# BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON FAMILIAL ERYTHROPHAGOCYTIC LYMPHOHISTIOCYTOSIS

By

Clifford Gregory Wong

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# ABSTRACT

## BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON FAMILIAL ERYTHROPHAGOCYTIC LYMPHOHISTIOCYTOSIS

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Children afflicted with familial Erythrophagocytic Lymphohistiocytosis (FEL), an inherited childhood disorder, were recently discovered to have defects in both humoral and cellular immunity, and the occurrence of a plasma inhibitor of in vitro lymphocyte blastogenesis. An autopsy examination of the liver of one FEL patient revealed an abnormally high amount of uncharacterized lipid material accumulating locally in the hepatocytes and in the infiltrating macrophages.

Lipid analysis of the FEL liver was performed by isolation and separation of the total lipids by Folch solvent partitioning, silicic acid column chromatography, and analytical thin-layer chromatography (TLC). The levels of neutral lipids were found to be increased 2-fold over normal levels, as determined by gravimetric, colorimetric, and gas-liquid chromatographic methods. This increase was primarily in triglyceride content with a modest increase in total cholesterol lipids. The cholesterol ester content in total cholesterol lipids was markedly reduced from 29% (in normal liver) to 2%. No alteration in the individual triglyceride species was found. Analysis of the neutral glycolipids and phospholipids by thin-layer chromatography revealed no abnormalities in their TLC patterns. The major lipid abnormality in FEL was discovered in the water-soluble (ganglioside) lipid fraction where lipid-bound sialic acid, as determined by the

resorcinol colorimetric assay for sialic acid, was over 11-fold higher than normal. The thin-layer chromatographic analysis of the total ganglioside fraction (Folch upper phase) revealed a general increase in all ganglioside species with an apparent 100-fold increase in lipid-bound sialic acid material with TLC mobility similar to $GM_2$.

Lysosomal glycosylhydrolase assays were conducted in liver, leukocytes, and fibroblasts of normal and FEL patients in order to determine whether the apparent ganglioside accumulation in FEL liver was due to a lysosomal enzyme deficiency. No enzyme deficiencies were found in FEL leukocytes or fibroblasts, although β-galactosidase activity in the one FEL liver examined was significantly reduced (25% of normal levels). The nature of the β-galactosidase activity decrease was investigated by means of mixing experiments with normal and FEL liver homogenates, cellulose acetate electrophoresis, and heat inactivation studies. These studies revealed the absence of a soluble inhibitor, no alteration in electrophoretic mobility of the FEL β-galactosidase isoenzymes, and the absence of a residual heat-stable isoenzyme that might have been responsible for the lowered activity in FEL liver. It was concluded that the reduction in β-galactosidase activity was not due to an enzyme defect, was only localized in liver, was not a generalized enzyme deficiency, and thus, was not the primary metabolic defect.

FEL and normal liver gangliosides were preparatively isolated by chloroform:methanol solvent extraction, DEAE-Sephadex anion exchange column chromatography, Iatrobead silicic acid column chromatography, and thin-layer chromatography for immunological testing. Two putative ganglioside fractions from normal liver and one from FEL liver were found to inhibit (at 2 μg/ml) in vitro antigen-stimulated lymphocyte mitogenesis, but not lectin-stimulated (Con A) mitogenesis. Initial characterization of these gangliosides by combined gas-liquid

chromatography-mass spectrometry revealed a probable lactotetraose or lactoneotetraose core sequence for the oligosaccharide moiety of the glycolipid.

Initial studies with the FEL plasma inhibitor did not reveal the presence of an accumulating ganglioside, although immunosuppressive activity was found in a fraction isolated by preparative TLC with similar mobility to the FEL liver ganglioside inhibitor.

These studies have demonstrated for the first time highly specific and potent immunosuppressive lipid-bound sialic acid compounds in FEL and normal liver with chromatographic and chemical properties similar to gangliosides that may be involved in the pathogenesis of an inherited immunological disorder.

Dedicated

to

my parents:
to my mother, for her faith and love;
and to my father, in memory of

## ACKNOWLEDGEMENTS

I wish to sincerely thank all of my friends, without whose aid this work could never have been completed: Drs. Bruce Macher, Bader Siddiqui, and John Klock, for their physical and moral support; my colleagues in the lab, for their invaluable advice and assistance; Charles Caldwell, for his much appreciated artistry; and especially, Drs. Charles C. Sweeley and Stephan Ladisch, for their long-suffering patience, incredible faith, and guidance.

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# LIST OF ABBREVIATIONS

## Miscellaneous

| | |
|---|---|
| GLC-MS | gas-liquid chromatography-mass spectrometry |
| GLC | gas-liquid chromatography |
| MS | mass spectrometry |
| TLC | thin-layer chromatography |
| Con A | concanavalin A |
| pNP- | p-nitrophenyl- |
| RBC | red blood cell |
| 4-MU | 4-methylumbelliferone |
| 4-MU- | 4-methylumbelliferyl- |
| FEL | Familial Erthyrophagocytic Histiocytosis |
| PHA | phytohemagglutinin |
| PWM | pokeweed mitogen |
| SKSD | Streptokinase-streptodornase |
| PBL | peripheral blood lymphocytes |
| NeuNAc | N-acetylneuraminic acid, a sialic acid |
| NeuNGl | N-glycolylneuraminic acid |
| Gal | galactose |
| Glc | glucose |
| Man | mannose |
| Fuc | fucose |
| GlcUA | glucuronic acid |
| GlcNAc | N-acetylglucosamine |
| Cer | ceramide |
| TMS | trimethylsilyl |
| PAS | periodic acid-Schiff Reagent |

## Glycosphingolipids

| | |
|---|---|
| GL-1 | glucosylceramide |
| GL-2 | lactosylceramide |
| GL-3 | Galα1→4Galβ1→4Glcβ1→1'Cer |
| GL-4 | GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1'Cer (globoside) |
| GL-5 | GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1'Cer (Forssman Hapten) |

# INTRODUCTION

Familial Erythrophagocytic Lymphohistiocytosis (FEL), first described by Farquhar and Claireaux (1), is an inherited, fatal childhood disorder characterized by anorexia, hepatosplenomegaly, jaundice, thrombocytopenia, liver dysfunction, and hyperlipidemia (2-4). A striking autopsy feature is a widespread histiocytic infiltration of liver, spleen, lymph nodes, bone marrow, lungs, and of the gastrointestinal, genitourinary, and central nervous systems, accompanied by erythrophagocytosis at the infiltration sites. The age at onset ranges from 2 weeks to 7 years, and the average survival time is generally 6 weeks from the onset of the illness with the children succumbing either to bleeding, sepsis, or lymphocytic meningitis. The disorder is believed to be inherited as an autosomal recessive trait (5).

In a recent study of 4 FEL children, Ladisch et al. (6) found an immunological deficiency syndrome which included defects in both humoral and cell-mediated immunity and a plasma inhibitor of in vitro lymphocyte blastogenesis. The humoral immunity defects included low antibody titers after previous immunizations and an impaired ability to respond to primary immunizations. However, immunoglobulin levels (IgA, IgM, IgG) were normal in these patients. Although a normal proportion of T and B lymphocytes were present in these patients, defects in cellular immunity were shown by anergy and by the inability of the patients' lymphocytes to proliferate in response to specific antigens; yet, responses to lectin mitogens and allogeneic cells (mixed leukocyte cultures) were normal. The level of inhibitory activity of FEL plasmas appeared to

be proportional to the degree of hyperlipidemia (measured in triglyceride levels) present.

Localized fatty changes were observed in the liver autopsy examination of one FEL patient along with an accumulation of uncharacterized lipid in the infiltrating histiocytes.

At present, the etiology of this disorder remains an enigma. The question on whether the immunodeficiency is the primary defect in FEL or merely secondary to another primary pathogenic mechanism remains unanswered and is the focal point of this study.

From the discoveries of an immunodeficiency syndrome, the inheritance of the disorder as an autosomal recessive trait, the localized fatty changes in liver along with the accumulation of lipid in the infiltrating macrophages, and a circulating inhibitor of in vitro lymphocyte blastogenesis whose potency was apparently related to the degree of hyperlipidemia, a rationale was established for the investigation of lipids in FEL liver and plasma to determine the possible presence of a lipid or glycolipid storage disease, and the effects of such a possible storage product on the observed immunodeficiency. If a storage problem was to be discovered, a search for the metabolic basis of the accumulation, particularly in the likelihood of a catabolic defect, would be conducted along with the investigation of the liver lysosomal enzymes.

# LITERATURE REVIEW

## I. The Gangliosides

### A. General Background

Glycosphingolipids, first discovered by Thudichum (8) in 1874, are amphipathic molecules composed of three basic components: a long-chain base, a fatty acid moiety, and a carbohydrate moiety which may vary in length from a single monosaccharide to a complex, branched oligosaccharide of 30-60 glycose units (9-10). The principal long-chain base of most mammalian glycosphingolipids is 4-sphingenine (commonly called sphingosine), to which saturated or unsaturated fatty acids and their α-hydroxy derivatives, varying in length from 14 to 26 carbon units, are covalently joined via amide linkages. The long-chain base and fatty acid components together constitute the hydrophobic portion (ceramide) of the glycosphingolipid that is anchored in bilayer membranes. Carbohydrate units constitute the hydrophilic portion of the glycosphingolipids and are covalently joined to the C-1 hydroxyl group of the ceramide molecule by glycosidic linkages. The carbohydrate moiety is the major determinant that expresses the wide structural and functional diversity of the glycosphingolipids.

Glycosphingolipids can be divided into three main groups as determined by the chemical nature of their carbohydrate moiety: neutral (i.e. non-polar, non-acidic) glycosphingolipids, sulfatoglycosphingolipids (commonly called sulfatides), and the gangliosides. The neutral and sulfated glycosphingolipids have been reviewed extensively elsewhere (11-15), and will not be discussed further in depth here.

Gangliosides are terrestrial and marine animal glycosphingolipids containing the unique acidic carbohydrate, sialic acid. They are principally located in the outer surface of plasma membranes of mammalian cells and, together with sialylated glycoproteins, are the main source of the negative charge observed on cell surfaces.

Originally identified by Klenk (16) in 1935 in his investigations of brain lipids in patients with Tay-Sachs Disease and Nieman-Pick Disease, the sialic acid-containing glycosphingolipids were soon discovered in normal brain (17) and were consequently named gangliosides by Klenk (18) because they were believed to be localized specifically in ganglion cells. Yamakawa and Suzuki (19) later reported their occurrence in extraneural tissues and fluids, after which, extensive research into ganglioside structure and function has evolved.

The concentration of gangliosides in all animal species is highest in brain, where they were first discovered. Since they are located at the cell surface and also in the synaptic membranes of the central nervous system, their function is of particular interest to neurochemists, especially in relation to the elucidation of synaptic transmission mechanisms at the molecular level. Metabolic disorders of gangliosides, such as Tay-Sachs Disease and $GM_1$ gangliosidosis, are of special interest in regard to their relationship to developmental neurobiology and mental retardation.

In extraneural tissues, gangliosides are believed to occur primarily on the cell surface where they are assumed to be important in intercellular recognition. They may also be involved in a variety of cell surface functions such as the regulation of metabolism, growth, differentiation, behavior, malignant transformation, viral infection (20-22); and as surface receptors for protein hormones (thyroid stimulating hormone, luteinizing hormone, human chorionic gonadotropin) (23-25), Sendai virus (26-27), interferon (28-29), serotonin (30), and

bacterial toxins, e.g. staphylococcal α toxin (31), botulinum toxin (32), tetanus toxin (33-34), and especially cholera toxin (35-38). A list of the bacterial toxins and their putative ganglioside receptors can be found in Table 1 (21).

Table 1. Bacterial Toxins and their Ganglioside Receptors

| Bacterial Toxin | Receptor |
|---|---|
| cholera toxin | $GM_1$ |
| tetanus toxin | $GD_{1b}$,$GT_{1b}$ |
| botulinum toxin | trisialoganglioside |
| staphylococcal α toxin | sialylparagloboside |

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sialylparagloboside= NeuNAcβ2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1'Cer

Sialic acid, is a generic term that encompasses a variety of derivatives of neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranoso-nic acid), which usually occur naturally as either N-acetylneuraminic acid or N-glycolylneuraminic acid. The O-acetyl derivatives of the sialic acids have also been found in bacteria and animals. Only the N-acetylneuraminic acid form of sialic acid has been found in human tissues, although N-acetyl-4-O-acetylneuraminic acid has been reported in human urine (39) and bile (40). The sialic acid residues in gangliosides are joined to the oligosaccharide moiety by α-ketosidic linkages (α2→3 or α2→6) to galactose residues, and to each other via α2→8 linkages (15).

## B. Nomenclature

Adoption of an appropriate nomenclature descriptive of the carbohydrate moiety of glycosphingolipids has been a confusing and complex issue, as complex as the structural nature of the oligosaccharide moieties themselves. Most early systems of nomenclature were developed to designate individual components of

human brain gangliosides which were observed in thin-layer or paper chromatographic systems. The most commonly used system for gangliosides was that of Svennerholm (41) which was fairly easy to remember and useful for description of the major brain gangliosides. However, due to the discoveries of more complex gangliosides and the extraneural gangliosides whose carbohydrate sequences differed in isomeric glycosidic linkages as well as in their amino sugar components, Svennerholm's nomenclature system became less appropriate and extremely limited in its description of the more structurally complex gangliosides. Wiegandt (42) developed an alternative system which employed the use of trivial names to denote the different families of the oligosaccharides that have been found to occur in glycosphingolipids. Borrowing partly from Wiegandt, the IUPAC-IUB Commission on Biochemical Nomenclature (43) and the Commission on Nomenclature of Organic Compounds have considered a systematic nomenclature for the various oligosaccharide families which are summarized here in Table 2. A more detailed description of the nomenclature and the use of the prefixes and structure abbreviations is given in a review by Sweeley and Siddiqui (15).

Table 2. Oligosaccharide Structural Families in Glycosphingolipids

| Name (prefix) | Structure |
|---|---|
| lacto | Galβ1→3GlcNAcβ1→3Galβ1→4Glc→ |
| lactoneo | Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ |
| ganglio | Galβ1→3GalNAcβ1→4Galβ1→4Glc→ |
| globo | GalNAcβ1→3Galα1→4Galβ1→4Glc→ |
| globoiso | GalNAcβ1→3Galα1→3Galβ1→4Glc→ |
| muco | Galβ1→3Galβ1→4Galβ1→4Glc→ |
| gala | GalNAc?1→3Gal?1→4Galα1→4Gal→ |

The prefixes given in Table 2 imply the entire structure of the root oligosaccharide up to the tetrasaccharide level, including the carbohydrate

sequence as well as the positions and anomeric configuration of the glycosidic linkages. So far, sialic acid sugars have only been discovered on the ganglio, gala, and lactoneo oligosaccharide families or series in human tissue.

C. Occurrence

Due to the large number of gangliosides (over 40) that have been discovered, only the extraneural gangliosides and the major brain gangliosides of human origin will be reviewed here. A more comprehensive review of all human neural and extraneural gangliosides and glycosphingolipids can be found elsewhere (15,44).

The major distinguishing feature of extraneural gangliosides is the presence of N-acetylglucosamine as the amino sugar component in the oligosaccharide moiety, whereas neural gangliosides only contain N-acetylgalactosamine. The possible occurrence of N-acetylglucosamine-containing gangliosides was first noted by Klenk and Lauenstein (45) in 1952 in a glycolipid hydrolysate obtained from bovine erythrocytes. Yamakawa et al. (46) subsequently reported the presence of GlcNAc in human, sheep, goat, rabbit, and bovine erythrocyte glycosphingolipids, and found glucosamine-containing gangliosides in their partially-purified glycosphingolipid fractions (47). The first isolation of a homogenous glucosamine-containing ganglioside was reported by Kuhn and Wiegandt (48) in 1964. With this final proof of the existence of glucosamine-containing gangliosides, a new possibility for ganglioside structures different from those of the major neural gangliosides was established. In Table 3, the structure and occurrence of selected, known human brain gangliosides, in increasing order of complexity up to the trisialogangliosides, and extraneural gangliosides are presented.

The fucogangliosides are of particular interest because of their serological and possible immunological properties. Glycolipid blood group antigenic activities

Table 3. Structure and Occurrence of Human Gangliosides

| Structure | Trivial Name | Source |
|---|---|---|
| NeuNAcα2→3Galβ1→1'Cer | $GM_4$ | brain,myelin (49,50) |
| NeuNAcα2→3Galβ1→4Glcβ1→1'Cer | $GM_3$ | brain,viscera,erythrocytes (51-54) |
| NeuNAcα2→8NeuNAcα2→3Galβ1→4Glcβ1→1'Cer | $GD_3$ | brain,viscera,placenta (55-57) |
| GalNAcβ1→4Gal(3←2αNeuNAc)β1→4Glcβ1→1'Cer | $GM_2$ | brain,viscera (58-61) |
| GalNAcβ1→4Gal(3←2αNeuNAc8←2αNeuNAc)β1→4Glcβ1→1'Cer | $GD_2$ | brain (62) |
| Galβ1→3GalNAcβ1→4Gal(3←2αNeuNAc)β1→4Glcβ1→1'Cer | $GM_1$ | brain,viscera (61,63) |
| NeuNAcα2→3Galβ1→3GalNAcβ1→4Gal(3←2αNeuNAc)β1→4Glcβ1→1'Cer | $GD_{1a}$ | brain,viscera (57,61,63) |
| Galβ1→3GalNAcβ1→4Gal(3←2αNeuNAc8←2αNeuNAc)β1→4Glcβ1→1'Cer | $GD_{1b}$ | brain (64) |
| NeuNAcα2→3Galβ1→3GalNAcβ1→4Gal(3←2αNeuNAc8←2αNeuNAc)β1→4Glcβ1→1'Cer | $GT_{1b}$ | brain (41) |
| NeuNAcα2→8NeuNAcα2→3Galβ1→3GalNAcβ1→4Gal(3←2αNeuNAc)β1→4Glcβ1→1'Cer | $GT_{1a}$ | brain (65) |
| Fucα1→2Galβ1→3GalNAcβ1→4Gal(3←2αNeuNAc)β1→4Glcβ1→1'Cer | $GM_1$-Fuc | bovine brain,thyroid (66-67) human brain (68) |
| Fucα1→2Galβ1→3GalNAcβ1→4Gal(3←2αNeuNAc8←2αNeuNAc)β1→Glcβ1→1'Cer | $GD_{1a}$-Fuc | human brain (69) |

Table 3 (cont'd.).

| Structure | Trivial Name | Source |
|---|---|---|
| NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1'Cer | sialyl-paraglobo-side | viscera,plasma,muscle,brain peripheral nerve (61,70-73) |
| NeuNAcα2→6Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1'Cer | | viscera (61) |
| NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1'Cer | | spleen (61,74) |
| NeuNAcα2→3Galβ1→4GlcNAc(3←1αFuc)β1→3Galβ1→4Glcβ1→1'Cer | | kidney (57) |
| NeuNAcα2→8NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1'Cer | | kidney,colon (75-76) |
| Galα1→3Galβ1→4GlcNAcβ1→6 NeuNAcα2→3Galβ1→4GlcNAcβ1→3 Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1'Cer | | erythrocyte blood group I (77) |
| Fucα1→2Galβ1→4GlcNAcβ1→6 NeuNAcα2→3Galβ1→4GlcNAcβ1→3 Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1'Cer | | erythrocyte blood group H (78) |

(ABO, Lewis, Ii, $PP^k$ antigens) have formerly been associated with neutral glycosphingolipids of the globo, isoglobo, muco, lacto, and lactoneo core oligosaccharide families (15). Recent studies have demonstrated the existence of fucogangliosides with blood group activities (77-80), and established a possible metabolic relationship between extraneural gangliosides with lactoneo sequences and the blood group fucolipids due to their common oliogosaccharide core structure.

Wiegandt first identified a fucosylated ganglioside with a fucoganglio-pentaose structure;

Fuc$\alpha$1→2Gal$\beta$1→3GalNAc$\beta$1→4Gal(3←2$\alpha$NeuNGl)$\beta$1→4Glc→,

from bovine liver (61). Fucogangliosides with similar ganglio core structures have since been isolated from boar testes (81), bovine brain (Fuc-$GD_{1a}$) (67,82), and pig adipose tissue (83). Fucogangliosides have also been found in rat hepatoma cells (84). The occurrence of human fucogangliosides is listed in Table 3.

D. Methodology- Isolation Techniques

Although gangliosides were identified in human neural tissue as early as 1935 (16), isolation and detailed characterization of specific gangliosides awaited the development of mild and effective extraction procedures and improved chromatographic methods.

Isolated gangliosides are water-soluble, yet water or aqueous salt solutions will not extract gangliosides from tissue (85). Gangliosides are bound to tissue by ionic bonds between proteins and the negatively-charged carboxyl groups of sialic acid. Therefore, these bonds require polar solvents for their disruption. On the other hand, non-polar solvents, such as chloroform, are also required to dissociate hydrophobic bonds between the ceramide moiety of gangliosides and the hydrophobic portions of membrane proteins and lipids (86). For this reason,

extraction methods with mixtures of chloroform and methanol have proven to be quite effective.

The most widely used procedure for glycosphingolipid and ganglioside extraction is that described by Folch et al. (87). This procedure involves the extraction of homogenized tissues with 20 volumes of chloroform:methanol (2:1), addition of an aqueous solution to the extract to form a biphasic solution, removal of the aqueous phase containing the gangliosides accompanied by additional solvent washing of the aqueous phase, reduction in volume of the aqueous phase, dialysis, and finally lyophilization of the aqueous extract. Because of the inability of the chloroform:methanol extraction to remove all the gangliosides from tissue (only 90% of total gangliosides with higher losses of the more polar gangliosides), Suzuki (88) devised a double extraction modification of the Folch procedure. The residue was re-extracted with 10 volumes of chloroform:methanol (1:2) to remove all of the more polar gangliosides, followed by aqueous partition and washings of the lower phase with "pure solvents upper phase" to quantitatively partition over the less polar gangliosides into the aqueous phase. The major drawback to both of these chloroform:methanol solvent partition methods was still the incomplete recovery of the less polar gangliosides ($GM_3$ and $GM_2$) from the organic lower phase.

Tettamanti et al. (89) described a modification of a tetrahydrofuran solvent extraction procedure originally introduced by Trams and Lauter (90). This procedure involved an aqueous salt solution:tetrahydrofuran:ether partition of the aqueous tetrahydrofuran lipid extract. Washing, reduction of volume, dialysis, and lyophilization of the aqueous phase was performed in an manner analogous to the Folch method.

Fractionation of the lyophilized ganglioside preparation into individual species was conducted by DEAE-cellulose chromatography (91) in which

gangliosides were eluted in increasing order of their sialic acid content, and/or by Anasil S (magnesia-silica gel) column chromatography (92) and preparative thin-layer chromatography. Because of the inherent problems of loss of the less polar gangliosides with either of the solvent extraction-aqueous partition methods described above and also possible loss of gangliosides during dialysis (93), other workers have retained the chloroform:methanol extraction but omitted the aqueous partition and dialysis. Sandhoff et al. (94) described a preparative TLC procedure to separate gangliosides from total lipids using two-dimensional, two-solvent systems. Rouser et al. (95) described methods that utilize DEAE-cellulose column chromatography to separate gangliosides from total lipids.

Recent technological developments have improved the efficiency, recovery, and resolution of ganglioside isolation methods. Anion exchange column chromatography has been extensively used in the rapid, one-step separation of gangliosides from total lipid extracts. DEAE-cellulose has been replaced by other anion exchange support media, such as DEAE-Sephadex (50), DEAE-Sepharose (96), DEAE-silica gel (97), and Spherosil-DEAE-Dextran (98), all of which exhibit superior ease of handling, flow rates, binding capacity, and resolution of gangliosides according to their sialic acid content. Recoveries of gangliosides with these anion exchange methods have been reported to be from 90% to 98%. Upon isolation of the ganglioside fractions, the gangliosides are further separated into individual components by silicic acid column chromatography with Iatrobeads, which are porous, silica spheres of relatively large particle size (60 micron diameter) (99). Because of their rigidity and crush-resistance to relatively high packing pressures, Iatrobeads can be packed into longer and thinner columns than conventional silica gels, resulting in higher number of theoretical plates and improved separation characteristics with little or no effect on flow rates (100). Elutions are performed with chloroform:methanol:water gradients.

Thin-layer chromatography of gangliosides has also been improved with the development of Silica Gel 60 (101) and High Resolution Silica Gel 60 (102-103) to replace the old standards, Silica Gels G and H (14,92). Increased resolution of individual gangliosides, faster development times, and increased sensitivity requiring less amounts of gangliosides for qualitative analysis are some of the benefits of the new developments in thin-layer chromatographic methods for ganglioside analysis.

Direct TLC separation of gangliosides from brain total lipid extracts has been done by Harth et al. (104). Chloroform:methanol extracts were directly applied on to Silica Gel 60 TLC plates and developed in one dimension with three different solvent systems in a special sandwich chamber. At the end of the development, individual gangliosides could be detected and were completely separated from the other neutral lipids and phospholipids, which migrated ahead of $GM_3$ and $GM_4$ ganglioside.

A simple and rapid method for ganglioside isolation from small amounts of tissues (1 mg) was recently developed by Irwin and Irwin (105) to overcome problems encountered with the Harth procedure. Because of the presence of interfering substances which complicated quantitative assays of the gangliosides and relatively low ratios of ganglioside content to total lipids in non-neural tissues, a prior purification of gangliosides was required before TLC analysis. Total chloroform:methanol (2:1) lipid extracts were applied onto Pasteur pipet columns of silicic acid (Unisil). Non-gangliosidic lipids were eluted with a chloroform:methanol solvent, and the gangliosides were subsequently eluted with a chloroform:methanol:water solvent. Comparisons to gangliosides obtained by the Suzuki modification (88) of the Folch procedure were highly favorable with recoveries of gangliosides averaging 89%.

High pressure liquid chromatography (HPLC) techniques have been applied

in attempts to develop a rapid (25-40 min), simple, and highly sensitive method for the analysis of individual neutral glycosphingolipids and gangliosides. Detection of the glycolipids was accomplished by either U.V. absorption of the perbenzoylated derivatives (106-107) or of the N-p-nitrobenzoylated derivatives of O-acetylated glycolipids (108-109). The detector responses were linear from 70 pmoles to 30 nmoles for the benzoylated derivatives and 10 pmoles to 60 nmoles for the N-p-nitrobenzoylated, O-acetylated derivatives. The major drawbacks to these techniques so far have been the requirement of prior partial purification of the glycolipids from the total lipid extracts before derivatization and limited applications toward preparative isolations of individual glycolipids. Tjaden et al. (110) has described an HPLC system for the semi-preparative isolation of underivatized, partially purified neutral glycolipids and gangliosides. Detection was performed by a flame ionization-moving wire detector, and detector responses were linear from 2 to 200 μg of glycolipid. Of the three HPLC methods described, the Tjaden method has been shown to be most promising in the ready application of HPLC to preparative isolations of gangliosides. However, refinements in the prior clean-up of the ganglioside preparations and improved recoveries are still required before HPLC methods attain wider acceptance.

The use of the new anion exchange supports in conjunction with the refinements in silicic acid column chromatography and TLC have given researchers a powerful tool in their attempts to isolate and characterize minor individual gangliosides in animal tissues hitherto undetectable due to their small quantities and similar chromatographic properties with the more predominant gangliosides present. Iwamori and Nagai (96), using a technique involving DEAE-Sepharose separation of ganglioside fractions and subsequent development of each fraction on an one-dimensional TLC system to obtain a "ganglioside map", have discovered the presence of at least 24 new minor gangliosides in human brain. Feizi et al. (79)

have reported the presence of at least 10 previously undetected gangliosides in human erythrocytes using sequential DEAE-Sephadex and Iatrobead chromatography of crude erythrocyte lipid extracts.

## E. Methodology- Characterization Techniques

### 1) Carbohydrate Composition

The most common method for the quantitation and identification of the carbohydrate components in glycosphingolipids is the methanolysis procedure as described by Vance and Sweeley (111) and Chambers and Clamp (112). Hydrolysis of the entire oligosaccharide and ceramide moieties of the glycosphingolipids (at least 50 μg) is achieved by heating at 80° for 18-24 hr in 0.75 N methanolic HCl (anhydrous). The hydrolyzed carbohydrate products (in the form of methyl glycosides and methyl ketosides, methyl esters of neuraminic acid) are converted to their trimethylsilyl derivatives and analyzed by GLC (111). Acetic anhydride is added to the hydrolysis products prior to trimethylsilylation in order to re-N-acetylate the hexosamines and sialic acids. In this procedure, N-acetyl and N-glycolyl neuraminic acids cannot be differentiated because of their N-deacylation during methanolysis and conversion to the N-acetyl methyl neuraminides after re-N-acetylation with acetic anhydride. However, NeuNAc and NeuNGl can be determined individually by GLC after mild methanolysis conditions (0.05 N methanolic HCl) of the ganglioside sample.

