	-	0.8
16:0	13.4	3.0	4.9	9.7	9.9	6.6	2.4	5.4	3.2	8.6	5.5	10.7
17:0	0.7	1.8	-	0.7	-	1.3	-	1.9	-	1.7	-	1.4
18:0	14.7	64.0	6.6	3.3	3.5	4.3	2.3	3.4	1.6	5.5	4.6	10.9
18:1	1.4	21.9	4.1	1.5	tr.	1.1	tr.	1.2	1.0	1.1	tr.	3.5
18:2	-	3.4	-	0.7	-	1.1	-	1.7	-	1.4	-	-
20:0	12.9	2.2	7.0	7.0	2.8	7.1	-	6.2	1.3	5.8	4.5	5.3
22:0	29.2	tr.	7.5	34.7	13.5	35.7	14.4	37.6	20.6	33.9	21.1	23.0
22:1	tr.	tr.	14.6	4.2	3.3	4.2	2.6	tr.	-	4.1	5.3	-
23:0	4.0	-	5.4	3.2	-	3.0	-	3.5	4.0	3.4	3.9	9.9
24:0	22.4	-	27.0	20.1	40.9	20.3	48.1	29.6	49.1	21.3	38.1	18.9
24:1	tr.	-	12.8	11.8	23.4	13.0	28.2	6.7	18.8	10.5	5.1	9.0
Others	0.7	3.0	10.0	1.0	2.5	0.6	2.0	0.6	0.4	3.2	12.0	5.8

[a]Fatty acids are denoted as chain length:number of double bonds. The amounts found are given as a percentage of total area on gas chromatograms.

9. Concentrations of Platelet Glycosphingolipids

The composition of the platelet glycosphingolipid fraction is shown in Table 9, in which the average from duplicate analyses are reported. It is evident that GL-2a was the major neutral glycosphingolipid in the platelet lipids, and accounted for 64% of the total neutral glycosphingolipid fraction. There was an appreciable amount of G_{M3}, GL-3a and GL-4 in platelets too, but GL-1a was present in rather small amounts as compared with other tissues.

10. Platelet Gangliosides

Total lipids, extracted from 73 units of trypsinized platelets, were partitioned at the National Red Cross Blood Research Laboratory according to the method of Folch et al. (232) using 0.75% of NaCl solution. In so doing, the upper phase was accidentally discarded by Dr. Jamieson. Partition of the lipid with a salt solution suppresses the loss of polar lipids into the upper phase, but it leads to considerable losses of the less polar gangliosides (such as G_{M3}) into the chloroform phase (264). Since deionized water produces maximal partition of extraneural gangliosides (such as kidney) into the upper phase (264), the lower phase was partitioned a second time, using water according to the method of Folch et al. Thus it was hoped that some of the platelet gangliosides could be isolated for structural analysis.

On TLC (Figure 7, lane 2) the water-soluble platelet lipids from the upper phase revealed 3 bands (A, B, and C; in the order of their distance from the solvent front). Band A was the major component and the fastest moving band. Gas-liquid chromatograms of the trimethylsilylated methyl glycosides derived from these bands showed a ratio of galactose to glucose of 1.04, 1.03 and 0.86 for Band A, B and C, respectively. The molar ratio of N-acylneuraminic acid to glucose was 0.83, 0.53 and 0.40, respectively. In addition, N-acetylgalactosamine was shown to be present in Band C and exhibited a ratio of N-acetylgalactosamine to glucose of 0.40.

Table 14 shows the normal fatty acid distribution of platelet gangliosides from the upper phase. Of the normal acids, $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{24:1}$ were predominant in Band A; fatty acids with a chain length greater than $C_{20:0}$ constituted 80% of the total normal acids. Palmitate and stearate were the major acids in Bands B and C and accounted for 59 and 65% of the total. There was substantially less $C_{22:0}$, $C_{24:0}$ and $C_{24:1}$ than in the mixture for the A band. N-acetylneuraminic acid was detected in Band A. Sphingosine was the major long-chain base in Bands A and B, along with a trace of sphinganine.

Because of the unusually high molar ratio of N-acetylneuraminic acid to glucose from Band A, the possibility was not ruled out that Band A was a disialohematoside (G_{D3}

Table 14. Fatty Acid Composition of Human Platelet Gangliosides from Folch Upper Phase[a]

Component	Band A	Band B	Band c
	% of total fatty acids		
14:0	1.4	3.5	3.9
15:0	0.7	1.7	1.9
16:0	13.5	27.7	30.8
17:0	tr.	1.4	1.5
18:0	6.9	13.8	15.3
18:1	5.0	7.7	8.5
18:2	---	---	---
20:0	10.0	3.4	3.8
22:0	39.5	12.5	13.9
22:1	tr.	---	tr.
23:0	2.8	10.1	tr.
24:0	13.3	11.2	12.5
24:1	5.2	2.1	2.4
others	1.8	4.9	4.4

[a]Fatty acids are denoted as chain length:number of double bonds. The amounts are given as a percentage of total area on gas chromatograms.

ganglioside). Ganglioside A was subjected to mass spectrometric analysis in the direct probe inlet of an LKB 9000 single-focusing mass spectrometer according to a previously published method (265). Ions from N-acetylneuraminic acid were observed at m/e 173, 186 and 205. No detectable peaks were observed at m/e 261 and 274 which rules out the possibility of N-glycolyl forms (a shift of ion location by 88 mass units). The mass spectra also disclosed the presence of sphingosine (m/e 311) as the major long-chain base with sphinganine (m/e 313) as the minor component.

These results suggest that ganglioside A is an N-acetylhematoside, and ganglioside B is probably also a hematoside with a different fatty acid composition. Ganglioside C is assumed to be similar to G_{M2}, but this needs to be verified by more detailed analyses. No other slow-migrating gangliosides (G_{D1a}, G_{D1b} and T_{T1}) were detected, but it is likely that these gangliosides were lost in the first aqueous salt partition.

11. Platelet Sphingolipids

a. Ceramide

Ceramide from trypsinized platelets was further purified by thin-layer chromatography (see Method). A single band was obtained, suggesting that there were no ceramides containing α-hydroxy fatty acids. After acid-catalyzed methanolysis, the sphingosine content and ester groups were determined

colorimetrically. The molar ratios of bases to esters were 0.77/1.00 and 0.80/1.00 in duplicate analyses. 4-Sphingenine (83.2%) and sphinganine (9.8%) were the long-chain bases present. The fatty acids were mainly 20:0, 22:0 and 24:0.

b. Sphingomyelin

Sphingomyelin was obtained from the crude phospholipid fraction isolated by silicic acid chromatography. 4-Sphingenine (75%) was the major long-chain base, sphinganine (15%) was also present, and minor peaks were identified as hexadecasphingenine (5%), heptadecasphingenine (2%) and octadecasphingadienine (3%). Platelet sphingomyelin contained a high proportion of the long-chain saturated fatty acids 22:0 (20.3%), 24:0 (8.2%) and 24:1 (24.0%).

12. Sphingolipid Content of Trypsin-treated and Non-treated Platelets

An attempt was made to determine whether there was a difference between the glycosphingolipid content of trypsin-treated and non-treated platelets. As shown in Table 9, the concentration of hematoside was much lower in platelets that were not treated with trypsin; otherwise, the composition was not changed by trypsin treatment. A more detailed investigation of the effect of proteolytic enzymes on the G_{M3} and ceramide levels in platelets is summarized in Table 15. The concentration of G_{M3} was increased in all of the treated platelets. Ceramide levels were unaffected by pre-treatment of the platelets with the proteolytic enzymes.

Table 15. Concentration of Ceramide and Hematoside in Platelets Treated with Proteolytic Enzymes[a]

Lipid Component	Control	Trypsin	Chymotrypsin	Thrombin
		µmoles/g TL		
G_{M3}	0.26	0.46	0.73	1.25
Ceramide[b]	9.85	9.44	8.59	9.08

[a]These analyses were carried out on separate batches of approximately 3 units of platelet concentrate (1 platelet unit is from 450 ml of whole blood), incubated with trypsin (1 mg/unit), thrombin (100 NIH units/unit), and chymotrypsin (1 mg/unit), respectively, at 37°C for 30 min (except chymotrypsin which was incubated for 60 min) in 0.85% saline at pH 6.7 before extraction of the lipids. The control was incubated in the same way without added proteolytic enzyme.

[b]Average of two determinations, each in duplicate.

The concentration of ceramide (washed platelets) was surprisingly high and accounted for 55% of the observed total sphingolipids including G_{M3}.

13. Platelet Phospholipids

Platelet phospholipids were studied by TLC using two different techniques. Development in two dimensions was utilized for identification only, while the one dimensional system was used for preparative isolation of individual components. Phosphatidylcholine (230.4 µmoles P/g total lipid), phosphatidylethanolamine (128.4 µmoles P/g total lipid) and sphingomyelin (131.6 µmoles P/gm total lipid) were the major

phospholipids in this mixture. Phosphatidylinositol could not be found in the methanol fraction from the silicic acid column. This is probably due to the fact that phosphatidylinositol can be eluted from the silicic acid column in acetone-methanol 9:1 (v/v) fraction (105), and is destroyed by the mild alkaline hydrolysis step. Furthermore, initial studies indicated the presence of inositol in a ganglioside fraction from upper phase of Folch wash. The inositol was not free, however, since direct trimethylsilylation of the intact lipid gave no inositol peaks on GLC. Nor was free inositol liberated from lyophilized lipid of upper phase by mild alkali-catalyzed methanolysis. It has been concluded that the upper phase lipid fraction contained a phosphoinositide.

14. Fatty Acid Composition of Platelet Phospholipids

The major fatty acids in phosphatidylcholine and phosphatidylethanolamine were 16:0 (26.6 and 4.0%), 18:0 (18.9 and 20.4%), 18:1 (30.6 and 7.9%) and 20:4 (15.1 and 34.9%) whereas phosphatidylserine contained mostly 18:0 (47.9%), 18:1 (15.9%) and 20:4 (15.0%). The phosphatidylethanolamine fraction also contained dimethylacetals of 16:0 (5.8%) and 18:0 (10.0%) derived from the plasmalogen phosphatidylethanolamine.

15. Platelet Neutral Lipids

Beside ceramide, the platelet neutral lipid fraction consisted of triglycerides, cholesterol, cholesterol esters and free fatty acids that were recovered from the chloroform fraction after silicic acid chromatography.

Cholesterol, cholesterol ester, triglyceride and free fatty acids were identified by TLC. Palmitate (33.4%), stearate (29.2%), oleate (23.6%) and linoleate (4.2%) were the major fatty acids found in the free fatty acid fraction, while cholesterol esters and triglycerides contained 16:0 (15.7 and 32.4%), 16:1 (30.2 and 10.3%), 18:0 (7.1 and 15.9%) and 18:1 (32.9 and 20.0%) as the major constituents. Triglycerides contained some linoleate (6.2%) as well, but this component was nearly absent in the cholesterol ester fraction.

C. Isolation and Quantitative Determination of Porcine Platelet Glycosphingolipids

1. Porcine Platelet Concentrates

Porcine platelet concentrates were obtained from ten liters of whole blood by differential centrifugation. The final suspension contained 2.78×10^7 platelets, 3230 erythrocytes and 1480 leukocytes per mm^3. Homogeneity of the platelet concentrates was observed by microscopic examination of the air-dried smear prepared from the suspension. Electron microscopic examination of the suspension showed a detailed

structure of the platelets similar to those reported previously (266,267).

2. Porcine Platelet Ceramides

Analysis of the ceramide fraction (chloroform-methanol 98:2) revealed three separate bands, designated ceramide A, B, and C. A typical preparative TLC of the ceramide fraction is shown in Figure 15. Band A was most abundant, followed by B and C (very faint bands). Band A migrated in the same area as the ceramide standard containing normal fatty acids. Band B was assumed to be the same general type of ceramide with different chain lengths of acyl groups. Band C was the slowest moving band, and was assumed to be due to the presence of α-hydroxy fatty acids as the acyl moieties, since it had the same R_F values as the ceramide standard containing hydroxy fatty acids.

3. Glycosphingolipid Content of Porcine Platelets

Glycosphingolipids were isolated from crude total lipid (300 mg) from 1.89 g of platelets by a combination of silicic acid and thin-layer chromatography. Thin-layer chromatography disclosed the presence of four neutral glycosphingolipids and hematoside. In addition, an extra band was observed between GL-2a and GL-3a. Previous experience indicated that sulfatide exhibited similar TLC behavior in the neutral solvent system.

Figure 15. Thin-layer chromatography of ceramides from normal porcine platelets.

The ceramide fraction, isolated from the total lipids by silicic acid chromatography (chloroform-methanol 98:2 fraction), were further purified on a 250μ silica gel G Quantum plate with chloroform-methanol 95:5 (v/v) as the developing solvent. Lane 1, reference standard ceramide containing normal fatty acids. In lane 2, reference standard ceramide containing 2-hydroxy fatty acids. In lane 3, ceramides from porcine platelets.

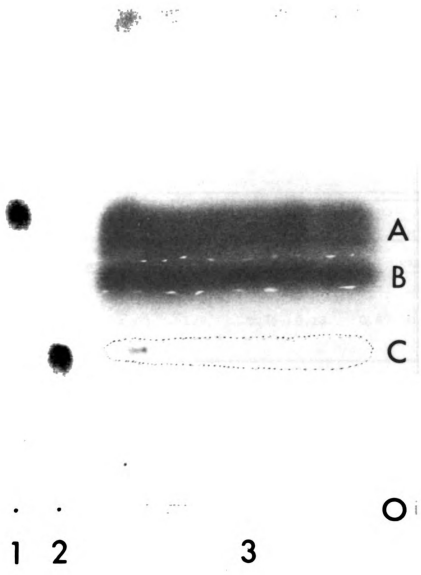


Figure 15

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Gas-liquid chromatography of the methyl glycosides derived from these lipids gave molar ratios indicating these fractions were GL-1a, GL-2a, GL-3a, GL-4 and G_{M3}. The molar ratio and concentration of each of the glycosphingolipids are given in Table 16. It is evident that GL-3a was the major neutral glycosphingolipid in the porcine platelet lipids, accounting for 52% of the total neutral glycosphingolipid fraction.

Table 16. Concentration of Glycosphingolipids in Porcine Platelets

GL-1a		GL-2a		GL-3a		GL-4			G_{M3}	
μmoles g TL	Gal Glc	μmoles g TL	Gal Glc	μmoles g TL	Gal Glc	GalNAc Glc	μmoles g TL	Gal Glc	NANA Glc	μmoles g TL
0.38	1.2	0.42	1.9	1.05	1.7	0.70	0.18	0.87	0.60	0.24

D. Globoside Concentration in Fetal Pigs

Total lipids were extracted from the erythrocytes of the gilt (5.2 ml), 90-day fetus (3.0 ml) and 45-day fetus (1.0 ml), respectively. Glycosphingolipids were isolated and purified by TLC as before. The concentration of GL-4 was determined quantitatively and is summarized below in Table 17. It is obvious that the red cell GL-4 concentration in the 45-day fetus is low when compared to the normal adult which is usually

Table 17. Concentration of Globoside in Fetal Pig Erythrocytes

	Gal / Glc	GalNAc / Glc	nmoles / ml RBC
Gilt	2.00	0.72	234.6
90-day fetus	2.07	0.75	166.7
45-day fetus	2.02	0.81	130.0

around 20 μmoles/100 ml red blood cells, whereas the concentration of this glycolipid was at the lower normal range in red cells from a 90-day fetus. If one takes into account the contamination of the fetal blood (45-day) by the gilt's blood during sampling, the actual GL-4 concentration is probably lower than that reported here, but this possibility must be confirmed.

E. Radioactive Glucocerebroside

1. Isolation of Glucosylsphingosine

The purity of the hydrolyzed product from Gaucher spleen GL-1a was determined by TLC (chloroform-methanol-2.5 NNH_4OH 60:40:9) after silicic acid chromatography, using a glycosphingolipid mixture (GL-1a, GL-2a, GL-3a and GL-4) and galactosylsphingosine (psychosine) as standards. As shown in Figure 16, glucosylsphingosine migrated slightly ahead of the galactosylsphingosine. The chloroform-methanol (8.5:1.5)

Figure 16. Thin-layer chromatography of the hydrolysis product from Gaucher spleen Glucosylceramide.

In lane 1, methanol eluate from silicic acid chromatography containing exclusively glucosylsphingosine. In lane 2, the chloroform-methanol (8.5:1.5) fraction from silicic acid chromatography containing some glucosylceramide and a small amount of unhydrolyzed GL-1a. In land 3, reference standard glycosphingolipids from top to bottom are GL-1a, GL-2a, GL-3a and GL-4. In lane 4, standard galactosylsphingosine (psychosine). The plate was developed in chloroform-methanol-2.5 N NH_4OH (60:40:9).

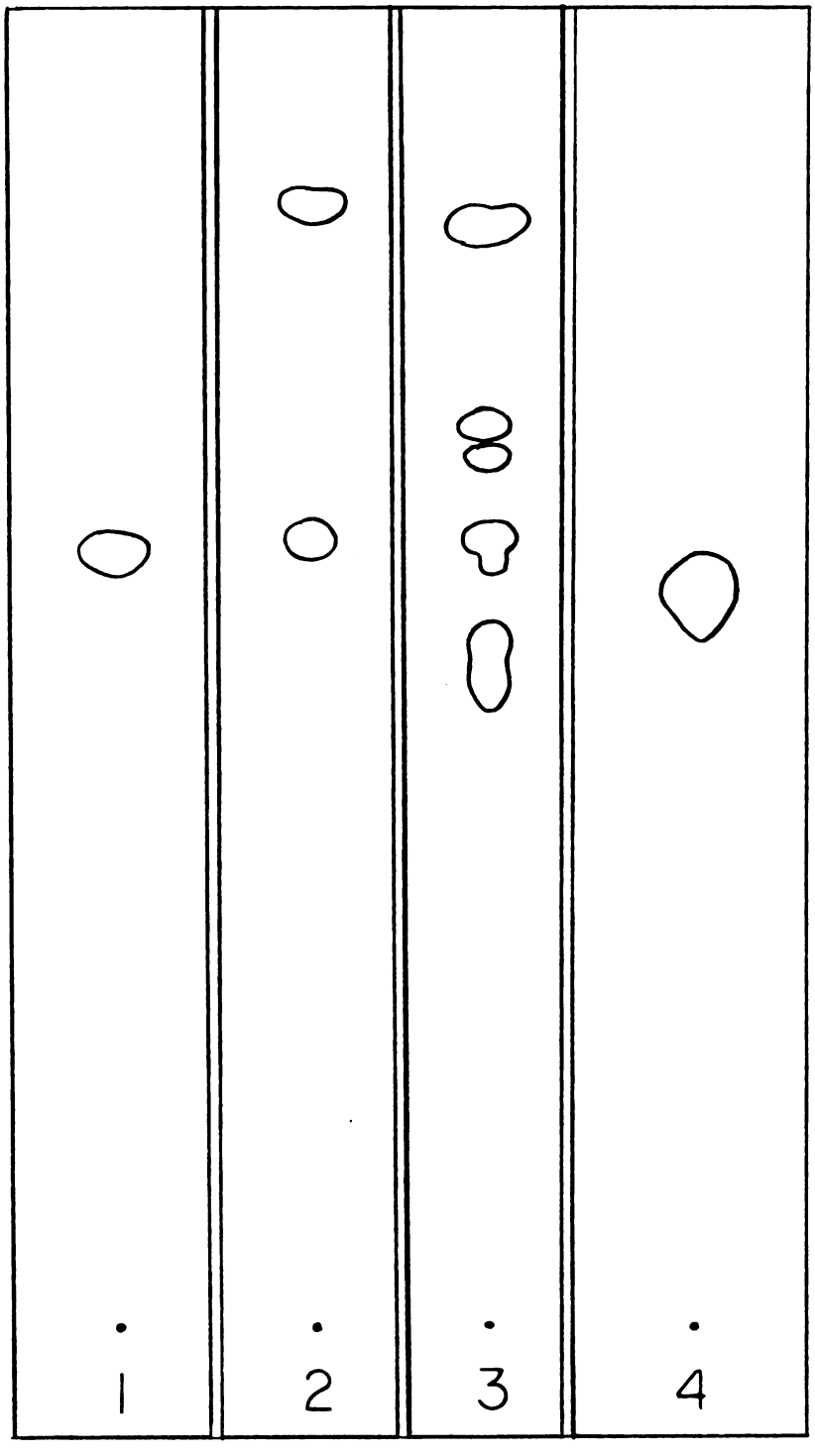


Figure 16

fraction contained some glucosylsphingosine and a small amount of unhydrolyzed GL-1a, while the methanol eluate contained exclusively glucosylsphingosine. After preparative TLC, the combined weight of the recovered glucosylsphingosine was 7.9 mg (40% yield).

2. Radioactive Glucocerebroside ([^{14}C] stearic acid)

The product of the coupling reaction was isolated as described under Methods by combined silicic acid chromatography and TLC on silica gel G. After development of the plate, the labeled GL-1a was eluted from the gel and weighed. The material (4 mg) was dissolved in 4 ml of chloroform-methanol 2:1 and 10 µl was removed for radioactivity counting. There was 456,567 cpm in 10 µl of material and hence 183 x 10^6 cpm in 4 ml of total material. With a counting efficiency of 96%, 79.7 µCi of radioactivity was recovered in the 4 mg sample, indicating a specific activity of 0.02 µCi/µg. Assuming a molecular weight of 727 (stearoylpsychosine), the specific activity of the radioactive GL-1a was 14.5 µCi/µmole.

3. Proof of the Radioactive Glucocerebroside

To prove that the synthesized radioactive GL-1a was composed of glucose, sphingosine, and fatty acids in a 1:1:1 molar ratio, the product was subjected to acid-catalyzed methanolysis and the individual components were separated and examined by GLC. Figure 17 shows the presence of long-chain bases (tracing at upper right corner) and methyl glucosides

Figure 17. Gas-liquid chromatography of trimethylsilyl methyl glycosides and sphingosines from [^{14}C] glucosylceramide.

The methyl glycosides and long-chain bases of [^{14}C]Gl-1a were recovered after acid-catalyzed methanolysis by solvent partition and trimethylsilylated with pyridine-hexamethyldisilazane-trimethylchlorosilane (10:4:2). The trimethylsilyl derivatives were analyzed with a Hewlett-Packard 402 Gas Chromatograph equipped with a 6 ft. 3% SE-30 column. The trimethylsilyl methyl glycosides were analyzed at 165°C isothermally. The O-trimethylsilyl derivatives of long-chain bases were analyzed by linear temperature programming on the same column, with an initial temperature of 160°C and a programming rate of 2°C/min to an upper temperature of 260°C. Top tracing, long-chain bases from [^{14}C] GL-1a. Bottom tracing, methyl glucoside from [^{14}C]GL-1a, mannitol was added as a marker.

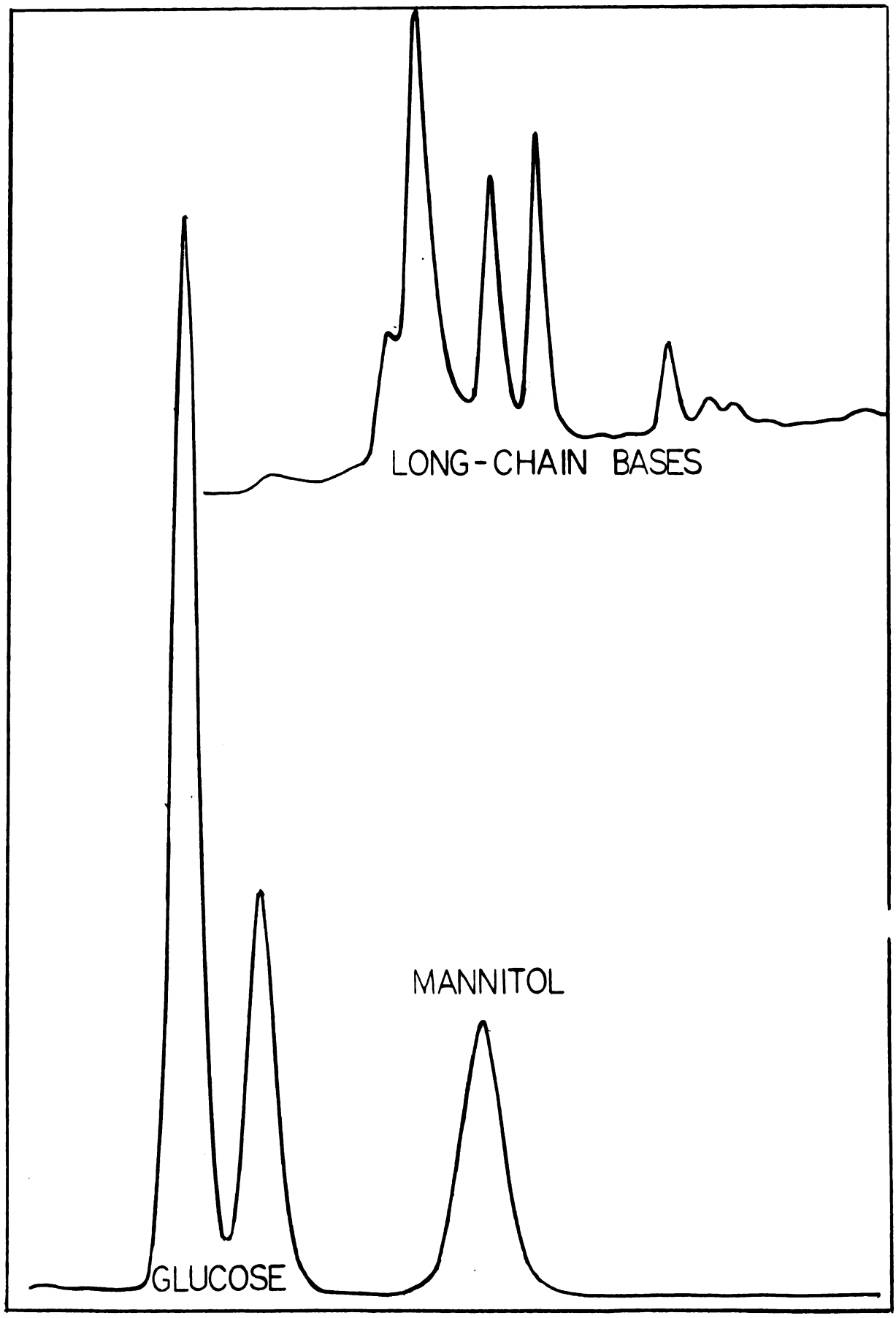


Figure 17

(tracing at lower left corner). Figure 18 shows the fatty acid methyl ester derived from the synthesized radioactive compound. Stearic acid was the only fatty acid present.

In addition, the fatty acid methyl ester was monitored for radioactivity. An aliquot of the isolated $C_{18:0}$ fatty acid was injected into a 3% SE-30 column in an F and M Model 400 Gas-liquid Chromatograph equipped with a Barber-Coleman Model 5190 Radioactivity Monitor. Radioactivity was associated exclusively with methyl stearate from the GLC column. The amount of radioactivity was determined by counting 0.5 µl of a 0.5 ml sample in the scintillation counter. The registered counts were 215,200. The amount of radioactivity was calculated to be 9.4 µCi in the 0.5 mg sample. The recovery of ^{14}C-label in the hexane extracts after acid-catalyzed methanolysis was estimated to be approximately 95%. These results indicate that the synthetic product of the coupling reaction is a cerebroside containing glucose, sphingosine and radioactive stearic acid.

4. In Vivo Experiment

After establishing the identity of the synthetic GL-1a, an experiment was planned to administer the labeled GL-1a intravenously into a pig so that the turnover of plasma and erythrocyte GL-1a could be studied by sampling blood according to a prescribed time course. A 10 kg Yorkshire pig was chosen from the Swine Research Center. The labeled GL-1a was

Figure 18. Gas-liquid chromatography of fatty acid methyl ester from [^{14}C]glucosylceramide.

The fatty acid methyl ester of [^{14}C]GL-1a was recovered from the acidic methanolysate by hexane estraction. The methyl ester was then analyzed with a Hewlett-Packard 402 Gas Chromatograph equipped with a 6 ft. 15% ethylene glycol adipate column maintained at 190°C. Top tracing, standard fatty acid methyl esters: peak 1, $C_{16:0}$; peak 2, $C_{18:0}$; and peak 3, $C_{20:0}$. Bottom tracing, stearic acid methyl ester derived from [^{14}C]GL-1a after acid-catalyzed methanolysis.

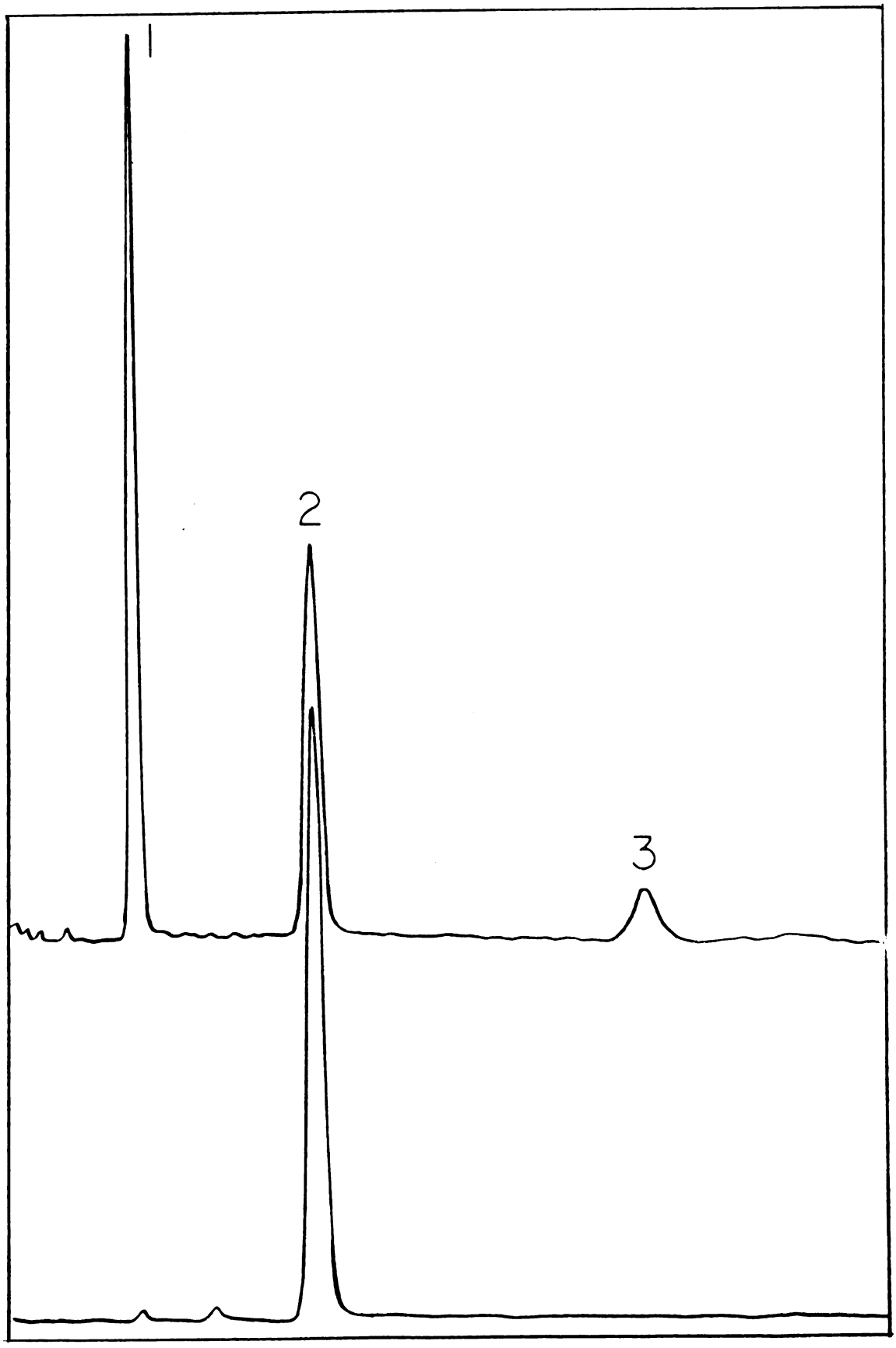


Figure 18

dissolved in 1 ml of ethanol and injected into the restrained pig, but unfortunately, one of pig's feet got loose during the injection and subsequently displaced the needle at the site of injection. The experiment was discontinued.

F. In Vitro Study

Incubation of intact red cells in plasma containing [^{14}C] glucose led to the incorporation of labeled glucose mainly into GL-1a of the neutral glycosphingolipid fraction of the red cells and, to a lesser extent, into GL-2a, as shown in Table 18. GL-3a contained very little radioactivity and no significant amount of activity was detected in GL-4. The radioactivity in GL-1a was localized mainly in the hexose moiety, whereas the amount of label recovered in the long-chain base and fatty acid fractions was progressively greater with increasing size of the oligosaccharide group. In GL-3a about 50% of the radioactivity was in the fatty acid fraction.

Analyses of the neutral glycosphingolipid fractions from plasma revealed significant incorporation of [^{14}C] glucose into GL-1a. The label was found exclusively in the hexose moiety, as shown in Table 19. The amount of label present in the long-chain bases and fatty acids did not show any increase with increasing chain length of the carbohydrate units.

Table 18. Incorporation of [^{14}C]Glucose into Neutral Glycosphingolipids of Pig Erythrocytes 20 Days Post-Induction of Anemia

The inbucation flask contained 100 ml of whole blood and 20 μCi of [^{14}C] glucose; incubation was done under 100% O_2 for 2 hr at 37°C. The red cell suspension was further fractionated according to age by ultracentrifugation. The lipids of red cells (13 ml) were fractionated on a 4 g silicic acid column. The identity of the neutral glycosphingolipid fractions was established by TLC and quantitated by GLC. Specific activity is expressed as cpm/μmole of lipid component.

Fraction	nmoles/ml	Specific Activity			Radioactivity Distribution (%)		
		Hexose	Base	Fatty Acid	Hexose	Base	Fatty Acid
GL-1a	7.7	940	120	70	83	11	6
GL-2a	10.0	77	54	23	67	23	10
GL-3a	44.6	16	24	35	45	22	33
GL-4	103.1	5	6	7	61	18	21

Table 19. Incorporation of [^{14}C]Glucose into Neutral Glycosphingolipids of Pig Plasma 20 Days Post-Induction of Anemia

The incubation condition was identical to the red cells. The lipids of plasma (50 ml) was also fractionated on a 4 g silicic acid column. Specific activity is expressed as cpm/μmole of lipid component.

Fractions	nmoles/ml	Specific Activity			Radioactivity Distribution (%)		
		Hexose	Base	Fatty Acid	Hexose	Base	Fatty Acid
GL-1a	8.0	2473	30	15	98	1	1
GL-2a	4.4	14	57	24	26	52	22
GL-3a	1.8	44	267	22	31	63	5
GL-4	2.2	32	209	218	23	38	39

These results suggest that the reticulocyte-rich cells are capable of incorporating labeled glucose into the hexose moiety of GL-1a biosynthetically. The synthetic mechanism would probably involve the enzyme glucosyl transferase and ceramide as the substrate, since porcine erythrocytes were shown to contain an appreciable amount of ceramides (9). The data may account for the early source of GL-1a observed previously in the in vivo experiment (45). Recently, similar results had been observed with normal rat red cells in vitro (268). Sloviter (269) has suggested that reticulocytes can synthesize glycosphingolipids, and it is uncertain whether the reticulocytes or erythrocytes were responsible for the biosynthetic activity in this study. The separation of the red cells according to age by ultracentrifugation technique was not complete due to overloading of the red cell suspension. It is likely that the erythrocyte fraction used in the incubation contained most of the reticulocyte population and perhaps some other cells.

The recovery of a significant amount of label in erythrocyte GL-2a suggests that this lipid can also be synthesized in vitro, presumably by stepwise reaction of ceramide with glucosyl and galactosyl transferases. The lack of significant radioactivity in GL-3a and GL-4 indicates that further synthesis does not occur during the 2 hr incubation time.

G. Red Cell Fractionation

1. Separation by Age of Erythrocytes from Normal and Anemic Blood

Separation of a red cell population into groups having different specific gravities was obtained by ultracentrifugation in discontinuous density gradient of bovine serum albumin, devised by Piomelli et al. (259). In this technique, the youngest cells (reticulocyte-rich), which are dense and larger in size, remain on top of the gradient, while the older cells (reticulocyte-poor) are more dense and remain in the lower part of the gradient.

A preliminary experiment with normal and anemic canine blood disclosed six bands after ultracentrifugation, as shown in Figure 19. In the normal dog (tube A), two very faint bands were observed at the top; most of the cells were congregated at Band 3 and 4 with some in Band 5 and 6 and a few cells were at the bottom of the tube (Band 7). In the anemic dog (tube B), the reverse was true. Most of the cells were in Band 1, 2 and 3, some were in 4, and a faint ring of cells were observed in Bands 5 and 6. Each band was recovered and washed extensively with saline, and the volume of packed cells was visually estimated in a calibrated centrifuge tube. Band 7 was not estimated.

No attempts were made to recover cells in between these discrete bands. Cells derived from the individual bands of the anemic dog were suspended in saline and air-dried smears

Figure 19. Separation of young and mature erythrocytes from normal and anemic dogs.

Approximately 1.2 ml each of red cell suspensions from normal and anemic dogs (reticulocyte count, 15-20%) were centrifuged on 6 layers of albumin solution ranging in specific gravity of 1.075-1.100 in cellulose nitrate tubes (1" x 3 1/2"). The tubes were centrifuged at 4°C for 30 min at 25,000 rpm in a Beckman Model LS-50 Ultracentrifuge with a 25.1 swinging bucket. The red cells were fractionated into 7 bands. Band 7 at the bottom of the tube is partly hidden from the picture. A small percentage of cells is dispersed in the albumin layers. A, normal dog. B, anemic dog.

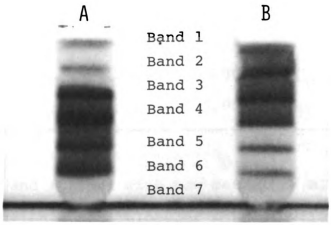


Figure 19

Table 20. Discontinuous Density Gradient Ultracentrifugation of Canine Blood

Bands	Volume of Packed Cells (ml)	
	Normal	Anemic
1	0.05	0.25
2	0.05	0.50
3	0.30	0.10
4	0.45	0.03
5	0.06	0.05
6	0.05	0.05

were prepared and stained with new methylene blue for microscopic examination. Reticulocytes were prevalent in Band 1 and a few were observed in Band 2, but no reticulocytes were seen in Band 3. The cells from Band 1 were large, and the cell size seemed to decrease from Band 1 to Band 6. Photomicrographs were made of these slides; unfortunately, they did not turn out.

2. Separation of Young and Mature Erythrocytes from Normal and Anemic Pig Blood

A similar experiment was carried out with normal and anemic porcine erythrocytes to determine whether pig red cells would behave in the same way as dog erythrocytes in the albumin gradients. The profile of red cell separation by gradient ultracentrifugation was exactly identical to that of

canine red cells. The hemoglobin content of each individual red cell band is listed in Table 21.

3. Electronic Sizing of Fractionated Red Cells

The red cell size distribution of individual bands was obtained by a Celloscope on a blood sample 30 days after giving the glucose label. Mean channel number of each layer was calculated as described in Methods, and is listed below (see Table 22). There was a significant difference in the size of Bands 2 and 5. The small differences observed between the middle bands are probably due to experimental errors.

4. Checking Hemolysis of Erythrocytes in Albumin Solutions

After incubating the pig erythrocytes with the respective albumin solutions at 4°C for 30 min, the cells were washed with saline and centrifuged. The supernatent solutions seemed to be clear. The hemoglobin content of each supernatant solution was recorded and summarized in Table 23.

In addition the Soret band of the albumin and supernatant solutions was examined at 416 nm against saline as blank. The results indicate that there was no significant amount of hemolysis of the red cells in the albumin solutions (see Table 24).

Table 21. Hemoglobin Concentration of Individual Fractions Derived from Normal and Anemic Porcine Blood.

	Normal Pig			Anemic Pig		
Band	Hb[a] (gm %)	% of total	% Hb[a] of each fraction in original sample	Hb[a] (gm %)	% of total	% Hb[a] of each fraction in original sample
1	0.2	1.9	1.8	1.5	17.2	18.7
2	0.2	1.9	1.8	1.3	14.9	16.2
3	1.6	14.8	14.6	2.9	33.3	36.2
4	6.8	62.9	61.8	2.1	24.1	26.2
5	1.3	12.0	11.8	0.5	5.8	6.2
6	0.7	6.5	6.4	0.4	4.6	5.0

[a]Hemoglobin

Table 22. Mean Channel Number of Individual Bands Fractionated by Density Gradient Ultracentrifugation

Bands	Mean Channel Number
2	52
3	49
4	50
5	48
6	45

Table 23. Hemoglobin Content of the Supernatant Solutions

Albumin Concentration (%)	Optical Density of Supernate
30	0.000
32	0.005
34	0.002
36	---
38	0.000
40	0.002

Table 24. Soret Band of the Supernatant Solutions

Albumin Concentration (%)	Optical Density	
	Albumin	Supernate
30	0.506	0.541
32	0.564	0.545
34	0.626	0.580
36	0.607	---
38	0.760	0.730
40	1.53	1.849

H. In Vivo Study

1. Induction of Anemia in Pig 123-6

In pig 123-6 rapid blood regeneration was induced by phlebotomy. The reticulocyte count is the simplest and most effective means of measuring bone marrow activity, since the reticulocyte numbers in the peripheral blood increase to a degree which is related to the severity of the anemia produced (270,271). The hematological profile of this anemic pig is shown in Figure 20. When 10% or more of the blood volume was removed daily from the pig, considerable reticulocytosis developed. The close correlation between reticulocytes, hemoglobin and hematocrit was apparent. Reticulocyte count was expressed as absolute percentage (271), and the error of reticulocyte measurement was estimated to be $\pm$ 5-10%. No attempt was made to classify reticulocytes on the basis of relative maturity.

To assess bone marrow production, correction of the absolute reticulocyte count was made according to the calculation described by Hillman and Finch (271) for humans, using an average packed cell volume and reticulocyte count (absolute %) of 25 and 24, respectively, derived from the results obtained on the first ten days after administration of the ^{14}C-labeled glucose. A corrected reticulocyte count of 12 was obtained, which directly reflects the rate of red cell production. In this connection, the pig was making blood at

Figure 20. Development of anemia in pig 123-6 during the induction period and throughout the *in vivo* study.

Phlebotomy was performed daily from the start of bleeding through day 10. The volume of blood withdrawn daily equaled approximately 10% of the estimated blood volumes. The weight of the pig was maintained constant by restricting the intake of the rations during this period. A single dose of 10 mCi [U-^{14}C]glucose was given intravenously on day 0, and the pig was allowed to gain weight at will from day 11 until the end of the 81-day period.

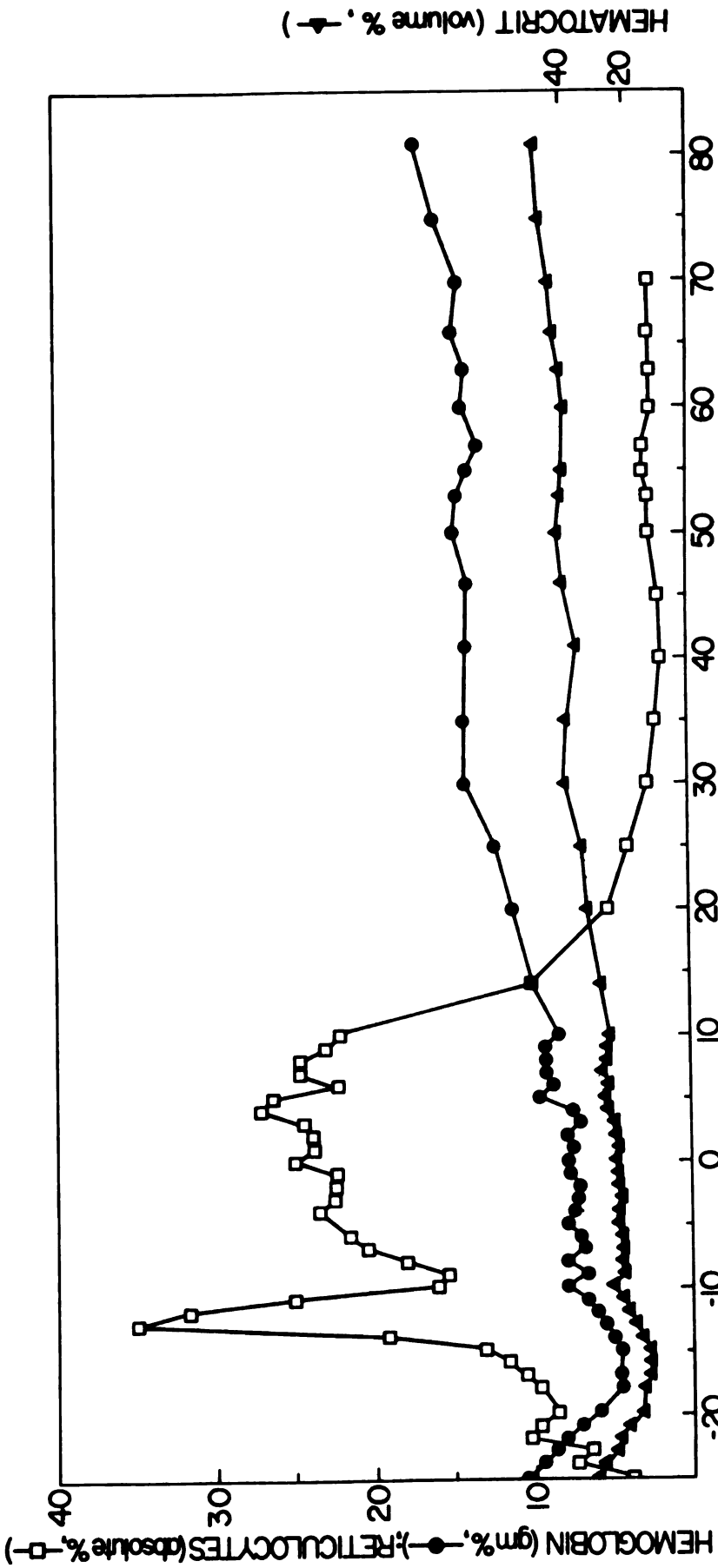

HEMOGLOBIN (gm%, —●—); RETICULOCYTES (absolute %, —□—)
HEMATOCRIT (volume %, —▲—)
TIME (DAYS)
10
20
30
40
-20
-10
0
10
20
30
40
50
60
70
80

Figure 20

a rate approximately twelve times higher than normal. Nevertheless, one should approach this value with caution, since it is based on the assumption that the means of correcting reticulocyte count in humans can be similarly applied to the pig. At any rate, pig 123-6 did not appear to be defective in erythropoiesis and the bone marrow seemed to be quite active in response to the bleedings.

Figure 21 is a microscopic view of the dried blood smears prepared on Day 0 (A, before the bleeding starts) and at 8 (B), 12 (C), and 25 (D) days post-induction of anemia. A relatively high concentration of reticulocytes were present on the 8th, 12th, and 25th days; some of the reticulocytes, which were rather large in size, were probably macroreticulocytes (immature reticulocytes). Various degrees of anisocytosis and poikilocytosis were also observed.

Starting on Day 18, the reticulocytes began to oscillate within a fairly narrow range (20-27%) while the hemoglobin and hematocrit rebounded to 6-8 g % and 20-25 volume %, respectively (Figure 20). To maintain a steady anemic state in the pig, daily removal of blood samples was necessary. After intravenous injection of the radioisotope on Day 25 (which is Day 0 for the <u>in vivo</u> experiment), the weight and the anemic condition of the pig was maintained constant for an additional 10 days by daily bleedings and restriction of ration. Beginning on Day 11 through Day 81, the weight of the pig was allowed to gain by lifting the diet regimen.

Figure 21. Photomicrographs of blood cells from Pig 123-6 during induction of anemia.

Air-dried smears of freshly drawn heparinized blood stained with supravital stain new methylene blue. The smears were examined under oil-immersion. A, day 0 (before bleeding starts); B, 8 days post-phlebotomy; C, 12 days post-phlebotomy; D, 25 days post-phlebotomy. The large cells containing heavily staining, aggregated clumps of reticulum represent young reticulocytes. The cells containing smaller amounts of reticulum in "punctate" foci are reticulocytes in an advanced stage of maturation.

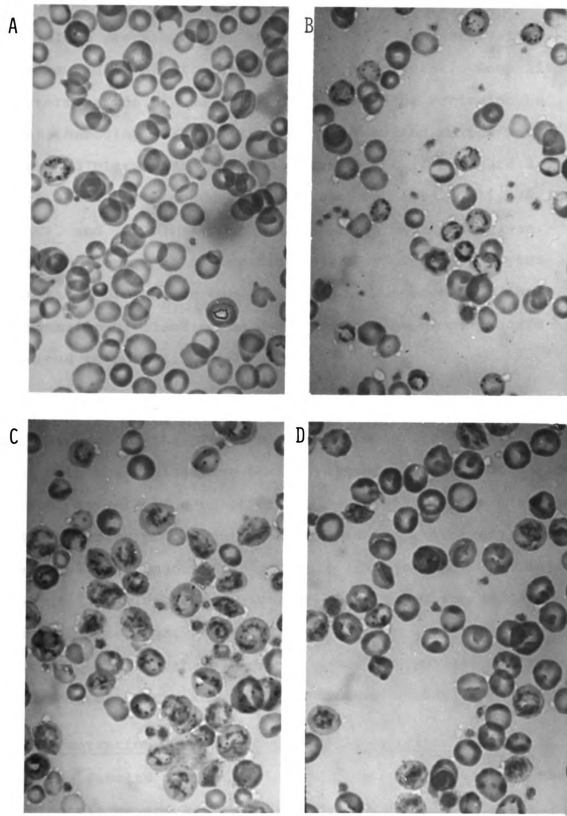


Figure 21

Blood was sampled at approximately 5 day intervals, withdrawing 40 ml at each prescribed time point. Soon after the termination of the daily phlebotomy, the reticulocyte count, hemoglobin and hematocrit all returned to the normal range and plateaued. This is also evident from Figure 22, which shows the blood smears obtained on Days 10 (A), 30 (B), 50 (C), and 70 (D) after the radioactive label was given. It can be seen that a significant number of reticulocytes were still present on Day 10; however, subsequent to the termination of bleeding on daily basis, the blood picture returned to normal.

2. Administration of Radioisotope

Because of the previous experience in the in vivo experiment with the radioactive GL-1a, routine practices of saline injections were made on other available pigs at the swine barn and a standard procedure was developed to prevent any more of the previously encountered problems in injection. The injection of [^{14}C]glucose was given on Day 0 after the initial start of bleedings, as indicated in Figure 20. The injection went smoothly and the pig was in excellent condition afterwards, except for a few wobbling minutes on her feet.

3. Concentration of Porcine Blood Sphingolipids

The results of TLC and GLC analyses indicated that the porcine plasma and erythrocytes contained the same neutral glycosphingolipids as previously described (45). In addition,

Figure 22. Photomicrographs of blood cells from Pig 123-6 during the in vivo study.

Air-dried smears of freshly drawn heparinized blood stained with supravital stain new methylene stain. A, 10 days after injection of [U-^{14}C] glucose as a pulse label. B, 30 days post-injection of the label; C, 50 days post-injection of the label; D, 70 days post-injection of the label.

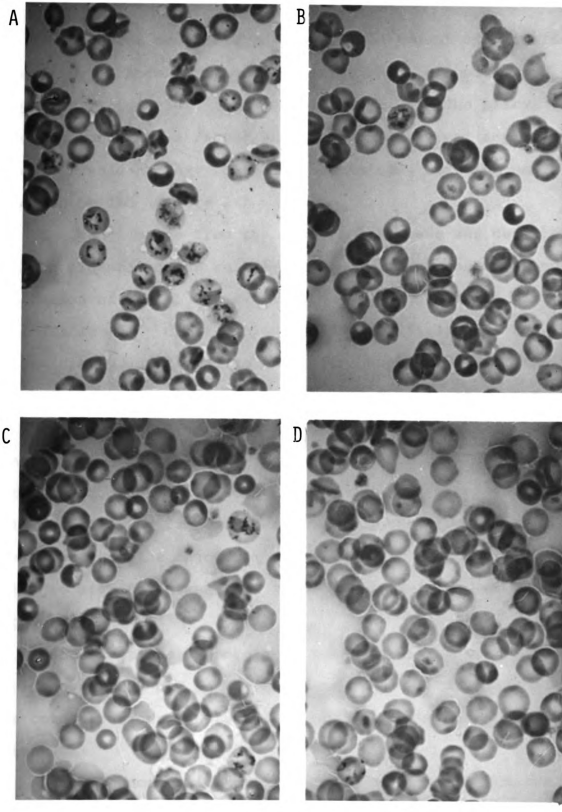


Figure 22

the erythrocytes contained an appreciable amount of ceramides consisting of normal fatty acids and a small proportion of α-hydroxy fatty acids, as shown in Figure 23. The plasma fraction contained a rather small amount of normal acyl-type ceramide; no α-hydroxy fatty acid-containing ceramide was detected by TLC (Figure 24).

G_{M3} ganglioside from the Folch lower phase was detected in both plasma and red cell fractions of the blood. The concentration of plasma G_{M3} was in the same range as the plasma GL-4 (Table 25). G_{M2} ganglioside (Figure 25, lane 4, Band B) was partially identified as the major ganglioside from the Folch upper phase of plasma by TLC, and G_{M3} was also present as expected (Band A). In porcine erythrocytes (Figure 25, lane 3) G_{M2} ganglioside was the major component from the upper phase, along with some G_{M3} (Band A) and G_{M1} (Band C). This is different from human red cell gangliosides (upper phase) wherein the major component (Band X) was reported (117) to migrate between G_{M2} and G_{M1}, as shown in lane 2 of Figure 25.

Concentrations of porcine plasma and erythrocyte sphingolipids over a 81-day period are summarized in Table 25. The concentrations generally did not fluctuate to any great extent.

Specific activities of porcine plasma and erythrocyte sphingolipids over a period of 81 days are tabulated in Tables 26 and 27.

Figure 23. Thin-layer chromatography of erythrocyte ceramides from Pig 123-6.

The ceramide fraction, isolated from the total lipids by silicic acid chromatography (chloroform-methanol 98:2 fraction), were further purified on a 250μ silica gel G Quantum plate with chloroform-methanol (95:5) as the developing solvent. In lane 1, reference ceramide standard containing normal fatty acids. In lane 2, reference ceramide standard containing 2-hydroxy fatty acids. In lanes 3, 4, and 5, erythrocyte ceramides from pig 123-6 obtained on days 81, 70, and 66 respectively.

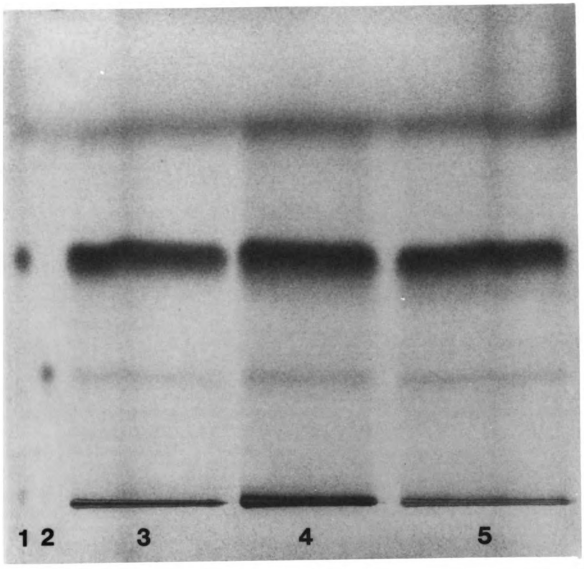


Figure 23

Figure 24. Thin-layer chromatography of plasma ceramide from Pig 123-6.