Hexoses and hexosamines can also be estimated as alditol acetates (114). The glycolipids undergo acetolysis and acid hydrolysis and conversion of the monosaccharide products to their alditols by borohydride reduction. The alditols are peracetylated and analyzed by GLC. However, sialic acids are destroyed by this procedure.

2) Fatty Acid and Long-Chain Base Composition

The most common procedure for the quantitative and qualitative analysis of long-chain bases is that described by Gaver and Sweeley (115) wherein the free base (sphingosine) is liberated from the glycosphingolipid by aqueous methanolic HCl hydrolysis. The free sphingosine is trimethylsilylated and analyzed by GLC. The intact ceramide moiety can also be studied by combined GLC-MS (116), which provides information about the actual molecular species in the ceramide mixture. This information cannot be obtained by separate analysis of the fatty acid fraction and sphingosine fractions liberated by methanolysis.

Quantitation of long-chain bases can also be achieved by colorimetric (117) or fluorimetric methods (118), which are extremely sensitive and reliable in the 1-100 nmole range.

Fatty acids are estimated and identified as their methyl esters by GLC after hexane extraction of the methanol hydrolysates following methanolysis in methanolic HCl. One half of the fatty acid mixture is trimethylsilylated to convert any α-hydroxyacyl methyl esters to volatile derivatives, which is then analyzed by GLC and compared to the GLC results obtained from the untreated hexane extract. Some GLC liquid phases, such as 15% polyethylene glycol-adipate polyester allow for the simultaneous separation and analysis of normal and α-hydroxy fatty acids, although the two fatty acid classes are generally separated by TLC before GLC analysis. Detailed information on the relative retention behavior of a wide variety of normal fatty acids (119) and α-hydroxy fatty acids (120) has been published in excellent reviews.

3) Determination of Carbohydrate Sequence and Anomeric Linkages

Specific exoglycosylhydrolases, which catalyze the hydrolysis of non-reducing terminal sugars, are used to determine the sequence as well as the

anomeric configuration of the glycosidic linkages in the oligosaccharide moiety of glycolipids. Bile salt or synthetic detergents are often required to solubilize the glycolipids in an aqueous media in the form of mixed micelles, which the enzymes can presumably attack. The products can be identified by thin-layer, paper, or gas-liquid chromatography. Enzymes that have been used in this manner are α-galactosidase, β-galactosidase, α-fucosidase, β-N-acetylhexosaminidase, β-glucosidase, and neuraminidase, all of which have been purified from various animal, plant, and bacterial sources (121-122).

Partial chemical hydrolysis (63) and enzymatic hydrolysis with specific endoglycosylhydrolases (122) have also been used to obtain information about the sequence of carbohydrates, particularly of the more complex, branched oligosaccharide structures.

Neuraminidase from C. perfringens and V. cholerae can hydrolyze all of the sialic acid residues of gangliosides except those (in $GM_1$ and $GM_2$) that are linked to the internal galactose of the oligosaccharide moiety with adjacent GalNAc or Gal-GalNAc residues substituted at the C-4 position of galactose. The neuraminidase-resistance of the internal sialic acid is assumed to be due to steric hindrance by the hexosamine residue (63). Susceptibility to neuraminidase is achieved after the Gal-GalNAc or GalNAc residues have been removed from $GM_1$ and $GM_2$, respectively (123). Thus, susceptibility to neuraminidase attack can be used as a means to locate the relative positions of NeuNAc residues on the oligosaccharide structure of gangliosides. The resistant neuraminic acid of $GM_1$ or $GM_2$ has been shown to be hydrolyzable by C. perfringens in the presence of bile salts (124), or if the gangliosides are incubated with the enzyme below the critical micelle concentration (125). Sugano et al. (126) has recently demonstrated the susceptibility of $GM_1$ to Arthrobacter ureafaciens neuraminidase in the absence of bile salts and independent of the ganglioside concentration in the incubation

mixture.

Nuclear magnetic resonance (NMR) spectroscopy can be used to determine the configuration of the anomeric linkages of glycosphingolipids. Anomeric proton signals are more easily resolved when the oligosaccharide moiety is trimethylsilylated or dissolved in $D_2O$ (15). Falk et al. (127) have recently reported the proton NMR spectra of permethylated, $LiAlH_4$-reduced glycosphingolipids and were able to distinguish between lacto and lactoneo oligosaccharide sequences. Because of the high sensitivity of their NMR method, Falk et al. have discussed the possibility of intact glycolipid structure elucidation with combined NMR-MS analysis on a microscale level (100 μg).

Natural abundance Fourier-transform $^{13}C$-NMR spectra of gangliosides and neutral glycosphingolipids were also reported (128-130). Although all anomeric carbons can easily be assigned, the major drawback with $^{13}C$-NMR has been the large amounts of sample required for study (50-400 mg). Application of this technique appears to be oriented toward ganglioside involvement in membrane perturbation studies, ganglioside cation binding effects, and lipid-lipid interactions in model membranes.

The anomeric configuration of glycosidic linkages in the oligosaccharide moiety can also be determined by chromium trioxide oxidation of the peracetylated glycolipid (131). All β-glycosides are oxidized while the α-glycosides remain unaffected. Identification of the unreacted sugars is conducted by GLC analysis of their alditol acetates following chromium trioxide oxidation, acetolysis and hydrolysis, borohydride reduction, and acetylation of the intact glycosphingolipid. Samples of only 100-300 μg are required for studies, but special care must be taken to completely acetylate the oligosaccharide and to remove all mild acid-labile groups, such as sialic acid, prior to acetylation and chromium trioxidation.

4) Determination of Position of Glycosidic Linkages

A widely-used procedure for the determination of the positions of glycosidic linkages in glycosphingolipids combines permethylation of unsubstituted hydroxyl groups on the carbohydrates, acid hydrolysis, borohydride reduction, acetylation of the partially methylated alditol products, and analysis of the partially methylated alditol acetates by combined GLC-MS (132). Identification of the alditol acetates is performed by GLC and analysis of the distinctive fragmentation patterns of the methylated alditol acetates at carbon positions with O-methyl substitutions (133). This method is well-suited for microscale work as good results have been obtained with amounts as low as 100 μg of glycolipid.

Substitutions on sialic acids can be determined with an analogous procedure to that described for the neutral sugars by permethylation of the glycolipid, methanolysis with 0.5 N methanolic HCl at $80^{\circ}$ to obtain the methyl ester, N-methyl, methyl neuraminide, acetylation of the partially methylated sugar, and GLC-MS analysis of the partially methylated, acetylated products (134).

5) Mass Spectrometry of Intact Glycosphingolipids

Early mass spectral studies of the intact glycosphingolipids involved analysis of the poly-O-trimethylsilyl derivatives (116,135). Limitations were apparent due to the complicated mass spectra obtained by this method and the high molecular weight of the poly-trimethylsilyl derivatives which approached the mass scan range limits of the instrument, and as a result, allowed only simple glycosphingolipids to be analyzed.

Karlsson et al. (136-137) later described the mass spectra of a number of permethylated, $LiAlH_4$-reduced intact glycosphingolipids and gangliosides. Conclusive information concerning the molecular ion, number of sugar residues, sequence and branching of the carbohydrate chain, and the identification of

individual ceramide species in the glycolipid were obtained (138). Although samples as small as 100 μg have been analyzed by this method, normal ranges for sample size were 0.5-1 mg. The limits of the mass scan range and the availability of high molecular weight calibration standards are the only limitations to this method.

6) Analysis of Intact Oligosaccharides Derived from Glycosphingolipids

Because of the high molecular weight of the more complex glycosphingolipids, carbohydrate studies can be performed on the oligosaccharides obtained by ozonolysis (139) or osmium tetroxide-periodate oxidation (140) of the double bond in 4-sphingenine and 4-eicosasphingenine of the glycolipid. The liberated oligosaccharides are reduced with borohydride and analyzed as either their permethyl, peracetyl, or trimethylsilyl derivatives by direct probe mass spectrometry in either the electron impact or chemical ionization mode (141). Quantities of oligosaccharides as low as 0.5 mg can be analyzed by this method.

F. Glycosphingolipid and Ganglioside Anabolism

It is generally accepted that glycosphingolipids are synthesized by a multi-glycosyltransferase system (142) via the sequential addition of monosaccharides to the ceramide moiety at the non-reducing end of the growing oligosaccharide chain. The pH optimum and divalent metal ion requirements are similar to those for glycoprotein synthesis. However, no evidence has been shown for the role of polyisoprenoid intermediates in mammalian glycolipid biosynthesis, although this biosynthetic mechanism has been demonstrated for glycoprotein anabolism. A brief summary of the glycolipid biosynthetic pathways of the globo, lacto, lactoneo, and ganglio families is presented in Figure 1. A more detailed review of the biosynthetic pathways and of the enzymes involved in glycosphingolipid

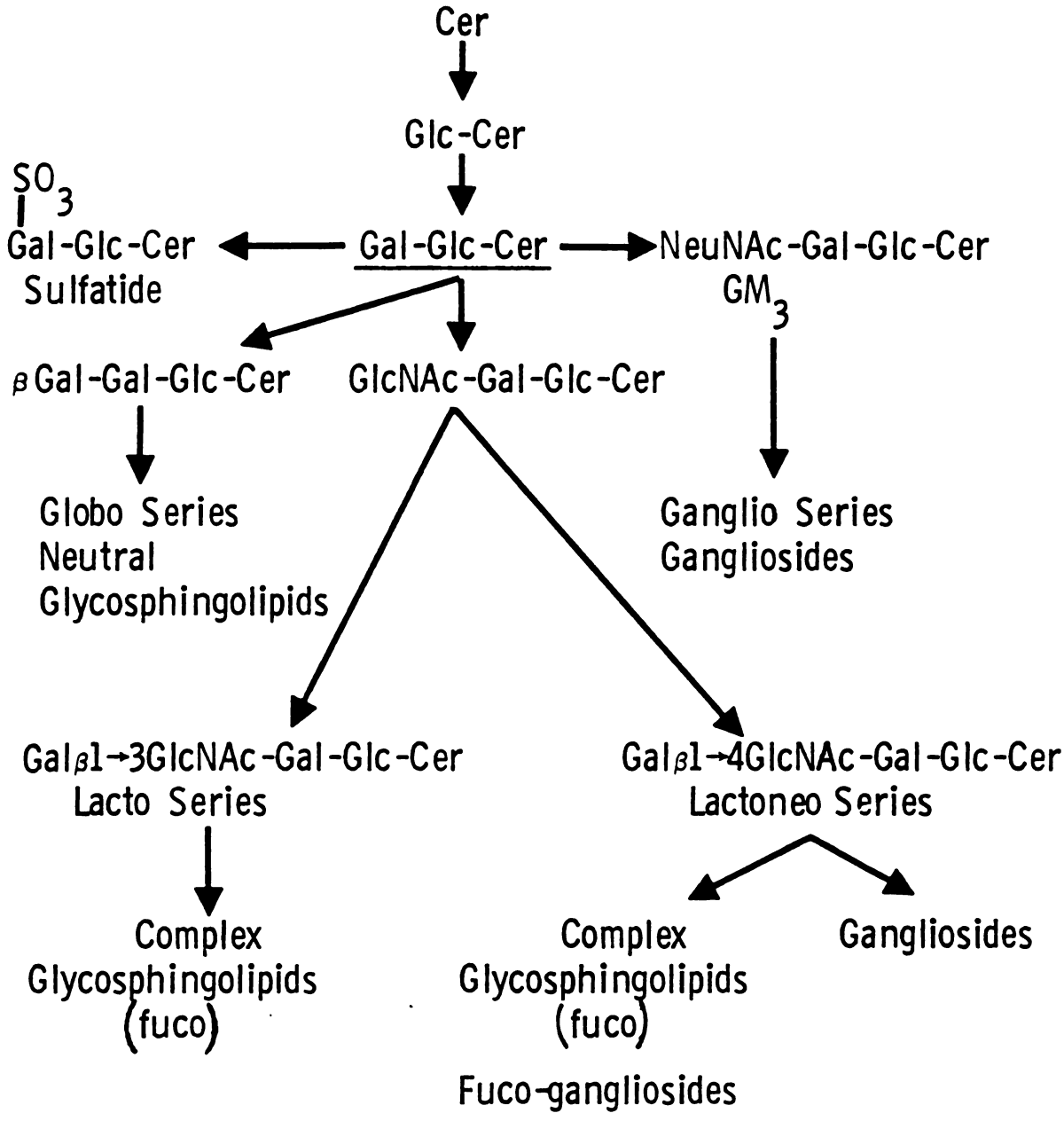


Figure 1. General Pathways for Ganglioside Biosynthesis

anabolism has been done by others (130,144-145).

Lactosylceramide is the critical branching point for the biosynthesis of a number of glycolipid families. The addition of either a sulfate group, α-galactosyl residue, β-N-acetylglucosaminyl residue, or sialyl residue will determine the metabolic pathway and fate for the individual lactosylceramide molecule. The glycosylation of lactosylceramide appears to be primarily tissue-specific rather than substrate-specific.

## G. Glycosphingolipid and Ganglioside Catabolism

Glycosphingolipids are sequentially catabolized by exoglycosylhydrolases, and there is no direct evidence for the existence of endoglycosylhydrolase catabolism in man analogous to glycoprotein or glycosaminoglycan degradation (146), although bacterial endoglycosylhydrolases have been used in structural studies of human glycosphingolipids. Evidence for the sequential exoglycosylhydrolase mode for glycolipid catabolism is shown in the various glycosphingolipidoses by the storage of specific glycolipids in lysosomes in which a specific exoglycosylhydrolase is defective.

Since glycolipid glycosylhydrolases have acidic pH optima and the glycosphingolipidoses are lysosomal storage disorders, it is assumed that glycolipids, as well as glycoproteins and glycosaminoglycans, are normally catabolized within lysosomes. Glycoprotein and glycolipid catabolism has been demonstrated with intact lysosomal preparations by various workers (147). Many theories on the mode of action of the lysosomal glycosylhydrolases have been proposed. On the basis of studies on mucolipidosis II ("I-cell" disease), it had been formerly proposed by Neufeld et al. (148) that the lysosomal enzymes are normally secreted and taken up by adjacent cells by specific receptor-mediated pinocytosis.

Recent findings by Natowicz and Sly (149) have shown that 6-phosphomannose residues on lysosomal hydrolases were required for intercellular pinocytosis. They proposed a different hypothesis to explain the biogenesis of lysosomal enzymes and the findings in "I-cell" disease in which the 6-phosphomannose recognition marker in lysosomal hydrolases serves as an intracellular traffic signal that directs high-uptake forms of lysosomal enzymes to the lysosome and prevents their secretion (150). In "I-cell" disease, this marker is presumably missing. Other theories for modes of action are protease activation of latent hydrolases (147), chondroitin sulfate regulation within lysosomes (151), association of the hydrolase in membrane-bound multienzyme complexes (123), and the involvement of glycoprotein co-factors in the specific hydrolysis of glycolipids (152-154).

The defects in glycolipid metabolism presented in the glycosphingolipidoses are practically all of lysosomal glycosylhydrolase origin. Excellent reviews on the metabolic defects of the glycosphingolipidoses, the mucopolysaccharidoses, and the glycoprotein storage diseases have been presented elsewhere (147,155-160).

The metabolic defects associated with ganglioside metabolism are illustrated in Figure 2. The glucosamine-containing gangliosides have not been implicated in any glycosphingolipidoses nor have they been found to accumulate in other glycosphingolipidoses.

The known glycosphingolipidoses associated with defects in either glycosylhydrolases or glycosyltransferases are presented in Table 4 (161).

The mucopolysaccharidoses and mucolipidoses are listed in Table 5 (159,162-164).

1) Role of Neuraminidase in Glycosphingolipid Catabolism

Most studies of the enzymatic release of sialic acid from gangliosides have involved the use of microbial sialidases. Most human neuraminidase preparations

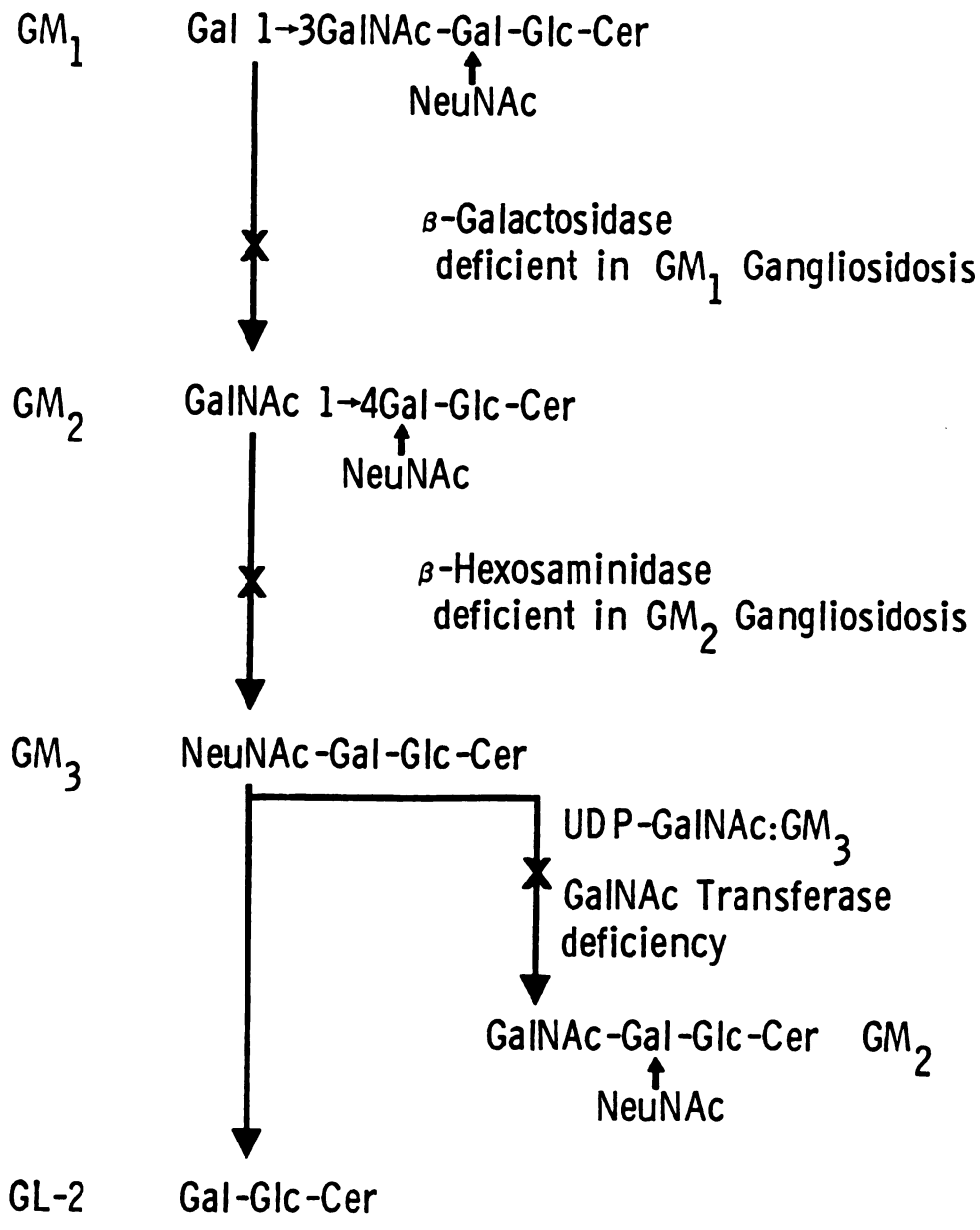


Figure 2. Enzyme Defects in Ganglioside Metabolism

Table 4. The Glycosphingolipidoses

| Disorder | Stored Material | Enzymic Defect |
|---|---|---|
| Globoid Cell Leukodystrophy | Gal-Cer, Gal-sphingosine | galactosylceramide β-galactosidase |
| Metachromatic Leukodystrophy | $SO_3$-Gal-Cer, $SO_3$-Gal-Glc-Cer (sulfatides) | arylsulfatase A |
| Multiple Sulfatase Deficiency | $SO_3$-Gal-Cer, $SO_3$-Gal-Glc-Cer cholesterol-sulfate, sulfated glycosaminoglycans | arylsulfatase A,B,C |
| Gaucher's Disease | Glc-Cer Glc-sphingosine | glucosylceramide β-glucosidase |
| Fabry's Disease | Galα1→4Gal-Glc-Cer Galα1→4Gal-Cer | α-galactosidase A |
| $GM_1$ Gangliosidosis | $GM_1$, galactose-rich oligosaccarides, keratan sulfate | $GM_1$ ganglioside β-galactosidase |
| Tay-Sachs Disease | $GM_2$ | β-hexosaminidase A |
| Sandhoff's Disease | $GM_2$, asialo-$GM_2$, GalNAcβ1→4Gal-Gal-Glc-Cer (globoside) | β-hexosaminidase A and B, asialo-$GM_2$ |
| $GM_3$ Gangliosidosis | $GM_3$, $GD_3$ | UDP-GalNAc:$GM_3$ GalNac transferase |

Table 5. The Mucopolysaccharidoses and Mucolipidoses

| Disorder | Stored Material | Enzymic Defect |
|---|---|---|
| Mucopolysaccharidosis IH (Hurler) | heparan sulfate, dermatan sulfate | α-iduronidase |
| Mucopolysaccharidosis IS (Scheie) | heparan sulfate, dermatan sulfate | α-iduronidase |
| Mucopolysaccharidosis II (Hunter) | heparan sulfate, dermatan sulfate | iduronosulfate sulfatase |
| Mucopolysaccharidosis IIIA (Sanfilippo A) | heparan sulfate | sulfamidase |
| Mucopolysaccharidosis IIIB (Sanfilippo B) | heparan sulfate | α-N-acetylglucosaminidase |
| Mucopolysaccharidosis IIIC (Sanfilippo C) | heparan sulfate | α-glucosaminidase? |
| Mucopolysaccharidosis IV (Morquio) | keratan sulfate, chondroitin sulfate | N-acetylhexosaminide -6-$SO_3$ sulfatase |
| Mucopolysaccharidosis VI (Maroteaux-Lamy) | dermatan sulfate | N-acetylhexosaminide -4-$SO_3$ sulfatase, arylsulfatase B |
| Mucopolysaccharidosis VII | dermatan sulfate, heparan sulfate, chondroitin sulfate | β-glucuronidase |
| Mucopolysaccharidosis F | fucosyl-glycolipid, -glycosaminoglycan, -oligosaccharide | α-fucosidase |
| Mucolipidosis I | uncharacterized glycolipid, mucopolysaccharide, oligosaccharide, sialy-oligosaccharidura. | α-neuraminidase? |
| Mucolipidosis II (I-cell Disease) | same as Mucolipidosis I | several lysosomal enzymes low, but high in media, serum |
| Mucolipidosis III | same as Mucolipidosis I | same as in Mucolipidosis II |

can liberate the external sialic acids from $GT_{1a}$,$GD_{1a}$, and $GM_3$, but not the sialic acid residue of $GM_1$ or $GM_2$, thus raising the possibility of two different neuraminidases with separate specificities. The issue on the presence of two distinct neuraminidases in human tissues has not been settled due to the lack of homogenous neuraminidase preparations.

A purified (3500-fold) neuraminidase preparation (164) has been demonstrated to have activity towards $GM_2$ as well as other monosialogangliosides, polysialogangliosides, and fetuin. However, this enzyme preparation failed to catabolize the stored $GM_2$ in Tay Sachs brain. This failure has been attributed to the fact that the mutant inactive hexosaminidase A binds to the substrate and thus prevents hydrolysis by neuraminidase (165). The activity of the other neuraminidases is unaffected in Tay-Sachs brain and current opinion seems to favor the existence of two distinct neuraminidases. However, the possibility of the requirement of a missing lipid component or glycoprotein activator similar in nature to that found for the catabolism of $GM_1$ or $GM_2$ ganglioside by β-galactosidase or hexosaminidase A (152-153) cannot be discounted. This missing component could account for the inability of a neuraminidase to hydrolyze the sialyl residues on the gangliosides.

Although neuraminidase will hyrolyze gangliosides in either the dispersed or micellar form, their activities are probably optimal at the plasma membrane level, where they appear to be membrane bound. The membrane-bound preparations of neuraminidases also appear to act preferentially on ganglioside substrates rather than on glycoproteins.

Touster et al. (169) and Schengrund et al. (170) had located the neuraminidases and their ganglioside substrates surprisingly on plasma membranes rather than in lysosomes as expected. Schengrund et al. (170) also found brain neuraminidase to be localized in synaptic membranes along with gangliosides and

their sialyltransferases. Their findings raised the possibility that neurotransmitters could be bound and released by gangliosides following the alternate actions of sialylation and de-sialylation of gangliosides at the synaptic junctions.

Presently, no human disease has been attributed to a specific deficiency of glycolipid neuraminidase activity. Neuraminidase deficiency in fibroblasts and leukocytes has been reported in the mucolipidoses with heavy excretions of sialyloligosaccharides in urine (164,166). However, examinations of autopsy solid tissues revealed normal levels of neuraminidase activity (167) and normal levels of gangliosides and protein-bound sialic acid (168). Neuraminidase, therefore, does not appear to be the key metabolic defect in the mucolipidoses. It must be noted however, that all neuraminidase assays in these studies were conducted with synthetic substrates, neuraminyl-lactose, or fetuin. Ganglioside substrates were never investigated.

2) Role of β-Galactosidase in Glycosphingolipid Catabolism

$GM_1$ ganglioside, its asialo form, and various oligosaccharides and mucopolysaccharides can be hydrolyzed by a β-galactosidase that can be detected with synthetic substrates. A complete deficiency of β-galactosidase activity for both synthetic and natural substrates is found in $GM_1$ gangliosidosis (171). There appear to be two other distinct lysosomal β-galactosidases, one specific for only galactosylceramide and galactosyldiglyceride, as demonstrated in Krabbe's disease (172), and one specific for lactosylceramide (173).

The activity of $GM_1$ and synthetic substrate β-galactosidase has been found to be less than 2% of normal in patients with Type 1 (infantile) $GM_1$ gangliosidosis, whereas in the Type 2 (juvenile) form, the β-galactosidase activity can vary from 5% to 30% of normal (171). These results, along with demonstrations that glycopeptides, oligosaccharides, and keratan sulfate are stored

in this disease, suggest that one common β-galactosidase can hydrolyze them all. From starch gel electrophoretic analysis of the β-galactosidase isoenzymes, all 3 isoenzymes (A,B,C) were absent in Type 1 $GM_1$ gangliosidosis, but only B and C were absent in Type 2. The A isoenzyme, which has the most negative charge, was the major isoenzyme in all tissue studied with more than 70% of the total β-galactosidase activity.

β-Galactosidase levels, as determined with respect to synthetic substrates, are abnormal in other diseases. Öckerman et al.(174) and MacBrinn et al. (175) reported a β-galactosidase deficiency in Hurler's disease, but Kint et al. (176) later demonstrated that the apparent β-galactosidase deficiency was due to the inhibition of synthetic β-galactosidase activity by chondroitin sulfate, which binds to the enzyme and changes its electrophoretic pattern.

In the mucolipidoses, β-galactosidase levels were reported deficient (177-179) in leukocytes and fibroblasts. However, subsequent studies have shown that the apparent β-galactosidase deficiency was not the major inherited, metabolic defect in the mucolipidoses (180-181), since the parents of the patients had normal β-galactosidase activity. Also, a number of glycosylhydrolase activities, along with that of β-galactosidase, were reported elevated in the serum of the patients, pointing to a possible lysosomal uptake defect reported for "I-cell" disease (182). Interestingly, $GM_1$ β-galactosidase levels were reported deficient in "I-cell" disease liver and leukocytes, but the enzyme activities were found normal in the plasma and leukocytes of parents of the patients (180). As a result, it was concluded that the cause of the observed $GM_1$ β-galactosidase deficiency was still uncertain, possibly deriving from inhibition by the uncharacterized storage material.