The ceramide fraction, isolated from the total lipids by silicic acid chromatography (chloroform-methanol 98:2 fraction), were further purified on a 250μ silica gel G Quantum plate with chloroform-methanol (95:5) as the developing solvent. In lane 1, reference ceramide standards containing normal fatty acids (fast-moving component) and 2-hydroxy fatty acids (slow-moving component). In lanes 2, 3, 4, and 5, plasma ceramide from pig 123-6 obtained on days 14, 10, 9, and 8 respectively.

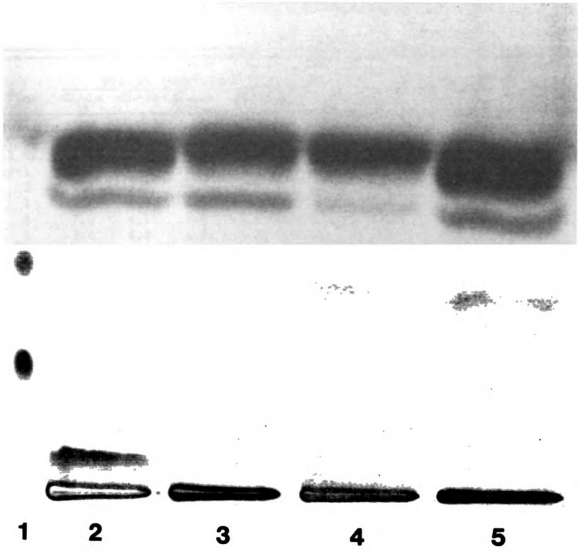


Figure 24

Table 25. Concentrations of Plasma and Erythrocyte Sphingolipids from Pig 123-6 During the Metabolic Experiment

Time	Plasma (nmoles/ml)					RBC (nmoles/ml)		
	GL-1a	GL-2a	GL-3a	GL-4	G_{M3}	GL-4	Cer-HFA	Cer-NFA
6 hr.	9.2	2.6	2.3	2.1	1.6	250	1.4	20.6
12 hr.	8.7	2.9	2.3	2.0	1.4		1.6	19.9
Day-1	8.7	4.4	2.2	2.1	1.5	258	1.5	22.2
2	6.3	3.0	2.2	2.4	1.3	192	1.9	23.0
3	7.8	3.9	1.9	2.8	1.7	232	2.5	22.4
4	7.9	4.1	2.0	1.8	1.9	205	1.4	22.7
5	7.6	4.2	1.8	1.8	1.8	210	1.1	23.1
6	6.3	5.3	2.3	2.5	1.6		1.8	21.7
7	7.4	4.4	1.8	2.2	1.6	206	1.6	23.1
8	6.8	3.9	1.8	2.2	1.9		2.1	20.1
9	6.8	4.7	3.0	2.3	2.0	223	2.6	18.9
10	5.8	3.8	1.5	2.1	1.7		1.3	13.8
14	5.3	4.3	2.6	2.8	1.8		1.1	18.2
20	8.3	4.5	2.5	2.8	1.9		---	---
25	6.9	3.6	3.4	3.3	2.0		1.1	17.8
30	6.8	4.3	---	2.6	---		1.8	21.6
35	7.5	4.4	2.1	2.4	2.0		1.6	19.7
41	7.8	3.0	3.3	2.7	1.5		2.3	19.2
46	7.4	2.6	2.4	2.0	1.6		1.6	19.9
50	4.8	3.7	3.5	1.9	1.8		1.6	23.6
53	7.9	2.7	3.7	1.8	1.4		0.9	13.4
55	5.0	3.6	2.2	2.9	1.4		1.2	11.3
57	5.2	3.8	2.5	1.8	1.3		1.2	21.0
60	8.0	3.8	3.1	2.0	1.5		3.1	24.1
63	8.4	2.8	2.3	1.9	1.7		1.4	19.5
66	5.8	3.2	2.3	2.4	1.4		0.9	13.4
70	9.1	4.0	3.0	2.0	1.7		---	10.7
75	9.2	2.8	2.7	1.3	1.2		1.5	10.8
81	6.9	3.0	3.1	2.7	---		1.9	16.0
Mean	7.2	3.7	2.5	2.3	1.6	222	1.6	19.0

Figure 25. Thin-layer chromatography of Folch upper phase gangliosides from normal human erythrocytes, normal porcine plasma and erythrocytes.

The Folch upper phases from human erythrocytes (17 ml), porcine erythrocytes (15 ml) and porcine plasma (15 ml) were dialyzed, hydrolyzed by mild alkali and dialyzed again in the cold for 72 hr. The crude ganglioside mixtures were purified by TLC, using 250μ Uniplate (heat activiated at 120°C for 30 min) and developed in two-solvent sequential system. Solvent systems were chloroform-methanol-1.25 N NH_4OH (60:40:9) and n-propanol-water (70:30). In lane 1, reference standard ganglioside mixture containing mono-, di-, and trisialoganglioside. In lane 2, gangliosides from human erythrocytes which contained hematoside (A), and the major ganglioside (X), structure unknown. In lane 3, porcine erythrocyte gangliosides partially and tentatively identified as hematoside (A), G_{M2} (B), and G_{M1} (C). In lane 4, porcine plasma gangliosides partially and tentatively identified as hematoside (A), and G_{M2} (B).

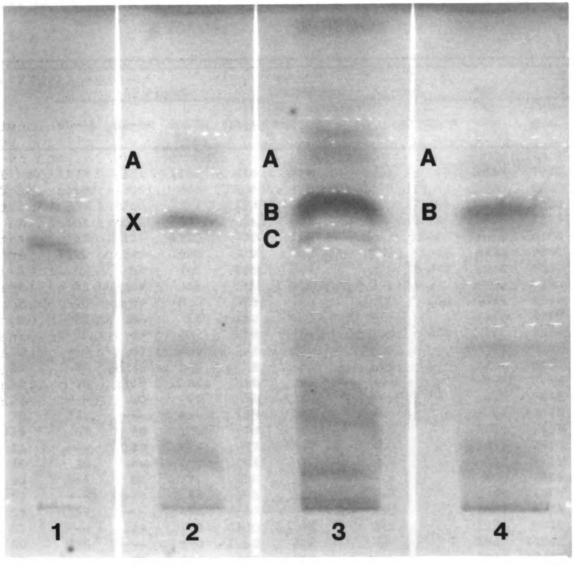


Figure 25

Table 26. Specific Activities (cpm/μmole) of Plasma and Erythrocyte Sphingolipids

Time	Plasma				GL-4 (corrected for anemic volume)		RBC		
	GL-1a	GL-2a	GL-3a	GL-4		G_{M3}	GL-4	Cer-NFA	Cer-HFA
6 hr.	1132	351	1165	418	418	3053	23	1597	4984
12 hr.	1118	305	843	350	350	1174		2086	9535
Day 1	709	260	2291	1320	1320	1786	485	1173	5050
2	502	252	1587	901	901	674	1247	1020	3297
3	85	171	1390	720	720	328	1268	1618	6335
4	1100	145	932	910	910	199	1076	1333	7301
5	82	64	761	855	847	199	828	1340	8590
6	73	52	356	329	329	131		1482	6236
7	75	73	670	395	348	95	608	1079	5793
8	68	94	315	180	182	---		1070	4713
9	28	39	430	298	301	16	420	2112	6364
10	50	63	469	208	208	56		1791	8291
14	25	39	145	79	73	20		1738	5457
20	38	118	164	110	81	---		---	---
25	---	20	99	31	23	---		1426	8444
30	141	---	---	153	116	---		1667	10328
35	67	29	156	78	60	166		1326	7139
41	---	139	84	77	57	69		1311	2590
46	38	---	154	106	77	958		759	7119
50	515	81	171	156	115	---		800	7365
53	---	---	54	173	129	---		1349	23005
55	153	---	464	65	48	90		1682	6735
57	---	243	122	130	96	315		1236	9370
60	336	---	390	1262	928	---		1645	44541
63	226	89	424	797	588	---		2847	40427
66	---	282	665	207	154	---		1404	---
70	144	---	147	274	206	---		3497	---
75	---	---	200	199	148	2472		1474	8502
81	37	---	175	200	149	---		3464	3741

Table 27. Specific Activities (cpm/ml) of Plasma and Erythrocyte Sphingolipids

Time	Plasma GL-1a	Plasma GL-2a	Plasma GL-3a	Plasma GL-4	RBC GL-4 (corrected for anemic volume	Plasma Cer-NFA	RBC Cer-NFA	RBC Cer-HFA	Plasma Upper Phase Ganglioside	RBC Upper Phase Gangliosides
6 hr	50	19	13	27	27	---	75	13	56	25
12 hr	96	21	29	103	103	1.6	132	32	41	26
Day 1	57	15	50	465	465	3.0	78	13	35	40
2	28	11	86	1018	1018	4.0	61	9	69	85
3	62	21	121	1299	1299	2.0	121	26	32	79
4	32	17	107	1079	1079	2.3	79	16	28	94
5	26	18	96	952	952	2.0	74	18	19	103
6	25	21	87	737	744	1.4	73	19	16	62
7	18	11	93	783	790	1.8	50	13	16	64
8	25	11	54	581	586	1.8	45	13	10	100
9	33	12	53	559	550	2.4	64	19	17	86
10	25	22	37	281	281	3.0	40	16	20	---
14	41	8	44	375	442	1.4	46	10	10	30
20	---	9	18	317	373	1.8	70	---	10	---
25	9	6	21	208	250	1.0	19	5	10	31
30	32	9	18	203	235	1.4	33	14	11	17
35	16	5	16	182	213	1.0	28	3	9	19
41	26	14	14	170	198	1.5	23	11	6	62
46	32	31	12	130	152	1.4	17	5	10	13
50	82	34	29	171	200	1.0	21	---	9	58
53	33	8	13	107	124	1,3	15	27	11	42
55	29	6	15	136	160	1.3	16	4	7	33
57	47	28	10	120	141	1.2	16	3	8	40
60	41	75	14	136	161	2.0	17	---	9	44
63	133	91	13	118	138	1.0	13	3	22	32
66	15	---	6	67	78	1.3	17	8	7	44
70	53	34	29	67	78	1.2	57	14	39	34
75	15	4	5	29	34	0.9	16	---	23	25
81	104	45	2	79	92	1.0	59	---	14	34

4. Metabolism of Porcine Blood Sphingolipids

As stated in the Methods, specific activities were expressed as cpm/ml of plasma or red cells and/or cpm/μmole hexose; in the latter case, the specific activity of an individual hexose moiety was obtained by dividing by the number of monosaccharide residues present in the particular glycosphingolipid, since it had been shown previously in several studies that the sugar moieties are equally labeled (45,179). Turnover curves for each of the sphingolipids were constructed from linear and/or semilogarithmic plots of specific activities versus time. The vertical bars at each data point represent the accumulated random errors for various steps in the procedure.

Table 28. Random Errors for Various Steps in the Procedure

Procedure		% Error
Measurement of plasma and RBC volume		2.0
Lipid extraction and silicic acid column chromatography		0.5
Mild alkaline hydrolysis and dialysis		0.5
TLC and elution of individual fractions		1.0
Acid-catalyzed methanolysis and GLC		2.0
Pipetting		2.0
Sphingosine assay		2.0
Radioactivity counting (Beckman LS-150 Manual)		
	Counts	
	100	20.0
	200	15.0
	400	10.0
	800	7.0
	1600	5.0
	4000	3.0

The estimation of the error of a computed result R from the errors of the component terms A, B, and C is summarized below:

If R is calculated as product or quotient where R = AB/C, then the random error for R is equal to $\{(S_A/A)^2 + (S_B/B)^2 + (S_C/C)^2\}^{\frac{1}{2}}$ where R is the calculated result; A, B, and C are the measured quantities from which R is derived. S_A, S_B and S_C are the absolute standard errors o A, B, and C.

To maintain an anemic pig in a state of hematopoietic equilibrium for an extended period of time is difficult; hence, the pig was allowed to gain weight after the initial 10-day period and corrections were made in the calculations for the large increase in blood volume that occurred after the initial 10 days.

5. Incorporation of Labeled Glucose into GL-4

The labeled glucose was rapidly incorporated into hexose, sphingosine and fatty acid moieties of plasma GL-4 (Table 29), which reached a maximum specific activity at 24 hr after the initial pulse label (Figure 26) and then declined rapidly for the next 10 days. The specific activity of plasma GL-4 remained rather constant over the next 35 days until around Day 50, when significant relabeling occurred throughout the whole molecule, reaching maximum activity on Day 60.

Semi-logarithmic plots of plasma GL-4 specific activity versus time for the first 10 days and at Day 60 subsequent

Table 29. Specific Activities of Hexose, Fatty Acid, and Sphingosine Moieties from Plasma GL-1a and GL-4

Time	Specific Activities (cpm/μmole)					
	GL-1a			GL-4		
	Sugar	Base	FAME	Sugar	Base	FAME
6 hr	1132	70	113	418	331	428
12 hr	1118	101	101	350	220	580
Day 1	709	166	207	1320	1199	562
2	502	380	354	901	561	714
3	85	165	249	720	793	470
4	1100	169	174	910	1005	1580
5	82	211	116	855	714	740
6	73	182	116	329	603	256
7	75	149	124	395	1289	960
8	68	95	34	180	1969	911
9	28	118	111	298	267	350
10	50	124	160	208	254	392
14	77	116	99	79	198	158
20	38	56	44	110	897	189
25	---	---	---	31	401	123
30	141	141	130	153	427	183
35	67	150	80	78	312	150
41	---	4	2	77	1497	413
46	38	171	57	106	1830	704
50	515	676	162	156	389	233
53	---	---	---	173	108	260
55	153	688	420	65	392	196
57	---	---	---	130	695	695
60	336	773	202	1262	2825	689
63	226	339	225	797	1328	1593
66	---	---	---	207	826	275
70	144	168	125	274	1205	657
75	---	---	---	199	1789	1391
81	37	45	22	200	1900	300

Figure 26. Turnover of plasma and erythrocyte globoside in Pig 123-6.

Linear plot of specific activities of GL-4, GalNAc-(1→3)-Gal-(1→4)-Gal-(1→4)-Glc-ceramide, from plasma (cpm/μmole hexose) and erythrocyte (cpm/ml) versus time over an 81-day period.

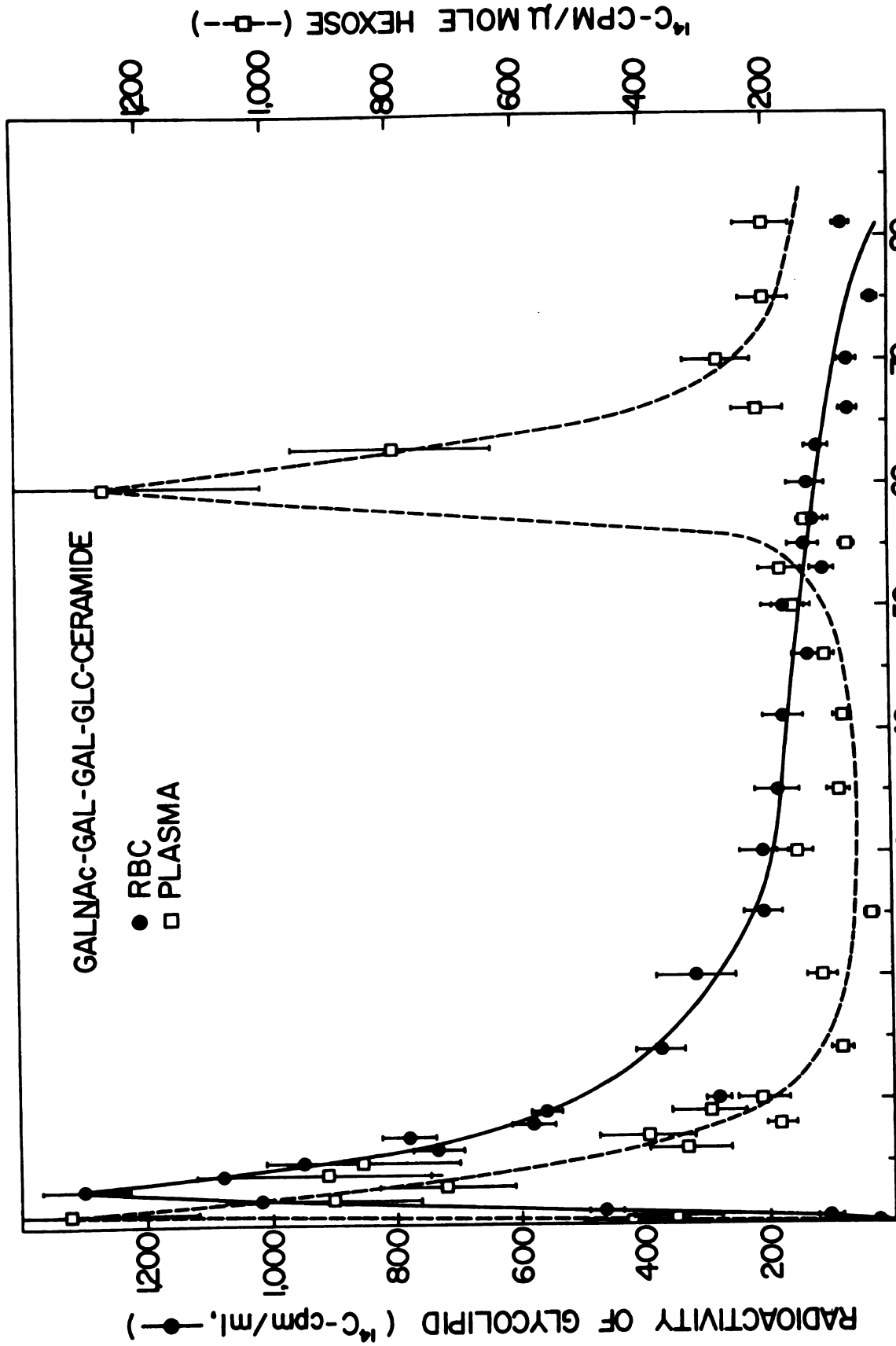

RADIOACTIVITY OF GLYCOLIPID (^{14}C-cpm/ml, —●—)
^{14}C-CPM/μ MOLE HEXOSE (--□--)
GALNAc-GAL-GAL-GLC-CERAMIDE
● RBC
□ PLASMA
TIME (DAYS)
200
400
600
800
1,000
1,200
10
20
30
40
50
60
70
80

Figure 26

to maximum incorporation revealed a decline in radioactivity at a rate suggesting half times of approximately 3.6 and 5.0 days, respectively. A linear plot of specific activity (cpm/ml) *versus* time revealed that the daily increment of incorporation of label into the erythrocyte GL-4 (Figure 26) was slower and the GL-4 did not reach maximum specific activity until 3 days after the injection of [U-^{14}C]glucose. This corresponds well with the finding of Bush *et al.* (273) that the time of maximum incorporation of ^{59}Fe into the erythrocyte was 3 days and less for the normal and anemic pigs, respectively. After maximum incorporation was attained the specific activity fell steadily; approximately 65% of the label was lost during the next 10 days, after which there was a slow and gradual decrease until around Day 60 when a much more abrupt decline occurred.

Corrections for the increase in blood volume were based on the normal blood volume figures reported by Bush *et al.* (272), since attempts to correct for the volume increase on the basis of anemic pig blood volume (273) did not reveal any significant changes (Tables 26 and 27) in the profile of the turnover curves. There was no change in the specific activities between 6 hr and Day 14 for both plasma and erythrocyte GL-4. Between Days 20 and 81, the specific activities of erythrocyte GL-4 were raised slightly while the specific activities of plasma GL-4 declined by about 20%. However, the basic profiles of the two turnover curves remained the same.

Figure 27 shows the linear plot of specific activity (cpm/μmole hexose) versus time of erythrocyte GL-4 from seven data points obtained during the first 10 days subsequent to the initial pulse label. It can be seen that following the initial incorporation phase, there was an exponential decrease in specific activity of the glycolipid, while the hemoglobin and packed cell volume of the red cells remained rather constant throughout this period. If one compares the peak of radioactivity of erythrocyte GL-4 with that reported previously for the normal pig (45), there is an approximately 6-fold increase in the amount of ^{14}C incorporation in the red cells of the anemic pig.

The semilogarithmic plots of specific activities of samples obtained from the pig 123-6, plotted against time, are depicted in Figure 28. It can be seen that the part of the curve between Day 25 and Day 57 is not a flat plateau; instead, it follows a gradual exponential decline in specific activity which is terminated by a more precipitous drop (this is also evident from the linear plot in Figure 26). This suggests that the turnover of erythrocyte GL-4 represents more than one process. The exponential phase may be ascribed to random destruction, whereas another process, which is age-dependent, is evidenced by the precipitous terminal decline in specific activity.

Figure 28 also suggests that there are at least three GL-4 fractions (pools) in the red cell population which undergo

Figure 27. Turnover of red cell globoside in pig 123-6 over a 10-day period.

Linear plot showing the decay of the ^{14}C-labeled hexose moiety of red cell GL-4 in a period of 10 days post-injection of [U-^{14}C] glucose.

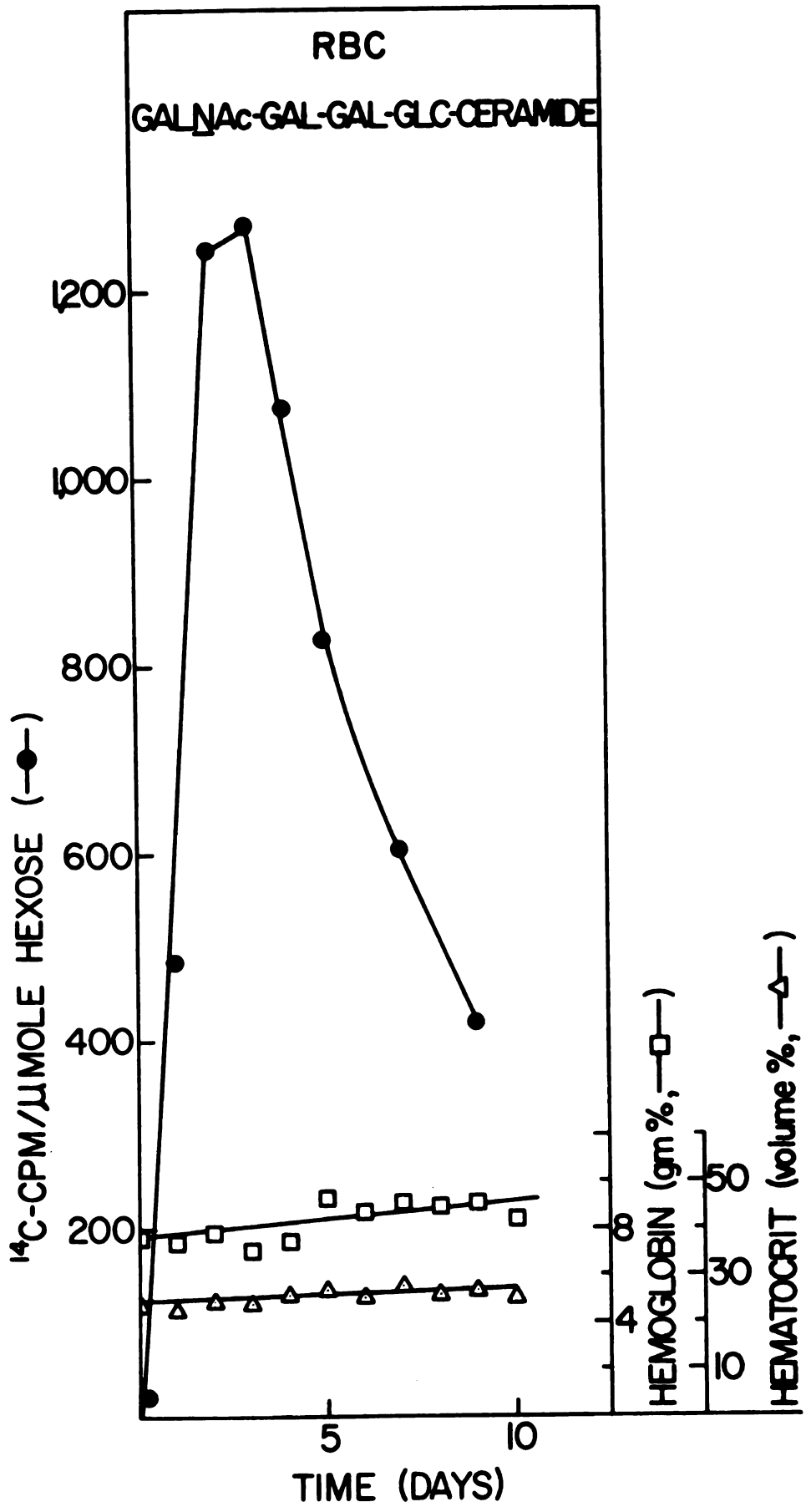

RBC
GALNAc-GAL-GAL-GLC-CERAMIDE
1200
1000
800
600
400
200
^{14}C-CPM/μMOLE HEXOSE (—●—)
HEMOGLOBIN (gm%, —□—)
HEMATOCRIT (volume%, —△—)
8
4
50
30
10
5
10
TIME (DAYS)

Figure 27

Figure 28. Turnover of erythrocyte globoside in pig 123-6.

Semilogarithmic plot of specific activity (cpm/ml) of GL-4 as a function of time emphasized the biphasic nature of the decay curve.

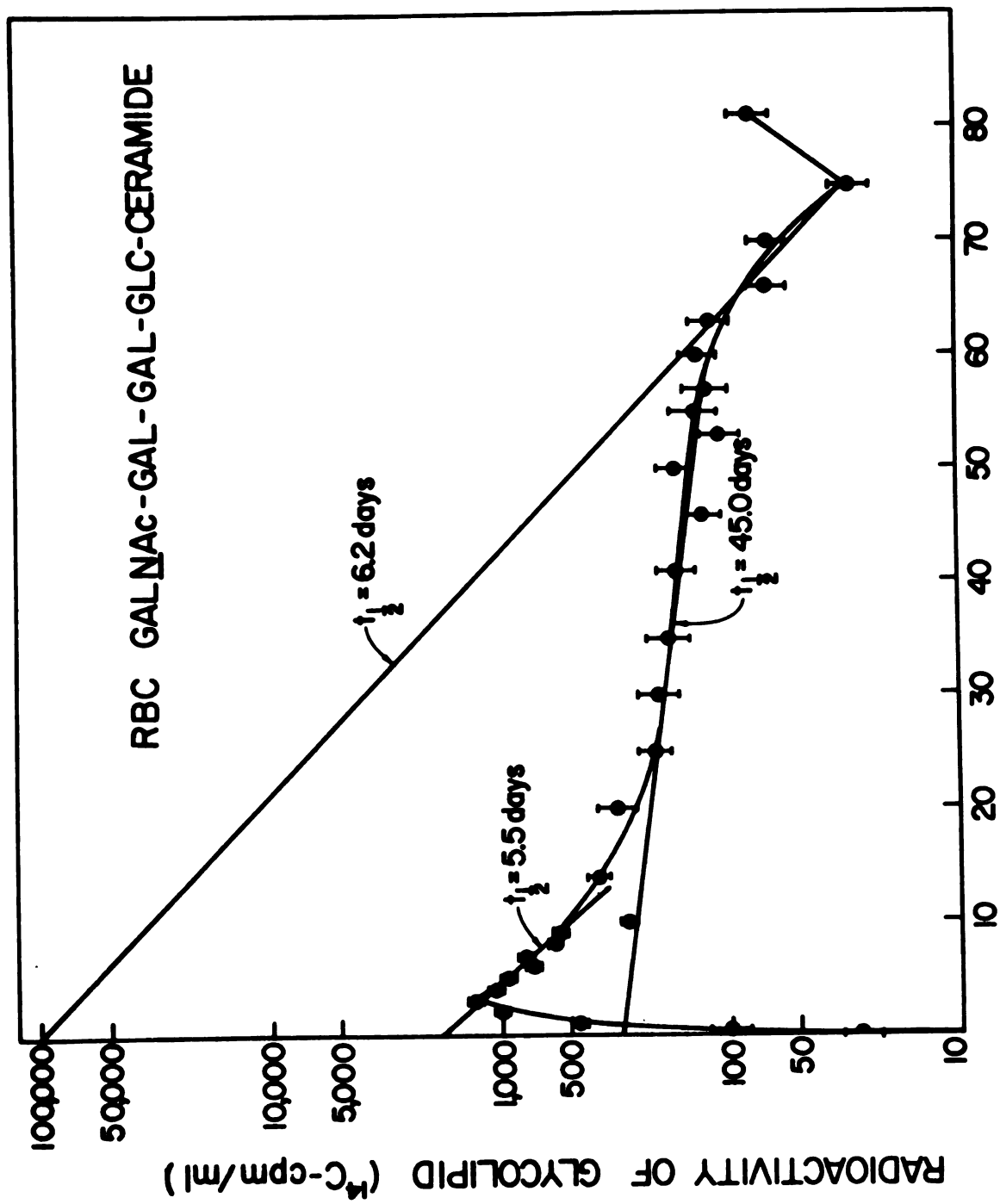

RBC GALNAc-GAL-GAL-GLC-CERAMIDE
$t_{\frac{1}{2}}$ = 6.2 days
$t_{\frac{1}{2}}$ = 5.5 days
$t_{\frac{1}{2}}$ = 45.0 days
RADIOACTIVITY OF GLYCOLIPID (^{14}C-cpm/ml)
100000
50000
10000
5000
1000
500
100
50
10
10
20
30
40
50
60
70
80
TIME (DAYS)

Figure 28

Figure 29. Turnover of plasma and erythrocyte trihexosylceramide in pig 123-6.

Linear plot of specific activities of GL-3a, Gal-(1→4)-Gal-(1→4)-Glc-ceramide, from plasma (cpm/μmole hexose) and erythrocyte (cpm/ml) as a function of time over an 81-day period.

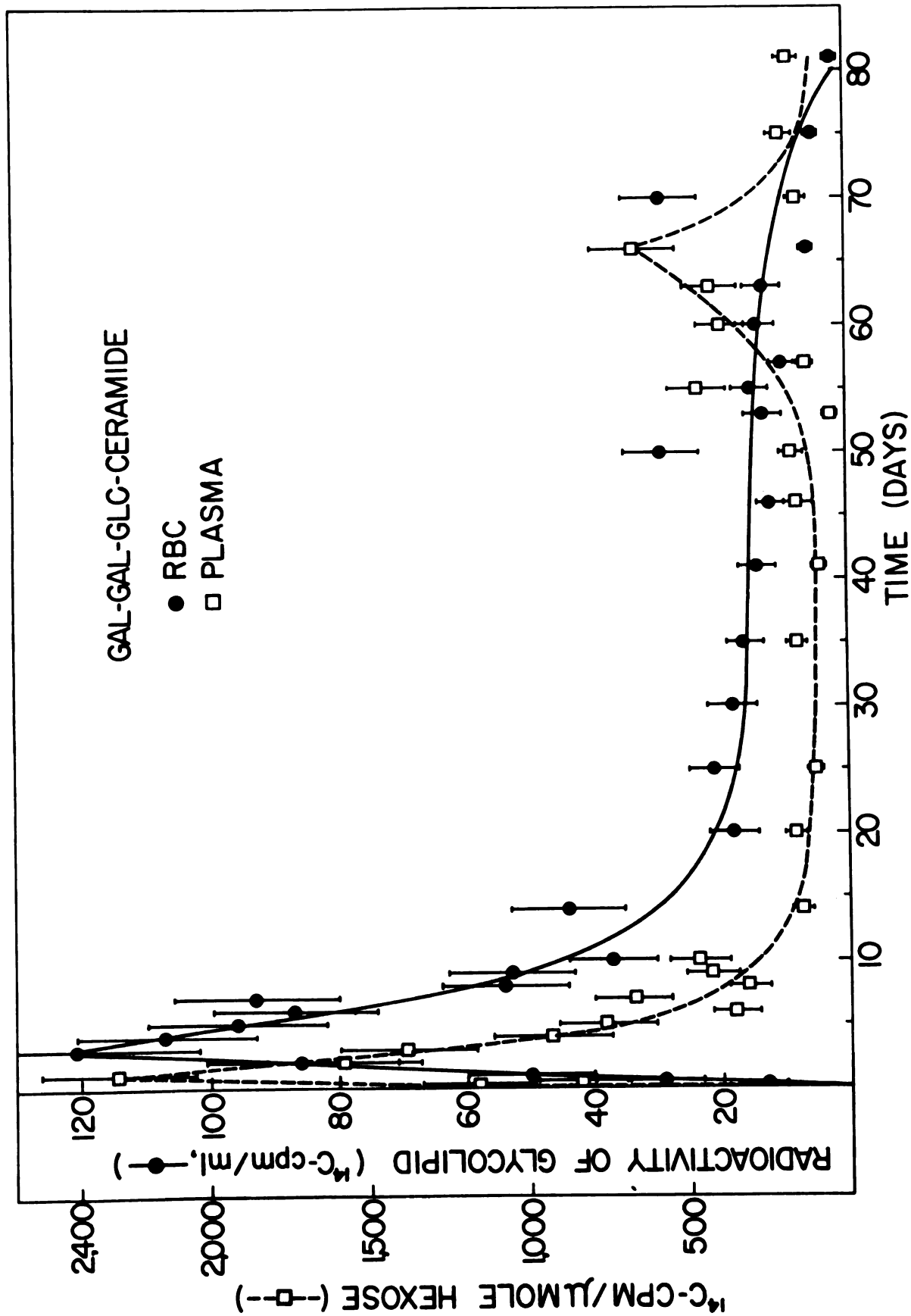

GAL-GAL-GLC-CERAMIDE
● RBC
□ PLASMA
TIME (DAYS)
RADIOACTIVITY OF GLYCOLIPID (¹⁴C-cpm/ml, —●—)
¹⁴C-CPM/μMOLE HEXOSE (--□--)

Figure 29

turnover at different rates. Within experimental inaccuracies, the rate of decrease of specific activity from Day 3 to Day 9 can be fitted to a single exponential component having a half-time of 5-6 days. If one assumes that GL-4 is an integral part of the red cell membrane and remains with the cell throughout its normal life span, the data can be correlated with a cellular mean life span of 8 days. It is likely that this represents turnover of reticulocytes. The value was estimated from the fact that the mean life time is 1.44 times the half-time when a process is represented by a single exponential decay (274,275). The rate of radioactivity decay from Day 25 to 57 can also be fitted to a single exponential component having a half-time of 45 days, which would indicate a mean cellular life span of 65 days for these cells. Finally, a mean life span of 9 days (t 1/2 = 6.2 days) can be obtained for the rate of decrease in specific activity of GL-4 from Day 60 to Day 75. It is interesting to note that the half-time of erythrocyte GL-4 between Days 60 and 75 is very similar to the half-time of reticulocytes subsequent to maximum labeling on Day 3.

6. Incorporation of Labeled Glucose into GL-3a

Labeled glucose was rapidly incorporated into plasma GL-3a (Figure 29), which reached a maximum specific activity at 24 hr. The specific activity then declined rapidly before reaching a plateau around Day 15. Significant relabeling did

not occur until Day 50, and GL-3a reached a new peak of specific activity on Day 65. A semilogarithmic plot of plasma GL-3a specific activity over the first 10-day period revealed a half-time of 3.5 days.

Erythrocyte GL-3a showed a similar pattern of incorporation (Figure 29) as that of red cell GL-4; except for differences in specific activities, the two curves were almost superimposable. Maximum incorporation of the radioactive label occurred on Day 3, followed by a decline with a loss of approximately 65% of the label during the next 10 days. After that the level of specific activity decreased slowly with time until around Day 60 when a precipitous loss occurred. A semilogarithmic plot of specific activity versus time (Figure 30) disclosed the presence of at least three GL-3a pools. The rate of decrease of specific activity from Day 3 to Day 9, Days 20-57 and Days 60-81 could be fitted to exponential components having half-times of 5.5, 45.0 and 6.3 days, respectively. This would indicate cellular mean life spans of 8, 65 and 9 days.

7. Incorporation of Labeled Glucose into GL-2a

Plasma GL-2a showed a rapid incorporation of the label (Figure 31), reaching a maximum specific activity after 6 hr, which was much earlier than observed with either GL-3a or GL-4, then the radioactivity was lost during the next 9 days in a similar manner as the other two plasma glycosphingolipids.

Figure 30. Turnover of erythrocyte trihexosylceramide in pig 123-6.

Semilogarithmic plot showing the turnover of ^{14}C-labeled GL-3a in porcine red cells as a function of time. Biphasic nature of the decay curve was apparent.

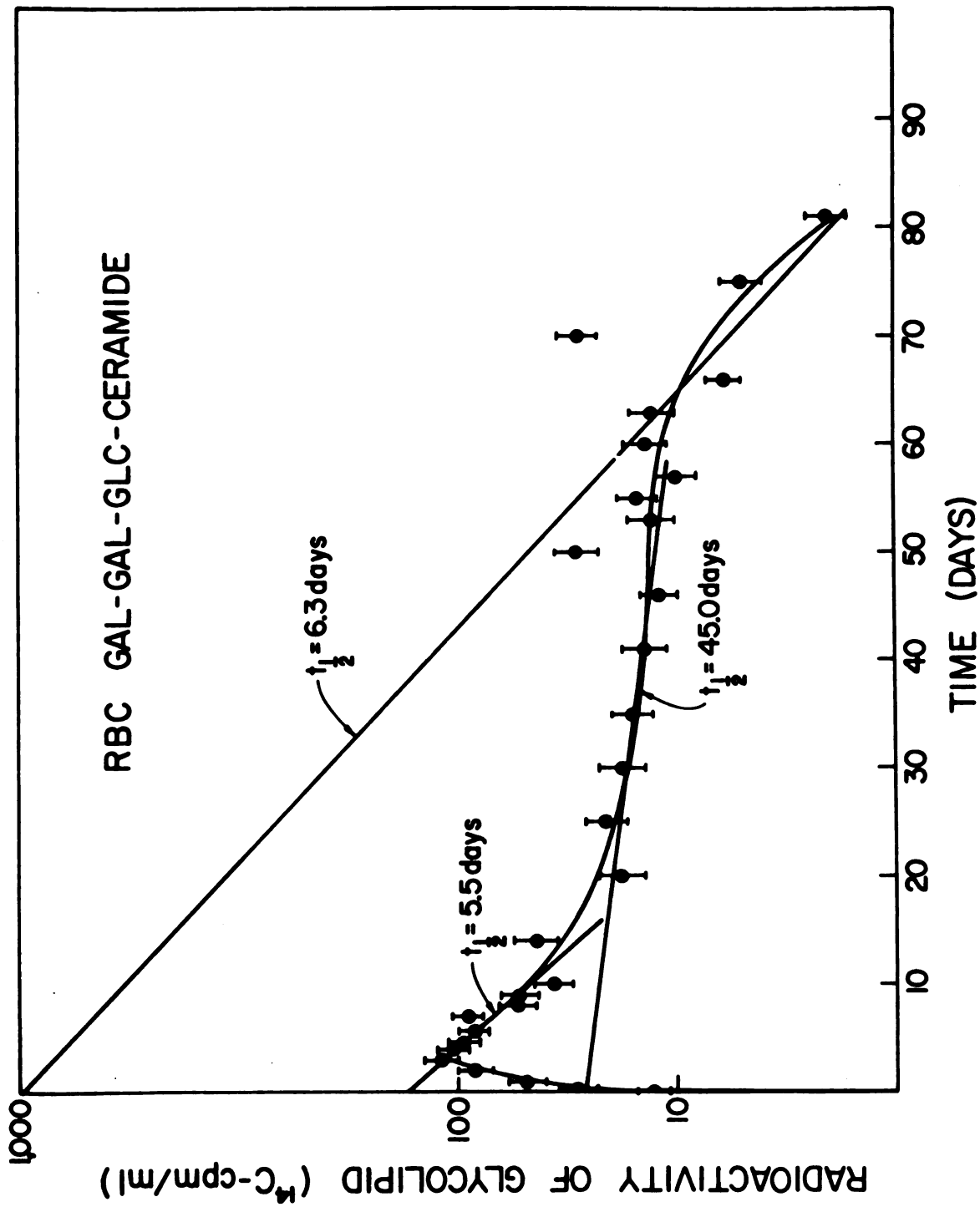

RBC GAL-GAL-GLC-CERAMIDE
$t_{\frac{1}{2}}$=6.3days
$t_{\frac{1}{2}}$=5.5days
$t_{\frac{1}{2}}$=45.0days
RADIOACTIVITY OF GLYCOLIPID (^{14}C-cpm/ml)
1000
100
10
10
20
30
40
50
60
70
80
90
TIME (DAYS)

Figure 30

Figure 31. Turnover of plasma and erythrocyte lactosylceramide in pig 123-6.

The specific activities of GL-2a, Gal-(1→4)-Glc-ceramide, from porcine plasma (cpm/μmole hexose) and erythrocyte (cpm/ml) were plotted linearly as a function of time over an 81-day period.

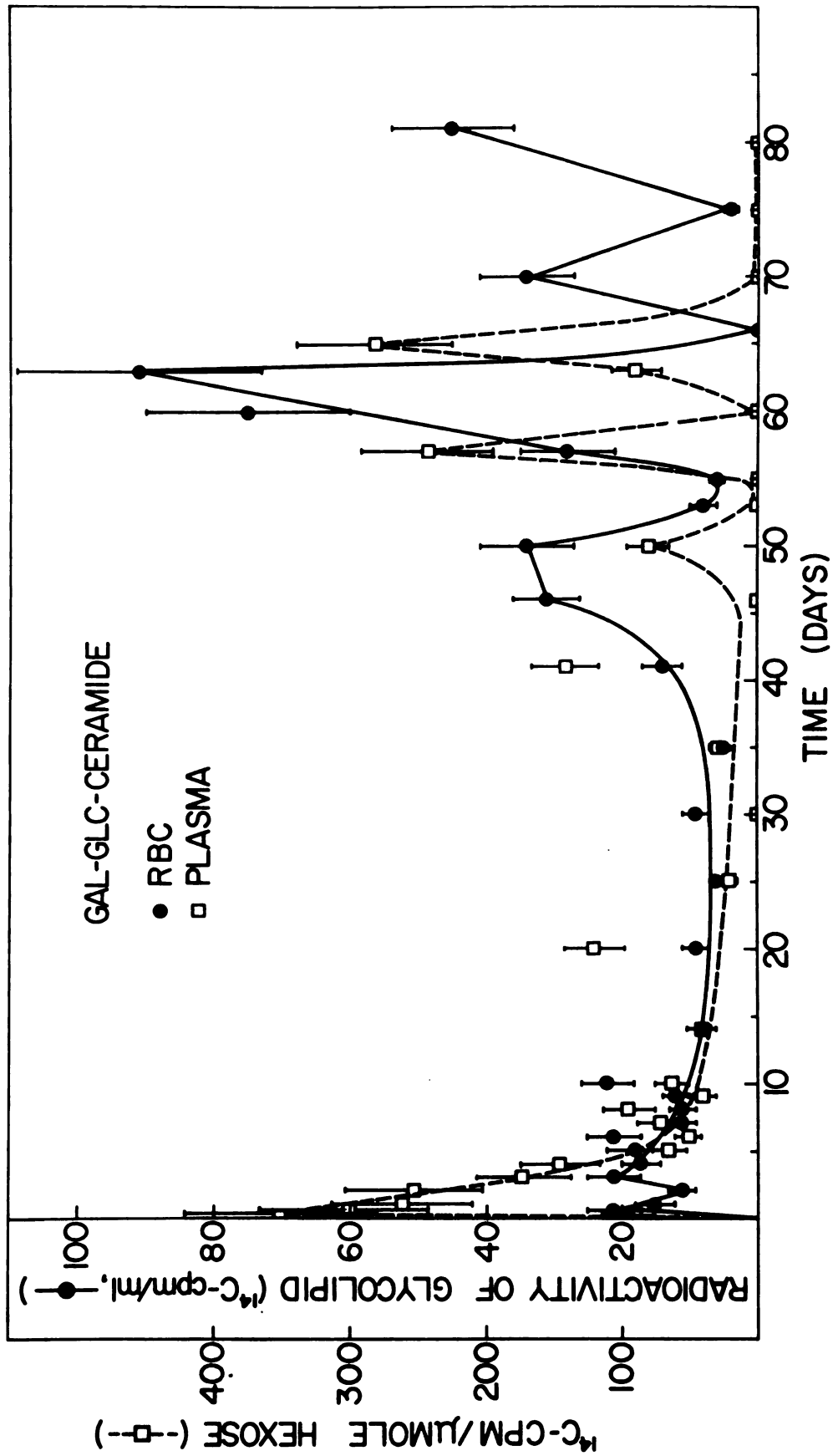

GAL-GLC-CERAMIDE
● RBC
□ PLASMA
TIME (DAYS)
RADIOACTIVITY OF GLYCOLIPID (^{14}C-cpm/ml, —●—)
^{14}C-CPM/μMOLE HEXOSE (--□--)
100
80
60
40
20
400
300
200
100
10
20
30
40
50
60
70
80

Figure 31

The half-time for turnover of GL-2a during this 10-day period was 3.0 days. The lipid was relabeled beginning on Day 50, peaking at Days 57 and 66, after which the specific activity declined nearly to zero after 70 days.

When red cell GL-2a was examined (Figure 31), two small peaks of maximum specific activity were observed at 12 hr and on Day 3, respectively. Approximately 50% of the label was lost during the next 20-day period, after which significant relabeling occurred with a complex pattern that showed maximum of radioactivity on Days 50, 63, 70, and 81. The turnover of GL-2a in red cells was different from that of GL-3a and GL-4 in several respects. First, there was an additional early peak of incorporation at 12 hr. Second, the radioactive peaks between Days 50 and 81 were more complex.

8. Incorporation of Labeled Glucose into GL-1a

The patterns of incorporation of label into plasma and erythrocyte GL-1a (Figure 32) were quite similar to those of GL-2a. Rapid incorporation of the label was observed immediately after administration of [U-^{14}C]glucose, with a peak of specific activity at 6 hr. Loss of this label occurred within the next 10 days and half-times of 0.75 and 7.5 days could be obtained from the semilogarithmic decay curve subsequent to maximal incorporation.

Erythrocyte GL-1a also showed rapid incorporation at 6 hr and on Day 3, and half-times of 0.8 day and 4.2 days could

Figure 32. Turnover of plasma and erythrocyte glucosylceramide in pig 123-6.

The specific activities of GL-1a, Glc-(1→1)-ceramide, from porcine plasma (cpm/μmole hexose) and erythrocyte (cpm/ml) were plotted linearly versus time over an 81-day period.

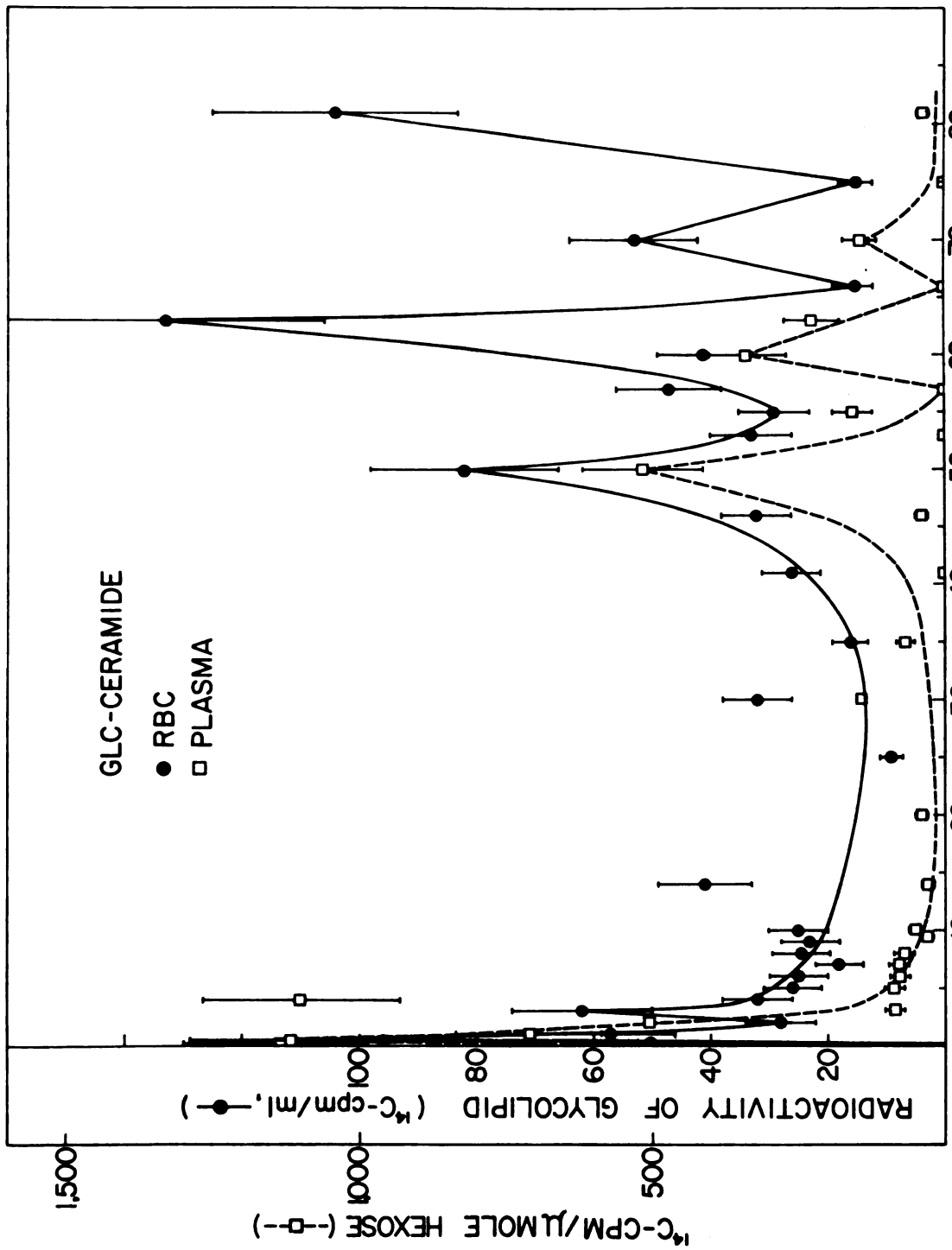

GLC-CERAMIDE
RBC
PLASMA
RADIOACTIVITY OF GLYCOLIPID (14C-cpm/ml.)
14C-CPM/μMOLE HEXOSE (--)
TIME (DAYS)
20
40
60
80
100
500
1000
1500
10
20
30
40
50
60
70
80

Figure 32

be obtained from the loss of radioactivity of these two early peaks on semilogarithmic plots of specific activity *versus* time. The specific activity of GL-1a hexose was 35% higher at 6 hr than it was at Day 3, indicating a much more active and rapid incorporation at the earlier time. The amount of radioactivity incorporated was higher in the plasma fraction than the corresponding red cells. The early peaks of incorporation from both plasma and red cell GL-1a were higher than those observed for the plasma and erythrocyte pools of GL-2a. Although this seems to be consistent with a precursor-product relationship, other criteria cannot be established and hence one cannot relate this to a classical precursor-product relationship.

Between Day 50 and Day 70, significant relabeling occurred in the plasma pool of GL-1a, reaching maximum specific activities on Days 50, 60, and 70. These peaks paralleled exactly the relabeling peaks observed in erythrocyte GL-1a, suggesting that the plasma and red cell membrane pools of GL-1a are in rapid exchange, which confirms the observation made in the *in vitro* experiment mentioned earlier.

9. Incorporation of Labeled Glucose into Folch Lower Phase G_{M3} Ganglioside

Maximum incorporation of label into plasma G_{M3} ganglioside occurred at 6 hr (Figure 33) and 90% of the label was lost during the next 10 days. A semilogarithmic plot of specific

Figure 33. Turnover of Folch lower phase G_{M3} ganglioside in plasma and erythrocyte of pig 123-6.

The specific activities of G_{M3} ganglioside (Folch lower phase) from porcine plasma (cpm/μmole hexose) and erythrocyte (cpm/ml) were plotted linearly as a function of time over an 81-day period.

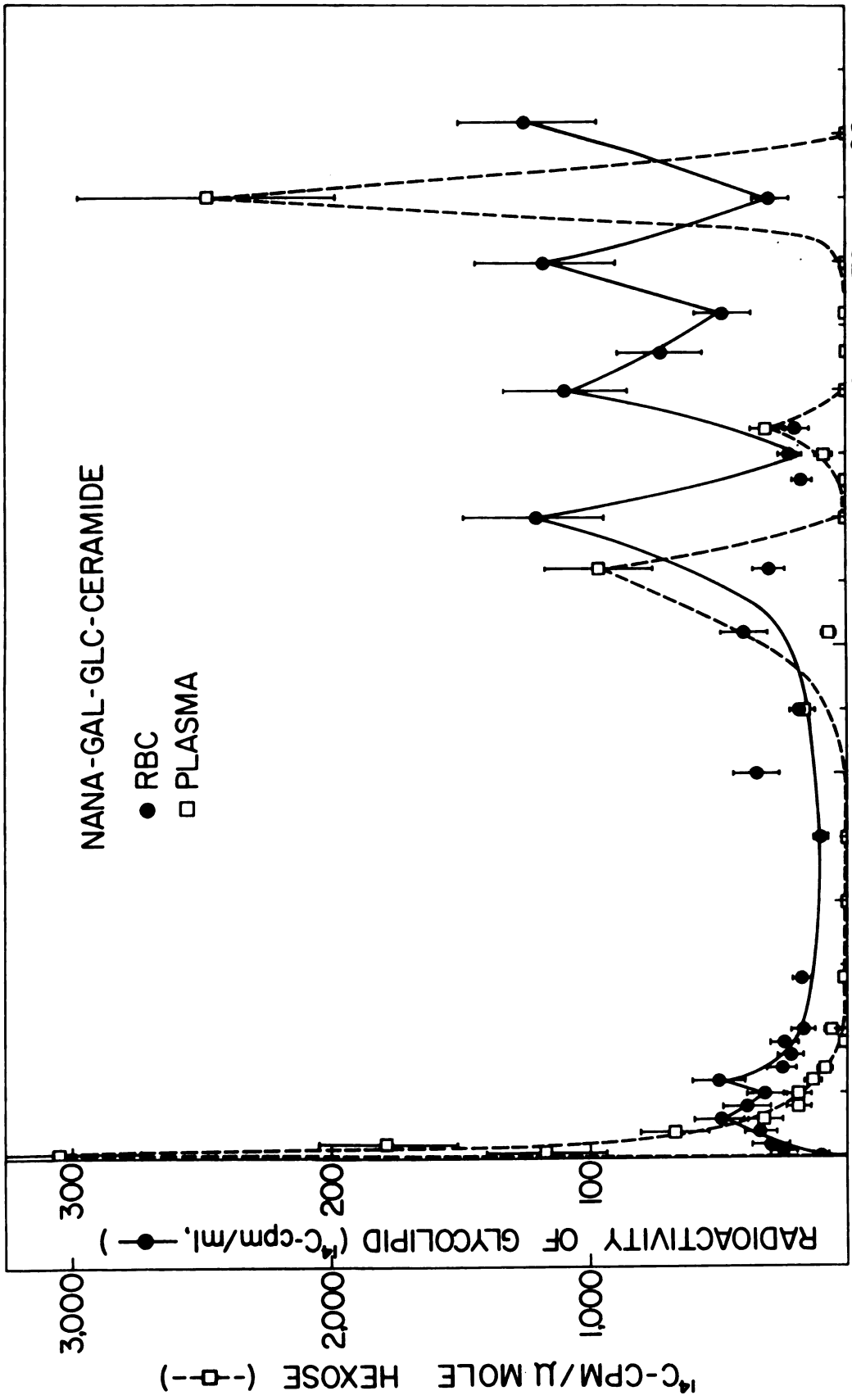

NANA-GAL-GLC-CERAMIDE
● RBC
□ PLASMA
TIME (DAYS)
RADIOACTIVITY OF GLYCOLIPID (¹⁴C-cpm/ml, —●—)
¹⁴C-CPM/μ MOLE HEXOSE (--□--)
1,000
2,000
3,000
100
200
300
10
20
30
40
50
60
70
80

Figure 33

activity versus time suggested a decay curve which was biphasic in nature, indicating that there were two pools of hematoside with half-times of 0.9 day and 1.9 days, respectively. There was no detectable label in plasma G_{M3} between Day 10 and Day 35, and significant relabeling did not occur until Days 46, 57, and 75. The specific activities of plasma G_{M3} during this period were higher than those of erythrocyte G_{M3}.