II. Immunoregulation by General Lipids and Lipoproteins

There has been increasing interest in the possible immunoregulatory role of lipids. Free fatty acids and their esters were the first classes of lipids to be extensively evaluated. These lipids have been shown to inhibit both in vitro and in vivo immune responses, including lymphocyte blastogenesis, lymphocyte cytotoxic responses, primary and secondary humoral immune responses, phagocytosis, and allograft rejection (183).

Prostaglandins, especially those of the E series, inhibited a wide range of immunological responses (184). A role for in vivo modulation of the immune response by endogenous prostaglandins has been demonstrated. In Hodgkin's disease, prostaglandin synthesis by a suppressor cell was shown to suppress the blastogenic response of lymphocytes to phytohemagglutinin mitogen. The inhibition of prostaglandin synthesis by indomethacin abrogated this suppression (185).

Studies in man defining an immunological defect due to a general alteration in lipid metabolism and levels of circulating lipids were first reported by Waddell et al. (186). They found that patients with Frederickson's Type IV and V hyperlipidemia, characterized by elevated levels of triglyceride and very low-density lipoprotein (Type IV), and chylomicrons (Type V) had a plasma suppressor of lymphocyte blastogenesis. Separation of lipids and lipoproteins from the plasma also removed the suppression.

Hyperlipidemia has also been found to be a constant characteristic of an inherited form of histiocytosis, familial erythrophagocytic lymphohistiocytosis (6). The immunological findings have been discussed in the introductory section of this thesis.

The study of the immunoregulatory properties of lipoproteins has been the effort of Edgington's group (187). Their first observations were the identification

of a low-density lipoprotein, associated with viral infection, which was capable of modulating the binding of T-cells to sheep erythrocytes (E-rosettes). They found that lymphocytes bear high affinity receptors for this lipoprotein species, and that occupation of these receptors inhibited E-rosette formation of lymphocytes. Subsequently, Curtiss and Edgington (188) defined a sub-fraction of low-density lipoprotein (called LDL-In for its inhibitory activity) which was capable of modulating the lymphocyte blastogenesis response to mitogens and allogeneic cells. This lipoprotein caused irreversible suppression of the blastogenic response when preincubated with responding lymphocytes for 24 hr prior to the addition of a stimulus of blastogenesis. These investigators demonstrated that the inhibitory effect of LDL-In was a direct, primary action on the unstimulated lymphocyte, mediated by a distinct lymphocyte receptor for LDL-In not identical with the known LDL receptor found on many types of cells, including lymphocytes (189).

In vivo studies with LDL-In have defined an inhibition of murine primary immune responses, as measured by the generation of plaque-forming (antibody-producing) splenic cells following immunization with sheep erythrocytes (190). Investigation of in vitro antibody synthesis induced by pokeweed mitogen stimulation of peripheral blood lymphocytes have also demonstrated the inhibitory effect of LDL-In. Their studies indicated that the target for LDL-In was the lymphocyte, and that both T and B lymphocyte functions were modulated by LDL-In (191). Studies of very low-density lipoproteins (VLDL) by Chisari (192) have demonstrated similar immunosuppressive activity for this lipoprotein.

Bieber et al. (193) have recently isolated a neutral glycosphingolipid derived from LDL in serum of Hodgkin's disease patients which inhibited E-rosette forming cells. Inhibition by LDL from normal serum was not found. The inhibitory activity was distinct from that of C-reactive protein and complement C1q. TLC analysis of the glycolipid identified the inhibitor as either a mono- or

diglycosylceramide. Interestingly, commercial preparations of galactosylceramide selectively inhibited Hodgkin's disease E-rosette forming cells.

Lipids have also been implicated in modulation of the host immune response to tumors. Using macrophage killing of tumor cells as an assay system, Chapman and Hibbs (194) have shown that a low-density lipoprotein present in human, murine, and fetal bovine serum was capable of inhibiting the normal tumoricidal response of activated macrophages. Schultz et al. (195) have shown that prostaglandins can also modulate the tumoricidal function of macrophages. Raz et al. (196) demonstrated inhibition of in vitro macrophage-tumor cell interaction by membrane vesicles which had been shed by the tumor cells, suggesting that the shedding may produce self-protection of the tumor cells from host immune destruction. Analogous inhibition of in vitro B-cell antibody synthesis was reported by Freimuth et al. (197) from the shed, high molecular weight (over 2 X $10^6$ daltons) membrane vesicles containing gangliosides with theta antigen activity from murine lymphoblastoid cell lines.

## III. Immunological Roles for Gangliosides

### A. Receptors and Antigenic Cell Surface Markers

The potential of glycolipids to act as cell surface antigens has been long recognized. Some of the well-studied glycolipid antigens include the Forssman antigen and the human blood group antigens (198).

The ganglioside nature of certain lymphoid cell surface markers has been reported. Esselman and Miller (199) demonstrated the gangliosidic nature of theta (Thy-1) antigen on mouse thymocytes, and recently, differentiated Thy 1.1 and Thy 1.2 antigenic activity from two distinct ganglioside fractions separated by TLC and differentially susceptible to neuraminidase degradation (200). The identity and structure of these gangliosides are unknown at present.

It has been reported that the macrophage receptor for the lymphokine, migration inhibition factor (MIF), is a ganglioside which is derived from the acidic, water-soluble glycolipid fraction of guinea pig macrophage total lipid extracts. Pretreatment of macrophages with macrophage derived glycolipids markedly enhanced their response to MIF, presumably by incorporation into macrophage plasma membrane. Because pretreatment of the glycolipids or the target macrophages with α-fucosidase or neuraminidase destroyed the macrophage responsiveness to MIF (201-202), it was thought that the MIF receptor was a fucoganglioside with TLC mobility between $GM_1$ and $GD_{1a}$ (203).

Other workers (204) have found that bovine brain mixed gangliosides contained a minor unidentified component that inactivated guinea pig and rat lymphocyte MIF and macrophage activating factor (MAF). The identity of the ganglioside was not established, but absorption studies with commercial preparations of purified $GM_1$, $GM_2$, $GM_3$, $GD_{1a}$, and $GT_1$ were negative.

Miura et al. (205) have recently reported the specific inhibition of MIF by a fucosylated glycolipid (RM) isolated from rat peritoneal macrophages which had the structure:

Galβ1→3Gal(2←1αFuc)β1→3GalNAcβ1→3Galβ1→4Glcβ1→1'Cer.

$GM_3$ and a blood group B-active glycolipid with identical structure with glycolipid RM except for a GlcNAc substitution were not inhibitory.

B. Immunoregulation by Gangliosides

Early studies (206) showed that the addition of mixed brain gangliosides to bacterial antigens prior to injection into rabbits resulted in a reduction in magnitude and duration of the primary response (IgM production) and complete inhibition of the secondary response (IgG production).

Miller and Esselman (207) first documented modulation of the in vitro immune response by gangliosides. The addition of $GM_1$ ganglioside to splenic cultures incubated with sheep erythrocytes depressed the anti-sheep hemolytic plaque response. They suggested that this effect was due to a direct effect on B lymphocyte terminal differentiation into antibody-producing plasma cells.

Studies of helper and suppressor T-cells in the mouse (208) demonstrated that the conditioned media of the mouse antigen-induced T-suppressor cells, which inhibited the primary in vitro immune response to a heterologous antigen, contained a glycolipid released by the T-cells. Treatment of the T-suppressor cell media with either anti-$GM_1$ or anti-Thy 1.2 would remove the inhibitory activity. Isolation of the inhibitory glycolipid by TLC revealed a glycolipid with a mobility similar to brain $GM_1$. Other glycolipids isolated from the media were not inhibitory. The inhibitory activity of this ganglioside on the B-cell response was found to be antigen non-specific, suggesting that the ganglioside might have important general immunoregulatory properties.

Lengle et al. (209) have demonstrated that lymphocyte blastogenic response to conconavalin A was inhibited by bovine brain gangliosides. These gangliosides caused reversible inhibition of RNA and DNA synthesis. Some structure-function data was obtained in that purified preparations of $GT_1$ gave the highest inhibition, followed by, in decreasing order of effectiveness, $GM_2$, $GD_{1a}$, and finally $GM_1$, which had very low inhibitory activity.

Ryan and Shinitsky (210) have also shown an inhibitory effect of bovine brain gangliosides on lymphocyte blastogenesis, although they claimed that the inhibition was exclusively towards B-cell response. The B-cell specificity was demonstrated by the fact that only E. coli lipopolysaccharide mitogen stimulation was inhibited, whereas phytohemagglutinin stimulation (T-cell specific) was unaffected at identical ganglioside concentrations.

Stewart et al. (211) recently reported that human brain gangliosides also have inhibitory activity toward lymphocyte blastogenesis as measured by con A-stimulation and the mixed leukocyte culture assay (MLC).

In summary, gangliosides have been found to have a variety of immunological roles:

1. They are antigenic in that specific antibodies can be directed against them.
2. They function as cell surface markers on murine thymocytes (theta antigen).
3. They are macrophage cell surface receptors for lymphokines, such as macrophage activating factor and migration inhibiton factor.
4. They are potent inhibitors of T and B lymphocyte blastogenesis and antibody synthesis, and as such, may be the biochemical mediator by which suppressor T-cells and tumor cells suppress immune responses.

# MATERIALS AND METHODS

## Materials

### Chemicals

Pharmacia (Piscataway, N. J.)
DEAE-Sephadex A-25
Dextran T 500

Pfanstiehl (Waukegan, Ill.)
N-acetylgalactosamine
N-acetylglucosamine
N-acetylmannosamine
galactose
glucose
fucose
mannose

Calbiochem (La Jolla, Ca.)
sodium taurocholate

Analtech (Newark, Delaware)
Silica Gel G TLC plates
Silica Gel H TLC plates

E. Merck (Cincinnati, Ohio)
Silica Gel 60 TLC plates
Silica Gel 60 High Performance TLC plates

Sigma (St. Louis, Mo.)
p-nitrophenyl-β-N-acetylgalactosaminide
p-nitrophenyl-β-N-acetylglucosaminide
p-nitrophenyl-β-galactoside
tyrosine
bovine serum albumin
sodium borohydride
galactose
glucose
stachyose
phenyl-α-N-acetylglucosaminide
N-acetylneuraminic acid
hexamethyldisilazane
trimethylchlorosilane
mannitol

dichlorodimethylsilane
glycine
tris-(hydroxymethyl)-aminomethane

Boehringer-Mannheim (Indianapolis, Ind.)
2(3'-methoxy)phenyl-α-N-acetylneuraminide

Eastman Kodak (Rochester, N.Y.)
m-methoxyphenol
p-nitrophenol

Koch-Light (Colnbrook, Buckinghamshire, England)
4-methylumbelliferyl-α-galactoside
4-methylumbelliferone
4-methylumbelliferyl-β-glucoside
4-methylumbelliferyl-β-galactoside
4-methylumbelliferyl-β-N-acetylglucosaminide
4-methylumbelliferyl-β-N-acetylgalactosamide
4-methylumbelliferyl-α-fucoside
4-methylumbelliferyl-α-mannoside
4-methylumbelliferyl-sulfate

A. H. Thomas (Philadelphia, Pa.)
dialysis tubing
Teflon screw-cap liners

Iatron Labs (Tokyo, Japan)
Iatrobeads 6RS 8060

Fisher (Fairlawn, N.J.)
orcinol
resorcinol
iodine
Redi-Plates (Silica Gel G TLC plates)
sulfuric acid
hydrochloric acid
acetic acid

Mallinckrodt (St. Louis, Mo.)
silver carbonate
sodium thiosulfate
citric acid
sodium citrate
sodium phosphate
sodium acetate

Matheson Gas (Joliet, Ill.)
hydrogen chloride

Harleco (Philadelphia, Pa.)
Folin-Ciocalteau reagent

Clarkson (Williamsport, Pa.)
Unisil (100-200 mesh)

Airco Industrial Gases (Montvale, N.J.)
hydrogen, 90.5% pure
compressed air
nitrogen, 99% pure
helium, 99% pure

Supelco (Bellefonte, Pa.)
$GM_3$ ganglioside
bovine brain mixed gangliosides
cholesterol
cholesterol oleate
oleic acid
phosphatidylcholine
phosphatidylethanolamine
phosphatidylserine
1-monopalmitin
1,2-dipalmitin
triolein
fatty acid methyl esters NIH standard mix
3% SE-30 on Supelcoport (80-100 mesh)
arachidic acid (C20:0)

Solvents

All solvents listed here are of reagent grade and re-distilled:

Mallinckrodt (St. Louis, Mo.)
methanol
chloroform
pyridine (stored over KOH pellets)
toluene
acetic anhydride
1-propanol
2-propanol
1-butanol
acetone
hexanes
petroleum ether(b.p. $30^{\circ}$-$75^{\circ}$)
diethyl ether

Commercial Solvents (Terre Haute, Ind.)
absolute ethanol

Tissues

Fibroblast cell lines, leukocytes, and plasma samples from patients were donated by Dr. Stephan Ladisch, UCLA Med. Ctr. Other normal and outdated plasma samples were donated by the American National Red Cross, Lansing, Mi. Human livers, spleens, and brains were donated by Dr. Stephan Ladisch and Dr. Allan Yates, Ohio State Univ.

## Methods

### I. Lysosomal Enzyme Studies

#### A. Preparation of Human Tissues and Cells

1) Frozen human liver or spleen samples were weighed and homogenized as described by Suzuki (212) in distilled water with a Polytron homogenizer for a pulse interval of 1 min repeated three times. The concentration of tissue in the final homogenate was 10% (w/v). The tissue cells were further disrupted by freeze-thawing three times and the mixture was centrifuged at 1,000 X g for 10 min to remove the insoluble cellular debris. Aliquots of the supernatant fraction were used for subsequent assays.

2) One to two ml of packed skin fibroblasts were subjected to hypotonic lysis by suspension in an equal volume of double-distilled water as described by Suzuki (212). The fibroblasts were further disrupted by freeze-thawing three times, centrifuged at 1100 X g for 5 min to precipitate the cellular debris, and aliquots taken from the supernatant fraction for enzyme assays.

3) Leukocytes were prepared as described by Snyder and Brady (213). Two ml of a 5% Dextran in isotonic saline (0.9% NaCl) solution was added to 10 ml of heparinized blood and the resulting mixture was allowed to settle at room temperature for 45 min. The leukocyte layer was carefully removed by Pasteur pipet and centrifuged for 10 min at 600 X g. The resulting pellet was resuspended and washed twice with 2 ml isotonic saline solution and centrifuged to obtain the pellet. The pellet was suspended in 3 ml of distilled water for 90 sec to lyse any remaining contaminant RBCs after which 1 ml of 3.6% NaCl solution was added to return the suspension to isotonicity. The leukocyte suspension was centrifuged at

600 X g for 10 min to obtain a RBC-free leukocyte pellet. This leukocyte preparation could either be stored frozen at -20° with 1 ml isotonic saline or lysed immediately by suspension in 1 ml distilled water, freeze-thawing three times, amd centrifugation at 1120 X g. The supernatant fraction was used for subsequent enzyme assays.

B. Lysosomal Glycosylhydrolase Assays

1) Assays with p-nitrophenylglycoside substrates

A typical assay mixture for the determination of lysosomal glycosylhydrolase activity with p-nitrophenylglycoside substrates contained: 50 µl of 0.6 M sodium citrate or sodium citrate-phosphate buffer, 200 µl of substrate(1-10 mM concentration) in a 13 mm X 100 mm test tube, 0-50 µl of enzyme, and distilled water to bring the total volume of the assay to 300 µl. The assay mixture was incubated at 37° for a time interval that ranged from 5 min to 3 hr. The reaction was stopped by the addition of 3 ml of 0.6 M potassium borate buffer (made by adjusting 0.6 M boric acid to pH 10.4 with a 5 M KOH solution). Controls were incubated at 37° without enzyme for appropriate time intervals, followed by the simultaneous addition of enzyme and stopping solution. Absorbance was read at 420 nm on a Gilford Model 250 Spectrophotometer. Due to large amounts of protein and insoluble matter in some liver homogenates, 1 ml of 1-pentanol/chloroform (1:5) was added to the final solution to precipitate proteinaceous matter at the interface. A standard curve of p-nitrophenol was linear from 0-300 nmoles. Duplicate assays were done at two different time points and at two different enzyme concentrations to insure linearity of the assay with respect to time and protein concentration.

2) Assays with 4-methylumbelliferylglycoside substrates

A typical assay mixture for the determination of lysosomal glycosylhydrolase activity with 4-methylumbelliferylglycoside substrates contained: 300 µl of a 1-10 mM substrate solution in 0.1 M sodium citrate, citrate-phosphate, or acetate buffer, 0-50 µl of enzyme in a 10 mm X 75 mm disposable test tube, and distilled water to bring the total volume of the assay mixture to 350 µl. After an incubation period at 37° for 0-6 hr, the reaction was stopped with 2 ml of a 0.2 M glycine-NaOH pH 10.8 buffer. Controls were incubated without enzyme and stopped with the simultaneous addition of enzyme and stopping solution. The liberated 4-methylumbelliferone was measured in an Aminco fluorometer-colorimeter Model J47439 with an excitation wavelength of 365 nm and an emission wavelength of 448 nm using the assay test tube directly as a cuvette. A standard curve of 4-methylumbelliferone gave a linear fluorescence response from 0-30 nmoles. Duplicate assays were done at two different time points and at two different enzyme concentrations to insure linearity of the assay with respect to time and protein concentration.

3) Measurement of neuraminidase activity

A typical assay mixture for the determination of neuraminidase activity (214) contained: 30 µl of a 10 mM 2(3'-methoxyphenyl)-α-N-acetylneuraminide solution in 0.1 M sodium acetate pH 5.0 buffer, 10-50 µl of enzyme, and additional acetate buffer mixed in a 10 mm X 75 mm disposable test tube to a final volume of 200 µl. The reaction mixture was incubated at 37° for intervals ranging from 10 min to 16 hr and stopped by the addition of 1.5 ml of 10% sodium carbonate. Upon addition of 0.2 ml of 2 N Folin-Ciocalteau phenol reagent, the tube contents were vortexed, allowed to stand for 20 min, and the absorbance read in a Gilford Model 250 spectrophotometer at a wavelength of 750 nm.

In the event of excessive interference from protein in the phenol reagent reaction, a modification of the technique was performed as follows (215): 0.2 ml of toluene was added instead of sodium carbonate as the stopping solution, the reaction mixture was vortexed and centrifuged in a Sorvall desk top centrifuge at 1000 X g for 5 min, and the toluene upper layer was removed by Pasteur pipet. This toluene extraction was repeated twice more at which point the toluene extract could either be stored at -20$^{o}$ or treated with 0.5 ml 10% sodium carbonate in order to extract the released methoxyphenol into the lower alkaline, aqueous phase. After carefully removing the toluene upper phase by Pasteur pipet, 0.25 ml of Folin-Ciocalteau phenol reagent (diluted 1:2 with water) was added, and the entire solution was vortexed and allowed to stand for 20 min. Using either tyrosine or m-methoxyphenol as a standard, linearity of absorbance at 750 nm was obtained from 0-300 nmoles. Duplicate assays were done at two different time points and at two different enzyme concentrations to insure linearity of the assay with respect to time and protein concentration.

4) Protein assays

All protein assays were done precisely as described by Lowry et al.(216). Using bovine serum albumin as standard, the assay gave a linear absorbance response at 750 nm from 0-80 μg of protein.

5) Cellulose acetate electrophoresis studies on liver β-galactosidases

Aliquots (30 μl) from the liver homogenates were applied by a special Gelman sample applicator onto 1 X 6 inch polyacetate strips (Gelman Sephraphore III) previously wetted in Gelman High Resolution tris-barbiturate pH 8.8 buffer and mounted on a Gelman electrophoresis apparatus model 51170 with dual chambers

containing the tris-barbiturate buffer. The strips were electrophoresed at 4° with a 250-volt electrical field across the strips (2.5 milliamperes per strip) for 50 min. To stain for enzymatic activity, the strips were carefully removed by tweezers from the apparatus and placed between a pair of 18.5 cm Whatman no. 1 filter papers wetted with 1 mM 4-methylumbelliferyl-β-galactoside in 0.1 M NaCl, Na citrate pH 4.5 buffer. The "sandwich" was placed in a large, covered Petri dish and incubated for 3 hr at 37°. Afterwards, the cellulose strips were transferred into another filter paper "sandwich" wetted with 0.2 M glycine-NaOH pH 10.8 buffer for 5 min. The strips were viewed under a portable long wavelength U.V. lamp for location of the β-galactosidases.

## II. Lipid Isolation and Characterization

### A. Lipid Extractions and Chromatography

#### 1) Folch Extraction of Human Liver

Lipid extractions as described by Folch et al.(87) were done on human livers as follows: A weighed, frozen sample of liver (10 g) was minced and homogenized with 20 volumes of chloroform:methanol (2:1) in sand by mortar and pestle. The homogenate was filtered through Whatman no.1 filter paper, the insoluble residue re-extracted again with the same volume of chloroform-methanol (2:1), refiltered, and the filtrates combined. A 0.05 M NaCl solution amounting to 20% of the pooled volume was added to the lipid extract in a large separatory funnel. After separation of the lipid extract into 2 phases upon standing overnight at 4°, the upper aqueous phase (40% of the total volume) was washed twice with theoretical lower phase (chloroform:methanol:water (86:14:1)), and the lower chloroform phase (60% of total volume) was washed twice with theoretical upper phase (chloroform:methanol:0.58% NaCl (3:48:47)) as described by Folch et al.(87).

The pooled upper phase and lower phase extracts were then evaporated in vacuo on a rotary evaporating apparatus .

2) Unisil Silicic Acid Column Chromatography

Unisil silicic acid column chromatography was used to fractionate the Folch lower phase lipids into 3 major classes. Unisil (20 g/g lipid) was activated at 80° overnight, slurried with chloroform, and quickly poured into a column with 3 bed volume washes of chloroform. The lower phase lipids were chromatographed and batch-eluted with 5 bed volumes of chloroform (neutral lipid fraction), 10 bed volumes of acetone:methanol (9:1) (neutral glycolipid fraction), and 8 bed volumes of methanol (phospholipid and other acidic lipids fraction), as described by Esselman et al. (217) and by Vance and Sweeley (111). Each of the 3 fractions was evaporated in vacuo and redissolved in 5 ml chloroform:methanol (2:1).

B. Quantitation and Characterization of Neutral Liver Lipids

1) Gravimetric quantitation of lipids

Total lipid extracts from the Folch lower phase and the 3 major lipid fractions derived from silicic acid column chromatography of the total liver lipids were weighed on a Mettler analytical balance. Solvents and water were thoroughly removed from the samples to be weighed by rotary evaporation in a 35° water bath, followed by further evaporation under a stream of nitrogen with 3 additions of absolute ethanol to remove all traces of water. The samples were further dried overnight in a desiccator in vacuo.

2) Qualitative Analysis of Liver Lipids

The 3 major lipid fractions were each taken up in 5 ml of chloroform:methanol (2:1) and aliquots spotted on TLC plates (activated for 1 hr at 110°) for further analysis. Silica Gel G, 250 micron thick plates were used for the analysis of both the neutral lipid and glycolipid fractions. Silica Gel H (no $CaSO_4$ binder), 250 micron thick plates were used for the analysis of the phospholipid fraction. The neutral lipids were developed in petroleum ether:diethyl ether:acetic acid (90:10:1), and the glycolipids and phospholipids were developed in chloroform:methanol:water (100:42:6). All lipids were visualized by iodine vapor for preparative TLC. For qualitative analysis (218), neutral lipids were visualized by $H_2SO_4$ charring and by $H_2SO_4$:acetic acid (1:1) spray reagent specific for cholesterol residues, glycolipids were visualized by orcinol-$H_2SO_4$ spray reagent, and phospholipids by molybdenum blue spray reagent.

3) Direct Probe Low Resolution Mass Spectrometry of Triglycerides

The triglycerides from the neutral lipid fraction (400 µl aliquot from 5 ml fraction) of both normal and patient liver were isolated by preparative TLC on silica gel G, 500 micron thick plates, developed in petroleum ether:diethyl ether:acetic acid (90:10:1) and visualized by iodine vapor. The triglyceride spots were scraped off by razor blade and poured into a 1.5 cm i.d. X 15 cm column plugged with a glass wool bed support, and the silica gel was washed with 5 column volumes of chloroform:methanol (2:1). The eluants were evaporated, redissolved in 1-2 ml chloroform:methanol (2:1), and aliquots taken for direct probe mass spectrometry and methanolysis. Mass spectra were obtained with a PDP-8/e computer-assisted Varian Model CH5 mass spectrometer using a direct probe, electron impact mode. Results were recorded on an U.V. oscillograph where the

peaks were mass assigned and integrated manually. Mass spectrometer conditions were as follows: probe temperature program 240°-300°, ionization energy 70 eV, accelerating voltage 3 kV, and ion source temperature 260°.

4) Gas Chromatographic Analysis of Fatty Acid Methyl Esters from Liver Triglycerides

Aliquots from the isolated triglycerides were subjected to methanolysis in 3 ml 0.75N HCl in methanol (methanolic HCl prepared as described by Chambers and Clamp (112)) at 80° for 24 hr. The hydrolysates were partitioned with 2 ml hexane to remove the fatty acid methyl esters, the hexane upper phase removed, and the hexane wash repeated twice more. The hexane washes were pooled, washed twice with 2 ml water, dried under a stream of nitrogen gas, and the residue redissolved in 100-200 µl hexane. Methyl arachidate (C20:0) was added as an internal standard for quantitation. Gas-liquid chromatography was performed on a F and M Hewlett-Packard Model 402 with dual flame ionization detectors on a 6 ft X 2 mm glass column containing 3% SE-30 on Supelcoport 80/100 mesh, operated at 170° isothermal. To calculate triglyceride molar content, the total fatty acid molar amount was divided by 3.

5) Quantitation of Cholesterol Lipids in Liver

Determination of cholesterol in liver was performed colorimetrically as described by Abel et al.(219). Total cholesterol was quantitated by assaying 200 µl aliquots from the whole neutral lipid fraction. Free cholesterol was quantitated following the isolation of the cholesterol by preparative TLC developed in petroleum ether:diethyl ether:acetic acid (90:10:1). Cholesterol ester amount was calculated as the difference between the total cholesterol and free cholesterol amount per equivalent weight of liver.

C. Isolation and Characterization of Gangliosides

1) Folch Upper Phase Lipids- TLC and Quantitation

The Folch upper phase lipid extract was lyophilized, redissolved in water, and dialyzed for 24 hr against 10 volumes of distilled water at $4^{o}$ with 4 changes of water, lyophilized, and redissolved in 1 ml chloroform:methanol (2:1). Insoluble material was removed by filtration through glass wool-stoppered Pasteur pipet columns. TLC analysis of the gangliosides was done on silica gel G 250 micron thick plates developed twice in chloroform:methanol:7%$NH_4OH$ (55:40:10) in paper-lined tanks saturated with developing solvent. The plates were visualized by both iodine vapor and by resorcinol spray reagent (14). Aliquots were taken from the 1 ml lipid solution for sialic acid colorimetric quantitation by resorcinol as described by Svennerholm (220) and modified by Spiro (221).

2) Ganglioside Isolation by DEAE-Sephadex Column Chromatography

The extraction of gangliosides from large amounts of human liver tissue was done by a slight modification of the procedure described by Ledeen et al.(50) and Ueno et al.(222).

DEAE-Sephadex A-25 was prepared for chromatography as follows: 2.2 g beads/g tissue were converted from the chloride to the acetate form by mixing into a slurry with chloroform:methanol:0.8 M sodium acetate (30:60:8). The slurry was decanted and the washing repeated 4 more times. The slurry was left to settle and stand overnight, after which the solution was again decanted and replaced by chloroform:methanol:water (30:60:8). Decantation and resuspension of the DEAE-Sephadex was repeated 3 times. The slurry was poured into a 3 cm i.d. X 35 cm glass column and washed with 4 column volumes of chloroform:methanol:water (30:60:8).

A 100-500 g sample of liver (wet weight) was homogenized with a Polytron homogenizer in 20 volumes of chloroform-methanol (1:1). After stirring overnight at 4°, the homogenate was filtered to remove the insoluble residue. The insoluble residue was re-extracted with 10 volumes of chloroform:methanol (1:1) and refiltered. The filtrates of both washes were combined and evaporated in vacuo. The lipid extract was redissolved in chloroform:methanol:water (30:60:8) and applied to a 3 cm i.d. X 35 cm column of pre-equilibrated DEAE-Sephadex A-25 (acetate form). Ten column volumes of the solvent followed by 2 column volumes of methanol were passed through the column to elute all non-acidic lipids. The acidic lipids (sulfatides, phospholipids, and gangliosides) were eluted with 9 column volumes of 0.2 M sodium acetate in methanol and then dried in vacuo. In order to remove phospholipids, alkaline methanolysis was performed by redissolving the extracted lipids in 0.5 N NaOH in methanol and incubating the mixture for 4 hr at 37°. The solution was neutralized with 5 N acetic acid in methanol and dried in vacuo. The residue was redissolved in water and dialyzed against distilled water at 4° with 4 changes over a period of 24 hr. The dialysate was lyophilized.