The incorporation of radioactive label into red cell G_{M3} ganglioside (Figure 33) was rather slow and did not reach a maximum specific activity until 3 days after administration of the labeled glucose, which was similar to the results observed with GL-3a and GL-4 of erythrocytes. At this time, the plasma G_{M3} fraction had reached its peak incorporation and the specific activities were rapidly decreasing. An additional peak of radioactivity was observed on Day 6 which was absent in the other glycosphingolipids examined so far.

The appearance of radioactivity between Day 40 and Day 81 was very similar to the patterns noticed previously for GL-2a and GL-1a; maximum relabeling was recorded on Days 50, 60, 70, and 81.

10. Incorporation of Labeled Glucose into Folch Upper Phase Gangliosides

The patterns of radioactivity incorporation into the upper phase gangliosides of both plasma and red cells bear some similarities to that observed in the G_{M3} ganglioside (Figure 34).

Figure 34. Turnover of Folch upper phase gangliosides from plasma and erythrocyte of pig 123-6.

The specific activities (cpm/ml) of Folch upper phase gangliosides from porcine plasma and erythrocyte were plotted as a function of time over an 81-day period.

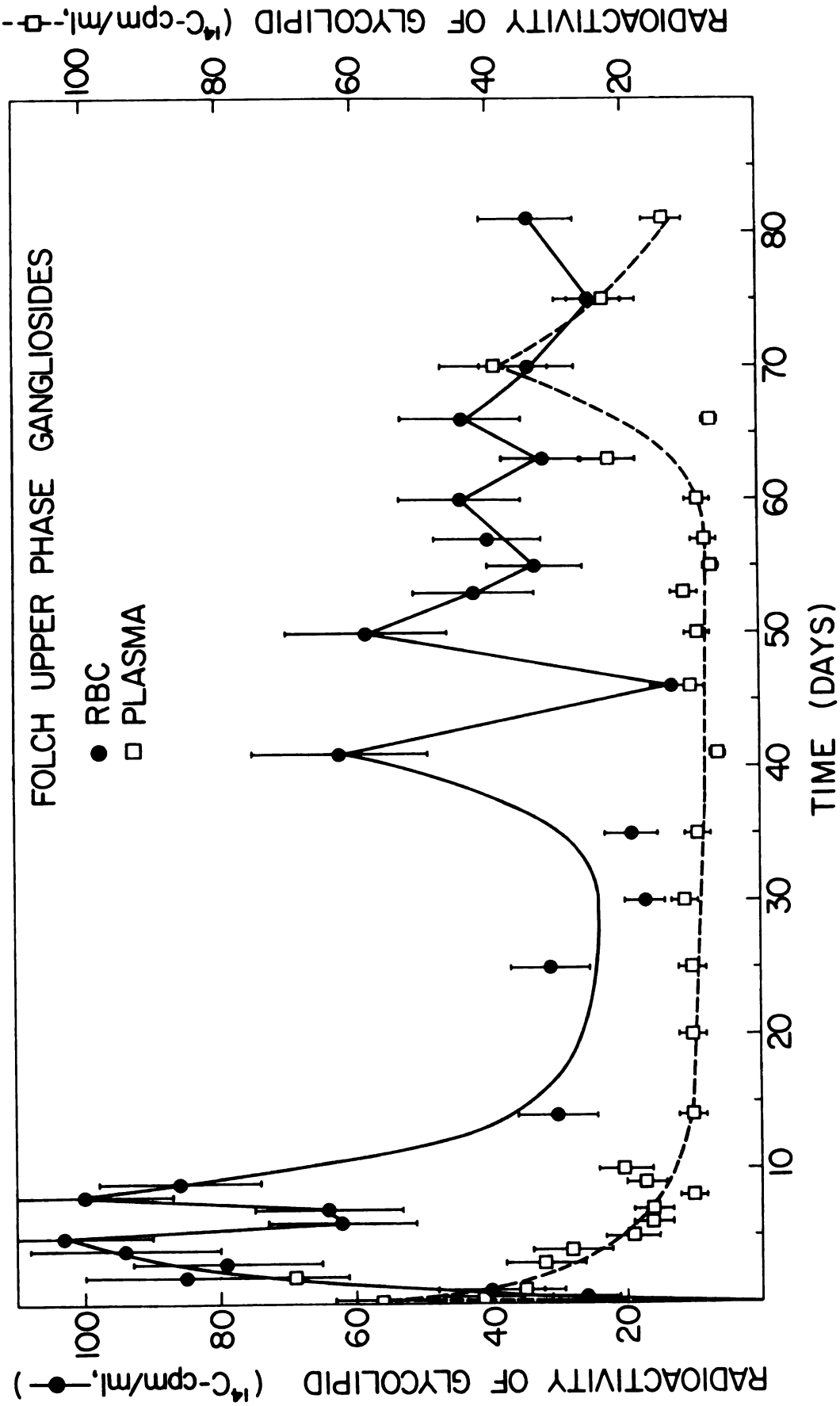

FOLCH UPPER PHASE GANGLIOSIDES
● RBC
□ PLASMA
RADIOACTIVITY OF GLYCOLIPID (¹⁴C-cpm/ml,—●—)
RADIOACTIVITY OF GLYCOLIPID (¹⁴C-cpm/ml,--□--)
TIME (DAYS)
20
40
60
80
100
10
20
30
40
50
60
70
80

Figure 34

The labeled glucose was incorporated rapidly, reaching maximum specific activity at 6 hr, and approximately 70% of this label was lost during the next 10 days. A semilogarithmic plot of specific activity versus time during this period clearly illustrates the biphasic nature of the decay curve, suggesting that there were two pools of glycosphingolipids with half-times of 0.9 day and 1.8 days, respectively. After the initial loss of the label from the plasma gangliosides, the specific activity remained rather constant for 50 days, then on Day 60 relabeling of this lipid occurred, reaching maximum specific activity at around Day 70. A semilogarithmic plot of the loss of the label versus time subsequent to Day 70 disclosed a half-time of 7.5 days.

When the upper phase gangliosides from erythrocytes were examined, maximum specific activities were detected on Day 3 and Day 8, followed by loss of approximately 70% of this label. Relabeling of the erythrocyte gangliosides are shown to occur around Day 35, and peaks of specific activities were detected on Days 41, 50, 60, 66, and 81. From the peaks of specific activities, the amount of radioactivity seemed to be decreasing from Day 41 to Day 81 with time during this period. Semilogarithms of the loss of label subsequent to Day 50 and Day 66 were plotted against time and revealed half-times of 6.3 and 11.2 days, respectively.

11. Incorporation of Labeled Glucose into Ceramides

Linear plots of specific activity (cpm/ml RBC) versus time for ceramide containing normal and α-hydroxy fatty acids (Figure 35) demonstrated rapid incorporation of the label at the early times similar to the results observed in GL-1a and GL-2a, namely, reaching maximum incorporation at 12 hr and on Day 3 and loss of approximately 50% of their label during the next 7 days. Relabeling of these two lipids occurred between Day 50 and Day 81; ceramide containing normal fatty acids exhibited peaks of maximum specific activities at Days 70 and 81, while α-hydroxy fatty acid-containing ceramide gave peaks of maximum relabeling at Days 53 and 70.

The ^{14}C-labeling of the ceramide fraction of plasma was negligible compared with the other glycosphingolipids (Figure 35).

When specific activity (cpm/μmole sphingosine bases) was plotted against time for the red cell ceramides (Figure 36) the profiles of radioactivity incorporation at the earlier times were shown to be essentially the same as mentioned immediately above. However, ceramide containing normal fatty acids was shown to be relabeled on Day 53 and Day 60 in addition to Days 70 and 81; while the fraction of ceramides containing α-hydroxy fatty acids exhibited peaks of relabeling on Days 53, 60 and 75. There were similarities in the patterns of relabeling of ceramide containing normal fatty acids, Gl-1a, GL-2a, and also G_M3.

Figure 35. Turnover of plasma and erythrocyte ceramides in pig 123-6.

The specific activities (cpm/ml) of ceramides from porcine plasma and erythrocyte were plotted linearly versus time over an 81-day period.

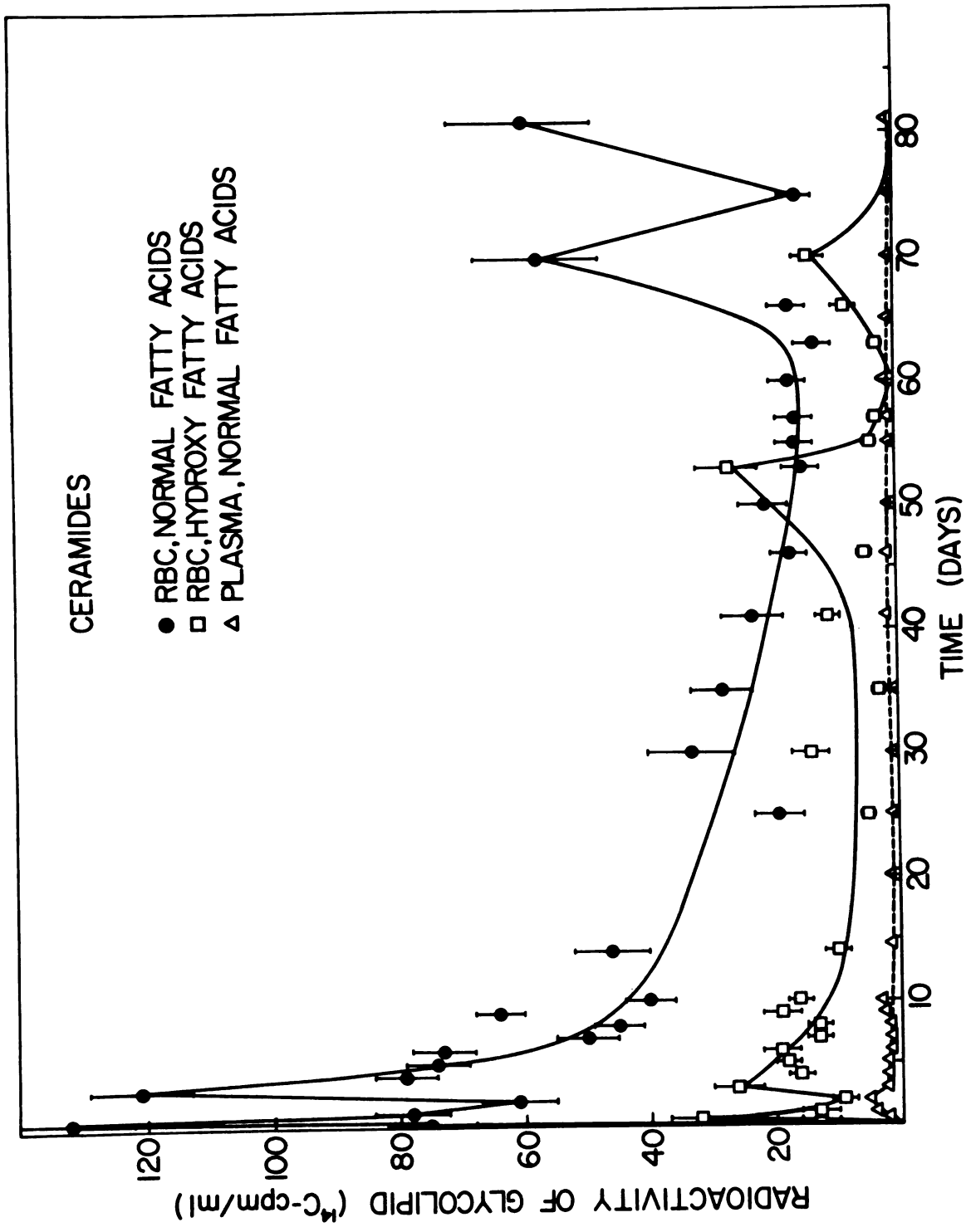

CERAMIDES
● RBC, NORMAL FATTY ACIDS
□ RBC, HYDROXY FATTY ACIDS
△ PLASMA, NORMAL FATTY ACIDS
RADIOACTIVITY OF GLYCOLIPID (^{14}C-cpm/ml)
120
100
80
60
40
20
10
20
30
40
50
60
70
80
TIME (DAYS)

Figure 35

Figure 36. Turnover of erythrocyte ceramides in pig 123-6.

A linear plot of specific activity (cpm/μmole sphingosine bases) versus time, showing the catabolism of the ^{14}C-labeled long-chain bases of red cell ceramides containing normal and 2-hydroxy fatty acids.

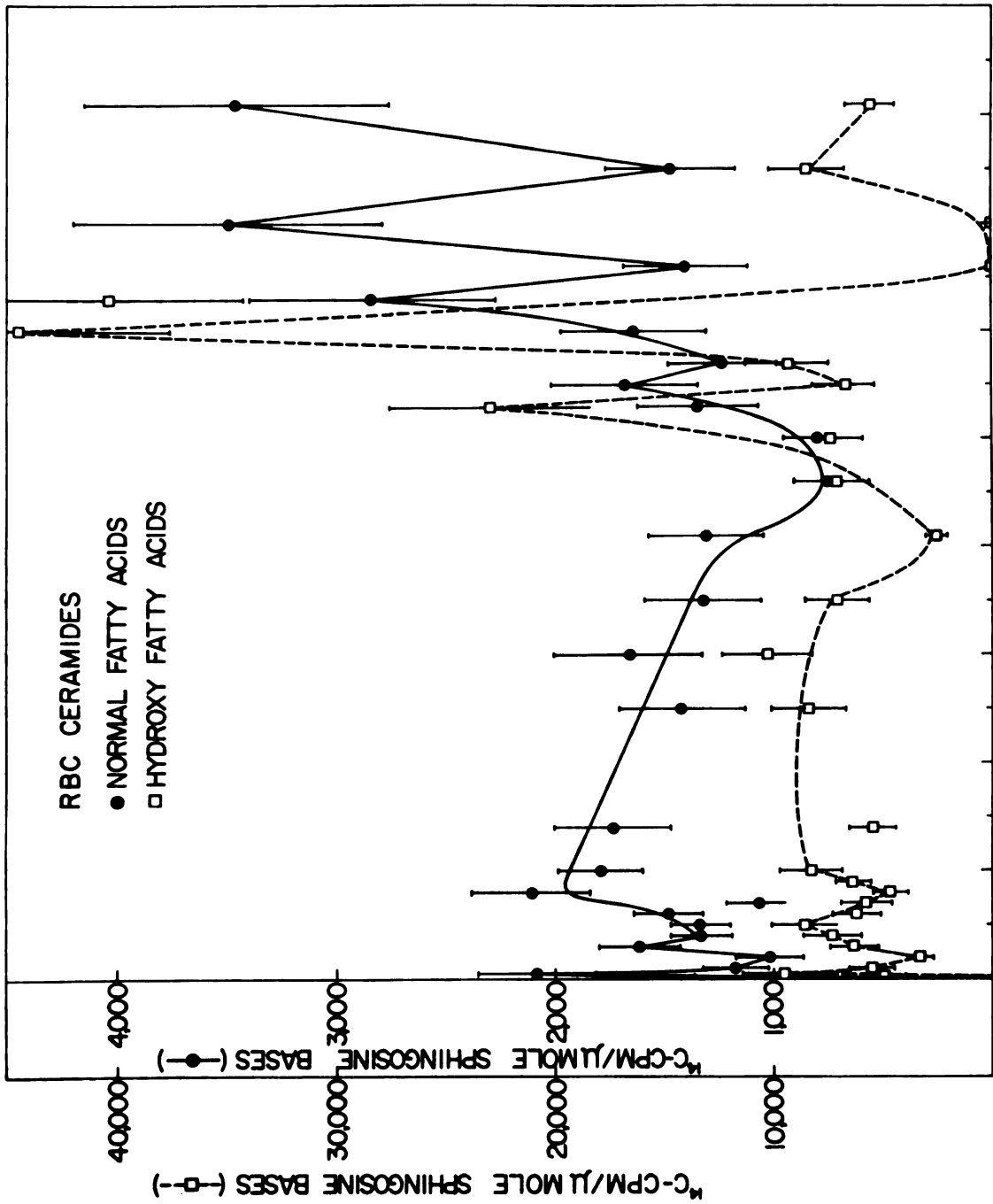

RBC CERAMIDES
● NORMAL FATTY ACIDS
□ HYDROXY FATTY ACIDS
TIME (DAYS)
10
20
30
40
50
60
70
80
^{14}C-CPM/μMOLE SPHINGOSINE BASES (—●—)
10,000
20,000
30,000
40,000
1000
2000
3000
4000
^{14}C-CPM/μMOLE SPHINGOSINE BASES (—□--)

Figure 36

12. Fractionation of Red Cells According to Age from Pig 123-6 During In Vivo Study

Fractionation of red cells into individual bands by the density gradient technique was performed according to the procedure outlined in Methods. Figure 37 shows the separation of red cells as bands after ultracentrifugation of the red cell samples obtained on Days 8 (A), 40 (B), and 70 (C), in a discontinuous density gradient of bovine albumin solution. On Day 8 (A) Bands 1 and 2 were clearly visible at the top of the gradient, representing reticulocytes and young cell fractions of the red cell population. On Day 40 and Day 70, these bands were hardly noticeable because the pig was no longer as anemic and no significant amount of reticulocytes were present at that time (Figure 20). This was also evident by the appearance of heavier bands at the lower half of the tube which is quite characteristic of the red cell separation patterns observed previously with normal pig and dog.

13. Globoside (GL-4) Concentration in Porcine Red Cells of Different Ages

The concentrations of erythrocyte GL-4 from 53 samples obtained between Days 2 and 81 are tabulated in Table 30. No changes with age in the relative proportions of the major erythrocyte glycosphingolipid component have been observed.

14. Globoside (GL-4) Turnover in the Fractionated Red Cells

Since GL-4 is the major porcine erythrocyte membrane glycosphingolipid, efforts were made to concentrate on the

Figure 37. Separation of young and mature erythrocytes from pig 123-6 during the *in vivo* study.

Two and one-half milliliters of porcine red cells were centrifuged on 6 layers of albumin solution ranging in specific gravity of 1.075-1.100 in cellulose nitrate tubes (1 1/2" x 3 1/2"). The tubes were centrifuged at 4°C for 30 min at 25,000 rpm in a Beckman Model LS-50 Ultracentrifuge with a 25.2 swinging bucket. A, 8 days post-injection of the radioactive label. B, 40 days post-injection of the radioactive label. C, 70 days post-injection of the radioactive label.

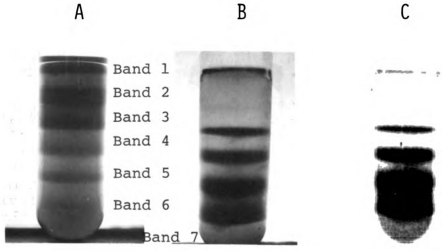


Figure 37

Table 30. Concentration and Specific Activities of Globoside from the Fractionated Red Cell Bands During the Metabolic Experiment

Days	Bands	ml. RBC used	μmoles / sample	nmoles / ml RBC	cpm (Hexoses) / sample	cpm (Bases) / sample	cpm (FAME) / sample	S.A.*	Hb	S.A.**
2	1	1.20	0.28	232	2582	317	412	2364	2.6	1013
	2	0.90	0.19	212	1388	123	162	1855	1.6	885
	3	1.40	0.29	208	456	43	49	399	3.3	141
3	1	1.50	0.33	219	1696	355	445	1399	2.0	882
	2	2.10	0.38	180	2342	264	314	1611	1.8	1353
	3	2.30	0.43	186	1314	122	156	799	2.2	621
4	1	1.50	0.31	205	380	226	249	330	1.8	228
	2	2.10	0.38	180	1794	364	412	1268	1.4	1383
	3	3.70	0.71	190	3172	349	518	1201	2.9	1181
	4	0.35	0.09	260	344	65	81	1017	0.5	743
5	1	0.55	0.13	245	46	37	66	96	1.3	39
	2	0.70	0.14	204	196	85	126	385	2.2	99
	3	1.30	0.24	180	868	139	202	1031	3.9	240
	4	0.90	0.16	183	552	84	121	939	2.0	309
6	1	1.30	0.26	204	84	78	101	90	1.8	53
	2	1.30	0.31	241	162	104	125	146	1.2	152
	3	2.40	0.42	175	1506	252	335	1011	1.7	1001
	4	0.42	0.09	218	234	58	72	721	0.5	529
7	1	0.70	0.14	194	28	41	47	59	0.9	36
	2	1.55	0.36	229	124	113	120	100	1.8	79
	3	4.20	0.96	229	2688	571	628	801	3.4	909
8	1	1.50	0.30	202	50	63	76	47	2.2	26
	2	4.30	0.85	197	1538	498	462	523	3.4	520
	3	1.40	0.31	222	666	160	167	616	2.0	383
10	1	2.00	0.44	219	58	121	109	40	2.0	35
	2	3.40	0.78	229	718	288	270	276	3.2	269
	3	2.00	0.32	158	748	194	216	709	2.4	374

14	1	1.00	0.25	250	42	52	77	59	1.4	42
	2	5.40	1.14	210	538	318	423	166	4.0	188
	3	1.00	0.21	211	292	82	107	484	1.1	372
20	1	0.40	0.11	271	30	25	21	121	0.4	132
	2	4.00	0.84	210	174	164	199	91	4.2	73
	3	2.10	0.41	196	270	108	132	289	2.5	190
30	4	0.27	0.07	242	22	18	27	188	0.4	123
	5	3.10	0.62	199	98	71	77	89	4.0	55
	6	1.80	0.28	150	78	40	55	158	2.8	62
	7	0.15	0.04	233	2	14	9	32	1.0	5
41	4	0.16	0.04	227	24	13	17	469	0.2	310
	5	2.20	0.45	204	48	47	53	76	2.4	57
	6	3.60	0.68	188	98	63	84	103	3.7	75
	7	0.15	0.04	245	16	24	25	310	2.6	18
50	5	2.10	0.42	201	14	30	35	27	3.0	15
	6	3.00	0.64	214	30	50	54	38	4.2	23
	7	0.05	0.01	206	12	9	19	947	2.0	20
60	5	2.00	0.40	202	8	23	29	19	2.5	12
	6	3.90	0.80	206	34	44	49	40	4.4	29
	7	0.16	0.04	248	10	15	24	239	4.0	10
70	5	2.40	0.48	202	16	24	25	36	2.8	25
	6	2.30	0.46	198	4	24	33	10	2.8	6
	7	0.14	0.02	166	20	26	22	942	1.3	67
81	5	0.79	0.15	192	16	33	20	131	1.3	61
	6	4.60	0.94	205	34	36	50	45	4.1	13
	7	0.50	0.09	172	10	15	23	144	5.8	9
Mean				209						

*S.A. = specific activity (cpm/μmole)

**S.A. = specific activity (cpm/ g Hb)

isolation of this lipid and study its turnover in the fractionated groups of red cells. Specific activities were expressed as cpm/μmole hexose and cpm of total hexoses/g hemoglobin. In both cases, specific activities were corrected for the increase in blood volume of the pig during the in vivo experiment. It will be noted that not all the bands were analyzed for radioactivity. Samples were randomly chosen at the beginning, middle and end of the experiment, with emphasis on analyses of the top and bottom layers of cells.

Table 30 summarizes the specific activities obtained from GL-4 of each individual group of red cells fractionated during the in vivo study. Labeled glucose was incorporated into the hexose, sphingosine and fatty acid moieties of the erythrocyte GL-4 molecule, although in the majority of cases, most of the label was found in the hexose residues.

The data in A of Figure 38 show that the radioactivity of GL-4 was concentrated in the top layers at the beginning of the experiment, then moved gradually downward to reach the bottom of the tube at 50 days. At Day 60, only 25% of radioactivity (from Day 50) was found to remain in Band 7. However, at Day 70, radioactivity reappeared in Band 7, reaching an almost identical specific activity as that of Day 50. This label was subsequently lost to approximately the same level as that of Day 60 by Day 81. During this period, relabeling seemed to occur in Band 5 also. Essentially similar results were obtained when specific activity was expressed as cpm of

Figure 38. Distribution of radioactivity of red cell globoside from pig 123-6 at time interval after the initial pulse label.

A, specific activity (cpm/μmole hexose) of ^{14}C-labeled GL-4 from porcine erythrocytes fractionated by ultracentrifugation on density gradient at various time intervals during the in vivo experiment. B, specific activity (cpm of total hexoses/g hemoglobin) of ^{14}C-labeled GL-4 from porcine erythrocytes fractionated by ultracentrifugation on density gradient at various time intervals during the in vivo study. The amount of recovered red cells in Bands 4-7 at the early times was too small to permit analysis.