3) Iatrobead Column Chromatography

The lyophilized lipids were fractionated by Iatrobead chromatography as described by Momoi et al. (99) utilizing a linear gradient of chloroform:methanol:water from 60:40:2 to 30:70:4 on a 2 cm i.d. X 24 cm glass column of Iatrobead silicic acid pre-equilibrated with the starting solvent. Small aliquots of each 10 ml fraction collected were qualitatively analyzed by development on silica gel 60 TLC plates in a chloroform:methanol: 0.2% $CaCl_2$ (60:40:9) solvent and appropriate fractions pooled. Some ganglioside fractions were further purified either by preparative TLC in the same developing solvent or by re-chromatography on the Iatrobead column. The final ganglioside fractions were

quantitated colorimetrically by the resorcinol assay (220-221) and shown to be free of contaminating substances by TLC.

4) Extraction of Plasma Gangliosides

The extraction of lipids from plasma (1-10 ml) was performed in a similar manner as that described above for liver ganglioside extraction with 20 volumes of chloroform:methanol (1:1). After the chloroform:methanol filtrates were pooled and dried in vacuo, the dried lipids were redissolved in the original volume (1-10 ml) with chloroform:methanol (1:1) solvent and applied on to silica gel 60 TLC plates. The plates were developed in three different solvent systems as described by Harth et al. (104) in order to separate the gangliosides from all other lipids. The gangliosides were identified by comparison with standard gangliosides and visualized by either iodine vapor or resorcinol spray reagent. They were scraped off the plate and eluted from the silica gel with successive washes of chloroform:methanol (1:1), chloroform:methanol:water (50:50:15), and methanol. The recovered gangliosides were quantitated by the resorcinol method as described earlier (220-221).

5) Carbohydrate and Fatty Acid Composition of Liver Immunosuppressive Substance

Aliquots of the ganglioside fractions that were immunosuppressive were further purified by preparative TLC on 200 micron thick silica gel 60 plates in the triple solvent system described by Harth et al.(104). The bands were visualized by iodine, scraped off, and eluted as described for the plasma gangliosides. The samples were dried in 1.3 cm X 10 cm Teflon-lined screw-capped test tubes and methanolyzed as described by Esselman et al. (217). Three ml of anhydrous 1 N HCl-methanol was added to the sample, the samples capped tightly, and the tubes

allowed to stand at 80° for 18 hr. The samples were cooled to room temperature and 2 ml hexane was added to extract the fatty acid methyl esters. The upper layer of hexane was removed by Pasteur pipet and the 2 ml hexane wash repeated twice more. The hexane washes were pooled and dried under a stream of nitrogen in separate 1 dram vials with Teflon-lined screw caps, whereas the methanol layer was neutralized by 10-50 mg $Ag_2CO_3$ powder added gradually into the methanol solution with frequent vortexing. When the methanol solution was pH 6 by litmus paper test, 300 µl of acetic anhydride was added and the mixture allowed to stand at room temperature for 8-12 hr to re-N-acetylate all amino sugars. The solution was centrifuged for 4 min at 400 X g and the methanol carefully transferred by Pasteur pipet into a 1 dram vial with Teflon-lined screw cap and dried under a stream of nitrogen. The AgCl precipitate was washed twice more with 1 ml methanol, centrifuged, and the washes transferred into the 1 dram vial. The methanol washes were finally dried under nitrogen and were ready for trimethylsilylation and GLC analysis. For sugar derivatization (223), 50-100 µl of TMS reagent (pyridine:hexamethyldisilazane:trimethylchlorosilane 10:4:2) was added to the hydrolyzed glycolipid samples, and the mixture allowed to stand at room temperature for 30 min before injection of 2-3 µl aliquots into a Hewlett-Packard Model 5840A gas liquid chromatograph for carbohydrate analysis.

The pooled hexane washes were dried under nitrogen in their vials, redissolved in 50-100 µl hexane, and 2-3 µl aliquots analyzed by GLC.

Conditions for the GLC analyses were as follows: nitrogen carrier gas flow-20 ml/min; temperature program- 140° to 240° at 3°/min (carbohydrate analysis), 140° to 240° at 5°/min (fatty acid analysis); glass column- 6 ft x 2 mm i.d. with 3% SE-30 on 80-100 mesh Supelcoport.

Identifications of the trimethylsilylated carbohydrates and fatty acid methyl esters detected by gas chromatography were confirmed by combined GLC-

MS on a Hewlett Packard Model 5895 using the same GLC conditions as described above with ionization energy- 70 eV, ion source temperature- 200°, and electron multiplier- 2200 volts.

6) Total Sialic Acid Determination in Liver by Gas-Liquid Chromatography

To determine the total sialic acid content in liver, 1-2 ml aliquots of 10% liver homogenates were lyophilized in Teflon-lined screw-capped test tubes. The dried samples were hydrolyzed in 4 ml 0.05 N HCl in methanol for 2 hr at 80° as described by Ledeen and Yu (224). After 3 washes with 6 ml hexane to remove fatty acid methyl esters, the methanol solution was centrifuged at 1000 X g for 10 min to pellet the insoluble matter, and the methanol was carefully removed by Pasteur pipet into a 1 dram vial with Teflon-lined screw cap. The insoluble matter was washed twice with 1 ml methanol, centrifuged, and the washes combined and dried in the vial. Then 50-100 nmoles of phenyl-α-N-acetylglucosaminide was added to the vial as an internal standard, and the entire content dried under nitrogen. The samples were trimethysilylated with 100-200 µl of the TMS reagent described earlier by Laine et al. (223). Finally, 2-3 µl aliquots of the derivatized sample were analyzed with a Hewlett-Packard Model 5840A gas chromatograph containing a 6 ft X 2 mm glass column of 3% SE-30 on 80-100 mesh Supelcoport operated at 215° isothermal.

D. Oligosaccharide Analysis

Oligosaccharides from liver and spleen were analyzed by a modification of the method described by Humbel and Collart (225). Five grams of tissue were homogenized in 10 ml distilled water for 1 min on a Polytron homogenizer to yield a 33% homogenate. The homogenates were centrifuged at 50,000 X g for 45 min at 4° to give a clear supernatant which was spotted in 10-20 µl aliquots onto silica

gel G 250 micron thick TLC plates (Fisher Redi-Plates). The plates were developed in butanol:acetic acid:water (3:3:2) and visualized by orcinol spray (14) for neutral sugar content and by resorcinol spray (14) for sialic acid content.

E. Ganglioside Immunosuppression of Lymphocyte Blastogenesis

Immunosuppressive activities of all gangliosides, presented in the form of liposomes, were tested by Dr. Stephan Ladisch using the procedure described by Muchmore and Blaese (226) by assessing the effect of the glycolipids on human lymphocyte in vitro proliferative responses.

The glycolipid-containing liposomes were prepared in the following manner: glycolipids to be tested were dissolved in a small amount of chloroform:methanol (1:1) in 4 ml glass vials. Chromatographically pure cholesterol and phosphatidylcholine in the same solvent were combined with the glycolipids in the respective ratio 5:5:1 by weight at room temperature. The solvent was evaporated under a stream of nitrogen and all final traces of solvent removed by further evaporation in vacuo. The lipid mixture thus prepared was resuspended with sterile 0.9% NaCl solution by sonication at $4^{o}$ for 5-20 min duration with pulse intervals of 1 min in a bath sonicator. The liposome-incorporated glycolipids were tested in the lymphocyte blastogenesis assay in final calculated ganglioside concentrations of 2-20 µg/ml, and added in 0.1 ml volumes/1.0 ml peripheral blood lymphocyte suspension.

The lymphocyte blastogenesis assay was carried out in the following manner: normal human peripheral blood was anticoagulated with preservative-free heparin, and subjected to ficoll-hypaque density gradient sedimentation by the method of Böyum (227). The peripheral blood lymphocytes (PBL) collected at the interface were washed three times and resuspended in medium (RPMI 1640 containing penicillin, streptomycin, and glutamine) containing 10% autologous

plasma before the addition of the test lipids for the assay. Controls for each experiment with test lipids were PBLs incubated in the carrier liposomes minus the test lipid, and PBLs in complete medium alone. The final concentration of the cell preparation was 1.35 X $10^6$ /ml, with 0.15 ml being plated per well. The appropriate antigen or tissue culture medium alone (unstimulated control wells) was added (0.05 ml) after an 18 hr preincubation of the cells with the liposomes being tested for immunoregulatory activity. All stimulants were used at concentrations giving optimal blastogenic responses. Stimulants used in the experiments were phytohemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM), Candida albicans antigen, streptokinase-streptodornase (SKSD), tetanus toxoid, and diphtheria toxoid. All incubations were performed at 37° in a humidified 5% $CO_2$-95% air atmosphere. Cultures were incubated for the previously determined optimum duration of culture: 3 days for mitogen responses and 6 days for antigen responses. At the end of the culture period, 0.5μCi tritiated thymidine was added, the incubation continued for 4.5 hr, and the cells harvested onto glass fiber filter paper using a Brandel automated harvester. Tritiated thymidine incorporation was measured as counts per minute on a Beckman β-counter. All tests were performed in triplicate and the mean net stimulation (compared to control wells containing medium instead of a stimulant) determined. Control wells (without a stimulant) were also counted to determine direct stimulatory or inhibitory activity of the added gangliosides. Other control wells were stained with trypan blue at the end of the culture period, and the viable cell count determined to eliminate non-specific cytotoxicity as a cause of apparent inhibition of the blastogenic response. A 50% inhibition of a blastogenic response in a test was considered indicative of the presence of suppressive activity. Furthermore, the presence of a dose-related inhibition by an added lipid was considered indicative of a suppressive effect.

# RESULTS

## I. Lysosomal Enzyme Studies

### A. Liver Lysosomal Glycosylhydrolases

Studies with liver lysosomal enzymes indicated a lower β-galactosidase activity in the patient's liver when compared to that of a normal liver, although his other enzyme levels were within the range of the normal liver levels of activity (Table 6). His α-mannosidase activity was also somewhat lower than normal , although not to the extent of the decreased levels of β-galactosidase. β-Galactosidase activity of the patient was 34% of normal levels on a per mg protein basis, but even lower (25% of normal) on the basis of an equivalent amount of liver. All other enzyme specific activities studied were also reduced when based on equivalent amounts of liver; however, their activities were not as reduced as that of β-galactosidase (Table 7). Classical lysosomal storage diseases generally present a deficiency of a given lysosomal enzyme with activities around 0.1-10% of normal levels. Such an obvious deficiency of any particular lysosomal glycosylhydrolase was not apparent here.

### B. Leukocyte Lysosomal Glycosylhydrolases

Table 8 lists the activities of selected leukocyte lysosomal enzymes from relatives of the FEL patient under study. The activities of these lysosomal enzymes of his relatives were all within the normal range and no clear deficiency of any enzyme activity could be demonstrated. Table 9 lists the results of a separate leukocyte preparation for β-galactosidase analysis in the patient's

Table 6. Liver Lysosomal Glycosylhydrolases- I

| Enzymes | Specific Activities* | | |
|---|---|---|---|
| | FEL | (% of normal) | Normal (n=1) |
| β-Galactosidase | 890 | (34) | 2640 |
| β-N-Acetylglucosaminidase | 3293 | (116) | 2835 |
| α-Galactosidase | 95.5 | (115) | 83.0 |
| α-N-Acetylgalactosaminidase | 14.7 | (68) | 21.7 |
| α-Neuraminidase | 2.33 | (94) | 2.48 |
| α-Fucosidase | 393 | (72) | 546 |
| β-Glucuronidase | 944 | (74) | 1280 |
| α-Mannosidase | 42.8 | (47) | 91.3 |

*nmoles substrate hydrolyzed/hr/mg protein. The standard error for all values was less than 10%.

Substrates used:
1 mM 4-MU-β-Gal in 0.1 M NaCl, Na citrate pH 4.5
1 mM 4-MU-α-Fuc in 0.1 M Na citrate-0.2 M Na phosphate pH 6.0
1 mM 4-MU-β-GlcNAc in 0.1 M Na citrate-0.2 M Na phosphate pH 4.4
10 mM pNP-α-GalNAc in 0.1 M Na citrate pH 5
1 mM 4-MU-α-Gal in 0.1 M Na citrate-0.2 M Na phosphate pH 4.4
10 mM 4-MU-β-GlcUA in 0.1 M Na acetate pH 4.8
5 mM 4-MU-α-Man in 0.1 M Na citrate-0.2 M Na phosphate pH 4.4

Table 7. Liver Lysosomal Glycosylhydrolases- II

| Enzymes | Specific Activities* | | |
|---|---|---|---|
| | FEL | (% of normal) | Normal (n=1) |
| β-Galactosidase | 35,600 | (25) | 145,200 |
| β-N-Acetylglucosaminidase | 247,000 | (77) | 319,000 |
| α-Galactosidase | 7,160 | (77) | 9,340 |
| α-N-Acetylgalactosaminidase | 1,102 | (45) | 2,440 |
| α-Neuraminidase | 261 | (68) | 385 |
| α-Fucosidase | 22,000 | (57) | 38,666 |
| β-Glucuronidase | 34,000 | (40) | 84,500 |
| α-Mannosidase | 2,400 | (37) | 6,450 |

*nmoles substrate hydrolyzed/hr/g equivalent weight liver. The standard error of all values was less than 10%.

Substrates used: c.f. Table 6.

Table 8. Leukocyte Lysosomal Glycosylhydrolases of the FEL Patient's Relatives

| Subject | Specific Activities* | | | | |
|---|---|---|---|---|---|
| | β-Gal'ase | α-Gal'ase | β-GlcNAc'ase | α-Man'ase | α-Fuc'ase |
| sister | 630 | 167 | 1748 | 212 | 84.6 |
| mother | 1033 | 226 | 2324 | 501 | 115.8 |
| father | 1296 | 263 | 4271 | 824 | 96.8 |
| maternal uncle | 640 | 191 | 2898 | 291 | 67.8 |
| uncle | 942 | 272 | 2725 | 372 | 41.4 |
| uncle | 962 | 241 | 3248 | 386 | 60.2 |
| normal adult | 692 | 188 | 3669 | 386 | 43.0 |
| normal adult | 1271 | 329 | 4057 | 571 | 114.0 |

*nmoles substrate hydrolyzed/hr/mg protein. The standard error for all values was less than 10%.
Substrates used:
1 mM 4-MU-β-GlcNAc in 0.1 M Na citrate, 0.2 M Na phosphate pH 4.4
1 mM 4-MU-β-Gal in 0.1 M NaCl, Na citrate pH 5.0
1 mM 4-MU-α-Gal in 0.1 M Na citrate, 0.2 M Na phosphate pH 4.4
10 mM pNP-α-Man in 0.1 M Na citrate pH 4.5
10 mM pNP-α-Fuc in 0.1 M Na citrate pH 4.5

Table 9. Leukocyte Lysosomal β-Galactosidase of the FEL Patient's Relatives

| Subject | Specific Activities* |
|---|---|
| sister | 422 |
| mother | 358 |
| father | 347 |
| uncle | 424 |
| uncle | 396 |
| uncle | 439 |
| grandmother | 454 |
| normal adult | 372 |

*nmoles substrate hydrolyzed/hr/mg protein. The standard error for all values was less than 10%.

Substrates used:
1 mM 4-MU-β-Gal in 0.1 M NaCl, Na citrate pH 5

Table 10. Leukocyte Lysosomal Glycosylhydrolases From Other FEL Patients

| Subject | Specific Activities* | |
|---|---|---|
| | β-Galactosidase | β-N-Acetylglucosaminidase |
| J. D. (FEL) | 894 | 5778 |
| Ju. C. (FEL) | 323 | 2298 |
| Je. C. (FEL) | 331 | 2104 |
| Normal | 672 | 3562 |
| Normal | 443 | 2511 |

*nmoles substrate hydrolyzed/hr/mg protein. The standard error for all values was less than 10%.

Substrates used:
1 mM 4-MU-β-Gal in 0.1 M NaCl, Na citrate pH 4.5
1 mM 4-MU-β-GlcNAc in 0.1 M Na citrate pH 4.5

relatives. The enzyme activities of all the relatives were close in value to the one normal adult tested. The lysosomal glycosylhydrolase levels of other FEL patients (Table 10) were examined to study the variation of enzyme activity in other patients similarly afflicted. Both β-galactosidase and β-hexosaminidase activities of the other FEL patients showed no real differences from the normal controls. The apparent increase of lysosomal enzyme activity of J.D. could be explained by the lower amounts of protein used in each assay (approximately 33% of the other patients' and normals' protein levels tested).

C. Fibroblast Lysosomal Glycosylhydrolases

The lysosomal enzyme activities from the fibroblast cell lines of several FEL patients, their parents, 2 $GM_1$ gangliosidosis lines, an agammaglobulinemia, and normal controls were compared. The results are listed in Table 11. No apparent lysosomal enzyme deficiencies were found although α-fucosidase and β-glucuronidase activities were elevated above normal ranges in most FEL patients.

D. Studies on the Nature of the Liver Lysosomal β-Galactosidase Deficiency

β-Galactosidase activity was measured over a wide pH range to determine whether the lowered enzyme activity in the FEL patient was due to a pH optimum shift of activity. As can be seen in Figure 3, results of this experiment revealed identical pH optimum curves for both normal and FEL liver β-galactosidase. Thus, the apparent low enzyme activity was not due to a shift in pH optimum for β-galactosidase activity.

To determine if the β-galactosidase deficiency was due to the presence of an enzyme inhibitor in the FEL liver, mixing experiments were conducted with equal amounts of normal and FEL liver homogenates. As can be seen in Figure 4, the enzyme activity of the mixed homogenates was median to the individual

Table 11. Fibroblast Lysosomal Glycosylhydrolases

| Cell line[2] | Enzyme Specific Activities[1] | | | | | | |
|---|---|---|---|---|---|---|---|
| | β-Gal'ase | α-Fuc'ase | α-Man'ase | β-Glc'ase | α-Gal'ase | β-GlcUA'ase | β-GlcNAc'ase |
| Ju.C. (FEL) | 539 | 91 | 103 | 272 | 95 | 215 | 4393 |
| Je.C. (FEL) | 453 | 81 | 70 | 247 | 90 | 147 | 3347 |
| G. (FEL) | 618 | 95 | 82 | 306 | 92 | 90 | 4348 |
| C. (FEL) | 421 | 44 | 122 | 350 | 48 | 50 | 3458 |
| K.C. (mother) | 458 | 72 | 60 | 271 | 66 | 144 | 3694 |
| S.C. (father) | 430 | 45 | 43 | 162 | 70 | 90 | 3167 |
| normal | 332 | 30 | 154 | 207 | 88 | 37 | 3094 |
| normal | 456 | 30 | 82 | 244 | 68 | 52 | 3156 |
| $GM_1$ Type 1 Gangliosidosis | 88 | 52 | 137 | 299 | 124 | 51 | 2946 |
| $GM_1$ Type 1 Gangliosidosis | 0 | 26 | 181 | 163 | 163 | 30 | 3261 |
| agammaglobulinemia | 364 | 54 | 68 | 190 | 69 | 70 | 3141 |

[1] nmoles of substrate hydrolyzed/hr/mg protein. Standard error of the average values was less than 10%.
[2] All cell lines were harvested after 4-10 passages in culture.

Substrates used (172,228):
1 mM 4-MU-α-Fuc in 0.1 M Na citrate, 0.2 M Na phosphate pH 6.0
10 mM 4-MU-β-GlcUA in 0.1 M Na acetate pH 4.8
5 mM 4-MU-α-Man in 0.1 M Na citrate, 0.2 M Na phosphate pH 4.4
1 mM 4-MU-β-GlcNAc in 0.1 M Na citrate, 0.2 M Na phosphate pH 4.4
5 mM 4-MU-β-Glc in 0.1 M Na citrate, 0.2 M Na phosphate pH 5.8 (each assay contained 500 μg Na taurocholate and 50 μg oleic acid)
1 mM 4-MU-β-Gal in 0.1 M NaCl, Na citrate pH 4.5
10 mM 4-MU-α-Gal in 0.1 M Na citrate, 0.2 M Na phosphate pH 4.4

Figure 3. Liver β-Galactosidase Activity vs. pH

Aliquots (30 µl) from normal liver (●——●) and FEL liver (○——○) were assayed at 37° for 10 and 15 min incubations in duplicate and the average activities were reported. Substrate concentrations used in all assays were 1 mM 4-methylumbelliferyl-β-galactoside in 0.1 M NaCl, Na citrate, 0.2 M Na phosphate buffer. Units of activity were defined as nmoles of substrate hydrolyzed per hr.

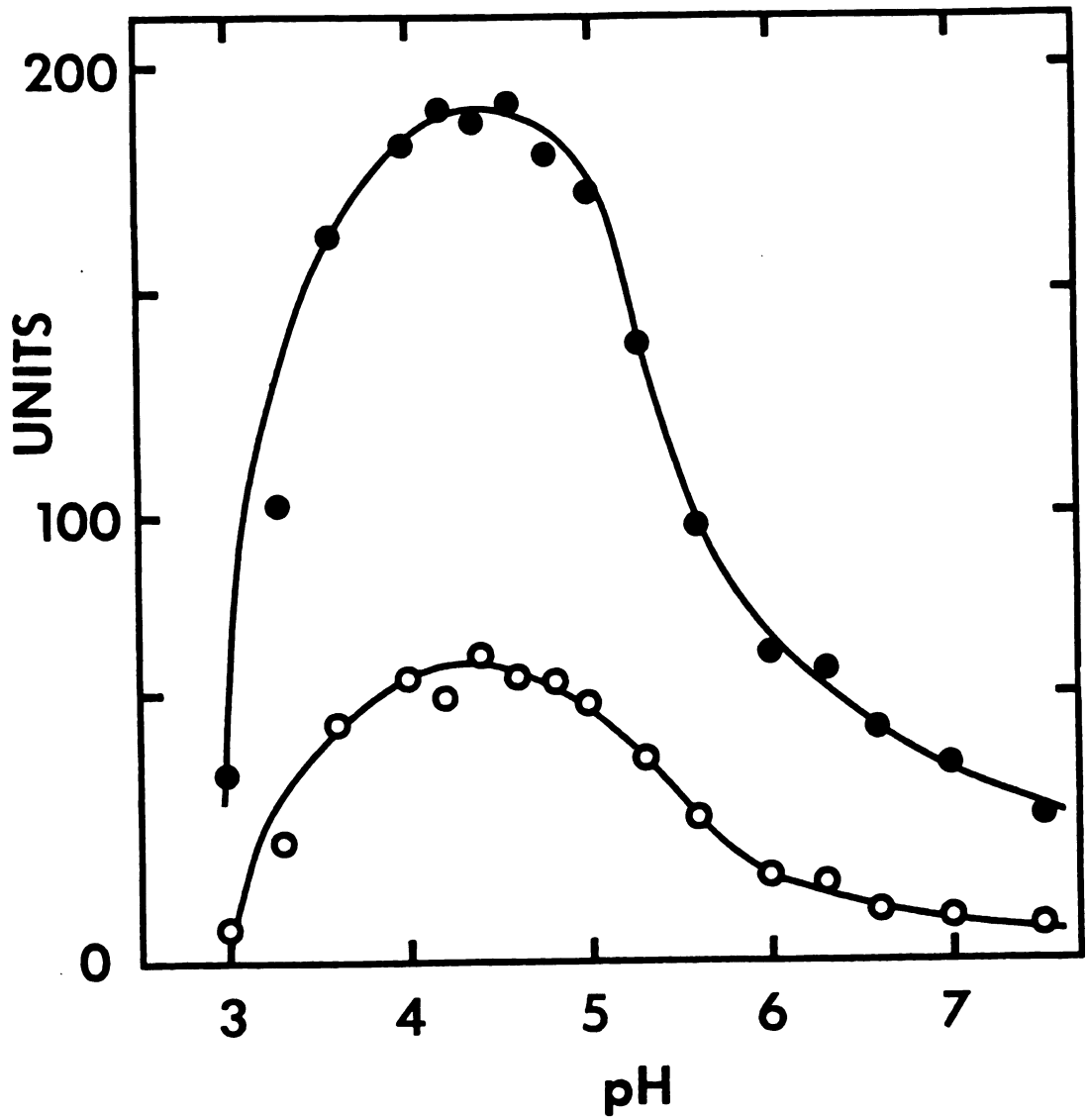


Figure 3.

Figure 4. Normal and FEL Liver β-Galactosidase Mixing Experiments

Enzyme activities were measure at different time intervals for normal liver (●), FEL liver (○), and mixed (50% of each) normal and FEL liver (+) homogenates. Aliquots (20 µl) were incubated at 37°, and the averages of duplicate asays were reported. Substrate concentration in all assays was identical to that described in Figure 3.

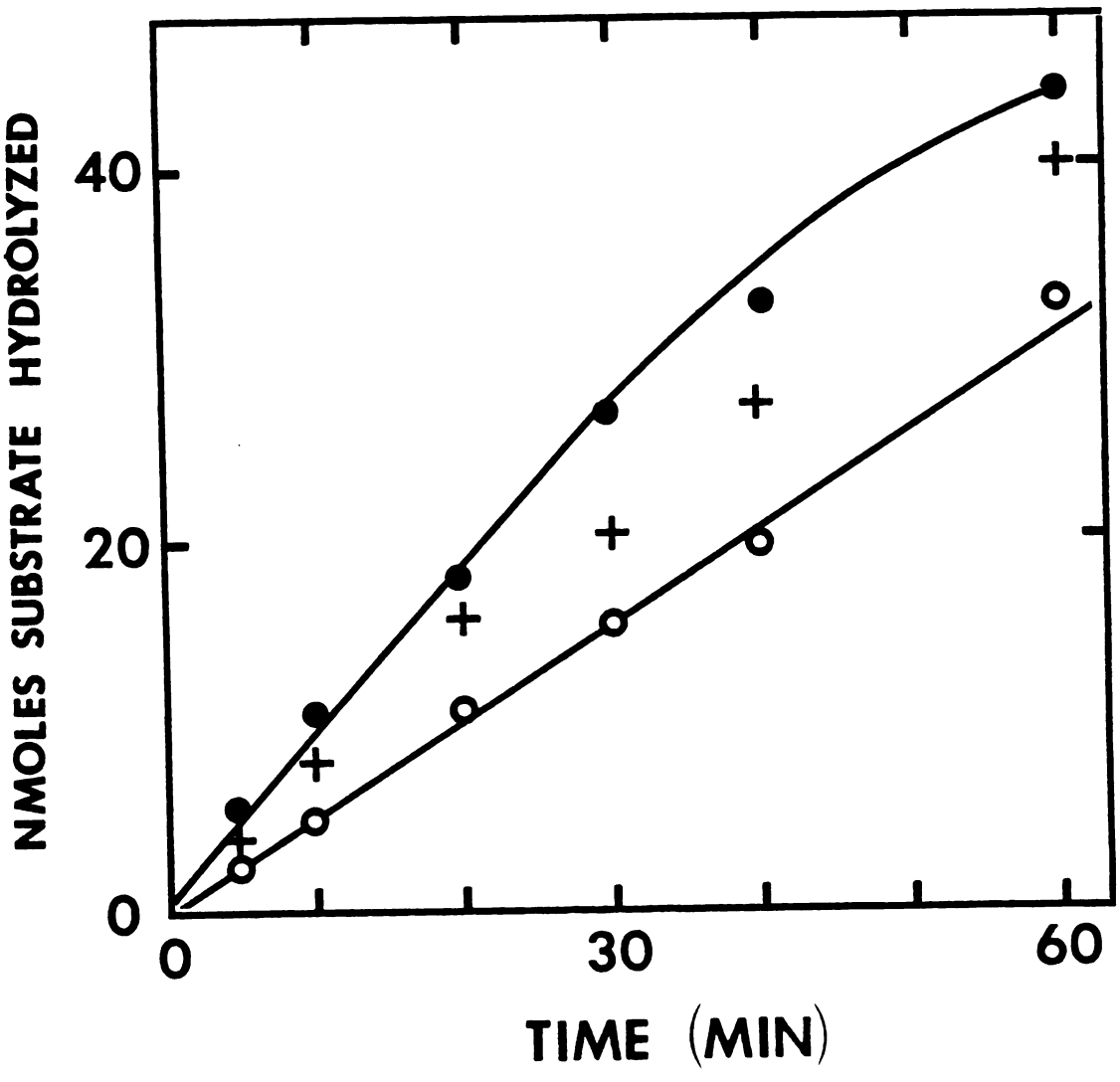


Figure 4.

activity levels of the normal and FEL liver homogenates, indicating the absence of an inhibitory substance. The effect of mixing increasing amounts of normal and FEL liver homogenates together (Figure 5) resulted also in a median level of β-galactosidase activity compared to the normal and FEL homogenates tested alone. The indication here was, again, the absence of an inhibitory substance at various concentrations of homogenates, and that the absence of inhibitory activity was not dependent on its concentration in the assay.

Heat inactivation studies (Figure 6) were done to determine whether the decreased FEL liver β-galactosidase activity was due to the presence of a residual, heat-stable isoenzyme. In both normal and FEL liver enzymes, the decrease in enzyme activity due to pre-incubation at $42^{o}$ was identical (73% reduction of activity), demonstrating that the FEL activity was not due solely to the presence of a heat-stable isoenzyme.

Cellulose acetate electrophoresis of the liver β-galactosidases (Figure 7) revealed 2 major isoenzyme forms (A and B), as visualized by activity staining with the fluorescent substrate, 4-methylumbelliferyl-β-galactoside. There was no alteration in electrophoretic mobility of the FEL liver β-galactosidase isoenzymes. A decrease in the level of β-galactosidase A activity could easily be discerned, but due to the relatively low level of the B isoenzyme activity, a decrease in β-galactosidase B could not be determined with certainty.

The effect of exogenous sialic acid addition on β-galactosidase activity was investigated (Figure 8) at 1 X, 10 X, and 100 X the total sialic acid concentration found in the FEL liver lipid extract. Inhibition of enzyme activity was not found at any level of added sialic acid, although a slight decrease was seen in FEL and normal liver at 0.01 mM NeuNAc.

Figure 5. Normal and FEL Liver β-Galactosidase Mixing Experiments with Increasing Homogenate Concentrations

Enzyme activities were measured for normal liver (●), FEL liver (○), and mixed (composed 50% of each) normal and FEL liver homogenates (+). Increasing aliquots of liver homogenates were added to the standard β-galactosidase assay described in Figure 3. The assays were incubated for 5 and 10 min, and the averages of duplicate assays at the two time points were reported. Units were defined as nmoles substrate hydrolyzed per hr.

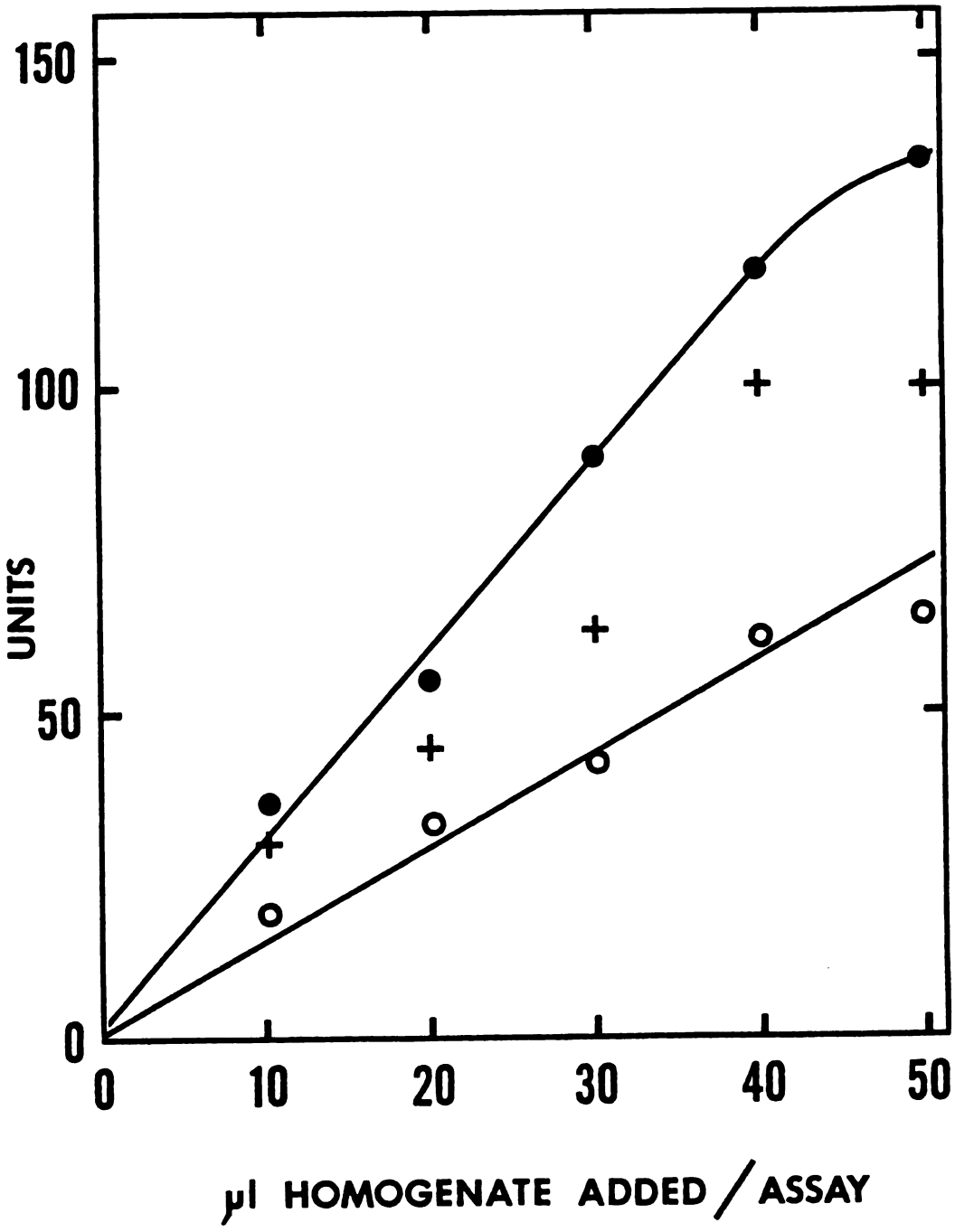


Figure 5.

Figure 6. Heat Lability for Liver β-Galactosidases

Normal liver (●—●) and FEL liver (○—○) homogenates were preincubated at 42° for the time durations described followed by the standard β-galactosidase assay described in Figure 3. The numbers in parentheses were the percentages of total enzyme activity remaining after preincubation. All values reported were averages of duplicate assays run at 10 and 20 min.

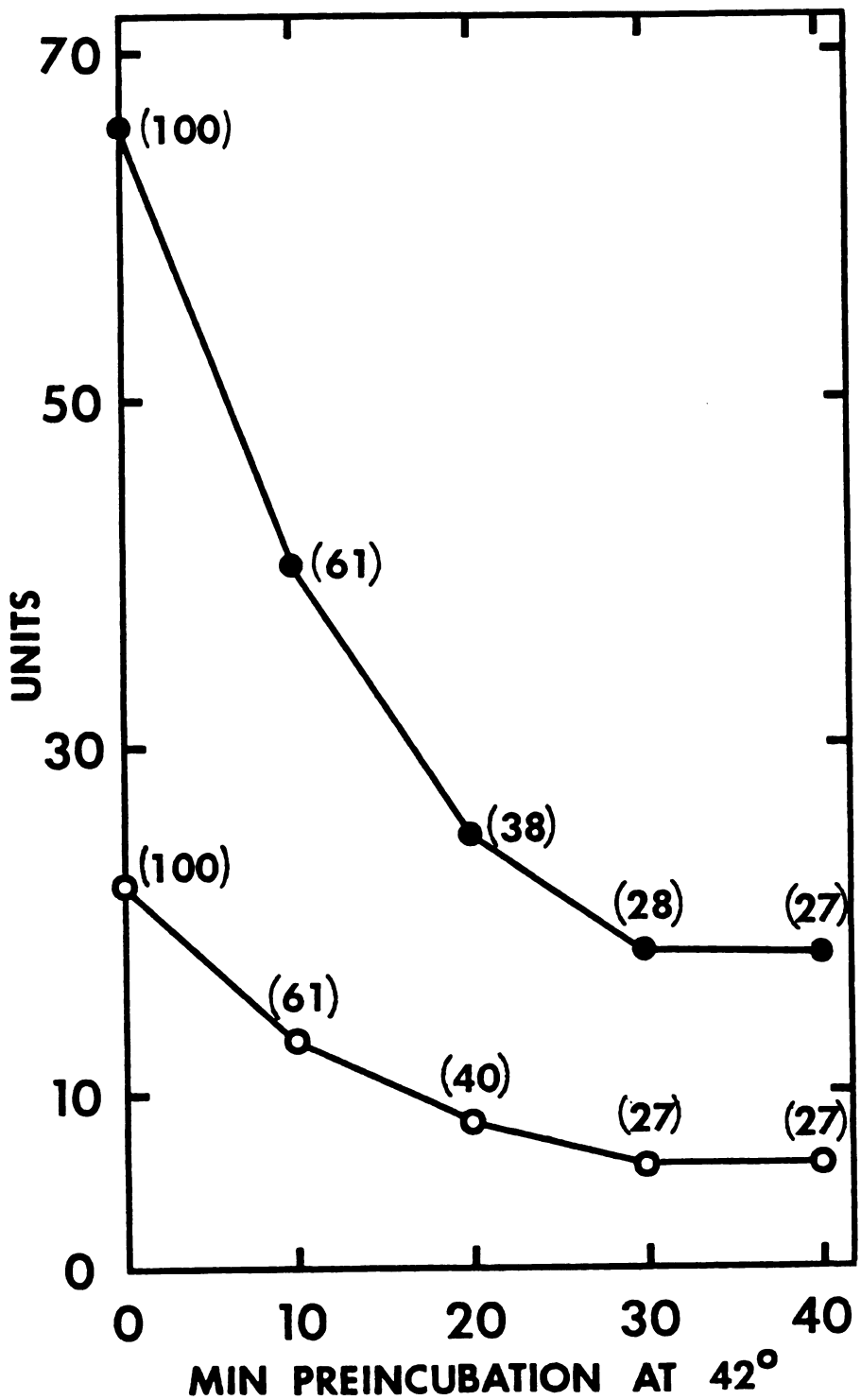
70
50
30
10
0
UNITS
(100)
(61)
(38)
(28)
(27)
(100)
(61)
(40)
(27)
(27)
0
10
20
30
40
MIN PREINCUBATION AT 42°

Figure 6.

Figure 7. Cellulose Acetate Electrophoresis of Liver β-Galactosidases

Normal and FEL liver lysosomal β-galactosidases were visualized by fluorescent activity staining of the polyacetate strips as described in Methods.

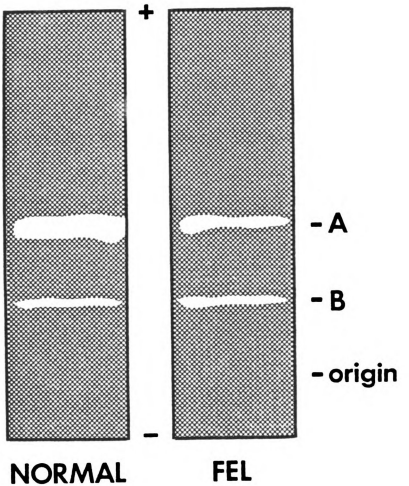


Figure 7.

Figure 8. Effect of Exogenous N-Acetylneuraminic Acid on Liver β-Galactosidase Activity

Equal aliquots from normal liver ( ● ) and FEL liver ( ○ ) homogenates were incubated for 10 and 20 min with exogenous sialic acid. Activities were reported as averages of duplicate assays at the 2 time points. Concentrations of sialic acid reported were the final concentrations in each assay. Assay conditions were identical to those in Figure 3. Units of activity were defined as nmoles of substrate hydrolyzed per hr.

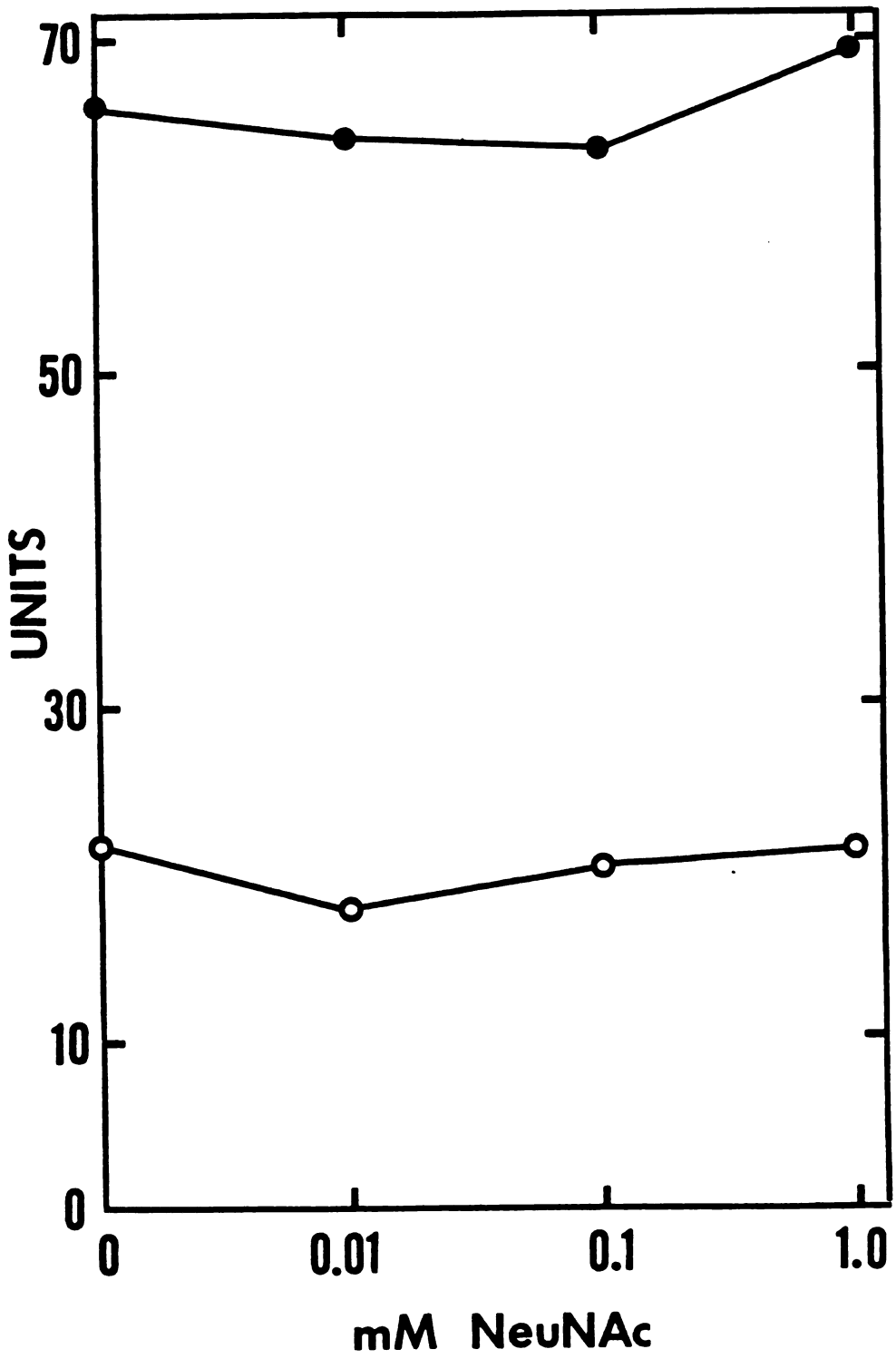


Figure 8.

## II. Liver Lipid Studies

### A. Lipid Composition of Folch Extracts

The Folch lower phase liver lipids were separated into 3 major fractions consisting of a neutral lipid fraction, a glycolipid fraction, and a phospholipid fraction by Unisil silicic acid chromatography as described in Methods. The quantities of each fraction in normal and FEL liver were determined gravimetrically (Table 12). Total FEL liver lipids were elevated 142% over the normal liver levels, this increase deriving completely from the neutral lipid fraction (167% of normal liver neutral lipids levels). Analysing the neutral lipids further, this elevation appeared to originate from the large increases in triglycerides and cholesterol lipids (187% and 158%, respectively, of normal liver levels) as seen in Table 13. Interestingly, free cholesterol in FEL liver was elevated to such an extent (218% of normal liver levels) that it accounted for almost all the cholesterol lipids present. Free cholesterol comprised 71% of normal liver total cholesterol lipids and an abnormally high 98% of FEL liver total cholesterol lipids. TLC analysis (Figures 9-11) of the 3 lipid fractions of the FEL and normal liver isolated by Unisil silicic acid chromatography revealed no apparent alterations in lipid profiles except in the FEL patient's neutral lipid fraction, where a large increase of triglycerides was seen. An increase in free cholesterol was also discernable on the TLC plate. In Figure 10, the orcinol-positive bandcorresponding to GL-5 was due to $GM_3$ ganglioside carried over into the Folch lower phase partition. The - symbol marked bands that were phospholipid contaminants in the acetone-methanol wash. Mass spectral analysis (Table 14) and fatty acid analysis (Table 15) were performed on the isolated triglycerides to confirm the identity of the triglycerides and to determine whether any alterations in the fatty acid constituents had occurred. The results demonstrated that there were no major changes in the relative distribution of individual triglyceride species

Table 12. Total Liver Lipid Composition

g/g wet weight liver

| Lipid Fraction | Normal* | FEL |
|---|---|---|
| Total Lipids | 0.074 ± 0.003 | 0.105 ± 0.014 |
| Neutral Lipids | 0.048 ± 0.001 | 0.080 ± 0.002 |
| Glycolipids | 0.004 ± 0.001 | 0.005 ± 0.001 |
| Phospholipids | 0.021 ± 0.001 | 0.020 ± 0.002 |

*All values are averages of duplicate samples and are reported with their standard error.

Table 13. Neutral Lipid Fraction Composition

μmoles/ g wet weight liver[1]

| | Normal | FEL |
|---|---|---|
| Free Cholesterol | 8.7 ± 0.5 | 19.0 ± 0.7 |
| Total Cholesterol | 12.3 ± 0.7 | 19.4 ± 0.8 |
| Total Triglycerides | 23.9 ± 3.0 | 44.6 ± 5.1 |
| Total Triglycerides (mg/g wet weight liver)[2] | 20.3 ± 2.1 | 37.9 ± 4.0 |

[1]All values are averages of duplicate samples and are reported with their standard error.

[2]Average molecular weight from M.S. and GLC data was 850.

Figure 9. Thin-Layer Chromatography of Liver Neutral Lipids

Normal liver (lane 1) and FEL liver (lane 2) neutral lipids from the Folch extracts were chromatographed along with standard neutral lipids. In lane 3 (top to bottom), the standard lipids were triolein, oleic acid, and 1,2-dipalmitin; in lane 4, cholesterol; and in lane 5, 1-monopalmitin. The + denotes spots that gave a positive reaction for cholesterol lipids with $H_2SO_4$:acetic acid spray reagent. The TLC plate was also visualized by $H_2SO_4$ charring at $130^{o}$ for general lipid visualization.

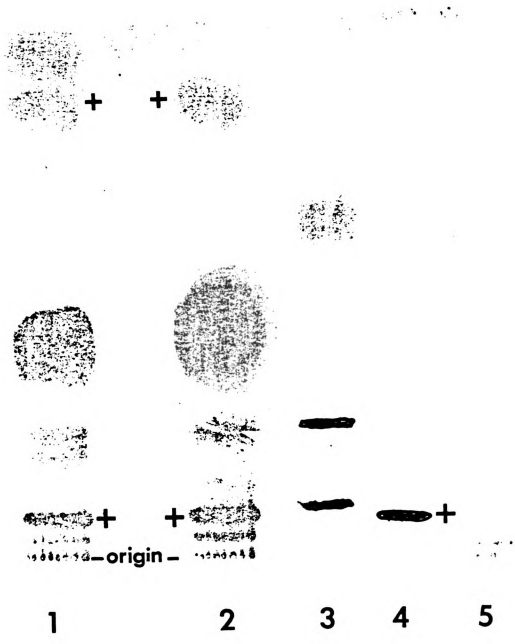


Figure 9.

Figure 10. Thin-Layer Chromatography of Liver Neutral Glycosphingolipids

Equivalent amounts of FEL (lane 1) and normal (lane 2) liver neutral glycolipids were chromatographed along with a mixed glycolipid standard derived from horse kidney. The symbol (-) denoted bands which did not give a positive reaction for the presence of carbohydrate with orcinol spray reagent.

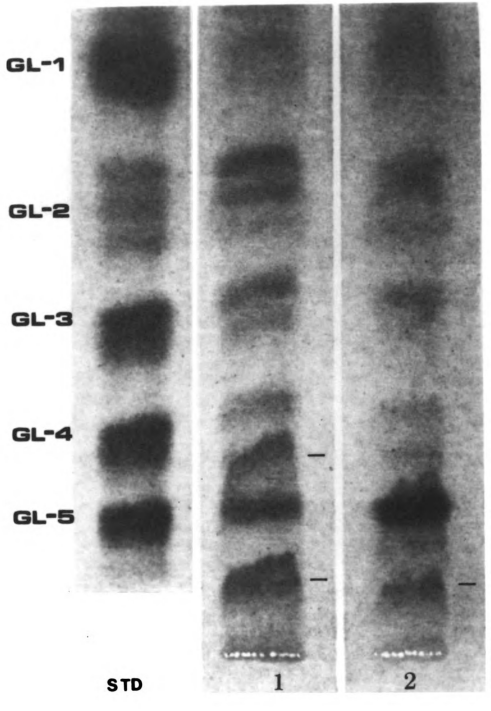


Figure 10.

Figure 11. Thin-Layer Chromatography of Liver Phospholipids

Normal liver (lane 1) and FEL liver (lane 2) phospholipids from the Folch lower phase extracts were developed and visualized as described in Methods. The standard phospholipids run were sphingomyelin (lane 3), phospatidylcholine (lane 4), phosphatidylethanolamine (lane 4), and phosphatidylserine (lane 5).

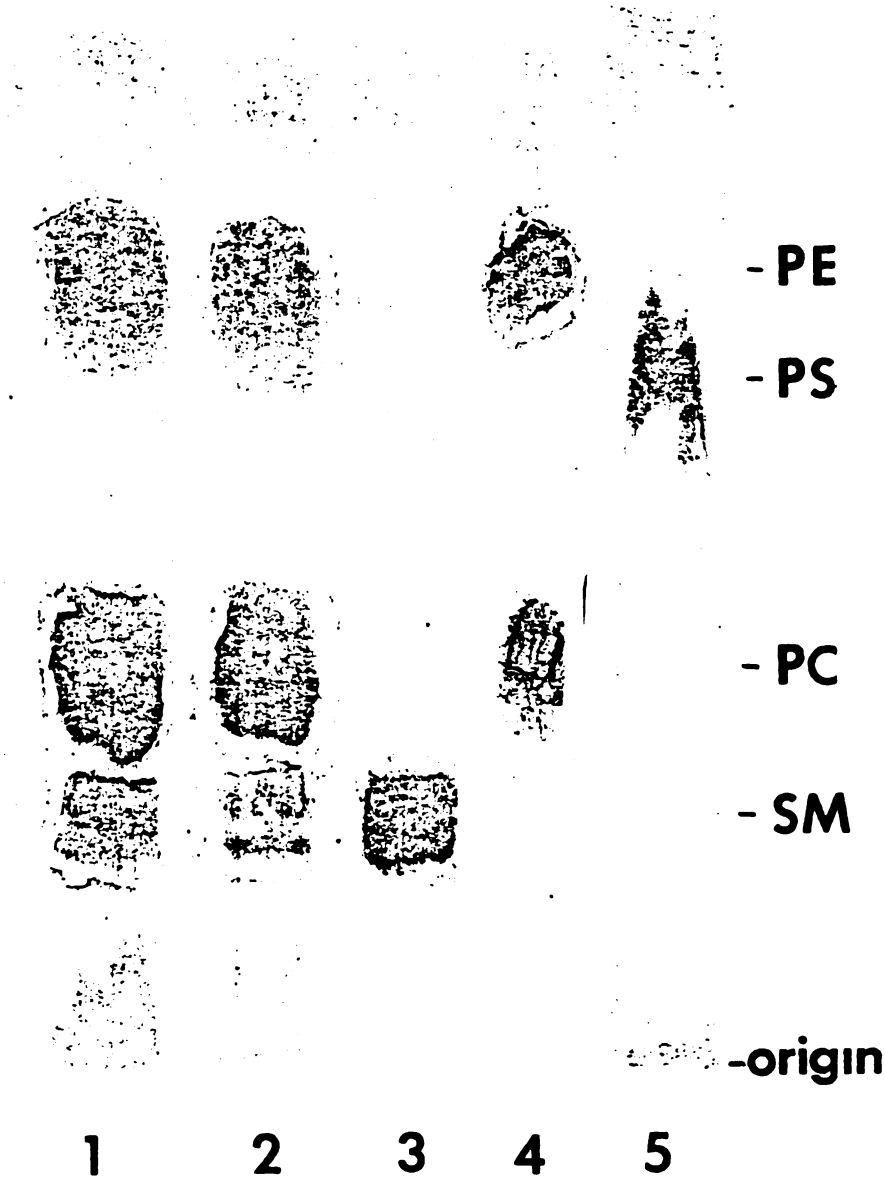


Figure 11.

Table 14. Distribution of Triglycerides in Liver

| Molecular Weight | Probable* Species | Normal | FEL |
|---|---|---|---|
| 800 | 16:1/16:1/16:1 | 1.7 | 3.6 |
| 804 | 16:0/16:0/16:1 | 2.8 | 2.6 |
| 828 | 16:1/16:1/18:1 | 10.8 | 7.7 |
| 830 | 16:1/16:1/18:0 or 16:0/18:1/16:1 | 7.9 | 9.0 |
| 832 | 16:0/16:0/18:1 or 18:0/16:0/16:1 | 4.5 | 7.5 |
| 834 | 16:0/16:0/18:0 | 2.0 | 1.5 |
| 856 | 18:1/18:1/16:1 | 21.8 | 17.0 |
| 858 | 18:1/18:1/16:0 or 18:0/18:1/16:1 | 22.5 | 19.2 |
| 860 | 18:0/18:0/16:1 or 18:0/16:0/18:1 | 6.2 | 7.3 |
| 884 | 18:1/18:1/18:1 | 11.3 | 15.5 |
| 886 | 18:1/18:1/18:0 | 5.7 | 6.4 |
| 888 | 18:0/18:0/18:1 | 2.8 | 1.8 |

*From calculated ion intensity measurements of the triglycerides mass ions $M^+$ and $(M-18)^+$ ions ($-H_2O$). Fatty acids composition of the triglycerides are designated in terms of chain length:number of double bonds.

Table 15. Fatty Acid Composition of Liver Triglycerides

| | Per Cent Distribution | |
|---|---|---|
| Fatty Acid | Normal | FEL |
| 14:0 | 2.3 | 2.0 |
| 16:0 | 31.6 | 32.0 |
| 16:1 | 6.8 | 7.6 |
| 18:0 | 6.7 | 7.6 |
| 18:1 | 52.6 | 50.7 |

Table 16. Total NeuNAc Content in Human Liver

| | Nanomoles per g Liver | |
|---|---|---|
| | Normal | FEL |
| Total liver[1] | 1720 ± 178 | 2230 ± 260 |
| Folch upper phase extract[2] | 8.9 ± 4.4 | 101.3 ± 15.0 |

All values are averages of duplicate assays on duplicate samples and are reported with their standard error.

[1]Calculated by GLC analyses as described in Methods.
[2]Calculated by resorcinol colorimetric assay.

type nor in the distribution of component fatty acids of the liver triglycerides in FEL liver compared to normal liver. Calculations on Table 14 to determine the relative composition of the individual fatty acids in the liver triglycerides corroborate quite well the values determined by GLC in Table 15, especially if it is assumed that those triglyceride species of a given molecular weight with multiple species possibilities are predominantly composed of the palmitic acid-containing species. Data for the quantitation of triglycerides in Table 13 and Table 15 were derived and quantitated from gas-liquid chromatographic data of the methanolyzed products of the isolated triglycerides using methyl arachidate as an internal standard . No endogenous methyl arachidate was detected in the fatty acid methyl ester products obtained from the methanolysis of the triglycerides.