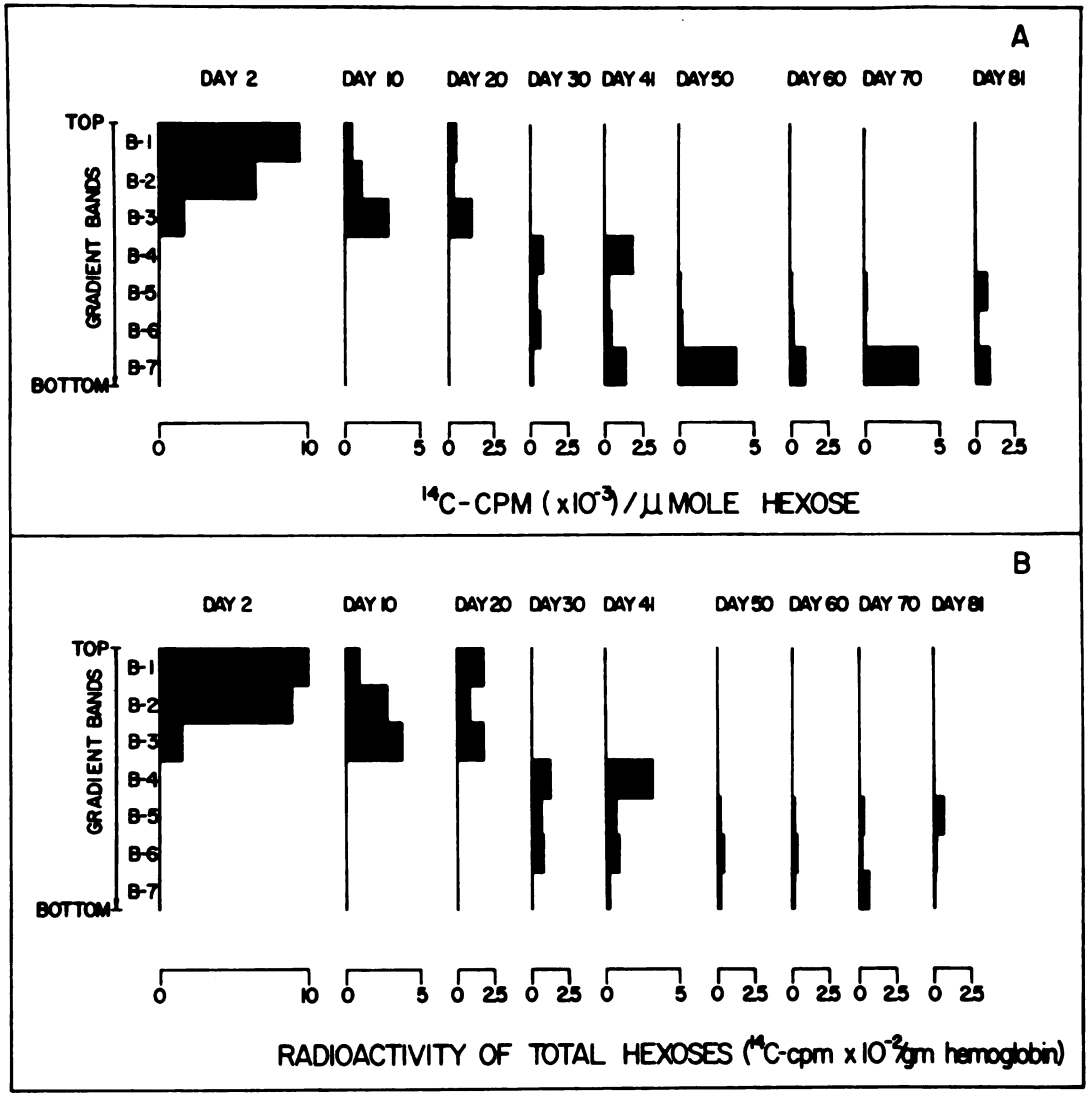

A
DAY 2
DAY 10
DAY 20
DAY 30
DAY 41
DAY 50
DAY 60
DAY 70
DAY 81
TOP
B-1
B-2
B-3
B-4
B-5
B-6
B-7
BOTTOM
GRADIENT BANDS
0 10
0 5
0 25
0 25
0 25
0 5
0 25
0 5
0 25
^{14}C-CPM (x10^{-3}) / µ MOLE HEXOSE
B
DAY 2
DAY 10
DAY 20
DAY 30
DAY 41
DAY 50
DAY 60
DAY 70
DAY 81
TOP
B-1
B-2
B-3
B-4
B-5
B-6
B-7
BOTTOM
GRADIENT BANDS
0 10
0 5
0 25
0 25
0 5
0 25
0 25
0 25
0 25
RADIOACTIVITY OF TOTAL HEXOSES (^{14}C-cpm x 10^{-2}/gm hemoglobin)

Figure 38

total hexoses/gm hemoglobin, as shown in Figure 38, B. The distribution of radioactive GL-4 from individual bands at various time intervals was quite similar to that observed in A of Figure 38, except that the radioactive label was not concentrated in the bottom layer until Day 70. There was no appreciable radioactivity detected at Day 81. The specific activity of Band 5 seemed to increase with time between Days 60 and 81.

Figure 39 is another variation of presenting the data shown in Figure 38, B. Essentially similar comments can be made.

Figure 40 is a linear plot of the specific activity (cpm/μmole hexose) versus time of the data from Table 30. In A of Figure 40, specific activities of Bands 1, 2, and 3 were plotted from Day 2 to Day 20 whereas specific activities of Bands 5, 6, and 7 were plotted from Day 30 to Day 81 as demonstrated in Figure 40, B. The [U-^{14}C]glucose was rapidly incorporated into GL-4 of the top layer, reaching maximum specific activity 48 hr after the initial pulse label. The label then disappeared rapidly until, at Day 6, almost none was detectable. Approximately 98% of this label was lost during this time period, suggesting that the turnover rate of GL-4 of this reticulocyte-rich fraction was very rapid. The remaining 2% of the radioactivity was maintained throughout the rest of the 20-day period. Incorporation of the radioactive label into GL-4 of Band 2 was quite rapid, reaching a

Figure 39. Bar graph presentation of the red cell globoside specific activity from pig 123-6 at various time intervals after administration of the label.

Porcine erythrocytes were fractionated by ultracentrifugation on density gradient. Left, represents the specific activity of GL-4 from Bands 1, 2, and 3 beginning at Day 2 through Day 14. Right, represents the specific activity of GL-4 from Bands 5, 6, and 7 between 30 and 81 days post-injection of the radioactive glucose.

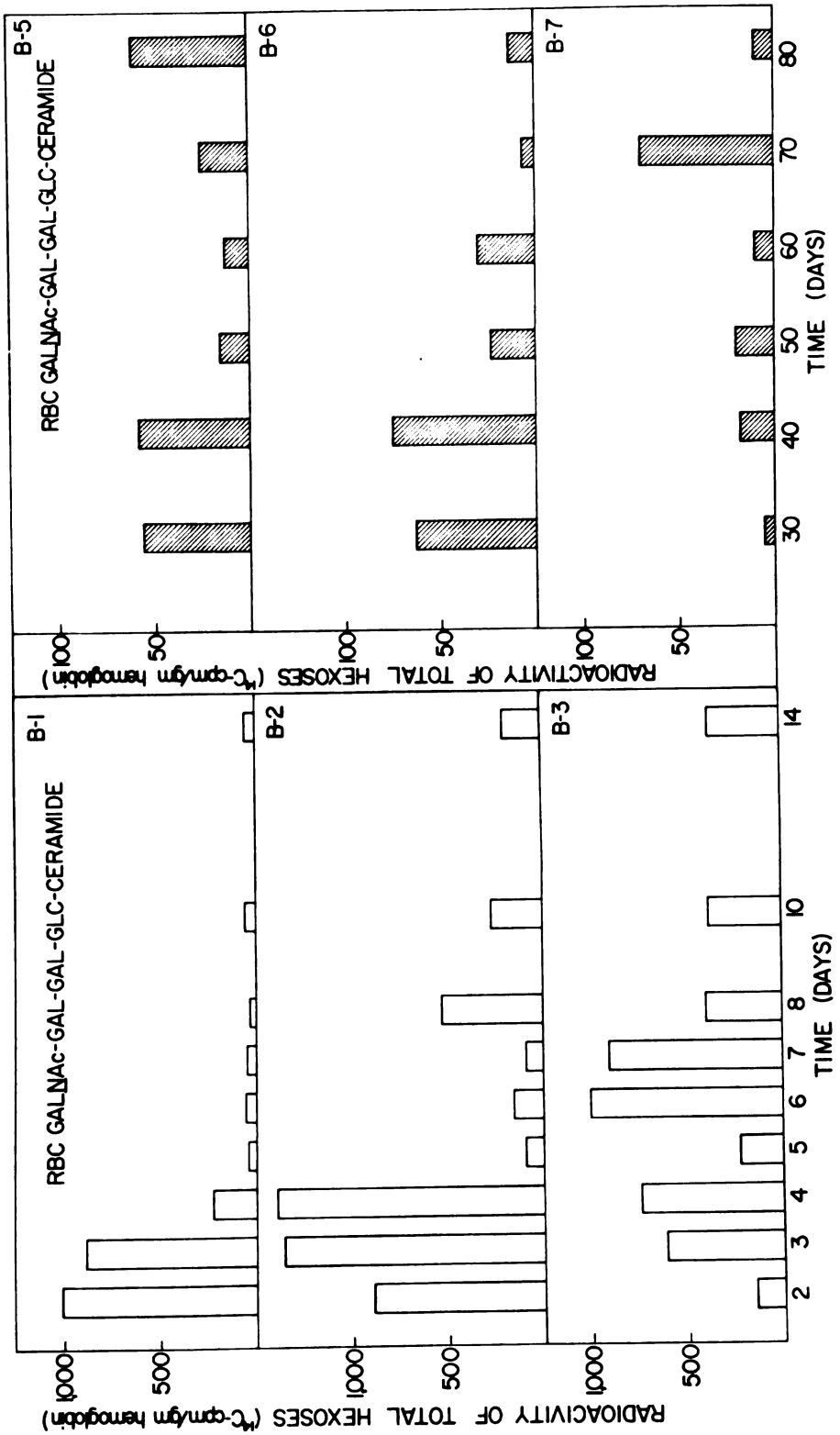

B-1
RBC GALNAc-GAL-GAL-GLC-CERAMIDE
B-2
B-3
B-5
RBC GALNAc-GAL-GAL-GLC-CERAMIDE
B-6
B-7
RADIOACTIVITY OF TOTAL HEXOSES (¹⁴C-cpm/gm hemoglobin)
TIME (DAYS)

Figure 39

Figure 40. Globoside turnover in individually fractionated groups of porcine erythrocytes.

A linear plot of the specific activity as a function of time for GL-4 derived from individually fractionated band of cells by ultracentrifugation on density gradient. A, represents the turnover of GL-4 from cells of Bands 1, 2, and 3 between Day 2 and 20. B, represents the turnover of GL-4 from cells of Bands 5, 6, and 7 between Day 20 and Day 81 at 10 days apart.

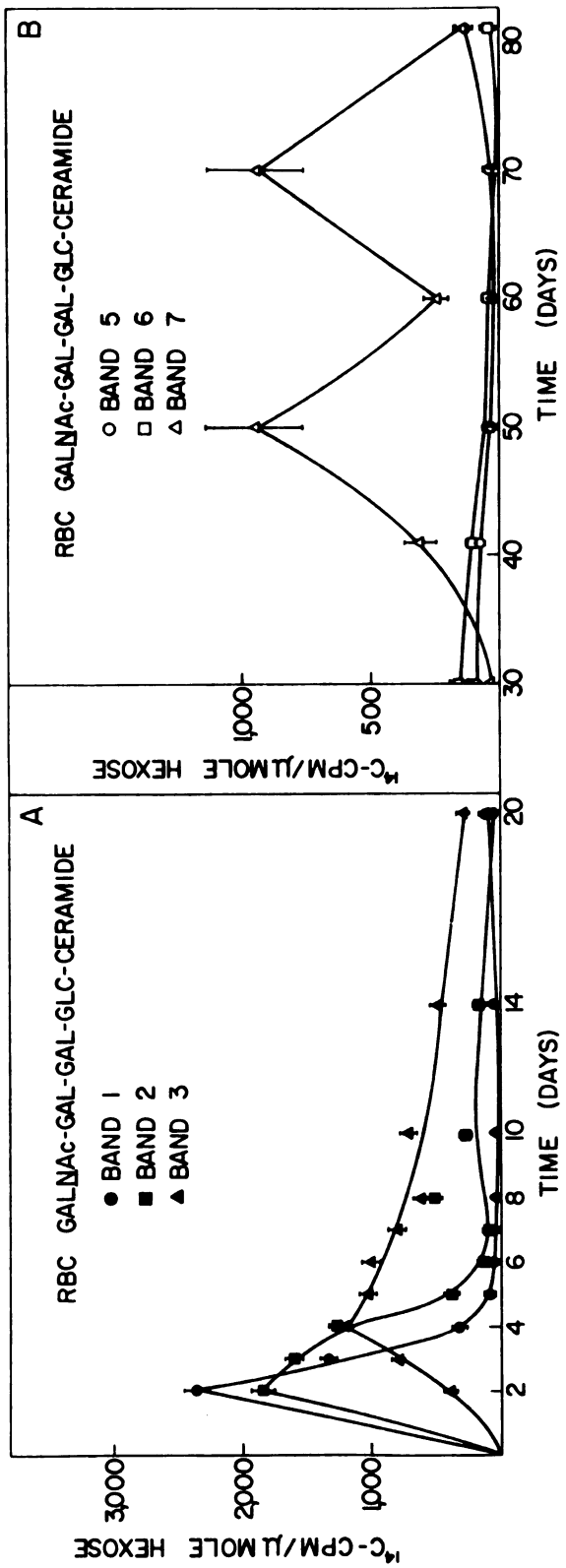

A
RBC GALNAc-GAL-GAL-GLC-CERAMIDE
● BAND 1
■ BAND 2
▲ BAND 3
^{14}C-CPM/μMOLE HEXOSE
TIME (DAYS)
B
RBC GALNAc-GAL-GAL-GLC-CERAMIDE
○ BAND 5
□ BAND 6
△ BAND 7
^{14}C-CPM/μMOLE HEXOSE
TIME (DAYS)

Figure 40

peak of incorporation also at 3 days. Loss of label subsequent to maximum incorporation was only a little slower and approximately 92% of the label was lost by Day 6. The specific activity remained rather constant thereafter for the remaining 14 days. Incorporation of radioactive glucose into GL-4 of Band 3 began at the time when the specific activity of GL-4 from Band 2 had reached its peak of incorporation and was beginning to lose its label. The rate of incorporation in this band was rather slow and its maximum specific activity was not reached until 4 days. Furthermore, loss of label subsequent to maximum incorporation was a gradual process since only approximately 50% was lost by Day 10 and 25% of the label remained at Day 20.

The specific activities from Bands 5 and 6 seemed to decrease gradually with time. A notable exception is Band 7, where relabeling of GL-4 seemed to occur, reaching maximum specific activity at Days 50 and 70.

Semilogarithmic plots of specific activity (cpm/μmole hexose) versus time (Figure 41) revealed biphasic decay curves, observed above, suggesting that there were at least two pools of GL-4 in the fractionated red cell bands (1, 2, and 3). Bands 1 and 2 showed a very rapid turnover of the GL-4 pool, having half-times of 0.75 day and 1 day, respectively. The turnover rate of the second pool was slower, with half-times of 3.3 and 5.5 days, respectively, for these two groups of cells. The two pools of GL-4 from Band 3 seemed to have a

Figure 41. Globoside turnover in the top three bands of porcine erythrocytes fractionated by density gradient ultracentrifugation.

Semilogarithmic plot of specific activity as a function of time for GL-4 derived from cells fractionated by ultracentrifugation on density gradient. Left, turnover of GL-4 in Band 1, Middle, turnover of GL-4 in Band 2. Right, turnover of GL-4 in Band 3. The biphasic nature of the decay curves indicated that there were two pools of GL-4 present in each group of cells.

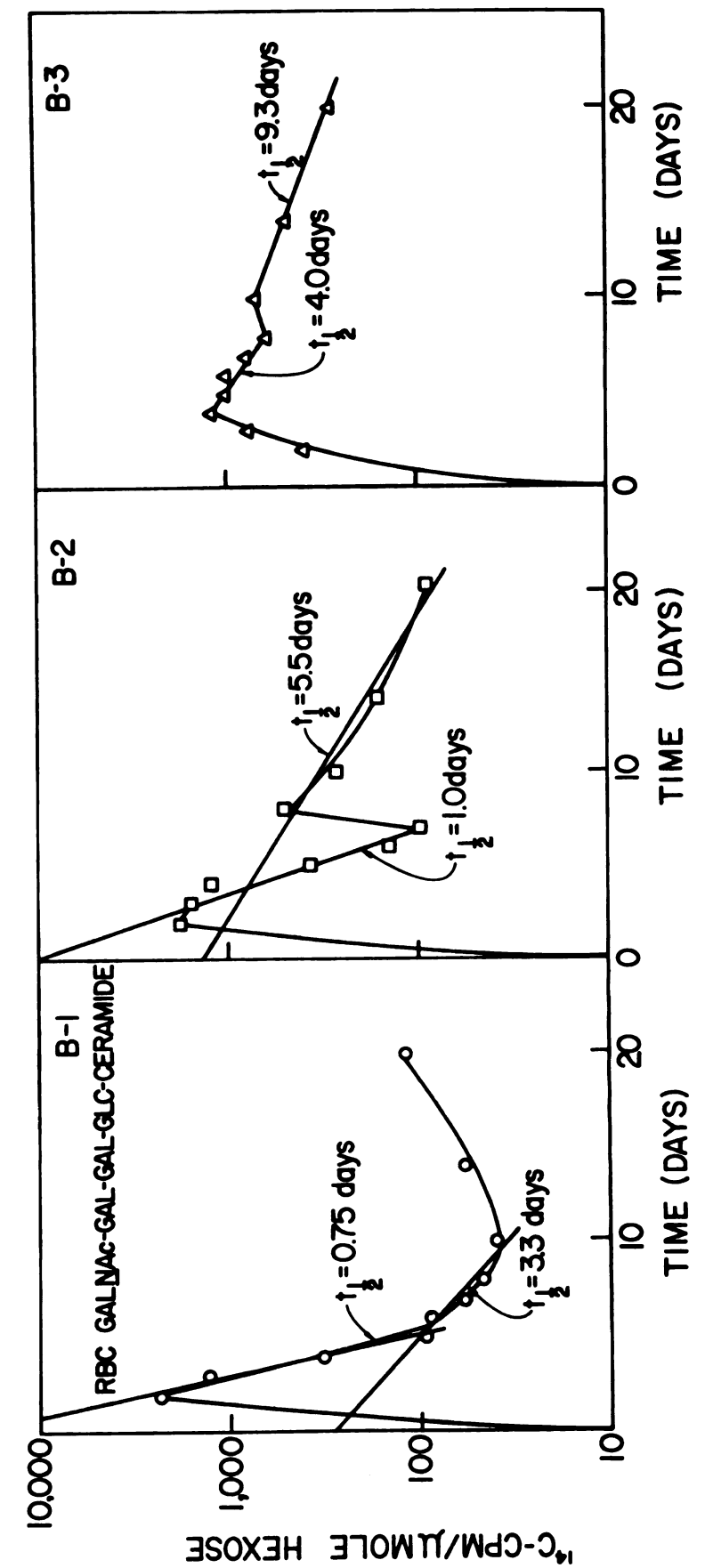

B-1
RBC GALNAc-GAL-GAL-GLC-CERAMIDE
B-2
B-3
$t_{\frac{1}{2}}$=0.75 days
$t_{\frac{1}{2}}$=3.3 days
$t_{\frac{1}{2}}$=5.5days
$t_{\frac{1}{2}}$=1.0days
$t_{\frac{1}{2}}$=9.3days
$t_{\frac{1}{2}}$=4.0days
TIME (DAYS)
TIME (DAYS)
TIME (DAYS)
^{14}C-CPM/μMOLE HEXOSE
10,000
1,000
100
10
0
10
20

Figure 41

much slower turnover time; the half-times were 4.0 and 9.3 days for the two corresponding pools.

15. Turnover Values of Plasma and Erythrocyte Glycosphingolipids

Turnover values were determined from semilogarithmic plots of specific activity (cpm/μmole hexose) versus time over the period subsequent to maximum labeling. The semilogarithmic plot provides the half-time (t 1/2) (275), from which turnover times can be calculated (t_t = 1.44 t 1/2). Turnover rates (p) can then be calculated from the total circulating pool sizes of the glycosphingolipid (r) and the turnover time (t_t) from the simple relationship described by Zilversmit (275), as shown below.

$$p = \frac{r}{t_t}$$

The calculated turnover values for the plasma and erythrocyte glycosphingolipids are summarized in Table 31. Because of the biconcave nature of the turnover curves, two turnover values (pool A and B) were obtained for both plasma GL-1a and G_{M3} ganglioside. In GL-1a, pool A turns over ten times more rapidly than pool B; in G_{M3} pool A has turnover twice as high. It is also apparent that GL-2a turns over more rapidly than GL-3a and GL-4. The turnover times of GL-2a, GL-3a, and GL-4 are approximately the same, whereas the turnover times of GL-1a and G_{M3} in pool A are much shorter than in pool B.

Table 31. Turnover Values of Porcine Plasma and Erythrocyte Glycosphingolipids

Glycolipid	Half-time[a] (days)	Turnover Time (days)	Total Pool Size[b] (μmoles)	Turnover Rate (μmoles/day)	Amount of Total Pool Synthesized per day (%)
Plasma GL-1a	0.75	1.08	5.98	5.54	93
	7.50	10.80	5.78	0.54	9
Plasma GL-2a	3.0	4.32	3.13	0.72	23
Plasma GL-3a	3.5	5.04	1.65	0.33	20
Plasma GL-4	3.6	5.18	1.74	0.34	20
Plasma G_{M3}	0.9	1.30	1.17	0.90	77
	1.9	2.74	1.38	0.50	36
RBC GL-4					
Band-1	0.75	1.08	70.66	65.43	93
	3.3	4.75	70.22	14.78	21
Band-2	1.0	1.44	67.74	47.04	69
	5.5	7.92	94.34	11.9	13
Band-3	4.0	5.76	66.48	11.5	17
	9.3	13.40	92.35	6.89	7

[a] t 1/2 was derived from semilogarithmic plot of specific activity (cpm/μmole hexose) *versus* time.

[b] Total pool size is calculated from plasma and erythrocyte volume reported by Bush *et al.* (272) for the anemic pig.

Approximately 20% of GL-2a, GL-3a, and GL-4 is metabolized per day, a finding that agrees quite well with previous studies in humans (222) and pig (45). This cannot be said for pool A of GL-1a and G_{M3}, however, since both pools are very active metabolically and approximately 90 and 80% of the total glycolipid in this pool are synthesized per day.

Table 31 also presents the turnover values of GL-4 derived from the red cell groups during the ultracentrifugation study. It is evident that two pools of GL-4 (designated as pool A and B) were present in each of the fractionated bands. The turnover times of both the A (early) and B (late) pools became longer as the cells aged, while the turnover rates were rapid in Band 1 but became less rapid with aging. This seems to indicate that the young cells were more metabolically active and thus turning over much faster than the older cells. This was also obvious from the percentage contribution to the total GL-4 pool each day listed in the last column of Table 31.

V. DISCUSSION

Since the initial discovery of globoside and hematoside in erythrocytes (8,68,120), much has been learned about the occurrence of various neutral and acidic glycosphingolipids in plasma, erythrocytes and to some extent, leukocytes. However, as an important member of the formed elements in blood, platelets have not been examined for glycosphingolipids in great detail. The first part of this thesis research was concerned with the isolation and structural characterization of platelet glycosphingolipids. In addition, studies were made of the composition of human plasma gangliosides.

A. Human Platelet Sphingolipids and Plasma Gangliosides

The results presented in this dissertation were obtained with a pooled sample of trypsinized platelets from 73 donors. Simultaneous analyses of the glycoprotein composition of these platelets necessitated the trypsin treatment; however, a comparison was made on a smaller scale of untreated platelets and platelets treated with different proteolytic enzymes.

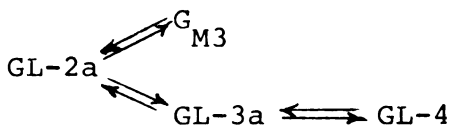
The solvent-soluble sphingolipid fraction of platelets consisted of sphingomyelin, ceramide, a family of neutral glycosphingolipids resembling those of plasma and erythrocytes (37), and G_{M3} ganglioside. Separation of the neutral glycosphingolipids and hematoside was easily accomplished on a single commercially available TLC plate. The molar ratios of sugars and permethylation studies coupled with use of specific glycosidases, confirmed that the major neutral glycosphingolipids were galactosyl(β1→4)-glucosylceramide (GL-2a), galactosyl(α1→4)-galactosyl(β1→4)glucosylceramide (GL-3a), and N-acetylgalactosaminyl(β1→3)-galactosyl(α1→4) galactosyl(β1→4)glucosylceramide (GL-4). The G_{M3} ganglioside consisted exclusively of N-acetylneuraminyl(2→3)-galactosyl-(1→4)glucosylceramide. The stereochemical configurations of the glycosidic linkages in GL-1 and G_{M3} ganglioside have not been determined.

The composition of the neutral glycosphingolipid fraction of human platelets was recently reported by Snyder, Desnick, and Krivit (224), who found that GL-2a (38-43%) and GL-4 (23-31%) were the major components. In my study, GL-2a was relatively much more dominant (64%) and GL-3a were present in about the same proportions (ca. 16%). The rather large amount of GL-2a present in platelets makes them quite unique among the formed elements of blood, and it is tempting to speculate that GL-2a may play a special role in the maintenance of platelet structural integrity or may be involved

in immunologic reactions, since the GL-2a from human epidermoid carcinoma explants in rats has been shown to have strong haptenic properties (276). GL-2a has also been shown to be the major neutral glycosphingolipid of human leukocytes (38, 39). Platelets and leukocytes differ substantially in this respect from erythrocytes, in which GL-4 is the major neutral glycosphingolipid (37). In porcine leukocytes, the concentration of GL-3a exceeds that of GL-2a (46).

Leukocytes are able to carry out the synthesis of GL-1a and GL-2a (40) as are cultured cells of bone marrow (147). No comparable experiments have been done with platelets to assess their enzymatic activity for glycosphingolipid biosynthesis.

The similarity of fatty acid composition in platelet GL-2a, GL-3a, GL-4 and G_{M3} seemed to support the concept of metabolic interconversion commonly believed to exist in glycosphingolipid metabolism, GL-2a has been indicated as an



intermediate in the biosynthesis of ganglioside and globoside (192). However, the marked differences in fatty acid composition observed in GL-1a and ceramides were indeed puzzling. In previous studies of porcine blood glycosphingolipids, the fatty acid composition of GL-1a was found to be significantly different from GL-4 (45,277); the latter glycolipid contained

appreciable proportions of 22:0 and 24:0 whereas more 16:0 and 18:0 were found to be present in GL-1a. Furthermore, in both porcine platelets and erythrocytes (45,278) the concentration of 16:0, 18:0, and 18:1 decrease with an increasing number of hexose units in the glycosphingolipids, whereas the concentrations of 22:0 and 24:0 increase. This does not seem to be the case with human platelet glycosphingolipids, which points to variations in species specificity among these cellular components.

It was previously reported that platelets contain G_{M3} ganglioside (223,224) but structural studies were not included in these earlier investigations. On the basis of molar ratios of sugars and permethylation studies, the structure of platelet G_{M3} ganglioside was found to be NANA-(2→3)-Gal-(1→4)-Glc-ceramide, which is exactly identical to the human plasma hematoside in structure.

The ratios of N-acetylneuraminic acid to glucose were somewhat lower than the theoretical value in both human plasma and platelet G_{M3}. Low recoveries of neuraminic acid are not uncommon (105), however, and may be the result of incomplete N-acetylation, partial acid destruction during methanolysis, overexposure to pyridine during the preparation of the trimethylsilyl derivatives (279), and loss during silicic acid chromatography (280) or gas-liquid chromatography (281). It is possible to obtain better relative values for neuraminic acid by using 0.5 N methanolic HCl during methanolysis and

exposing to pyridine in the trimethylsilylation reaction for a short period of time, since the neuraminic acid values decrease slowly with time (279).

The concentrations of the water-soluble gangliosides were not determined, and hence one cannot comment on the relative importance of G_{M3} compared with other platelet gangliosides. On the basis of a G_{M3} partition ratio of 0.4 between the upper and lower phases of a partition system (282), it was calculated that the total platelet concentration of G_{M3} was about 1.9 μmoles/g total lipid, a value that agrees reasonably well with the relative yields of G_{M3} given by Snyder et al. (224). The finding that G_{M3} is the major ganglioside along with some hexosamine-containing gangliosides in both human platelets and plasma has been confirmed recently by Marcus et al. (283) and Yip and Ledeen (284). However, individual ganglioside structures have not yet been determined, and the presence of glucosamine indicates that some of the structures must have different oligosaccharide moieties than those characteristic of brain.

Previous studies had demonstrated that the relatively non-polar neuraminic acid-containing glycolipids were the major representatives of the extraneural tissues (264). Hence, it is generally believed that the pattern of gangliosides in extraneural tissues is appreciably simpler than in the brain. However, recent studies by Puro (264) demonstrated the presence of several slow-moving gangliosides in a variety of

tissues. In pig platelets (278), 8 bands were detected in the ganglioside fraction by TLC. This finding, coupled with the detection of slow-moving minor gangliosides in both human plasma and platelets, further extend the observation of Puro from extraneural tissues to body fluids and its component. Thus, the ganglioside patterns in the extraneural tissues and fluid are at least as complex as those in the brain.