B. Ganglioside Studies

1) Isolation and Quantitation of Liver Gangliosides

Human liver gangliosides were isolated by DEAE-Sephadex column chromatography and fractionated by combined Iatrobead silicic acid column chromatography-preparative TLC into fractions based on their column elution times and TLC mobilities (Figure 12 and Figure 13). Total gangliosides and other sialic acid-containing components in liver were quantitated on the basis of sialic acid by GLC analysis of the mild methanolysis (0.05 N HCl-methanol hydrolysis at $80^{\circ}$) products of the human liver homogenates. Total gangliosides in the Folch upper phase lipids and those gangliosides fractionated by DEAE-Sephadex-Iatrobead column chromatography were quantitated by the colorimetric resorcinol assay for sialic acid. These results were summarized in Tables 16-18. Although the sialic acid content of the FEL liver was slightly elevated over the normal liver (1.3-fold), the major increase was seen in the Folch upper phase (11.4-fold) where the major sources of sialic acid were undoubtedly gangliosides. Thus, the increase of sialic

Figure 12. Ganglioside Fractions Isolated from Normal Liver



Aliquots of the ganglioside fractions isolated by DEAE-Sephadex and Iatrobead column chromatography were analyzed on Silica Gel 60 TLC plates which were developed in chloroform:methanol:0.2% $CaCl_2$ (60:40:9) and visualized with resorcinol spray reagent.

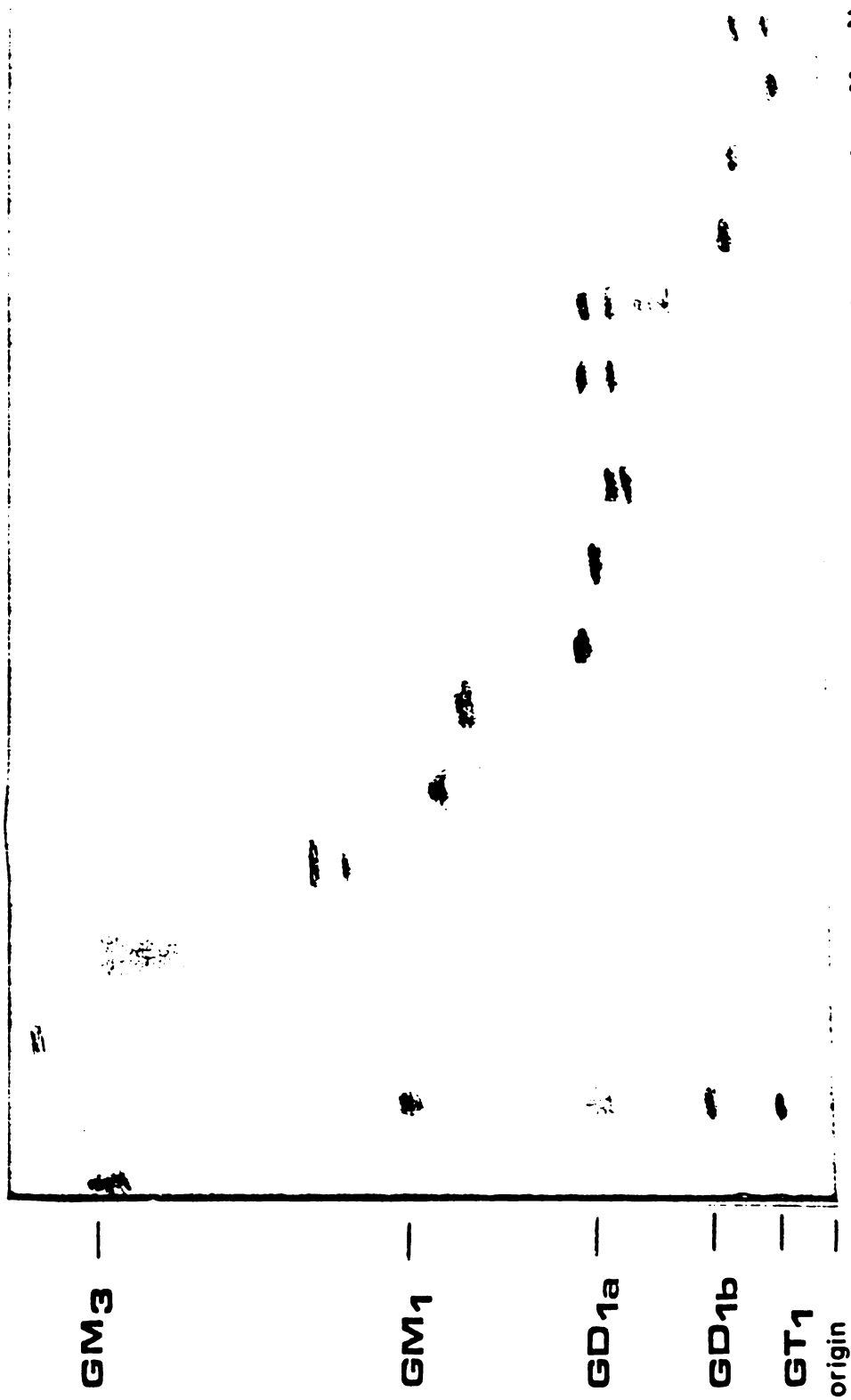


Figure 12.

Figure 13. Ganglioside Fractions Isolated from FEL Liver



Aliquots of the ganglioside fractions isolated by DEAE-Sephadex and Iatrobead column chromatography were analyzed on Silica Gel 60 TLC plates which were developed in chloroform:methanol:0.2% $CaCl_2$ (60:40:9) and visualized with resorcinol spray reagent.

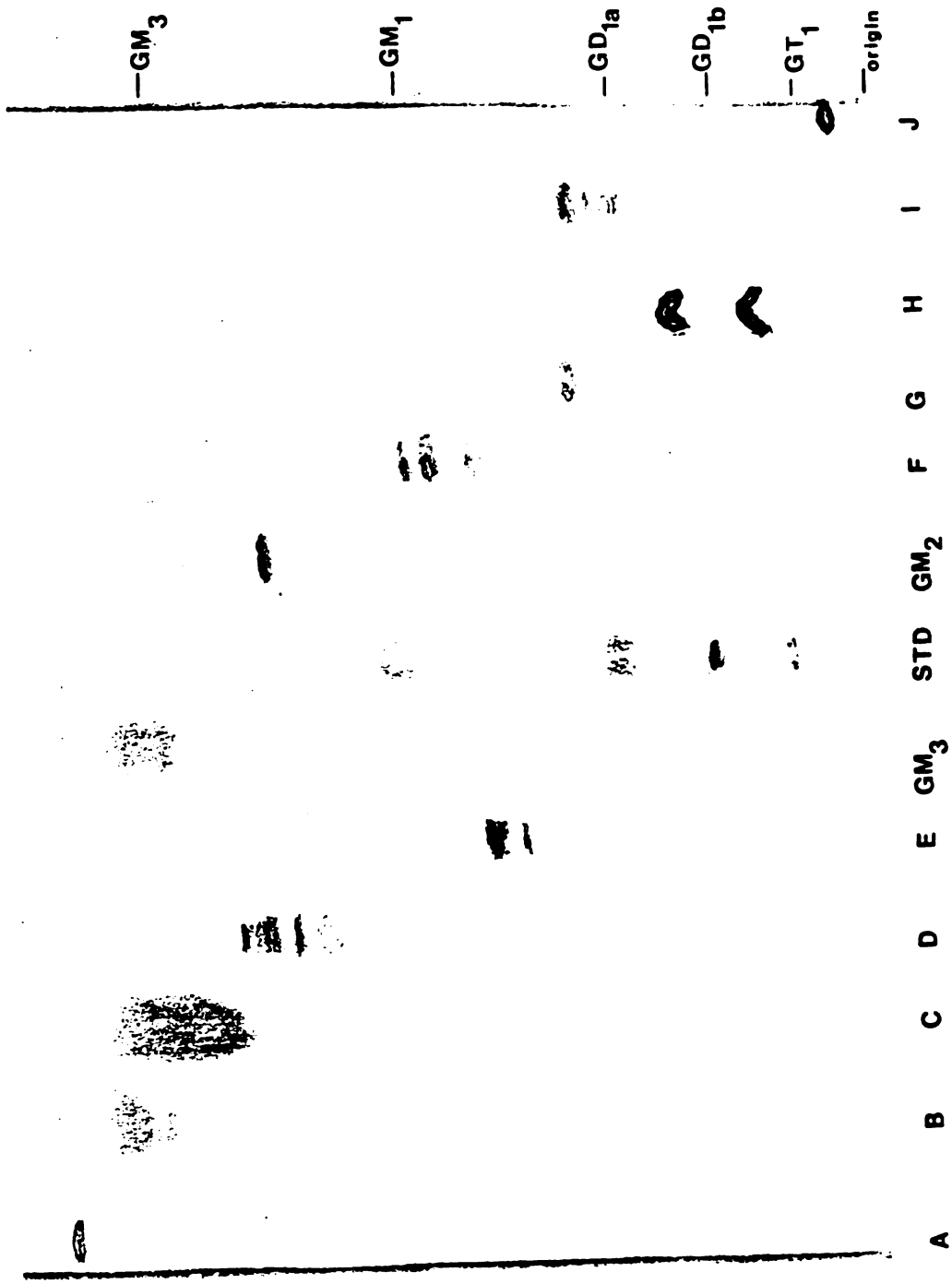


Figure 13.

Table 17. Quantitation of Normal Liver Ganglioside Fractions

| Fraction | TLC mobility similar to:[1] | Total NeuNAc (nmoles) | NeuNAc per g liver[2] (nmoles) | (approximate) µg ganglioside per g liver[2] |
|---|---|---|---|---|
| A | $GM_4$ | 53 | 0.11 | 0.11 |
| B | $GM_3$ | 413 | 0.85 | 1.02 |
| C | $GM_2$ | 84 | 0.17 | 0.28 |
| D | $GM_1$ | 5 | 0.01 | 0.01 |
| E | $GD_3$ | 680 | 1.40 | 0.99 |
| F | $GD_{1a}$ | 52 | 0.11 | 0.10 |
| G | $GD_{1a}$ | 19 | 0.04 | 0.04 |
| H | $GD_2$,$GD_{1a}$ | 30 | 0.06 | 0.05 |
| I | $GD_{1a}$ | 480 | 0.99 | 0.91 |
| J | $GD_{1a}$,$GT_{1a}$ | 23 | 0.05 | 0.04 |
| K | $GD_{1b}$ | 14 | 0.03 | 0.03 |
| L | $GD_{1b}$ | 440 | 0.91 | 0.82 |
| M | $GT_1$ | 100 | 0.21 | 0.13 |
| N | $GT_1$ | 23 | 0.05 | 0.03 |
| Total of all Fractions | --- | | 4.99 | 4.56 |
| Total Liver Gangliosides Assayed Before Fractionation | --- | | 13.0 | 19.5 |

[1]Compared to neural ganglioside TLC mobilities as described by Ueno et al.(222).

[2]Based on 485 g liver starting material and averages of duplicate resorcinol colorimetric assays. The standard error was less than 10% for all values.

Table 18. Quantitation of FEL Liver Ganglioside Fractions

| Fraction | TLC Mobility similar to:[1] | Total NeuNAc (nmoles) | NeuNAc per g liver[2] (nmoles) | (approximate) µg ganglioside per g liver[2] |
|---|---|---|---|---|
| A | $GM_4$ | 130 | 1.45 | 1.5 |
| B | $GM_3$ | 3990 | 45.0 | 54.0 |
| C | $GM_3$,$GM_2$ | 8920 | 101 | 122 |
| D | $GM_2$ | 1760 | 20.0 | 28.1 |
| E | $GD_3$ | 650 | 7.0 | 5.2 |
| F | $GM_1$,$GD_3$ | 820 | 9.0 | 14.9 |
| G | $GD_{1a}$,$GD_3$ | 150 | 2.0 | 1.5 |
| H | $GD_2$,$GD_{1b}$ | 40 | 0.5 | 0.4 |
| I | $GD_{1a}$,$GT_1$ | 340 | 4.0 | 3.8 |
| J | $GT_1$ | 170 | 2.0 | 1.3 |
| Total of all Fractions | | --- | 192 | 233 |

[1]Compared to neural ganglioside TLC mobilities as described by Ueno et al.(222).

[2]Based on 88 g liver starting material and average of duplicate resorcinol colorimetric assays. The standard error was less than 10% for all values.

acid-containing material in FEL liver was probably due to gangliosides, and this increase in gangliosides appeared to be heavily weighted toward the less complex gangliosides, e.g. $GM_3$ and $GM_2$. TLC analysis of the Folch upper phase lipids of the longer chain fatty acids compared to FEL liver gangliosides. FEL (Figure 14) confirmed the ganglioside increase, especially those with TLC mobilities between $GM_3$ and $GM_1$. The large discrepancy in the amount of gangliosides in normal liver before and after fractionation was due to an accidental loss of some ganglioside fractions during the DEAE-Sephadex column chromatography, and was not a reflection on the actual recoveries of gangliosides after DEAE-Sephadex and Iatrobead column chromatography. As a result, the $GM_3$ levels were abnormally low in normal liver compared to the work of others (54) who found that $GM_3$ comprised nearly 90% of the total amount of liver gangliosides. There was good agreement between the amounts calculated for gangliosides in FEL liver Folch upper phase and for the sum of total ganglioside fractions after the purification steps.

2) Immunosuppressive Activity of the Liver Gangliosides

The fractionated liver gangliosides were tested for immunosuppressive activity with the lymphocyte blastogenesis assay (Figure 15 and Figure 16). In the FEL liver gangliosides, two fractions were suppressive. One fraction (A) was found to be cytotoxic by trypan blue inclusion, low cell count, and Con A non-responsiveness of the test lymphocytes, but the other fraction (H) appeared to be truly immunosuppressive (high cell count and normal Con A response). In the normal liver gangliosides, 2 fractions were found to be immunosuppressive (H and L) and 4 fractions were considered cytotoxic (F,G,K, and M).

3) Characterization of the Liver Immunosuppressive Substances

Fraction H from FEL liver and fractions H and L from normal liver were

Figure 14. Thin-Layer Chromatography of Total Liver Gangliosides

Aliquots from the Folch upper phase lipid extracts were chromatographed with a $GM_3$ ganglioside standard and a standard bovine brain ganglioside mixture (STD). N1 and N2 contained 20 and 40 µl aliquots, respectively, from the normal liver extracts. F1 and F2 contained 20 and 40 µl aliquots, respectively, from the FEL liver extracts. The TLC plate was developed in chloroform:methanol:0.2% $CaCl_2$ (60:40:9) solvent and visualized with resorcinol spray reagent.

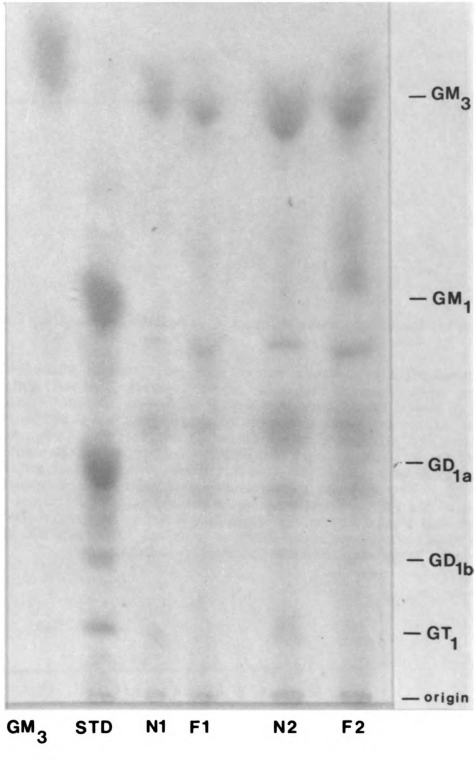


Figure 14.

Figure 15. Immunosuppressive Activities of Isolated Normal Liver Ganglioside Fractions

All lymphocyte mitogenesis assays were performed in triplicate. The average values were reported (net cpm) minus controls assayed without mitogen (Con A o——o) or antigenic stimulus (SKSD ●——●), and plotted on a logarithmic scale. All controls without mitogen or antigen gave less than 500 cpm background counts. The standard error on all assays was less than 10%. All lymphocytes tested for mitogenesis were obtained from a single normal adult donor. Final concentrations per assay for Con-A mitogen were 6 μg/ml and, for SKSD, 250 units/ml. Fraction 1 was a control assay with mitogens or antigenic stimuli added alone to the PBLs. Fraction 2 was a control assay with mitogens or antigenic stimuli and carrier liposomes without gangliosides. All gangliosides were assayed at a final concentration per assay of 2 μg/ml. Fractions F,G,K, and M were cytotoxic. Data was supplied by Dr. Stephan Ladisch.

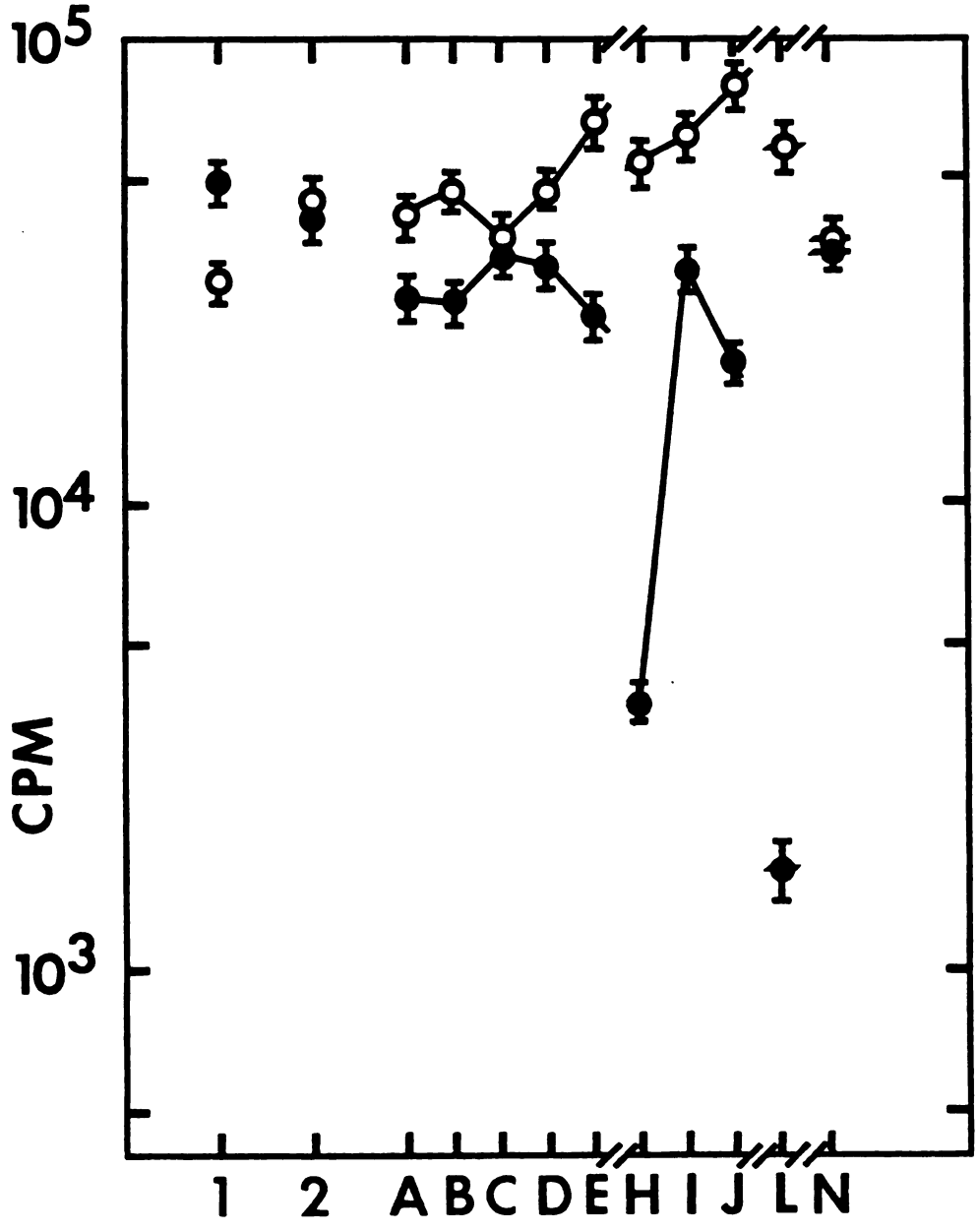


Figure 15.

Figure 16. Immunosuppressive Activities of Isolated FEL Liver Ganglioside Fractions

All lymphocyte mitogenesis assays were performed in triplicate. The average values were reported (net cpm) minus controls assayed without mitogen (Con A o——o) or antigenic stimulus (SKSD ●——●), and plotted on a logarithmic scale. All controls without mitogen or antigen gave less than 500 cpm background counts. The standard error on all assays was less than 10%. All lymphocytes tested for mitogenesis were obtained from a single normal adult donor. Final concentrations per assay for Con A-mitogen were 6 μg/ml and, for SKSD, 250 units/ml. Fraction 1 was a control assay with mitogens or antigenic stimuli added alone to the PBLs. Fraction 2 was a control assay with mitogens or antigenic stimuli and carrier liposomes without gangliosides. All gangliosides were assayed at a final concentration per assay of 2 μg/ml. Fraction A was cytotoxic. Data was supplied by Dr. Stephan Ladisch.

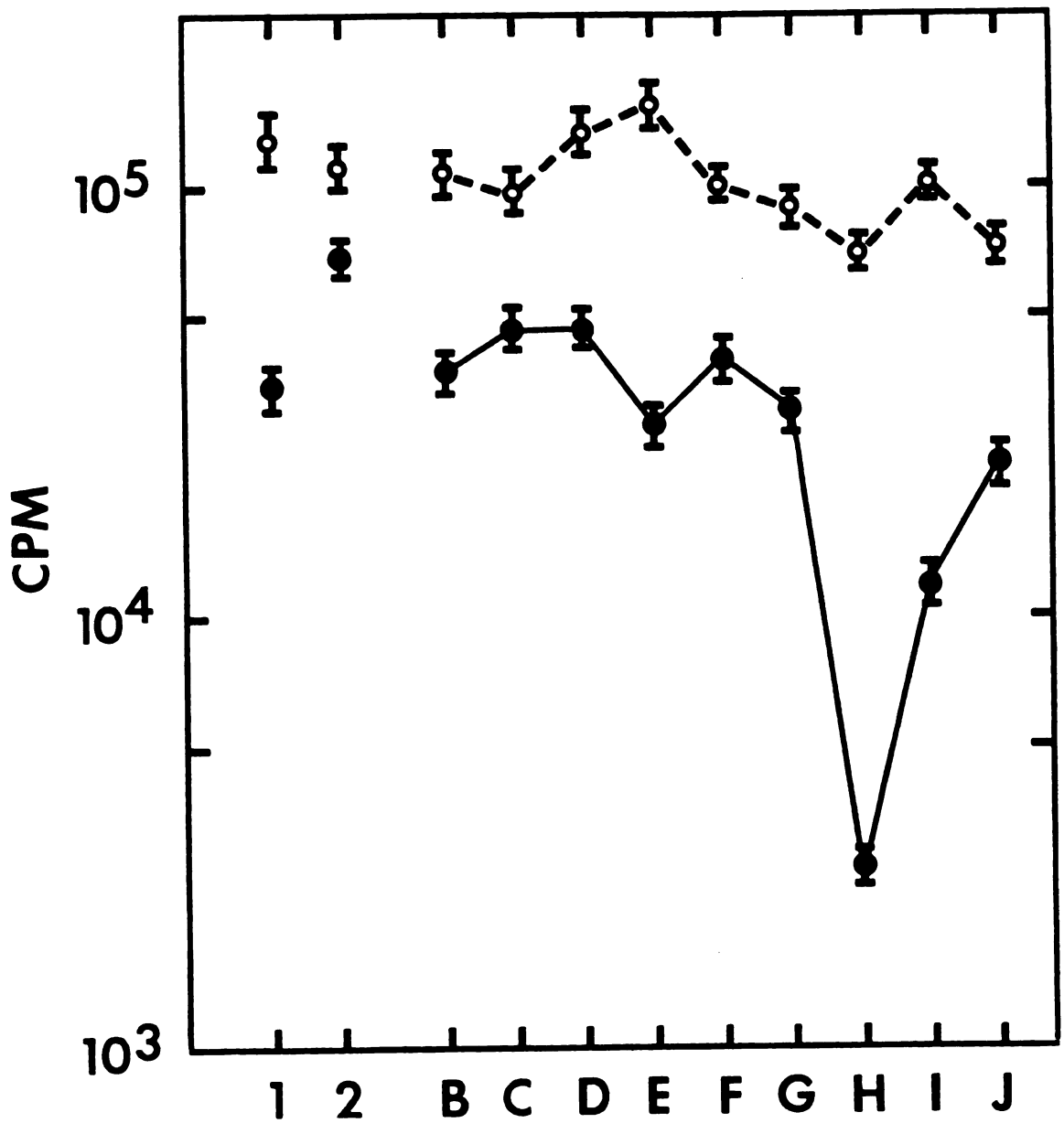


Figure 16.

methanolysed for analysis of their component carbohydrates and fatty acids by GLC-MS. Due to the extremely small amounts of the gangliosides analyzed (5-44 μg) and the possible heterogeneity of the ganglioside fractions analyzed, the carbohydrate and fatty acid composition data must be regarded as tentative until larger quantities of liver gangliosides can be obtained and their homogeneity demonstrated. The results of the analyses were summarized in Table 19 and Table 20.

The immunosuppressive ganglioside fractions from both normal and FEL livers were GlcNAc-containing gangliosides, which was consistent with the known general composition of gangliosides from visceral tissues as opposed to neural gangliosides, which were known to have only GalNAc as their counterpart in the carbohydrate sequence.

Preliminary indications from the carbohydrate data were that the immunosuppressive gangliosides had a carbohydrate sequence of the lacto or lactoneo series, most probably, Cer-Glc-Gal-GlcNAc-Gal with either 1 or 2 NeuNAc sugars ketoside-linked to either of the galactose moieties. Fractions H in both livers appeared to consist of monosialogangliosides while Fraction L in normal liver appeared to be a disialoganglioside. Bovine brain $GM_1$ ganglioside standard was analyzed for comparison of fatty acid and carbohydrate ratios. Since $GM_1$ was known to have the sequence Cer-Glc-Gal(←NeuNAc)-GalNAc-Gal, a comparison of its sugar ratios with those of the liver gangliosides revealed a close similarity. The high glucose ratios appeared to be from a contaminant carried along during the isolation procedure and, therefore, galactose and not glucose was used as the basis of the carbohydrate ratios determination. The glucose amount in Fraction L was too high (15 times the quantities of the other sugars analyzed) to be even considered. Fucose was not detected in any of the ganglioside fractions analyzed.

From the fatty acid analyses, the normal and FEL liver gangliosides were shown to contain palmitic acid (16:0) as their major component and stearic acid

Table 19. Carbohydrate Composition of Liver Immunosupppressive Fractions

| Sugars | Molar Ratios* | | | |
|---|---|---|---|---|
| | Normal Liver Fraction | | FEL Liver Fraction | $GM_1$ Ganglioside |
| | H | L | H | (Bovine Brain) |
| Gal | 1.00 | 1.00 | 1.00 | 1.00 |
| GlcNAc | 0.42 | 0.46 | 0.52 | — |
| GalNAc | — | — | — | 0.33 |
| NeuNAc | 0.38 | 0.77 | 0.45 | 0.42 |
| Glc | 1.60 | — | 1.30 | 0.67 |

*Based on galactose amount = 1.00

Table 20. Major Fatty Acid Composition of Liver Immunosuppressive Fractions

| Fatty Acid | Per Cent Distribution | | | |
|---|---|---|---|---|
| | Normal Liver[1] Fraction | | FEL Liver[1] Fraction | $GM_1$ Ganglioside[2] |
| | H | L | H | (Bovine Brain) |
| 14:0 | 12.1 | 9.4 | — | ----- |
| 16:0 | 63.1 | 57.0 | 38.2 | 1.9 |
| 16:1 | 5.6 | 5.0 | — | ---- |
| 18:0 | 16.7 | 23.5 | 25.8 | 81.6 |
| 18:1 | 2.5 | 5.0 | 6.7 | 2.9 |
| 20:0 | — | — | 6.7 | 4.4 |
| 22:0 | — | — | 9.0 | 9.2 |
| 24:0 | — | — | 13.5 | --- |

[1]calculated from ion intensities at m/e 87.
[2]calculated from GLC detector response.