The fatty acid distributions of both human plasma and platelet G_{M3} are different from the results reported for brain gangliosides in man (285-288) and vertebrates (288) wherein stearic acid comprised 80-90% of the total fatty acids. In the plasma and platelet G_{M3} ganglioside from lower phase the longer-chain fatty acids from C_{20}-C_{24} represented 68-79% of the total mixture, which is in accordance with some of the reported studies on extraneural gangliosides (113,289,290). In the upper phase, platelet gangliosides were shown to contain principally 16:0, 18:0, and 18:1, with the exception of ganglioside A which had more of 22:0.

The physiological significance of gangliosides is largely unknown. Inherited diseases with a storage of gangliosides in different tissues, especially in brain, are termed gangliosidoses (67). There have been only two cases found with an accumulation of ganglioside G_{M3} (52,291). There are many facts, however, supporting the view that gangliosides play a dynamic role in the function of the cell membrane and thus are

not merely structural lipids (292). As for G_{M3}, it has been suggested that this ganglioside may have an immunologic role in transformed cells (293); it may also be involved as a receptor site on platelet membranes for serotonin (294,295), and in platelet aggregation (224).

As it was stated earlier, a comparison was made on a smaller scale of untreated platelets and platelets treated with several proteolytic enzymes to determine whether the composition of the platelet sphingolipids was changed by preincubation with these enzymes. The yield of hematoside was substantially higher when the cells were trypsinized and was higher still after incubation with thrombin, but no other measurable changes were observed. It has been reported that thrombin (and also trypsin) produces striking stimulation of phosphatidylserine formation in platelets (296). When trypsin was preincubated with soybean trypsin inhibitor, the effect of trypsin was abolished, suggesting that the proteolytic action of trypsin may be required to produce the effect of phosphatidylserine formation, since trypsin inhibitor alone exerted no effect on phosphatidylserine formation (296). The mechanism(s) of the thrombin effect on the stimulation of both phosphatidylserine and G_{M3} ganglioside and its physiological significance are not clear at the present time.

Thrombin is a proteolytic enzyme which not only clots fibrinogen but also causes platelets to aggregate and undergo "viscous metamorphosis" (296). Trypsin has also been shown

to aggregate washed platelets (297) in a manner similar to that observed with thrombin. Both trypsin and chymotrypsin were obtained from Calbiochem and were classified as Grade A purity. Thrombin was from Parke, Davis and Co., and was presumably also of good quality. Nevertheless, the possibility of having some other substance(s) present in the thrombin preparation has not been completely ruled out and further studies are required to clarify this point.

Thrombin affects platelet metabolism in a number of ways. It causes an increase in ATPase activity and lactate production (298), and a decrease in the intracellular ADP and ATP levels (298-300). It is possible that the increased G_{M3} formation may be an indirect effect of the changes of intracellular sugar nucleotide levels by the action of thrombin on the platelet membrane.

Indirectly, the proteolytic enzymes may act on the platelet surface, splitting a target protein and producing an active peptide that might act as a messenger to stimulate the release reaction (301). During the release reaction, acid hydrolases are believed to be released in soluble form (302). If this is true, the possibility exists for the stepwise removal of sugar units from more complex gangliosides (such as G_{M2}, G_{M1}) by these hydrolases. However, examination of the gangliosides derived from the Folch upper phase by TLC did not reveal any discernable changes between control platelets and those treated with proteolytic enzymes.

Another possible explanation is that thrombin causes some conformational changes on the platelet membrane surface possibly by proteolytic action which might in turn alter the spatial configuration of enzymes, such as sialyltransferase, that will actively transfer NANA from some other macromolecules (such as the glycoproteins) to the receptor GL-2a, the major glycosphingolipid in platelets. It has been reported (303) that washed human platelets incubated with [^{14}C]CMP-NANA in the presence of homogenized rat liver as the source of sialyltransferase showed an increase in the amount of sialic acid bound to the platelet membrane. Furthermore, an increase in sialic acid bound to the

$$\text{Platelet} + [^{14}\text{C}]\text{CPM-NANA} \xrightarrow{\text{sialyltransferase}} \boxed{\text{Platelet}}\sim\sim \quad \text{sialic acid} + \text{CMP}$$

surface favors the aggregation of serotonin on platelets. Sialic acid has been implicated as a component of the receptor complex for serotonin on platelet membrane as well as on smooth muscle of the guinea-pig intestine (304). It has been suggested that G_{M3} ganglioside may be involved at the receptor site for serotonin on the platelet membrane (294, 295).

It is also possible that the burst of G_{M3} synthesis is involved in blood coagulation, since thrombin is an active participant in the clotting mechanism. It is of interest to note that phosphatidylserine thus far seems to be the best

replacement for platelet membranes in coagulation (305), although it is not as effective as the platelet membranes themselves (306). Nevertheless, it is uncertain whether or not G_{M3} ganglioside can replace the platelet membranes in the coagulation process and further study is required to evaluate this point.

An interesting fact, although not related to the thrombin effect, is that injection of bacterial neuraminidase intravenously causes thrombocytopenia in the rats (266). It was concluded that the neuraminidase cleaved the sialic acid from the platelet membrane, altering and deforming the surface characteristics and thus provoking removal of the damaged platelet from the circulation by the reticuloendothelial system. This seems to imply that the sialic acid-containing component plays an important role in the structural integrity of the platelet membrane. Another interesting aspect about neuraminidase is that it inhibits the release of histamine by rat mast cells in the IgE antibody-mediated reaction. In this reaction, the IgE antibody binds to the receptor site on the surface of the mast cell which in turn activates the release of histamine (307). Preliminary evidence (307) suggests that neuraminidase acts on the receptor site for IgE rather than on substrates involved in the reaction sequence (307). If this is true, then the receptor site would seem to contain sialic acid residue(s), probably a ganglioside as part of this receptor complex on the membranes of both

platelets and mast cells and other cells for that matter. In this regard, there seems to be a functional role for the presence of this ganglioside in a variety of extraneural tissues. Platelet is the only formed element of blood which exhibits the characteristics of accumulating serotonin (308) and dopamine (309). The mechanism of the release and transport of these amines to target organs or tissues in the blood stream is not clear at present.

Platelets may be unique among mammalian cells in the high proportion of total sphingolipids accounted for by free ceramide, which confirms recent reports by Krivit and Hammarström (225) for human platelets and by Heckers and Stoffel (278) for pig platelets. It is clear from a variety of studies that ceramides are common constituents of animal tissues, including erythrocytes (9), plasma (13), aorta (14), liver (5), spleen (6), lung (7), kidney (10), and brain (15). In none of these cases is the concentration of ceramide as high as it is in platelets, however. It is possible that the ceramide fraction represents accumulation from glycosphingolipid turnover especially since significant activities of various glycosyl hydrolases have been reported in platelets (224). The fatty acid composition of the various sphingolipid fractions does not support this theory, however. The ceramides contained significantly more 16:0 and 18:0 than any of the glycosphingolipids, while the level of 24:1 was much lower. The ceramide fraction also differed considerably in fatty acid

composition from that of ceramides (13) and other sphingolipids of human plasma, and was confirmed by Krivit and Hammarström (225,310). These results and the high concentration of the ceramide fraction suggest that its role in platelets may not be limited to that of an intermediate in the biosynthesis and degradation of more complex sphingolipids.

The yields of total phospholipids (65%) and neutral lipids (25%) in platelets and their composition were not remarkably different from the results reported by Marcus, Ullman and Safier (226). Phosphatidylcholine was the major phospholipid and free cholesterol accounted for most of the neutral lipid fraction. The proportion of sphingomyelin was approximately equal to that reported for erythrocytes and was considerably higher than that of normal lymphocytes and polymorphonuclear leukocytes (311). The fatty acid composition of the various phospholipids, triglycerides and cholesterol esters were in close agreement with the results obtained by Marcus et al. (226) including the interesting fact that the cholesterol ester fraction is practically devoid of 18:2. It was also noted that the phosphatidylethanolamine fraction contained about 20% of plasmalogen form, consisting primarily of molecular species containing 16:0 and 18:0 alkenyl groups, whereas the other phospholipids contained very much lower levels of plasmalogens.

B. Porcine Platelet Glycosphingolipids

Analyses of porcine platelets revealed that GL-3a was the major neutral glycosphingolipid present, which confirmed recent studies of Heckers and Stoffel (278). In addition, GL-1a, GL-2a, GL-4, and G_{M3} were also found. The presence of GL-4 in my samples may have been due to a small amount of erythrocytes in the platelet preparation, since this lipid was not found by Heckers and Stoffel. An interesting observation was that an extra band was detected by TLC, which migrated between GL-2a and GL-3a and had a R_F value similar to that of sulfatide. Heckers and Stoffel reported that sulfatide is one of the acidic glycoshpingolipids in porcine platelets. They also found that the major porcine platelet ganglioside consisted of a double band on TLC; one band contained a lactosylceramide residue and the other a diglucosylceramide residue which had never been found before. N-Glycolylneuraminic acid was found to be present in these lipids. G_{D3} was not detected in this study, because it is present exclusively in the Folch upper phase and was not analyzed in this case.

The chromatographic behavior and identification of the ceramides confirmed the findings by Heckers and Stoffel for porcine platelets. The fact that human and porcine platelets are both rich in ceramides further substantiate the importance of this class of lipid in platelet physiology.

It is interesting to note that GL-2a is the major membrane-bound glycosphingolipid in both human leukocytes and

platelets, whereas GL-3a is the major glycolipid of porcine leukocytes and platelets. Thus, there appears to a degree of species specificity in the composition of neutral glycosphingolipids of these cells which is not observed in the erythrocytes.

C. Fetal Erythrocyte GL-4

The fetal erythrocyte has been shown to possess a number of unique structural and metabolic properties (312). Some of these are related to cell age whereas others are unique to the fetal cell. Decreased survival of the fetal erythrocyte, to approximately 80 days, has been clearly demonstrated by many techniques (313). These findings may have important implications in certain neonatal diseases.

Many possibilities have been proposed for the decreased survival of fetal erythrocytes (312), and membrane lipid alteration is one of them, since lipids play an important role in the erythrocyte membrane function. As part of the interest in blood glycosphingolipid metabolism, an attempt was made to study the major glycosphingolipid composition of fetal red cells during development. In particular, the question was posed whether globoside is part of the erythrocyte membrane structural component during the early embroyonic stages or whether it is incorporated into the membrane structure only at certain stages of the developmental process such as near or at term.

It is not practical to obtain blood samples from human fetuses. Although the pig has a gestation period of ∿ 114 days (about 1/2 of humans), as an animal model it seems to provide all the necessary requirements from which a study of this nature can be performed. First of all, the pig also possesses GL-4 as the major erythrocyte glycosphingolipid. Secondly, the pig fetus is large enough in size so that adequate amounts of blood can be removed for analysis. And finally, in contrast to many other animals, the placenta of the pig is a rather primitive one (314). The chorion and the urterine mucosa lie in close contact with each other but are not fused, so they can easily be peeled apart. The maternal and fetal circulation are always separated.

No definite conclusions can be drawn from the analyses of erythrocyte GL-4 of the 45- and 90-day fetuses. However, the low GL-4 concentration found in the 45-day fetuses is of interest and may be significant when one considers the contamination of mother's blood in the sample. Hence, in actuality, the values may be lower than what was observed or GL-4 may actually be totally absent. If blood-sampling had been delayed until perhaps Day 52 or 55 (of gestation) it may have been possible to withdraw blood from the vessels (as that of the 90-day fetuses) without any difficulties and contamination.

D. Metabolic Study in Pig 123-6

The second half of this thesis is concerned with metabolism of neutral and acidic glycosphingolipids in an anemic pig. The primary objective was to study the metabolism of globoside as a function of erythrocyte senescence, and to determine the nature of interrelationships between plasma and red cell pools of glycolipids. One approach to study the turnover of globoside in red cell fractions involved separation of red cells by density gradient ultracentrifugation.

Induction of anemia in pig 123-6 was accomplished by daily bleeding. The data from the hematological profiles indicated that the pig responded well to the stress of bleeding with an active marrow production. The reticulocyte percentage was used to define the response of the anemic pig, and the hemolytic state was identified on the basis of a high circulating reticulocyte count. No reports were available for comparison on the induction of reticulocytosis in a pig with such a degree and for such a long period of time.

Separation of reticulocytes from the mature erythrocytes was achieved by density gradient ultracentrifugation. A discontinuous gradient offers the advantage over a continuous one is that larger quantities of red cells can be centrifuged. However, even with the degree of resolution obtained by density gradient ultracentrifugation, pure fractions containing 100% of cells of a given age cannot be obtained from normal

animal blood (259). In the discontinuous system used in the present study, it was estimated (259) that 95% of the reticulocytes were present in the top-most layer; the remaining 5% were found in the second layer, and only occasional reticulocytes were seen in any other layer. Hence, it is to be noted that the peripheral blood used in this turnover study was not a homogenous mixture, whereas in the density gradient experiment, individually fractionated groups of cells were analyzed.

Two types of red cell glycosphingolipid turnover curves were obtained from this in vivo study, one of which seemed to follow the normal red cell survival (GL-3a and GL-4), while the others did not (GL-2a, GL-1a, G_{M3}, upper phase gangliosides, and ceramides).

The results from the incorporation of the labeled glucose into both plasma GL-3a and GL-4 (Figures 26 and 29) confirm the previous observation (45) that there is an early synthesis of these two lipids, probably in the liver, which exhibits peaks of incorporation at 24 hr. This agrees well with studies of ^{32}P incorporation into canine plasma lecithin (315). The loss of specific activity subsequent to maximum incorporation was rapid in both lipids, presumably reflecting a loss of the labeled lipid from plasma to other tissues, as well as their catabolism and elimination from the animal.

The fact that the specific activities of both glycosphinolipids remained rather constant between Days 20 and 40 rules out any possibility of exchange between these lipid pools.

The reappearance of label in GL-3a and GL-4 began around Day 50, at a time when the specific activities of erythrocyte GL-3a and GL-4 were gradually declining. The plasma glycosphingolipids reached maximum relabeling around 60 days, at which time a precipitous decline of specific activities occurred in both red cell GL-3a and GL-4 fractions, indicating the possibility of a loss of red cell glycosphingolipid into the plasma without exchange. The plasma glycosphingolipids were relabeled around 60 days, which is the average reported life span of porcine erythrocytes (273).

The ^{14}C-labeling of the erythrocyte GL-3a and GL-4 shows a consistent pattern (Figures 26 and 27). Because of the similarities between these two lipids during metabolic turnover, they will be discussed together. Emphasis, however, will be placed on GL-4, and it can be said that the metabolic fate of GL-3a is very similar to GL-4. Both GL-3a and GL-4 decay curves are essentially composed of three identifiable pools of each lipid.

The specific activity of each red cell glycosphingolipid reaches a maximum peak at Day 3, which coincides with the approximate time of entrance of erythrocytes from the bone marrow into the peripheral circulation when pulse labels of radioactive iron and glycine are utilized (272,273). The data from the in vitro study did not reveal any evidence of GL-3a and GL-4 biosynthesis by the reticulocyte-rich cells; if any, it was insignificant. This was further confirmed by the

absence of any early peak of incorporation in the erythrocyte glycosphingolipid fractions before Day 3 from the in vivo study. It seems evident, therefore, that most if not all of the erythrocyte GL-3a and GL-4 is synthesized in the bone marrow, and that synthesis in the reticulated circulating red cells is qualitatively of minor significance.

It was previously observed (45) that approximately 60% of the label in red cell GL-3a (subsequent to maximum incorporation at Day 5) was lost by Day 9, while there was no such loss in the GL-4 fraction. In the present experiment with the anemic pig, such losses were demonstrated to occur in both glycosphingolipids. If one compares the turnover curves of GL-4 between the normal and anemic pig, it can be seen that the part of the curves between Days 30 to 81 are very similar indeed in both animals. The only difference which has definitely been established is the presence of the initial peak in the anemic pig, and such initial incorporation merely indicates the rate of uptake by new red cells of the radioactive glucose utilized in the biosynthesis of this membrane glycolipid. It has been demonstrated that incorporation of ^{59}Fe and [^{15}N] glycine into erythrocytes was faster in the anemic pig ( ) and rabbit ( ) than the normals. It is quite clear, then, that the difference observed in the slope and extent of GL-4 and GL-3a incorporation between the normal and anemic pig presumably reflects the uptake of label by young cell fraction(s) present in the marrow, most likely the precursors of reticulocytes.

The precipitous decrease of specific activities between Day 60 and Day 81 in red cell GL-3a and GL-4 is unique but not uncommon. Previous attempts using radioisotopes of [2-^{14}C]glycine, ^{59}Fe and [^{15}N]glycine in red cell survival studies of normal and anemic pig (272,316), human (317), dog (318), cat (319), and rabbit (320) has demonstrated such phenomena in the survival curves, and it was implicated to be the result of an age-dependent process, loss of label due to cell destruction. Had this in vivo experiment been continued beyond 81 days, it may have been possible to see a plateau after this abrupt decline, so that a sigmoidal (or S) shaped curve may be observed for this late period.

Several possibilities may be advanced to account for the loss of label observed subsequent to Day 3. Since the daily removal of blood from the pig was still enforced during the first 10 days, it is possible that the loss of specific activity may be associated with the amount of radioactivity removed by sampling. Table 32 shows that approximately 10-13% of the circulating blood was removed daily. From the packed cell volume of the blood samples obtained each day, an average of 11% can be calculated from the removal of red cells from the total pool. The percentage change in specific activities of both mixed population (cpm/ml) and fractionated red cells (cpm total hexoses/ml) varied considerably. Day to day changes were not constant. Hence, it seems that the decline of label is attributed to some other process or processes rather than

Table 32. Percentage of Blood Removed from Pig 123-6 Between Day 3 and Day 10

Days	Plasma Volume (ml)	RBC Volume (ml)	Total Blood Volume (ml)	Blood Removed (ml)	Blood Removed (%)	S.A.[a] Mixed Cells	S.A. Change (%)	S.A.[b] Band 1	S.A. Change Band 1 (%)	S.A.[b] Band 2	S.A. Change Band 2 (%)
2	742	256	998	115	12			2814		1896	
									-38		-24
3	754	261		100	10	1299		1734		1446	
							-17		-64		- 9
4	786	272	1058	120	11	1079		616		1322	
							-12		-32		-51
5	811	281	1092	124	11	952		417		651	
							-23		-45		-48
6	811	281	1092	125	11	737		229		340	
							-26		-17		-22
7	843	292	1135	150	13	783		191		265	
							-26		-24		
8	843	292	1135	135	12	581		145			
							- 4				
9	869	301	1170	120	10	559					
							-50				
10	869	301	1170	130	11	281					

[a] S.A.= Specific activity-counts per minute of GL-4 per ml RBC (mixed cells).

[b] S.A.= Specific acitivyt-counts per minute of total hexoses in GL-4 per ml RBC of fractionated bands.

the removal of a constant fraction of blood cells present. However, the possibility exists that the observed decrease in specific activity can be a combination of two different processes.

Since approximately 12% of the blood was removed daily (Table 32) during the early period, it seems that the amount of erythrocyte GL-4 radioactivity removed by sampling may be significant with respect to the total circulating red cell GL-4 radioactivity and this loss might affect the calculated t 1/2 values seriously. An attempt was made to correct for this. The correction used was that suggested by Brown and Eadie (318). If a_1 is the total circulating erythrocyte GL-4 radioactivity on Day 3 and if b_1 is the GL-4 radioactivity removed in a sample on that day, then the total circulating GL-4 radioactivity at time of sample on Day 4, a_2, must be divided by

$$\frac{a_1-b_1}{a_1}$$

Similarly the total circulating red cell GL-4 radioactivity shown on Day 5 must be divided by

$$\frac{(a_1-b_1)(a_2-b_2)}{a_1a_2}$$

etc. After correction, a half-time value of 5.0 days was obtained from the semilogarithmic plot of specific activity versus time.

An attempt was also made to take into consideration the replacement rate of red cell mass from the bone marrow. Normally, the replacing rate of red cells is 1/60 to its total red cell mass, since the life span of the porcine erthrocytes is approximately 60 days, whereas the plasma volume remains rather constant. In acute bleeding, the rate of replacement is believed to be around 8-10 times higher than normal in humans. No information is available about the replacement rate in pigs; however, if one assumes that an estimate of 10 times normal is also applicable to the pig, then the maximum rate of replacement in the pig is 1/6 of the red cell mass per day. On this basis, after correction, the semilogarithmic plot of specific activity versus time revealed a half-time value of 7.0 days for the decline of radioactivity between Days 3-10. The slope of the decay curve is less steep, but not to a great extent.

Another explanation is dilution of the existing radioactive red cell GL-4 by red cells containing GL-4 of low isotope content. Since the pig is still in the steady state of hematopoiesis, the hemoglobin, hematocrit and blood volume

was rather constant as expected (Figure 27). If the anemic condition were moderate (moderate dilution) one would expect to see a curve showing a gradual decay whereas, if the anemic condition were severe with lots of incoming cells from the marrow, then the decay would be more steep. In either case, the decay curves should not contain any breaks or plateau in the specific activity *versus* time plot. This was not observed, however.

A third, and more probable, explanation is loss of globoside-containing membrane fragments from the reticulocytes as they mature in the circulation. Red cells are known to lose membrane lipids as they age *in vivo* (321,322). This change occurs primarily during the first part of the cell's life span (321,258), perhaps due to regiculocyte maturation (323).

Although reticulocytes lack a nucleus and contain little, if any, rough or smooth endoplasmic reticulum, they possess mitochondria and free ribosomes (324). Glycolipids are not

found in mitochondria or membranes of the endoplasmic reticulum and they have not been found in the nucleus (325,326). Rather, this important class of lipids seems to be unique to the plasma membrane. The fact that globoside is confined to the erythrocyte plasma membrane rests on the demonstration that the lipid can be removed in its entirety in ghosts prepared from erythrocytes (8,68).

It has been established that under an anemic condition, premature delivery of marrow reticulocytes to the blood stream occurs in rat (327). These immature reticulocytes are termed macroreticulocytes; their presence was detected in pig 123-6 as expected. Recent studies (327,328) have investigated the maturation of these cells from animals during acute anemia. It was found that macroreticulocytes undergo normal in vivo maturation without an appreciable reduction in life span. The cell undergoes remolding within the circulation, involving loss of membrane and cell contents until it approaches approximately the size of the normal adult red cell. One-third of the plasma membrane lipids (cholesterol and phospholipids) was estimated to be lost during maturation due to factors operative in vivo (327). Spleen is one of these factors (327). The lipids were lost largely during the first two days of reticulocyte maturation and the process was completed by Day 5. Although red cells at this point were "mature" as defined by the reticulocyte stain, loss of membrane lipids and cell contents continued over the remaining 6 days.

It is unclear how long this process normally continued; however, from the extrapolation of the data, it was suggested that normal values would have been achieved by Days 20 to 25 (327).

In this respect, if one assumes that the life span of macroreticulocytes or reticulocytes is normal or near normal, as demonstrated by Ganzoni et al. (328) and Shattil and Cooper (327), then one can envisage the early decline of specific activity in red cell GL-4 subsequent to maximum incorporation as a result of loss of membrane components due to maturation. In a given situation, with a group of young cells containing GL-4 which are maximally labeled at Day 3, assume that it takes 11 days for these cells to mature during which time the globoside-containing membrane fragments and hence the radioactive label are being lost. Other cellular contents, such as mitochondria, etc. are being lost also but, since they do not contain any glycolipids, will not be considered here. Therefore, by Day 14, the majority of the young cells would have reached maturation and the specific activity should begin to be constant. If one also assumes that the maturation process will continue on until Day 25 as suggested by Shattil and Cooper (327), then all the cells will be normal adults and thus resuming a normal age-dependent process. Then, one would expect the specific activity of the red cell GL-4 to remain rather constant after Day 25, until such time when cell destruction occurs and the specific

activity of GL-4 will take a rather sharp drop. If this were the case, one would expect to see a turnover curve of red cell GL-4 quite similar to that shown in Figure 28 and 30. The slight decrease in specific activity observed between Days 25 and 60 is probably due to random destruction of red cells, since a perfectly normal youthful cell may meet with a fatal accident while speeding around the circulation. Random destruction of pig red cells has been implicated previously in survival study (273).

Half-times of 0.75 and 1.0 day were obtained from the GL-4 decay curves of Band 1 and Band 2 (Figure 41) during the period subsequent to maximum labeling. These data suggest that the reticulocyte GL-4 pools have a very short half-time and are turning over very rapidly. One may relate this loss of labeled GL-4 as evidence of a short-lived population. However, it should be realized that the detection of labeled GL-4 loss cannot be equated with cell death, since it may be due to loss of a portion of red cell component without actual cell destruction. If there were cell destruction one should observe reappearance of the GL-4 label in the plasma fraction at a time subsequent to the rapid loss of specific activity from red cell GL-4. No significant peak of relabeling was observed for either GL-3a or GL-4. Furthermore, Ganzoni et al. (328) could not demonstrate any significant shortening of rat blood cell life span by repeated red cell mass measurements or radioautography techniques. Hence, some other mechanism must

be involved to account for the loss of cell membrane and reduction in size without causing a loss of individual red cell.

Another interesting point is that Figure 40 shows quite clearly that as the specific activity of GL-4 is being lost from the reticulocyte fraction, there is a concomitant increase in the label of GL-4 of more mature red cells. This is quite evident between Band 2 and Band 3. In other words, the labeled GL-4 is progressing downward through the gradient with red cell aging, and hence the cells are not dying, but instead, maturing.

Figure 42 shows plots of μmoles hexose/g hemoglobin for GL-4 versus time on three of the individually fractionated red cell bands during the first eight days post-injection of the pulse label. As the specific activity of GL-4 declines (Table 30) the amount of GL-4 per gram hemoglobin also decreases, while the hemoglobin content and packed cell volume remain constant throughout this period. These data most strongly indicate that the loss of radioactivity from GL-4 of the young cells is most likely associated with plasma membrane loss due to maturation. However, it is to be noted that the percentage loss of GL-4 in early days is greater than that predicted for the reticulogyte maturation. Such loss perhaps represents a combination of processes such as cell maturation as well as random cell destruction and removal of cells by sampling.

There were three distinct pools of erythrocyte GL-3a and GL-4 as shown by the semilogarithmic plots of specific activity

Figure 42. Loss of labeled GL-4 from individual bands as a function of time.

Plots of amount of globoside per g hemoglobin as a function of time during the first 8-day period post-injection of the pulse label.