(18:0) as the next predominant fatty acid. The normal liver gangliosides appeared to contain relatively shorter chain fatty acids (14-18 carbon units) with an absence ofthe longer chain fatty acies compared to FEL liver gangliosides. FEL gangliosides contained predominantly long-chain fatty acids (18-24 carbon units) with an absence of the short-chain fatty acids, such as, myristic acid (14:0).

4) Plasma Gangliosides

Plasma gangliosides of the FEL patient were compared to those of normal subjects and other FEL patients (Figure 17 and Figure 18). The large resorcinol-positive spot near the origin of the lane corresponding to the FEL patient's plasma lipid extracts was initially believed to be a ganglioside accumulating in the plasma. However, studies with the other FEL plasmas as well as GLC analysis of the isolated band by preparative TLC identified the putative ganglioside as free sialic acid and as an apparent artifact of the prolonged storage of the FEL patient's plasma at $4^{o}$. Only in that one FEL plasma was a resorcinol-positive spot clearly detected along with $GM_3$. Interestingly, a strong iodine-staining band, moving about one centimeter above the origin, was seen in all the FEL plasmas analysed, but did not appear in any of the normal plasmas (Figure 17). This band has not been identified yet.

Quantitation of the major ganglioside ($GM_3$) of normal and FEL plasma (Table 21) revealed an abnormal increase in $GM_3$ in only one FEL patient (no. 127), although the significance of this increase was not known.

The large spots above $GM_3$ were due to neutral glycolipids and phospholipids which migrated just above the gangliosides in this particular solvent system. In all FEL plasmas, a large increase in these lipids could be seen in comparison to normal plasma. This increase was not unexpected as all FEL plasmas were known to be hyperlipidemic. Plasmas of normal and relatives of FEL

Figure 17. Human Plasma Gangliosides-I

Plasma gangliosides from 3 FEL patients (169,127, and FEL) and one control (153) were extracted and isolated as described in Methods. The total lipid extracts were chromatographed sequentially in three different solvent systems consisting of:
1) chloroform
2) chloroform:methanol:water (70:30:4)
3) chloroform:methanol:0.25% KCl (60:35:8)
The TLC plate was visualized with iodine vapor.

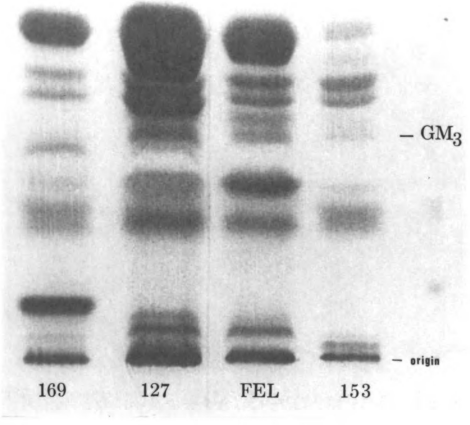


Figure 17.

Figure 18. Human Plasma Gangliosides-II

The plate seen here was the same plate described in Figure 17. The plate was visualized with resorcinol spray reagent for the detection of sialic acid-containing material. The symbol + marked the presence of sialic acid-containing substances in the plasma samples. Lane 1 contained a ganglioside standard ($GM_3$) and lane 2 contained a bovine brain ganglioside mixture.

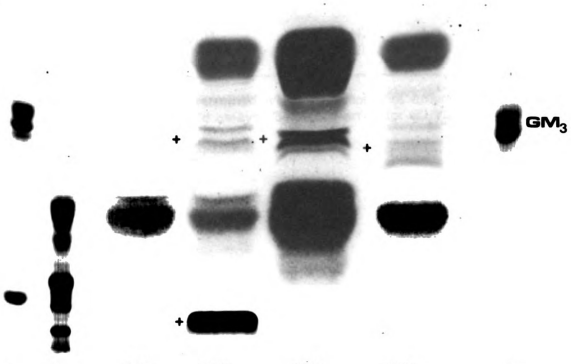


Figure 18.

Table 21. Gangliosides of Normal and FEL Plasmas

| Ganglioside | Nanomoles NeuNAc/ml Plasma<br>Plasma Sample<br>Normal | FEL | 127 | 169 |
|---|---|---|---|---|
| $GM_3$ | 10.1<br>± 6.2<br>n=4<br>range<br>3.7-16.8 | 7.8 | 50 | 6.0 |
| Putative Ganglioside near origin | N.D. | 88 | N.D. | N.D. |

All reported values were determined with the resorcinol colorimetric assay and were the averages of duplicate assays. Standard error for all values was less than 10%.
N.D.= not detectable

patients were similar to the one normal plasma lipid profile seen in Figure 17.

III. Liver and Spleen Oligosaccharides

The possibility of an oligosaccharide storage problem in the liver and spleen of the FEL patient was investigated (Figure 19). Results revealed the presence of mono-, di-, and trisaccharide species in all livers analyzed. Only monosaccharides were seen in all the spleens analyzed. There was no evidence of any abnormal storage of oligosaccharides in either FEL liver or spleen, nor were any sialic acid-containing oligosaccharides present. Free NeuNAc was also not detectable in spleen or liver homogenates, so its contribution to the increased sialic acid levels in FEL liver was negligible. Orcinol-positive spots at the origin of the TLC plate were probably due to glycopeptides or larger oligosaccharide species (greater than 12 glycose units).

Figure 19. Oligosaccharides from Human Liver and Spleen

Lane 1: $GM_3$ ganglioside (100 nmoles)
Lane 2: neuNAc (100 nmoles)
Lane 3: stachyose (tetrasaccaride—50 μg)
Lane 4: FEL liver
Lane 5-7: normal liver
Lane 8: FEL spleen
Lane 9-10: normal spleen

Aliquots (10 μl) from liver and spleen homogenates were spotted. The symbol (+) marked the presence of neuNAc-containing substances. All other spots visualized were carbohydrate-containing substances as determined with orcinol spray reagent.

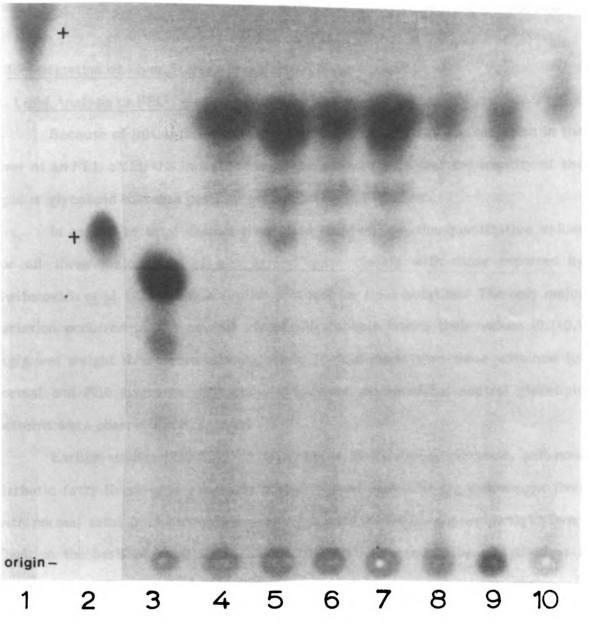


Figure 19.

# DISCUSSION

## I. Identification of Liver Storage Product

### A. Lipid Analysis of FEL Liver and Plasma

Because of initial pathological reports of localized fat accumulation in the liver of an FEL child, the initial focus of the study on FEL was the identity of the lipid or glycolipid that was possibly accumulating in FEL liver.

In regard to total normal liver lipid composition, the quantitative values for all three major lipid classes agreed quite closely with those reported by Kwiterovich et al. (229) using a similar protocol for lipid isolation. The only major variation occurred in the neutral glycolipid amounts where their values (0.1-0.4 mg/g wet weight liver) were approximately 10-fold lower than those obtained for normal and FEL livers in this study. However, no abnormal neutral glycolipid patterns were observed in FEL liver.

Earlier studies (230-231) on total lipids of alcoholic, cirrhotic, and non-cirrhotic fatty livers gave ranges of amounts from 0.08-0.30 g/g wet weight liver with normal total lipid amounts averaging around 0.03-0.06 g/g wet weight liver. Thus, on the basis of total lipid amount, the FEL liver could be classified as a moderate fatty liver at the lower end of the fatty liver range.

In the FEL liver examined, the increase in total lipids was entirely due to the neutral lipids (0.080 g/g wet weight liver) which was at the lower extreme of the range of fatty liver neutral lipids (0.09-0.32 g/g wet weight liver) (231). By comparison, normal ranges of neutral lipids (229,231) were 0.01-0.06 g/g wet

weight liver. Further examination of the FEL neutral lipids revealed 3 features: an almost 2-fold increase in triglycerides; a modest increase in total cholesterol; and a decrease of the percentage of esterified cholesterol to total cholesterol amount from 29% to 2%. Compared with other studies (229,231), the normal cholesterol ester/total cholesterol percentage range was 19-32%. In alcoholic fatty livers, although cholesterol lipids in general were slightly elevated (231), the cholesterol ester/total cholesterol percentage was still normal (28%). Thus, the decreased cholesterol ester percentage seen in FEL fatty liver was unique compared with alcholic fatty livers.

The reasons for the increase in triglycerides and decrease of cholesterol ester percentage will remain unsolved until further studies into the neutral lipids and their metabolism in FEL liver are conducted. One possible explanation for the cholesterol ester decrease in FEL liver is the extensive liver damage and necrosis due to the histiocytic infiltration. Autolysis of the cholesterol esters by intracellular cholesterol esterases due to the liver damage and necrosis may thus occur, resulting in the observed decreased levels of cholesterol esters, even though the total cholesterol level in FEL liver was not appreciably different from that in normal liver.

The low cholesterol ester amount in FEL liver may also be a reflection of the period of time during which the tissue remained in the body after the time of death and before the time of its removal for study and preservation. Approximately 8-9 hours elapsed before the liver was removed from the deceased during which time considerable autolysis may have occurred.

Although neutral lipids in all of the FEL plasmas in Figure 17 and Figure 18 were not studied in detail, all of the FEL plasmas presented there were hyperlipidemic with respect to total lipid content and high phospholipid content as seen in the TLC of total plasma lipids in Figure 17. The phospholipids in this figure

all migrated just above $GM_3$ in the solvent system used, and the neutral lipids migrated with the solvent front (not seen).

Free fatty acids were also investigated in FEL liver but they comprised only about 0.5% of the total fatty acids in normal liver (Table 22) and were similar in composition and quantity to free fatty acids in normal liver.

Table 22. Quantitation of Liver Fatty Acids

| | (μmoles/g liver) Normal | FEL |
|---|---|---|
| Free Fatty Acids (diazomethane methylation) | 0.398 | 0.394 |
| Total Fatty Acids (methanolysis products) | 71.8 | 133.9 |

Fatty acids were quantitated as their methyl esters by GLC as described in Methods.

Neutral glycolipids and phospholipids in the FEL liver were similar in profile and quantity as judged by TLC to normal liver and were not investigated further. Previous studies of phospholipid levels in alcoholic and cirrhotic fatty livers (230-231) did not show any significant elevations compared to normal liver levels. Thus, increases in neutral lipids of fatty livers did not necessitate a concomitant increase of other lipid classes.

Since the only major lipid changes in FEL occurred in the liver neutral lipid fractions and those changes were essentially quantitative and not due to changes in the internal composition, the Folch upper phase lipids were also studied to investigate the possible existence of an abnormally accumulating lipid. From TLC analyses and sialic acid quantitation of the Folch upper phase lipids a large general increase (11-fold) in liver lipid-bound sialic acid (presumably gangliosides) was

noted, predominantly of those gangliosides of higher TLC mobility ($GM_3$-$GM_2$). In these "simpler" gangliosides, the increase was apparently 100-fold. However, if an assumption of the amounts of gangliosides accidentally lost were attributed mainly to $GM_3$ (8 nmoles NeuNAc/g liver), and that $GM_3$ accounted for about 90% of the total gangliosides in normal liver (54,229), then the $GM_3$ increase would only be about 16-fold (8.85 vs. 146 nmoles NeuNAc/g liver). Nevertheless, $GM_2$ still appeared to be elevated by at least 100-fold. It must be noted, however, that the $GM_2$-like product was so identified solely by TLC mobility, and may actually be a heterogeneous mixture of $GM_3$-like gangliosides with different fatty acid constituents, especially of the α-hydroxy type which accounted for the multiplicity of $GM_3$ bands seen in normal human liver by others (54). Thus, the identity of the $GM_2$-like ganglioside, based on TLC comparative studies and not on individual characterization, must be treated as tentative.

Due to the interference from non-resorcinol positive materials in the Folch upper phase lipids during the one-dimensional TLC analysis of total gangliosides, a "ganglioside mapping" technique as described by Iwamori et al. (96) was employed to detect any ganglioside that was unique to the general liver ganglioside profile. This analytical method involved fractionation of the total liver lipid extract by DEAE-Sepharose column chromatography followed by TLC separation of the individual fractions eluted from the anion-exchange column with an ammonium acetate gradient. No unusual pattern was detected in the FEL liver aside from the noted general increase of gangliosides compared to normal liver.

The possibility of a generalized storage of gangliosides existing in other tissues was explored further by investigating the ganglioside levels of other tissues of the FEL patient available. FEL spleen and plasma did not reveal the presence of an abnormal ganglioside pattern, nor was there an increase in the level of any individual ganglioside component, as determined qualitatively by TLC. What was

originally believed to be a large amount of ganglioside with approximately $GD_{1b}$ mobility accumulating in one FEL patient's plasma (labelled "FEL" in Figure 18) turned out to be free sialic acid upon further experiments involving dialysis and GLC analysis of the isolated product. The resorcinol-positive band in this one FEL patient's plasma studied was not present in other FEL plasmas examined. In fact, the ganglioside patterns of other FEL plasmas were essentially normal, although one patient (no. 127) did have an abnormally high $GM_3$ level.

Although phospholipids were not quantitated in the FEL plasmas, it was apparent that all FEL plasmas examined were hyperlipidemic (6) in triglycerides (2 to 3.5-fold increase) and especially in phospholipid levels as seen in Figure 17. The high phospholipid and triglyceride levels and normal ganglioside levels observed in the FEL plasmas could not be readily explained, particularly when FEL liver phospholipid levels were normal, but triglyceride and ganglioside levels were high in FEL liver compared to normal liver. Lipoprotein metabolic dysfunction might be an important factor here.

B. Oligosaccharide Analysis of FEL Liver and Spleen

Oligosaccharides have been observed to accumulate in visceral tissues and urine of patients with $GM_1$ generalized gangliosidosis (225,232), $GM_2$ gangliosidosis (233,234) mannosidosis (235), fucosidosis (236), and the various mucolipidoses (I,II,III) (237-238), all of which are metabolic disorders in which a specific lysosomal glycohydrolase needed for glycoprotein, glycolipid, and mucopolysaccharide catabolism and was proven or presumed deficient. Therefore, FEL liver and spleen oligosaccharides were investigated in order to determine the possibility of an oligosaccharide storage disorder in FEL . No abnormalities in oligosaccharide type or amount were found in FEL spleen or liver, especially sialyl-oligosaccharides or free sialic acid, which might have accounted also for the 11-fold increase of sialic

acid-containing material in FEL liver. The absence of accumulating oligosaccharides would tend to rule out the possibility of FEL as a glycoprotein or mucopolysaccharide storage disease.

Liver mucopolysaccharides in FEL were not examined here since it was thought that any mucopolysaccharide accumulation would also be reflected in oligosaccharide accumulation. In addition, the clinical symptoms of the FEL patients did not match those of any of the known mucopolysaccaridoses, where neurological involvement and facial and skeletal deformities are a hallmark. Exploratory studies of the lysosomal arylsulfatases, which catabolize the sulfated mucopolysaccharides (161), revealed no enzyme deficiencies in the FEL fibroblasts studied. The total arylsulfatase assays were not conclusive, however, due to the non-linear response to protein amount. β-Glucuronidase, another enzyme involved in mucopolysaccharide catabolism, was normal and even elevated in some FEL fibroblasts compared to control fibroblasts. The evidence gathered thus far appears to rule out FEL as a mucopolysaccharidosis or mucolipidosis, although a more detailed look at the liver and urinary mucopolysaccharides and oligosaccharides should be undertaken before a final conclusion is reached.

## II. The Search for an Enzymatic Basis in FEL as a Storage Disease

### A. Lysosomal Enzymes in Liver, Leukocytes, and Fibroblasts

The lysosomal enzymes were studied to determine whether FEL was a new complex glycoconjugate storage disease with a classical lysosomal enzyme deficiency. The results in this work so far have shown no clear deficiency of any of the selected lysosomal enzymes tested, although β-galactosidase activity was apparently low in the FEL liver. The decreased β-galactosidase activity, however, was not seen in leukocytes and fibroblasts of the proband's relatives and other FEL patients. Leukocytes or fibroblasts of the main FEL patient of this study were not

available for comparison, so a generalized decrease of β-galactosidase activity in all tissues could not be determined. Initial studies on the FEL spleen did reveal some decrease in β-galactosidase activity (56%) compared to normal spleens, but the reduction was not as significant as in the FEL liver. As a whole, there appeared to be no conclusive evidence to support a hypothesis of generalized β-galactosidase deficiency in FEL.

In the comparison of normal and FEL liver lysosomal enzymes, only one normal liver preparation was used due limited availability at that time. Although one normal liver did not make a solid statistical basis for comparison, it served as an approximate gauge of normal levels of activity. FEL values within 50% of the normal liver enzyme activities were considered within the normal range variation. With the exception of β-galactosidase, α-N-acetylgalactosaminidase, and α-neuraminidase, all lysosomal enzyme activities that were tested in the normal and FEL liver were within literature values for normal controls (172,180,239-240). Literature values could not be found for normal liver α-neuraminidases, assayed by the method used here, or for α-N-acetylgalactosaminidase. Human liver α-galactosidase B isoenzyme has been shown to hydrolyze equally well α-galactoside and α-N-acetylgalactosaminide residues at the non-reducing terminal end of oligosaccharides (241), so the α-N-acetylgalactosamindase activity measured in these assays was probably a reflection of liver α-galactosidase activity. Liver lysosomal β-galactosidase levels as determined by others (176,242-243) were quite varied, (262 to 777 nmoles substrate cleaved/hr/mg protein or 34,800 to 97,200 nmoles substrate cleaved/hr/g wet weight liver) and demonstrated the need for the inclusion of normal control tissues in the clinical enzymatic evaluation of pathological tissue regardless of the uniformity of the assay procedure. Given the wide range of β-galactosidase levels, there was a distinct possibility that FEL liver β-galactosidase activity fell just in the lower end of the normal range and was,

therefore, not significantly deficient. The absence of a β-galactosidase decrease in other FEL leukocytes and fibroblasts indicated that the β-galactosidase deficiency seen in the one FEL liver under study might have been unique or perhaps localized only in the liver and that it was not a key genetic metabolic defect in FEL.

Heat inactivation studies by Ho and O'Brien (244) on β-galactosidase activities in Hurler's Syndrome, a mucopolysaccharidosis, demonstrated the absence of a heat-labile isoenzyme which accounted for the decrease of activity presented there. There was no analogous missing isoenzyme form in FEL liver, as both normal and FEL liver β-galactosidase activities were reduced by an equal percentage.

Exogenous sialic acid at concentrations equal to or exceeding the concentration found in FEL liver were added to normal and FEL liver homogenates to determine whether sialic acid itself would inhibit β-galactosidase activity. While no inhibition of activity was found, the possibility existed of some other unknown storage product in FEL which could cause enzyme inhibition. In the enzymatic studies of two mucopolysaccharidoses, Hunter's Disease and Sanfilippo Type B, with observed decreased β-galactosidase activity (19-28% of normal), Kint et al. (176) demonstrated that the lowered β-galactosidase levels were due to the tight binding of exogenous chondroitin sulfate to normal liver β-galactosidase, causing enzyme inhibition (50-60%) as well as an alteration in the enzyme's isoelectric point to that seen in Hunter and Sanfilippo liver β-galactosidase. Nevertheless, results of mixing experiments with normal and Hunter or Sanfilippo liver homogenates failed to reveal the presence of a soluble, dissociable inhibitor. In analogous experiments conducted in FEL liver, mixing experiments also demonstrated the absence of a soluble, dissociable inhibitor, and cellulose acetate electrophoresis of FEL β-galactosidase revealed no alteration in electrophoretic

mobility of FEL β-galactosidase, indicating that there was no similar binding of a mucopolysaccharide-like inhibitor to the enzyme which might increase the net negative charge on the enzyme.

β-Galactosidase levels as determined by synthetic substrates were deficient in a number of mucopolysaccharidoses (Types I, II, III) (175,244-246) and mucolipidoses (177-179). However, subsequent studies have shown that the mucopolysaccharidoses and mucolipidoses have different primary lysosomal enzyme defects, and that the apparent β-galactosidase deficiency was secondary (180-181,247). In FEL liver, the decreased β-galactosidase level might similarly be a secondary enzyme defect, and not the primary genetic mutation.

α-Neuraminidase, a key enzyme involved in ganglioside catabolism, was not appreciably decreased in FEL liver. However, the possibility of a defective or deficient neuraminidase isoenzyme specific for ganglioside substrates cannot be ruled out, since only synthetic substrates were used in the neuraminidase assay.

Currently, no human disease has been attributed to specific deficiency of glycolipid neuraminidase activity. Fibroblast and leukocytic neuraminidase deficiencies have been reported in various inborn metabolic disorders collectively termed the "sialidoses", which cover the mucolipidoses (164,166,178,237) and cherry red spot-myoclonus syndrome (248-249). In all of these inherited disorders, sialyl-oligosaccharides accumulate in fibroblasts and are secreted in large quantities in urine. Although a ganglioside storage problem might be expected to occur in these disorders, only normal ganglioside patterns and levels have been found in autopsied tissues (168). As a result, no defects in ganglioside catabolsim have been attributed to the sialidoses. Recent studies by Suzuki and Fukuoka (167) have demonstrated normal α-neuraminidase activity in autopsied tissues from patients, suggesting that either neuraminidase was not the primary metabolic enzyme defect, or the deficiency was only limited to certain tissues.

Heat-stable, glycoprotein activators of $GM_1$ β-galactosidase and $GM_2$ β-hexosaminidase A have recently been discovered in human liver and were partially characterized (152-153,250-251). These activators have been shown to be required for the in vitro enzymatic hydrolysis of $GM_1$ and $GM_2$ ganglioside in the absence of exogenous bile salt detergents. One might speculate on the possibility that the accumulation of gangliosides in FEL might be due to the absence of such an activator of ganglioside catabolism in vivo.

B. Anabolic Defects in FEL

A novel gangliosidosis was recently reported by Fishman et al. (252-253) in which an anabolic enzyme, UDP-GalNAc:$GM_3$ N-acetylgalactosaminyltransferase, was deficient. $GM_3$ and, to a lesser extent, $GD_3$ levels in brain and liver were elevated 4-fold above normal with a resultant absence of the higher ganglioside homologs. This gangliosidosis has been the only instance found where a lysosomal storage disease was caused by a defect in an anabolic enzyme. In investigating a similar anabolic defect in FEL, preliminary studies were conducted with CMP-NeuNAc sialyltransferase assays using lactosylceramide and bovine brain gangliosides ($GM_1$, $GD_{1a}$, $GT_1$) as acceptors. Although no significant differences were seen in sialyltransferase activities of FEL liver compared to normal liver, the activities were extremely low and non-linear with protein concentration. The results were, therefore, not considered a conclusive indicator of ganglioside sialyltransferase activities in liver. It must be noted that brain gangliosides were used as acceptors in these assays and not endogenous liver gangliosides, which have been shown to contain different carbohydrate sequences (lacto and lactoneo series) as well as external ketosidic linkage positions for the NeuNAc residues. Neural gangliosides have their NeuNAc residues on the internal portions of their carbohydrate chains. The inability of liver sialyltransferases to recognize brain

ganglioside substrates as acceptors was a definite possibility.

Studies with other glycosyltransferases and endogenous ganglioside acceptors were left for future work, after isolation of sufficient amounts of endogenous liver gangliosides for study and characterization.

## III. Immunosuppressive Activity and Structure of Liver Gangliosides

Immunological abnormalities have been demonstrated in patients with a variety of liver diseases of viral or toxic origin (254-256). These abnormalities occurred both in the humoral as well as in the cell-mediated immune systems and were not specific to any particular liver disease. Circulating plasma inhibitors of mitogen-stimulated lymphocyte blastogenesis were described and partially characterized by Wands and Dienstag (257) and Nakao et al. (258) to be anionic in nature with a molecular weight around 270,000 daltons and electrophoretic mobilities similar to the α-globulins.. A similar substance which also inhibited PBL blastogenic response response to allogeneic cells was found in the aqueous extracts of normal liver by Chisari (259) and Schumacher et al. (260). Their partially purified inhibitor had a molecular weight of approximately 65,000, was heat-labile, and had the electrophoretic mobility of a beta- or gamma-globulin. The inhibitor was not believed to be identical to the lipoprotein inhibitor of Curtiss and Edgington (188) due to the absence of cholesterol and triglyceride. The characterization of these inhibitors has not been fully completed due to the problems of impurities and heterogeneity of the inhibitors with respect to molecular weight and electrophoretic mobility. The possibility of the presence of a water-soluble glycolipid, such as a ganglioside, in these inhibitor preparations could not be ruled out. These results have established a firm connection between liver dysfunction and immune abnormalities, and have revealed possible mechanisms by

which the liver might regulate the immune response and the observed immune abnormalities.

Because of the reported association of FEL with an immunological deficiency syndrome which included cellular and humoral immunity defects and a plasma inhibitor of in vitro lymphocyte blastogenesis (6), immunological studies were conducted with the lipid components that were found to have the highest relative accumulation in FEL liver, namely, the lipid-bound sialic acid compounds (presumably gangliosides). Initial PBL blastogenesis inhibition studies (7) with the FEL liver ganglioside-enriched Folch upper phase lipids (at concentrations of 2 μg/ml) revealed an approximate 75% reduction in Con A mitogen responsiveness and an even more marked 98.5% reduction in responsiveness to SKSD antigen. As a result, investigations were directed toward the isolation of the individual FEL liver gangliosides to identify the specific ganglioside or ganglioside fraction that was responsible for the inhibitory activity. Normal liver gangliosides were also investigated to determine whether the immunosuppressive ganglioside was present as a naturally-occurring component of liver gangliosides.

The isolation methods for liver gangliosides described in the Methods section yielded ganglioside fractions that were free of proteins, neutral lipids, neutral glycolipids, sulfatides, and phospholipids. Of these fractions that were tested for immunosuppressive activity, one fraction (H) in both FEL and normal liver and one fraction (L) unique to normal liver were found to be immunosuppressive. The fractions H derived from both livers were of similar TLC mobility and the fraction L derived from normal liver appeared to contain a more complex structure (possibly more sialic acid residues) due to its lower TLC mobility.

Characterization of these immunosuppressive gangliosides (Table 19 and Table 20) is only tentative due to the low amounts that were available for analysis. With only approximately 5-6 nmoles (quantitated on the basis of sialic acid) of the

fractions H and 44 nmoles of fraction L, the sensitivity of the GLC method was stretched to its extreme limits of detection. As a result, only GLC-MS analysis of the trimethylsilylated methylglycosides (261-263), the trimethylsilylated methyl ketosides of NeuNAc (264), and the fatty acid methyl esters (265) could definitely confirm the identity of the methanolysis products by means of mass chromatography of specific ions and the mass spectra of the GLC peaks observed.

A list of the ion masses used for mass chromatographic identification of sub-microgram quantities in the liver immunosuppressive substance characterization studies is presented in Table 23.

Table 23. Ion Masses Used For Mass Chromatographic Analysis

| m/e | Diagnostic for: |
|---|---|
| 73 | any trimethylsilyl derivative |
| 420 | NeuNAc trimethylsilyl derivative |
| M-90 (392 for neutral sugars) (304 for fucose) | trimethylsilyl methyl glycosides |
| M-31 (267 for stearic acid) | normal fatty acid methyl esters |
| 87 | " " |
| 90 | α-hydroxy fatty acid methyl esters |
| 103 | " " |

The fatty acid composition of the analyzed liver gangliosides was less diverse than those found by Seyfried et al. (54), who found, in addition to the fatty acids seen in this study, components of the type C23:0, C24:1, and α-hydroxy fatty acids from C20:0 to C24:0. In fact, they found that the α-hydroxy fatty acid content in liver gangliosides was 43% of the total hematosides in liver. No α-hydroxy fatty acids were detected in this study even with the aid of mass chromatographic analysis of the GLC-MS data. Fatty acid analyses in other studies

of the more complex gangliosides in liver and other extraneural tissues (42,145) have shown a large predominance of C16:0, C22:0, C24:0, and C24:1 fatty acids with only minor traces of the hydroxylated forms. The absence of α-hydroxy fatty acids and the longer chain normal fatty acids might be explained by the extremely low amounts of ganglioside analyzed, so that only the major fatty acid components could be detected. The predominance of stearic acid (C18:0) in the fatty acid components of brain $GM_1$ ganglioside was typical for fatty acid patterns in other neural gangliosides.