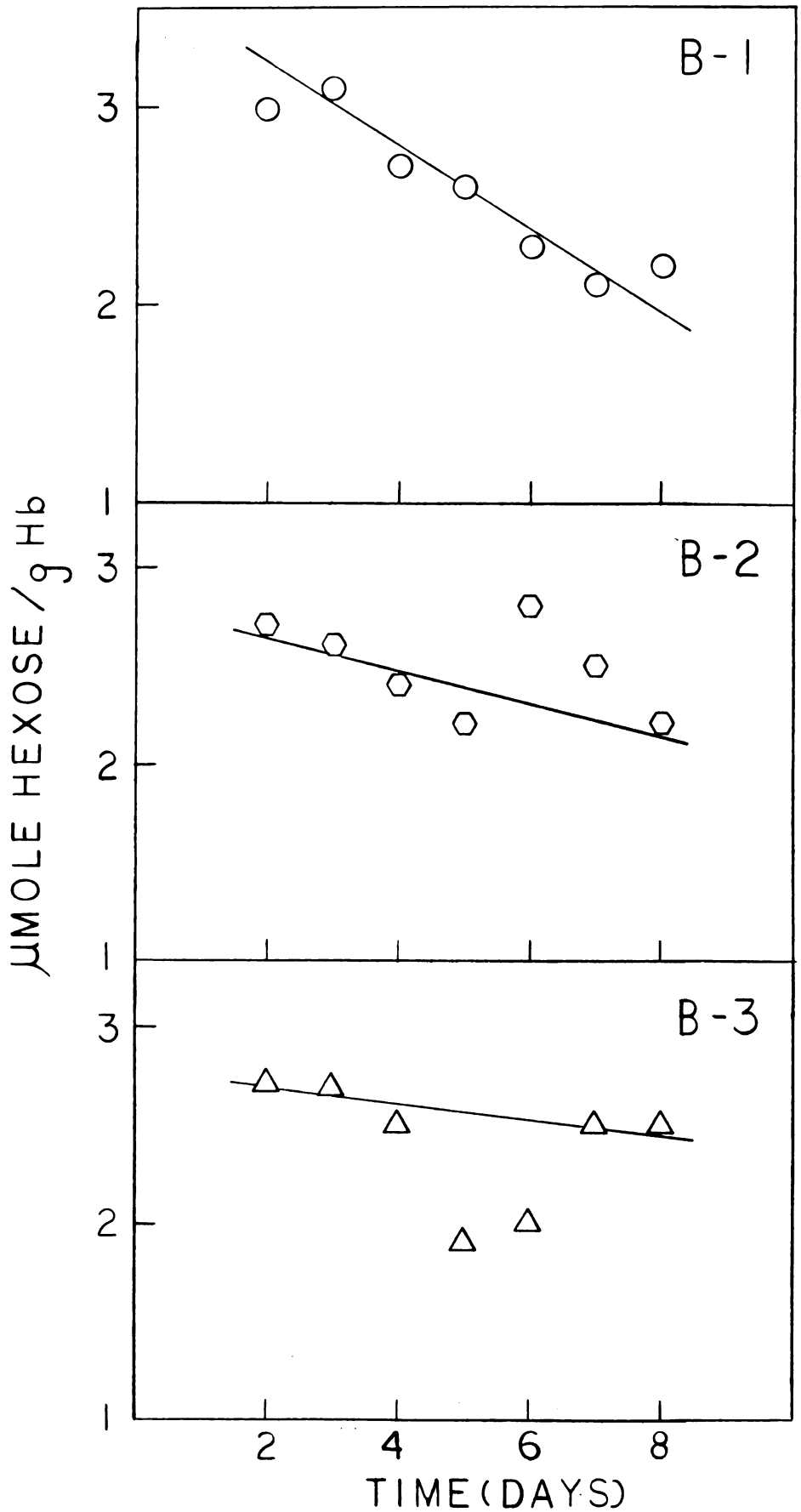


Figure 42

versus time (Figures 30 and 28). It is possible even more than three pools may exist, if one considers the slopes around the breaks of the curves. The presence of more than one pool of a given molecule, whether simple or complex, in a biological system is not uncommon. The turnover of human plasma cholesterol has been proposed to involve a two-pool system (329). From their studies of the turnover of myelin phospholipids in the adult and developing rat brain, Jungalwala and Dawson (330) presented supportive evidence that two pools also exist in myelin. Turnover studies of the glycoproteins in adult and developing rat brain (331) have also revealed the presence of at least two glycopeptide components with differing half-times. And recently, in studies on the characterization and metabolism of glycosphingolipids in normal human skin fibroblasts (332,222) and embryonic mouse fibroblasts (334) evidence was presented for two pools of glycosphingolipids. The results in this study suggest that red cell GL-3a and GL-4 each contain two major components which differ in turnover rate. The pool with a half-time of 5-6 days accounts for the more rapid decline which occurs between Day 3 and Day 10. It is to be noted that the apparent half-times obtained are probably maximum values, since the specific activities during this early period were derived from blood samples of heterogenous populations. The true half-time for the younger cell fractions is more likely to be less, as shown in Figure 41. The pool with a half-time of 45 days accounts for the decline in label between Day 25 and Day 57.

This slower half-time is an additional indication that GL-4 and GL-3a are components of red cell plasma membrane or components of structural units as classified by Burton (179).

In addition, a third pool was evident in both lipids, with a half-time of approximately 6 days, which accounts for the decline in specific activities between Days 60 and 81. The slope of the decay for GL-4 is very similar to that observed for the early period. This suggests that the pools of red cell GL-3a and GL-4 which turn over at the time of red cell senescence have a very similar t 1/2 to that observed earlier. Or one may speculate that the glycosphingolipids metabolized at this late period may have been derived from the corresponding red cell glycolipid pools from the early period which have gone through the maturation process and finally are being released from the membranes into the plasma before phagocytosis of the cells begins.

The finding of several GL-4 pools in the porcine erythrocytes may be correlated with the reports of Hanahan et al. (335) and Coles and Foote (277) that there are two forms of globoside. Both have the same basic structure of GalNAc-Gal-Gal-Glc-ceramide; the only difference is that one contains 2-hydroxy fatty acids while the other contains normal straight-chain fatty acids (335). 2-Hydroxy fatty acids were detected in other porcine erythrocyte glycosphingolipids too (277), but they are most abundant in red cell GL-4. The similarity in the 2-hydroxy fatty acid compositions of porcine erythrocyte GL-3a and GL-4 further suggests a close metabolic

relationship between these two lipids. In the present study, no attempts were made to separate the two forms of globoside or GL-3a on the basis of their fatty acid differences. However, these components may account for the different turnover rates observed in the in vivo study. It is possible that these two forms of globoside are synthesized from two different fatty acid pools at different "sites" or "pockets" of the erythrocyte membrane with different rates; one may reside at the surface membrane, while the other is imbedded in the membrane. It is also possible that these two forms of globoside are synthesized from a common pool of fatty acids, some of which are nonselectively hydroxylated. Whether one is a structural component and the other a functional component is uncertain.

The turnover curves of plasma GL-1a and GL-2a were similar but different from GL-3a and GL-4 in that maximum incorporation was detected much earlier, at 6 hr. Similar to GL-3a and GL-4 these two lipids were also relabeled between Day 50 and Day 70. However, one noticeable difference was the presence of several peaks during this period instead of one. One objective of this in vivo study was to study as many samples as possible throughout the prescribed time course, especially during the latter part of the time sequence, so that a better perspective could be obtained with regard to the turnover curves. The curves turned out to be more complex than anticipated. If one were to use the time points chosen

in a previous pig experiment (45) the decay curves of plasma GL-1a and GL-2a for the anemic pig would look quite similar to those observed in the normal pig, with a single broad peak of relabeling around Day 60.

The initial incorporation peak of radioactive precursor into plasma GL-1a and GL-2a at 6 hr indicates an early synthesis of these lipids, possibly in the liver. Earlier studies had indicated that triglyceride and cholesterol esters were labeled with radioactive linoleate at around 6 hr (336). Nevertheless, another source of these two plasma glycolipids should not be ignored, that is, through the exchange with its corresponding erythrocyte GL-1a and GL-2a pools, since the results from the in vitro study indicated that reticulocyte-rich red cells could synthesize GL-1a and GL-2a.

Erythrocyte GL-1a and GL-2a turned over quite similarly to the red cell GL-3a and GL-4 in several ways. First, both lipids exhibited a peak of incorporation at Day 3, indicating that bone marrow is one of the possible contributors to these two glycosphingolipid pools. This contradicts the previous finding (45) that bone marrow contributes significantly to the red cell GL-2a, GL-3a and GL-4, but not to GL-1a. The observation was based on the fact that no increase in specific activity was detected in GL-1a at the time when the erythrocytes were released from the marrow. Another similarity is that the label was rapidly lost and the specific activity was maintained rather constant between Days 10 and 40.

Several differences were also noticed. One was that in addition to the maximum specific activity detected at Day 3, both red cell GL-1a and GL-2a showed an additional rapid incorporation of radioactive label, reaching maximum specific activity at 12 hr. In GL-1a, the peak at 12 hr was 33% higher than the peak at Day 3; whereas in GL-2a the two peaks were of equal height. The presence of an additional early peak in erythrocyte GL-2a was not detected previously (45), whereas the early synthesis of GL-1a was noted and liver was implicated to be the possible contributing source. Erythrocyte GL-1a was also believed to derive from the plasma through rapid exchange (45). However, in view of the findings from the in vitro study, an additional source of erythrocyte GL-1a and GL-2a should be considered; that is, the synthesis of these lipids by red cells; and thus, indicating the presence of ceramide-β-glucosyltransferase and GL-1a-β-galactosyltransferase. These enzymes are probably membrane-bound. There was no evidence that red cell GL-2a acted as a substrate for the biosynthesis of erythrocyte GL-3a. Recently, similar results were obtained when rat erythrocytes were incubated with radioactive glucose (268). Since the plasma GL-1a had a higher specific activity than the corresponding erythrocyte GL-1a, it is possible that the red cell GL-1a labeled under the in vitro condition originated from the plasma, thus exhibiting a precursor-product relationship. Nevertheless, the in vivo studies conducted in the normal as well as the

anemic pig could not support such a theory. Furthermore, quantitative analyses revealed a significantly higher concentration of ceramides in porcine red cells during the study (1.9 μmoles/100 ml). It is difficult to envisage, contrary to the data from the in vitro study, that red cell GL-1a is derived from plasma and then galactosylated to form GL-2a, without taking advantage of the available ceramide pool present in the cells.

Another difference observed in the turnover curves of both erythrocyte GL-1a and GL-2a from that of GL-3a and GL-4 is the reappearance of label between 50 and 70 days. Relabeling in erythrocyte GL-2a was not noted previously. The data suggest that GL-2a as well as GL-1a do not follow the expected normal survival as was previously believed (45). Instead, the red cell GL-1a and GL-2a are probably in rapid equilibrium with the plasma counterparts. Figure 32 established clearly the nature of exchange between the plasma and erythrocyte GL-1a pool simply by the fact that these two turnover curves were parallel with each other, except for one point at Day 4. This point was omitted from the curve on the grounds that there were no other points on the slopes up to or after this point to make a peak. However, if there were a peak at Day 4 it would coincide rather closely with the corresponding erythrocyte GL-1a peak at Day 3 thus accounting for all peaks.

The turnover curve of plasma GL-2a (Figure 31), on the contrary, does not show an exchange equilibrium as ideal as

that of GL-1a. Notably, there is no exchange between the red cell and plasma GL-2a at Day 70. Since the results from the *in vitro* study did not give any clue in this respect, it is rather difficult to ascertain whether or not any exchange occurs. However, on the basis of the similarities in the specific activity profiles between GL-1a and GL-2a and their difference from GL-3a and GL-4, it is tempting to speculate that exchange between the GL-2a pools may occur. An unconfirmed report by Krivit and Kern (337) indicated that a similar type of experiment was performed on a rabbit using a pulse label of [^{14}C]glucose. It was found that rabbit erythrocyte GL-2a and GL-1a were apparently in rapid equilibrium with the plasma glycosphingolipids whereas a pentahexosylceramide, the major erythrocyte glycolipid of rabbit (Gal-Gal-GalNAc-Gal-Glc-ceramide), followed the expected red cell survival in the same manner as the porcine red cell GL-4.

The accumulating GL-1a and GL-3a in Gaucher's and Fabry's disease have been proposed to derive from erythrocyte glycosphingolipid metabolism (124). In Gaucher's disease, GL-1a was found to be elevated in both plasma and red cells. Since GL-1a is exchangeable between these two pools, it is believed that the excessive GL-1a present in erythrocyte is derived from plasma where this lipid accumulates due to the absence of glucosylceramide hydrolase to metabolize this catabolic product of complex glycosphingolipids. With respect

to the results obtained from the in vitro study, one might speculate that red cell may contribute additional GL-1a to the existing red cell pool simply by utilizing the adequate amount of ceramides that red cell provides for synthesis. It is interesting to note that red cell ceramide contains a significant proportion of 2-hydroxy fatty acids, whereas plasma ceramide does not. And yet, 2-hydroxy fatty acids occurred in significant quantities in GL-1a of the plasma low density lipoproteins. Hence, the possibility exists that the plasma GL-1a can be derived from liver, which had been shown to contain GL-1a with 2-hydroxy fatty acids. Alternatively, plasma GL-1a may be derived from GL-2a which originates either from red cell GL-2a through process of exchange or from GL-3a (and GL-4) released into the plasma when the red cells are catabolized. These red cell glycolipids contain high concentrations of 2-hydroxy fatty acids.

In Fabry's disease, GL-3a accumulates in plasma but not in the red cells. Hence, it might be concluded from the in vitro as well as the in vivo experiment that red cells do not synthesize GL-3a from GL-2a nor engage in any exchange reaction with the plasma pool of GL-3a. According to the model, red cell GL-3a remains with the cell throughout its life span; from the time of GL-3a being incorporated into the membrane structure of erythrocyte until the time of cell destruction, the level of this lipid should remain the same throughout any accumulation. But once it is released into the

plasma, this lipid cannot be metabolized further due to the absence of GL-3a-cleaving enzyme and hence it accumulates.

The red cell pools of G_{M3} and upper phase gangliosides were quite similar in parts of their turnover curves. Both lipids were maximally labeled at around Day 3 to Day 5, indicating that bone marrow is at least partially responsible for their synthesis. The gangliosides also showed an additional peak of incorporation at around 6-8 days. This could mean some newly labeled cells are entering the circulation from the marrow, since the pig is still in an active hematopoietic state. It could also represent reutilization of the label derived from the catabolism of other glycosphingolipids during the early turnover period (Day 3 or before), which had found its way back to the bone marrow via plasma.

Significant relabeling occurred during the late period for the gangliosides. There does not appear to be any exchange process involved, although several plasma peaks were observed in close parallel to those of red cells. Definite evidence for a classical exchange equilibrium between the plasma and erythrocyte pools of gangliosides cannot be established. It is interesting to note that maximum specific activity of relabeling was reached on Day 41, and the amount of radioactivity present in the other peaks during this late period seemed to decrease in size and height with time, which could mean a slow gradual decrease of specific activity. If so, this would indicate that the red cell upper phase

gangliosides were lost to the plasma without exchange, a situation rather similar to that observed in red cell GL-3a and GL-4. It is possible that such turnover represents a combination of processes involving several pools, one in exchange with plasma and one not, such probability is only a speculation and cannot be ascertained at this time.

The turnover curves of red cell ceramides (Figures 35 and 36) also showed that they were labeled within a short time after injection of the initial pulse label, and reached maximum specific activity in about 12 hr. This seems to indicate again that liver and perhaps the erythrocyte itself can account for the early synthesis. The fact that an additional peak of uptake was also observed at Day 3 further confirmed that bone marrow is one of the major sources for all of the sphingolipids studied thus far. The extent of synthesis, as judged from the peak size, varied between individual lipids from GL-4 to ceramide. The decay curves of red cell ceramides did not demonstrate that they followed the normal expected survival. There was a trough at around 40 days, suggesting loss of label, and subsequent increase in the specific activity betweeb Days 50 and 81. It is rather difficult to rationalize such a phenomenon since there is a substantial body of evidence, summarized by van Deenen and de Gier (338), that mature erythrocytes are incapable of carrying out anabolic lipid processes. No conclusion can be made with regard to the possibility of having an exchange process, since the specific

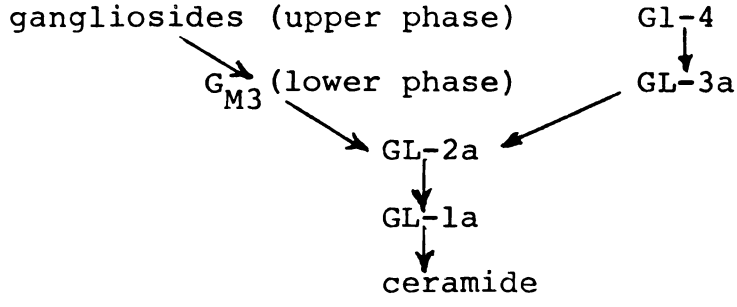
activities of the plasma ceramides were too low to permit any meaningful interpretation.

A summary of the results, showing maximum relabeling periods for each lipid, is given below.

Table 33. Time of Reappearance of Label in Sphingolipids During the In Vivo Study

Red Cell Sphingolipids	Days
Upper phase gangliosides	40, 50, 60, 66, 81
Lower phase G_{M3}	50, 60, 70, 81
GL-2a	50, 63, 70, 81
GL-1a	50, 63, 70, 81
Ceramide-NFA	55, 63, 70, 81
Ceramide-HFA	53, 60, 75

As can be seen, similarities exist in the pattern of relabeling among all of these lipids. The fact that ceramide became relabeled might be an indication of the catabolism of some of the complex glycosphingolipids, such as gangliosides and globoside.



Lipid loss has been reported during the course of *in vivo* aging (321,233) as well as during *in vitro* incubations of abnormal cells (339). In these instances, the loss of lipid has been found to involve all of the neutral and phospholipid components. The concomitant formation of small membrane fragments of varying sizes was also observed in some of these studies (340). Perhaps it is possible that these membrane fragments are adsorbed by circulating erythrocytes, or perhaps they can be incorporated into the membrane.

It is more likely that the cells which take up the ^{14}C-labeled tracer slowly will outlive, on the average, cells formed earlier, and will be present to a larger extent in the blood after the end of the major senescence phase, that is, the period of the sharp decrease in specific activity.

The results from the previous metabolic study in the normal pig (45) did not seem to support some of the discrepencies observed from the fatty acid analyses of the plasma and erythrocyte glycosphingolipids. It was postulated that the fatty acid composition of plasma and red cell GL-1a should be the same, because they exchange freely, and this was found to be the case and confirmed by the present study. The data from the *in vitro* study indicated that synthesis of GL-2a by the reticulocyte-rich cells can occur in addition to its formation in the bone marrow. Hence, some similarity in the distribution of fatty acids should exist between the red cell GL-1a and GL-2a. Previous reports on the analyses of normal

fatty acids clearly indicated that these lipids contained primarily 16 and 18 carbon acids, whereas red cell GL-3a and GL-4 contained mostly fatty acids of 20 carbons and higher. The percentage distribution between red cell GL-1a and GL-2a is not exactly identical, which is not unexpected for the reason given above and because GL-2a is also involved as an intermediate in the biosynthesis and catabolism of gangliosides and it should contain a rather heterogenous population of fatty acids. An interesting observation from the in vitro study is that the amount of label recovered in the fatty acid fraction seemed to increase with the size of the oligosaccharide chain (Table 18). It is possible that this represents the elongation of the fatty acid chain with the radioactive acetate derived from the matabolism of the labeled glucose, since the reticulocytes are known to possess the normal complements of TCA cycle (341) and the capability of incorporating fatty acids into neutral lipids (342).

Similarities in the distribution of 2-hydroxy fatty acids in red cell GL-2a, GL-3a and GL-4 have been demonstrated (277). However, although the biosynthesis of GL-3a and GL-4 have not shown in vitro by reticulocytes in whole blood, it does not rule out the possibility that GL-2a is an anabolic intermediate in the biosynthesis of GL-4. The process of step-wise addition of monosaccharides to GL-2a can very well be the mechanism of synthesis operational in the bone marrow.

Since quantitative determinations of the plasma and erythrocyte glycosphingolipids were made, the pool sizes of each lipid as well as the turnover rate and the fractional turnover rate could be calculated (275). Although the turnover rate for GL-2a differed somewhat from those of GL-3a and GL-4, there was close correlation with the total pool sizes. The proportion of GL-2a, GL-3a, and GL-4 synthesized each day was quite the same; approximately 21% of each plasma pool was newly synthesized each day. This finding was in good agreement with the data obtained from human (222) and pig plasma (45). Approximately 90% of plasma GL-1a was synthesized each day, which is considerably different from the value of 20% observed previously in the normal pig (45). This rapid turnover of plasma GL-1a could be the reflection of the rapid synthesis of GL-1a by the reticulocytes in circulation. Approximately 75% of plasma G_{M3} was shown to be metabolized each day, whether this represents the normal rate or not is uncertain. Since the turnover values of erythrocyte GL-4 from the normal pig are not available for direct comparison, it is evident from this study that the daily production of GL-4 is pronounced (90%) in the reticulocyte fraction (Table 30, Band 1) which is in keeping with the hemolytic state of the anemic pig. The amount metabolized each day for the more mature cells (Band 3) was much less.

Globoside is the major glycosphingolipid in human (222) and porcine (45) erythrocytes, and accounts for about 70% of

the total red cell glycolipids. The fact that globoside forms an integral part of the red cell membrane and follows the normal red cell survival is interesting indeed. Integrity of the red cell membrane can be disrupted by fragmentation, indirect enzyme hydrolysis or hemoglobin malfunction. If the cellular degradative hydrolases were involved, GL-4 would not be released into the plasma as a whole molecule at or around senescence. Hence, the possibility of direct intravascular disruption must be considered. It is tempting to speculate that the release of GL-4 from the erythrocyte membrane may cause an alteration in the protein-lipid configuration of the membrane complex in such a manner that the deformed erythrocyte will be recognized and hence removed from the circulation by the reticuloendothelial system, such as the spleen. However, it is evident that one must still understand more about the architecture and physiology of the red cell membrane before one can go directly from lipid analyses to an understanding of the erythrocyte aging process. For example, we still know very little about the functions and involvement of the red cell membrane proteins. Nevertheless, it is hoped that the results of metabolic experiment presented in this thesis may provide some insight into the function and structural organization of the red cell membrane. Firm conclusions are not yet justified, but as the body of data grows the image brightens.

VI. SUMMARY

Six fractions of sphingolipids were isolated from washed trypsinized human platelets. The concentrations and chemical structures were studied in detail. On the basis of sugar molar ratios, studies of permethylation products, and the action of stereospecific glycosidases three major neutral glycosphingolipids were identified, of which GL-2a was the most abundant type. In addition, GL-1a and G_{M3} ganglioside were also found. The structures of human plasma and platelet G_{M3} were found to be identical. Treatment of platelets with trypsin, chymotrypsin or thrombin increased the yield of G_{M3} ganglioside as compared with a control, while the level of ceramides was not changed.

In contrast to human platelets, porcine platelets contained GL-3a as the major neutral glycosphingolipid. Sulfatide and G_{M3} ganglioside were the acidic glycosphingolipids present in pig platelets. Both human and porcine platelets were found to be rich in ceramides, suggesting that this class of lipid may be unique to platelet physiology.

No definite conclusions can be drawn about the level of globoside in fetal red cells of pigs since the blood samples were contaminated. However, the level of this glycosphingolipid in the erythrocytes of 45-day old fetuses was low when

compared to values for new-born and adult pigs.

The results from the in vitro study indicated that reticulocyte-rich red cell fractions can synthesize GL-1a and, to some extent, GL-2a as well. Radioactive label was detected in the hexose moiety of plasma GL-1a also, suggesting either that plasma could synthesize GL-1a or that plasma GL-1a was derived from the red cell GL-1a by active exchange of the two pools. Porcine plasma and erythrocytes contained significant quantities of ceramides and G_{M3} ganglioside in addition to GL-1a, GL-2a, GL-3a and GL-4. A metabolic experiment to study the turnover of these sphingolipids in an anemic pig was conducted by injecting [^{14}C]glucose into the pig intravenously as a pulse label, and removing aliquots of blood samples for lipid analyses at frequent intervals throughout a period of 81 days. Turnover studies were performed on the plasma, a mixed population of red cells, and cells which had been fractionated into individual groups according to age by density gradient ultracentrifugation.

All of the plasma glycosphingolipids became labeled within a relatively short time after the initial pulse label, and reached maximum specific activities at 6 or 12 hr. The label was then rapidly lost and remained rather constant between Days 15 and 40. From Day 50 to Day 81, all of the glycosphingolipids in plasma were relabeled. Maximum specific activities for GL-3a and GL-4 were reached at around Day 60, which was the average life span of porcine erythrocytes.

The other plasma glycolipids showed several additional peaks of relabeling besides the one observed on Day 60 during the latent period. The presence of such complex peak is not clear at the moment.

Plasma and red cell GL-1a behaved essentially the same throughout the duration of the experiment; the two turnover curves paralleled each other and hence it was concluded that plasma and erythrocyte GL-1a pools were in rapid equilibrium. This finding was further supported by the data obtained from the in vitro study. The decay curves of plasma and erythrocyte GL-2a were not as superimposable as those observed for GL-1a, there was a peak of relabeling which could not be accounted for; however, on the basis of the similarities in turnover between GL-1a and GL-2a, it was tentatively concluded that plasma GL-2a may also be involved in an exchange reaction. This needs to be verified.

The rate of incorporation of isotope into all fractions of erythrocyte sphingolipids was faster in the anemic than the normal pig. A peak of incorporation was observed at Day 3 for all of the lipids studied, indicating that bone marrow is one of the prime sources of these lipids. In addition, a contribution by early synthesis was also evident for erythrocyte GL-1a and GL-2a. Possible sources for these syntheses could be the liver or red cell itself.

Two types of red cell glycosphingolipid turnover curves were observed; one seemed to follow the normal red cell

survival (GL-3a and GL-4) while the others did not (ceramides, GL-1a, GL-2a, lower phase G_{M3} ganglioside and upper phase gangliosides).

With regard to erythrocyte GL-4 (and GL-3a), the results obtained from the turnover studies of mixed cell population, and the fractionated individual groups of cells by density gradient ultracentrifugation, were consistent with the hypothesis that the radioactive glucose was rapidly incorporated into the membrane-bound GL-4 of the immature erythrocytes in the bone marrow. After being released from the marrow these cells lost a portion of their globoside-containing membrane as they matured in the peripheral circulation. The remodeling of the cells within the circulation continued until it approached the size of a normal adult cell, after which time the turnover of GL-4 remained rather constant. The membrane-bound GL-4 remained with the cell until the time of red cell senescence, and then was released directly into the circulation as a whole unit when cell destruction began. Thus, it became the major source of all four plasma neutral glycosphingolipids.

Erythrocyte GL-1a and GL-2a appeared to be in dynamic equilibrium with plasma glycolipids and did not follow the expected survival. No definite conclusion could be drawn with respect to the other sphingolipids, since the results seemed to imply that more than one process was present, one involves exchange and the other without exchange. However, from the

similarities in the turnover curves of these lipids, some metabolic interrelationships were evident.

This study also revealed that glycosphingolipids are a family of complex and heterogeneous lipids. At least two pools of each glycosphingolipid were present in the plasma and erythrocytes of porcine blood, with rapid and slow turnover rates. The semilogarithmic plots of specific activity versus time reveals half-times of 5.5, 45.0 and 6.3 days for red cell GL-3a; whereas erythrocyte GL-4 gave half-times of 5.5, 45.0 and 6.2 days, respectively.

When the globosides from fractionated red cell bands were examined, biphasic decay curves were also demonstrated. Half-times of 0.75, 1.0 and 4.0 days were obtained from the rapid turnover pools of Bands 1, 2 and 3; whereas 3.3, 5.5 and 9.3 days were obtained from the slow turnover pool of these cell bands. Calculated turnover rates were 65.4, 47.0 and 11.5 μmoles/day for the rapid turnover pools and 14.8, 1.9 and 6.9 μmoles/day for the slower pools of these respective bands.

Biphasic decay curves were also observed for the plasma GL-1a (t 1/2=0.75, 7.5 days) and G_{M3} (t 1/2=0.9, 1.9 days), again indicating the presence of rapid and slow turnover pools. Approximately 93% and 9% of GL-1a and 77% and 36% of G_{M3} were found to metabolize each day. This is in contrast to an average value of 21% found for the plasma GL-2a, GL-3a and GL-4.

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APPENDIX

LIST OF PUBLICATIONS

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Sweeley, C. C., and Tao, R. V. P. Gas Chromatographic Estimation of Carbohydrates in Glycosphingolipids. In: Methods in Carbohydrate Chemistry. R. L. Whistler and J. N. BeMiller (Editors), Vol. VI, Academic Press, New York, 8 (1972).

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