The possibility of other carbohydrate moieties present in the gangliosides analyzed, such as fucose, was minimal as combined GLC-MS analysis of the carbohydrates revealed no presence of either fucose or N-acetylgalactosamine. Comparison of the carbohydrate data with that of bovine brain ganglioside $GM_1$, revealing similar molar ratios for the trimethylsilylated sugars analyzed, indicated that the liver gangliosides probably had a lactotetraose or lactoneotetraose core sequence similar to that reported by Wiegandt (266). The positions of the NeuNAc residues on the FEL and normal liver gangliosides were not determined here, but their positions on the oligosaccharide moiety of the glycosphingolipid would be very important. An internal NeuNAc linkage on the oligosaccharide would stress the importance of a exo-β-galactosidase deficiency, while an external NeuNAc position on the non-reducing terminal galactose of the tetrasaccharide core structure would highlight the importance of a deficient neuraminidase as the enzymatic basis for the ganglioside accumulation seen in FEL liver.

Proposed structures of the monosialoganglioside (fractions H) with the possible NeuNAc ketoside linkages marked with parentheses would be:

Gal→GlcNAc→Gal(←NeuNAc)→Glc→Cer

or NeuNAc→Gal→GlcNAc→Gal→Glc→Cer

Proposed structures for the disialoganglioside (fraction L) would be:

NeuNAc→NeuNAc→Gal→GlcNAc→Gal→Glc→Cer

or NeuNAc→Gal→GlcNAc→Gal(←NeuNAc)→Glc→Cer

The assignment of the NeuNAc residues are based on known structures of glucosamine-containing gangliosides (Table 3). No internally-linked NeuNAc residues have yet been discovered in human extraneural lactoneo series gangliosides.

It must be remembered that the ganglioside structure assignment is only tentative. The sialylparagloboside structure assigned to fraction H cannot really be possible if judged by its TLC mobility, which suggests a disialo structure or possibly a lactoneohexaglycose core sequence for the ganglioside. The ganglioside fractions may also be a heterogeneous mixture of gangliosides with similar TLC mobilities. Further purification of the ganglioside fractions may eventually be required. Upon isolation of sufficient amounts for further study, the positions of NeuNAc residues on the oligosaccharide moiety can be determined either by mass spectral analysis of the intact permethylated glycolipid and/or susceptibility of the gangliosides to V. cholera or C. perfringens neuraminidases which would only hydrolyze the external (on the non-reducing terminus of the oligosaccharide moiety) NeuNAc residues.

There was a possibility of a contaminant in the isolated liver fractions, albeit a minor one, following the purification procedures in this study. Free sialic acid or neutral monosaccharides migrate to about the position of standard $GT_{1b}$ in the TLC solvent system employed. Larger oligosaccharides or glycopeptides would not move above the origin. Although the liver immunosuppressive fractions were not homogeneous, as seen by TLC analysis (Figure 12 and Figure 13), they did appear to contain only gangliosides, as only resorcinol-positive bands were visible.

Evidence for the identification of the liver immunosuppressive lipid components as gangliosides so far rests in their chromatographic behavior on silicic

acid column chromatography and thin-layer chromatography, and their retention on a DEAE-Sephadex anion exchange column, which indicates their acidic properties. Their solubility in chloroform:methanol (1:1), chloroform:methanol:water (30:60:8), and methanol point out their amphipathic nature as polar, water-soluble lipids, and their positive reaction with resorcinol identifies their sialic acid components. Subsequent studies with the effect of neuraminidase on the putative ganglioside fractions in regard to their TLC mobility and immunosuppressive activity should verify their tentative identification. Future work in the identification of the immunosupressive gangliosides will depend on obtaining sufficient amounts of both normal and FEL liver gangliosides. With sufficient material, methylation studies, total carbohydrate and sphingosine analysis, and anomeric studies of the carbohydrate linkages could be conducted to elucidate the structure of the immunosuppressive glycolipid.

IV. Identification of the FEL Plasma Immunosuppressive Factor

Since essentially all circulating gangliosides and neutral glycosphingolipids in plasma were shown to be intimately associated with lipoproteins (267-269), the hypothesis that the inhibitory activity associated with LDL could be accounted for by the ganglioside components (7) was tested by isolation of the ganglioside-enriched Folch upper phase plasma lipids and performance of the PBL mitogenic inhibition assay with the isolated lipids quantitated on the basis of sialic acid at 2, 5, and 10 μg/ml ganglioside concentration. Complete inhibition of the PBL mitogenic response to PHA, Con A, and PWM was found at 5 and 10 μg/ml, but only slight (1%) inhibition was seen at 2 μg/ml. A comparison of the work of Curtiss and Edgington (188) with whole intact LDL as inhibitor showed that if their inhibitory activity was based on the known concentration of ganglioside in LDL, the

effective inhibitory concentration of the gangliosides would be comparable to the studies performed with the lipoprotein-derived ganglioside fractions isolated in this study. These results with the Folch upper phase lipoprotein lipids could only be seen as preliminary due to the presence of a multitude of other lipids that also could be partitioned into the Folch upper phase; namely, phospholipids, sulfatides, neutral glycosphingolipids with three or more carbohydrate units length, and even small quantities of neutral lipids. Non-lipid materials such as nucleotides or small oligosaccharides could also be present. More definitive answers to the role of plasma gangliosides would depend on their isolation from other lipids and non-lipids, especially with improved microscale methods (105), which would be useful with pathological samples of limited quantity.

Preliminary investigations (7) into the nature of the FEL plasma inhibitor were conducted by isolation of the ganglioside fractions and the other lipids by preparative TLC (Figure 17), and testing their immunosuppressive activities at their equivalent concentrations in plasma before isolation. Initial results were promising as only the strong resorcinol-positive band corresponding to $GD_{1b}$ in the TLC analysis (from the plasma lipids labeled "FEL" in Figure 18) had immunosuppressive activity. The isolated neutral lipids were highly cytotoxic.

Further studies on the nature of the plasma inhibitor were attempted with permethylation analysis of the intact inhibitor for carbohydrate sequence determination, and with tests on the effect of specific exo-glycosylhydrolase treatment on inhibitory activity. These studies are still in progress.

Further purification attempts on the FEL plasma inhibitor revealed the putative $GD_{1b}$-like ganglioside to be free sialic acid, a finding confirmed by GLC analysis and the dialysability of the resorcinol-positive band. There was a small possibility that a ganglioside was present which co-migrated with free sialic acid. If this were true, the potency of the inhibitory factor would be greater than

originally believed. However, none of the other FEL plasmas analyzed (no. 127 and 169 in Figure 18) showed any detectable resorcinol-positive bands analogous to the one seen in the original FEL plasma (labeled "FEL" ) studied. Therefore, the identity of a ganglioside as the FEL immunosuppressive factor was still uncertain. The possibility exists that the putative plasma immunosuppressive ganglioside normally is present in nanogram or picogram quantities in plasma and is elevated to a barely detectable level in FEL plasma or liver as a result of the observed liver ganglioside accumulation. The elevated amount of the immunosuppressive ganglioside might just exceed the threshold level at which the ganglioside is immunosuppressive. The ganglioside may even be macrophage-derived and may be elevated to the threshold level due to the abnormal proliferation of histiocytes observed in this disorder. Future work will be directed at the study of macrophage gangliosides and the comparison of those gangliosides to those observed accumulating in FEL liver, particularly to the liver immunosuppressive ganglioside fractions.

An important note should be made here that free sialic acid was not immunosuppressive, indicating that the inhibitory activity was authentic even though the identity of the factor was unknown. In Figure 17, a strong iodine-positive band was seen of comparative TLC mobility to the sialic acid-containing band near the origin. This band was common to all the FEL plasmas analyzed and was co-purified along with the sialic acid band in the one FEL plasma tested for inhibitory activity. One might speculate that this band might be the inhibitory factor. Comparative studies of the immunosuppressive activity of isolated gangliosides and other lipids from other FEL plasmas and normal plasmas are still in progress.

PBL mitogenesis inhibition tests were also conducted with purified glycosaminoglycans. Keratin sulfate, chondroitin sulfate, and heparan sulfate had

no inhibitory activity at equivalent molar concentrations to sialic acid in the FEL plasma.

Recent work by others has demonstrated the presence of an uncharacterized immunosuppressive factor or factors in normal plasma (270-278), hyperlipidemia Type IV plasma (186), and more specifically in the plasma β-lipoproteins (186,188,279). Although some of these studies have tentatively identified the factor as an anionic, large-molecular weight protein with α- or β-globulin electrophoretic mobility, the exact nature of the inhibitory factor was still unclear due to the wide variance of biochemical properties attributed to it, as well as to the diverse methods of its isolation. As a result, the common identity or relationship of all these factors reported would be extremely difficult to ascertain.

Admittedly, the connection between plasma or LDL ganglioside and the immunosuppressive factor is currently circumstantial, and the connection between the FEL plasma inhibitor and ganglioside even more tenuous. However, the results obtained thus far justify further investigations of the immunoregulatory properties of plasma gangliosides as well as the liver gangliosides, which are the probable source of plasma gangliosides (271).

# SUMMARY

Biochemical studies were conducted on the autopsy liver and plasma of a child afflicted with the hereditary disorder, Familial Erythrophagocytic Histiocytosis. Lipid accumulation was found in localized regions of the liver as well as in the infiltrating macrophages. Humoral and cell-mediated immunity defects were noted, and a circulating immunosuppressive factor was found in the hyperlipidemic plasma. Studies were carried out to identify the accumulating lipid material in the liver and to ascertain its relationship to the plasma immunosuppressive factor.

Lipid analysis of the FEL liver revealed a mild fatty liver condition with a 2-fold increase in triglyceride levels along with a marked decrease in the ratio of cholesterol esters to free cholesterol. No abnormalities in the neutral glycolipid or phospholipid levels or patterns as determined by TLC were observed. High lipid-bound sialic acid levels, with over an 11-fold increase over the levels of a comparative normal liver were the most striking feature found in the FEL liver. Although a generalized increase of all ganglioside species was found, a major proportion of the gangliosides consisted of the "simple" $GM_3$ and $GM_2$ type. Preliminary analyses of spleen and plasma showed no concomitant increase in gangliosides even though the plasma was hyperlipidemic.

Investigations into other possible sources of the accumulating material were conducted and no accumulation of oligosaccharides or free sialic acid was found in liver. Mucopolysaccharides or glycopeptides in liver were not investigated as the occurrence of a mucopolysaccharidosis or glycopeptide storage disease was unlikely in FEL as presented here.

To check the possibility of a lysosomal storage disease with a specific glycosylhydrolase deficiency, numerous liver lysosomal enzymes were assayed. Of all the lysosomal enzymes tested, only β-galactosidase activity was found to be decreased to 25% of a comparable normal liver level. Studies on the nature of the reduced β-galactosidase activity revealed the absence of a soluble enzyme inhibitor. No alterations were observed in the electrophoretic pattern of the enzyme and in the pH optimum of activity, nor was a residual heat-stable isoenzyme form responsible for the low enzyme activity level seen in the FEL liver. The evidence pointed to a decrease in activity of all liver lysosomal β-galactosidase isoenzyme forms, although the decrease was not as marked as those observed in $GM_1$ gangliosidosis, a lysosomal storage disease with a characterized β-galactosidase deficiency. The low β-galactosidase activity measured in FEL liver was probably not the primary metabolic defect in FEL as fibroblasts and leukocytes from other FEL patients did not exhibit a similar decrease in enzyme activity. No lysosomal enzyme was seen to account for the localized increase of liver gangliosides thus far.

Studies on the inhibition of lymphocyte mitogenesis were conducted with isolated liver gangliosides to determine whether the accumulated gangliosides could account for the observed immune deficiency syndrome in FEL. Two ganglioside fractions from normal liver and one fraction from FEL liver were found to have immunosuppressive activity. The ganglioside immunosuppression was unique from the work of others in that the effective levels were lower than reported elsewhere, and in that only specific antigen-stimulated lymphocyte proliferative responses were inhibited by the gangliosides. Plant mitogen stimulation of PBL proliferation was unaffected.

Partial characterization of the gangliosides identified a common monosialo-ganglioside as the FEL and normal liver immunosuppressive factor and a

unique disialoganglioside in normal liver with either a lactoneotetraose or lactotetraose sequence for the core oligosaccharide moiety of the glycosphingolipid. However, the structure assignment is only tentative as the ganglioside fractions have not been shown to be homogeneous and the amounts available for characterization were suboptimal. Characterization of the plasma immunosuppressive factor is still in progress, but inhibitory activity could be demonstrated in the plasma lipid extracts.

The biochemical basis for FEL is still undetermined and appears to be rather complex in that neutral lipid metabolism, glycolipid metabolism, and immune dysfunction might all be somehow inter-related in the pathogenesis of this inherited disorder. Although the etiology of this disease has not been elucidated, important information in the identification of glycolipids in immunoregulation has been obtained. This is the first known demonstrable instance of possible human visceral ganglioside involvement in immunosuppression, as well as in the possible pathogenesis of an immune dysfunction. The results from this study join the ever-mounting evidence of others of an important biological role for glycosphingolipids in the regulation of mammalian immunological systems.

# APPENDICES

# APPENDIX A

## Case Presentation of the FEL Patient

The patient was a two-year-old boy who presented the classic symptoms of the disorder. These symptoms were recurrent fever, anorexia, anemia, hepatosplenomegaly, abnormal liver function, hyperlipidemia, and prominent erythrophagocytosis in the bone marrow and lymphoid tissues. Widespread tissue infiltration by morphologically normal cells of the macrophage series was also noted. Serum-glutamate-oxaloacetate-transaminase (SGOT), serum-glutamate-pyruvate-transaminase (SGPT), and serum-lactate-dehydrogenase (LDH) were elevated, and serum-haptoglobulin was low. Fibrinogen level was low and the fasting plasma triglyceride level was 420 mg/dl. Absolute leukocyte counts were normal. Total immunoglobulin levels were normal, but antibody titers after primary immunizations to various bacterial antigens and toxoids were abnormally low. Restoration of normal antibody titers to bacterial toxoids was achieved following booster immunizations. The immunological findings have been reported elsewhere (6). At the time of death, the patient reportedly had both bacterial infection (sites unknown) and evidence of liver damage (7). Summaries of the liver biopsy report, autopsy report, and clinical records, which are all available at the National Cancer Institute, were communicated to the author for the purpose of this investigation (7).

APPENDIX B

# [6] Analysis and Structural Characterization of Amino Sugars by Gas–Liquid Chromatography and Mass Spectrometry

By Clifford G. Wong, Sun-Sang Joseph Sung, and Charles C. Sweeley

*Department of Biochemistry, Michigan State University, East Lansing, Michigan*

## Introduction

Amino sugars are important components in the oligosaccharide structures of glycoproteins, glycosphingolipids, mucopolysaccharides, bacterial peptidoglycans, lipopolysaccharides, and antibiotic substances and in the free oligosaccharides of urine and milk. Within the past ten years, combined gas-liquid chromatography–mass spectrometry has become practical for the sub-microgram-scale identification and characterization of these complex carbohydrates. In this chapter are presented the retention behavior of several kinds of amino sugar derivatives on gas-liquid chromatography (glc) and the major ions produced from these substances by electron impact ionization mass spectrometry (ms).

The three most common types of derivatization for carbohydrates are acetylation, methylation, and trimethylsilylation. Although the preparation of acetyl derivatives of monosaccharides is a simple technique, there are a few complications. When dealing with alditols produced by borohydride reduction of sugars, borate complexes are formed and can interfere with the acetylation reaction (*1*). Thus, it is important to remove borate prior to the acetylation step. Another problem may arise in the possible decomposition of sugar acetates on the column, as reported by Bishop *et al.* (*2*), Perry (*3*) and Gunner *et al.* (*4*). Stellner *et al.* (*5*) have reported very poor recoveries of their partially methylated hexosaminitol acetates due to the inherent design of individual glc-ms models.

Trimethylsilylation of sugars, as reported by Sweeley *et al.* (*6*), is a simple and rapid method for derivatization. However, it must be kept in mind that the treatment of hexosamine hydrochlorides with trimethylchlorosilane and hexamethyldisilazane in pyridine will not yield silyl derivatives of the amino



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groups. However, use of *N,O*-bis(trimethylsilyl)acetamide (BSA) (*7*, *8*) or *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (*22*, *23*) as the trimethylsilylating reagent has been shown to effectively trimethylsilylate all functional groups.

Methylation is an important method for structure elucidation of complex polysaccharides (*9*). A convenient method for methylation of carbohydrates, giving very high yields of permethylated derivatives, has been developed by Hakomori (*10*, Vol. VI [64]). Complete methylation of all accessible functional groups, including the *N*-acetamido groups, can be accomplished in one step. The oligosaccharide products themselves have been analyzed by mass spectrometry up to molecular weights approaching 2,000. Subsequent acetylation of the acid-hydrolyzed and reduced alditols yields the partially methylated alditol acetates. Separation of these substances on an ECNSS-M GC column and MS analysis of the eluted components gives structural information of a polysaccharide in regard to glycosidic linkages and carbohydrate composition (*9*). The individual partially methylated alditol acetates are identified not only by their glc retention times, but also by their characteristic fragmentation patterns on the mass spectrometer.

## Procedures

### Equipment

Relative retention times of the various sugars reported here were determined with a Hewlett-Packard F & M Model 402 gas chromatograph equipped with a hydrogen flame ionization detector. The carrier gas was nitrogen, with flow rates between 40 and 50 ml/min. Glass columns containing various packings were 6 ft × 2 mm (i.d.).

The GC–MS runs were performed on an LKB 9000 gas chromatograph–mass spectrometer, interfaced to a PDP 8/e minicomputer (Digital Equipment Co., Maynard, Mass.) for data compilation and analysis. The mass spectrometer was operated at 70 eV with an accelerating voltage of 3.5 kV and an ion source temperature of 290°. The helium carrier gas flow rate was approximately 30 ml/min. Coiled glass columns were 6 ft × 2 mm (i.d.).

#### *2-Acetamido-2-deoxyalditols and 2-Amino-2-deoxyalditols*

In separate one-dram vials with Teflon-lined screw-caps, about 2.5 mg of each *N*-acetylhexosamine and hexosamine hydrochloride (Sigma Chem. Co., St. Louis, Mo. and Pfanstiehl Laboratories, Waukegan, Ill.) are mixed with 15 mg of sodium borohydride in 1.5 ml of water. The reaction is allowed to proceed overnight (6 h) at 4° and is stopped by the dropwise addition of

glacial acetic acid until the pH of the solution is acid (pH 2–3) and hydrogen gas can no longer be seen bubbling from the solution. The acidified solutions are then taken to dryness under a stream of nitrogen on a 50° water bath with successive additions of methanol (total volume approximately 20 ml) and evaporation to remove borate completely as the volatile trimethylborate ester. Finally, the dried residues are dissolved in 0.5 ml of water and used as stock solutions for derivatization.

*Methyl 2-Acetamido-2-deoxyglycosides*

Into a Teflon-lined, screw-capped test tube (10 × 1.3 cm) containing 5 mg of the *N*-acetylhexosamine, 3 ml of 0.75N anhydrous methanolic HCl (Vol. IV [21], Vol. VI [69], Vol. VII [34]) is added; and the mixture is heated at 80° for 3 h. Losses of solvent from leaky caps are minimized by momentarily loosening the cap after about 10 min heating to reduce the pressure. After methanolysis, powdered silver carbonate is added in small portions to neutralize the reaction mixture (pH 6 by litmus paper test).

For further conversion to *N*-acetyl derivatives, 0.3 ml of acetic anhydride is added to the tubes, and the reaction mixtures are kept at 20°–25° for 6 h. The mixtures are centrifuged; the supernatant fraction is transferred to a one-dram, Teflon-lined, screw-capped vial; and the solvent is removed by a stream of nitrogen. The silver chloride precipitate is washed twice with 2-ml portions of anhydrous methanol (Vol. VII [3]), and the combined supernatants are quantitatively transferred to one-dram vials and dried down under nitrogen. The methyl-2-acetamido-2-deoxyhexosides are redissolved in 1 ml of water and used as standards (5 mg/ml) for subsequent derivatization.

For some biological samples, an incubation time of 18–24 h is preferred for quantitative acid-catalyzed methanolysis.

*Partially Methylated Alditol Acetates*

Permethylation of carbohydrates is done under dry nitrogen by the method of Hakomori (*10*). Hexane is redistilled after refluxing with 20 g/l of barium oxide for 2 h and is stored over sodium. Dimethyl sulfoxide is dried by refluxing with 50 g/liter of barium oxide for 2 h, redistilled, and stored over molecular sieves (Vol. VI [64], Vol. VII [26]). All other solvents are redistilled. A sample (0.9 g of 57% oil emulsion) of sodium hydride (Alfa Inorganics, Beverly, Mass.) is washed 7 times with 15-ml portions of dry redistilled hexane. Dry redistilled dimethylsulfoxide (10 ml) is added and allowed to react at 65°–70° for about 90 min, until the bubbling of hydrogen ceased. The methylsulfinyl ion solution (0.5 ml) is added to a solution of 0.5 g of the sample in 0.5 ml of dimethylsulfoxide, and the mixture is allowed to react for 30 min with periodic sonication. Two ml of redistilled iodo-

methane (methyl iodide) (Pflatz and Bauer, Stamford, Conn.) is then slowly added, and the mixture is allowed to stand for 2 h at 20°–25°. The reaction mixtures are then mixed with 5 ml of chloroform and washed twice with 5 ml of water, once with 5 ml of a 20% solution of sodium thiosulfate ($Na_2S_2O_3$), and three times with water. The organic phases are evaporated to dryness under nitrogen with the aid of absolute ethanol to remove water by azeotropic distillation, and the residues are hydrolyzed in 0.5 ml of 0.5*N* $H_2SO_4$ in 95% acetic acid for 24 h at 85°. Water (0.5 ml) is then added, and heating is continued for an additional 5 h at 85°.

A small column containing 2 ml of Dowex 1X8 anion-exchange resin [acetate form] (50–100 mesh) is used to absorb the sulfate, the permethylated carbohydrates being eluted with 2–3 ml of acetic acid. The hydrolyzate is transferred to a 1-dram vial and evaporated to dryness under nitrogen. Reduction with 0.5 ml of sodium borohydride (10 mg/ml) for 2 h at 20–25° yields the partially methylated alditols. After the addition of several drops of glacial acetic acid, the solutions are dried under nitrogen. Borate is removed as its methyl ester, as described above, using 1–2 drops of acetic acid and 2 ml of methanol and heating in a 50° water bath for 5 min under a stream of nitrogen. Esterification is repeated three more times. The dried sample is acetylated in 0.5–1 ml of acetic anhydride for 60–90 min at 100°. After drying under nitrogen with the aid of toluene, the sample is dissolved in 2 ml of dichloromethane (methylene chloride), washed three times with 1–2 ml of water, redried under nitrogen, and redissolved in 0.5–1 ml of dichloromethane for GC and GC-MS analyses.

Partially methylated glucosaminitol (2-amino-2-deoxy-D-glucitol) acetates may be synthesized from D-glucosamine hydrochloride by the method of Tai *et al.* (*11*).

*Trimethylsilyl Derivatives*

*Reagent I.*—Pyridine (redistilled, stored over KOH), 10 volumes (Vol. II [43], [53], [63], [73]; Vol. IV [73]; Vol. VII [2]). Hexamethyldisilazane (commercial reagent), 4 volumes. Trimethylchlorosilane (commercial reagent), 2 volumes. The reagents are added to a 7-ml, screw-capped test tube with a Teflon-lined cap, mixed, and centrifuged. If moisture is excluded, the derivatizing solution can be used for 1 week.

*Reagent II.*—*N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (Pierce Chemical Co., Rockford, Illinois).

*2-Amino-2-deoxy-O-trimethylsilylhexosides.*—Reagent I (100 μl) is pipeted into dry, 1-dram, Teflon-lined, screw-capped vials containing 125 μg of amino sugar. The mixture is allowed to stand at 20°–25° for 30 min. An

appropriate aliquot (1–3 μl) is injected immediately into the gas chromatograph, for the *N*-trimethylsilyl hexosamine derivatives are present in appreciable amounts after 2 h at room temperature.

*2-Deoxy-2-trimethylsilylamino-O-trimethylsilylhexosides.*—Into 1-dram Teflon-lined screw-capped vials containing 125 μg of amino sugar is added 50 μl of dry pyridine, followed by 50 μl of BSTFA (Reagent II). The sealed vial is heated at 80° for 30 min, and an aliquot is injected into the GC. (Note: *N*-acetyl derivatives do not form any *N*-trimethylsilyl amide under these conditions).

*Acetate Derivatives*

Acetic anhydride (100 μl) and dry pyridine (100 μl) are added to dry, 1-dram, Teflon-lined, screw-capped vials containing 250 μg of amino sugar. The sealed vials are heated at 100° for 4 h, after which 2 ml of redistilled toluene is added and the mixture is dried by evaporation under a stream of nitrogen at 50°. This addition of toluene and subsequent evaporation are repeated once more to ensure the complete removal of acetic anhydride and pyridine. A solution of the acetylated sugar in 200 μl of dry, redistilled methylene chloride is used for GC analysis.

## Results

Tables I–XII are a summary of the relative GC retention times and the major ions found in the mass spectra of each denoted amino sugar. Since stereoisomers and anomers of the carbohydrate derivatives give similar mass spectra, with small differences in peak intensity, the mass spectrum of only one stereoisomer is given.

TABLE I

*Retention Times of the Peracetylated Amino Sugars*

| *Compound* | *Relative retention time*[a] | *Other references* |
|---|---|---|
| 2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucose[b] | 0.36, 2.57 | |
| 2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-galactose[b] | 0.37, 0.43, 2.53, 2.77 | |
| 2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-mannose[b] | 0.46, 2.33, 2.48, 2.90 | |
| 2-Acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-glucitol[c] | 1.76 | (*12*) |
| 2-Acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-galactitol[c] | 2.34 | (*12*) |
| 2-Acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-mannitol[c] | 2.83 | (*12*) |

[a] Retention times are relative to 1,2,3,4,5,6,7-hepta-*O*-acetylperseitol on a column of 3% Poly A-103 on Gas Chrom Q 100/120 mesh (Applied Science Laboratories, Inc., State College, Pa.).

[b] Isothermal at 200°; internal standard retention time was 11 min.

[c] Isothermal at 210°; internal standard retention time was 6.8 min.

TABLE II[a]

*Retention Times of the 2-Acetamido-2-deoxy-O-trimethylsilyl Sugars*

| *Compound*[a] | *Relative retention time*[b] |
|---|---|
| 2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-trimethylsilylglucopyranoside | 0.93, 1.69 |
| 2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-trimethylsilylgalactopyranoside | 1.35, 1.56 |
| 2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-trimethylsilylmannopyranoside | 0.95, 1.27 |
| Methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-trimethylsilylglucopyranoside | 1.31, 1.41,-1.56 |
| Methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-trimethylsilylgalactopyranoside | 1.14, 1.37 |
| Methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-trimethylsilylmannopyranoside | 0.94, 1.60 |
| 2-Acetamido-2-deoxy-1,3,4,5,6-penta-*O*-trimethylsilylglucitol | 1.69 |
| 2-Acetamido-2-deoxy-1,3,4,5,6-penta-*O*-trimethylsilylgalactitol | 1.77 |
| 2-Acetamido-2-deoxy-1,3,4,5,6-penta-*O*-trimethylsilylmannitol | 1.88 |

[a] Derivatized with either Reagent I or II.

[b] Retention times are relative to 1,2,3,4,5,6-hexa-*O*-trimethylsilylmannitol (10.5 min) on 3% SP 2100 on Supelcoport 80/100 mesh (Supelco, Inc., Bellefonte, Pa.) at column temperature of 180° isothermal.

TABLE III

*Retention Times of the 2-Amino-2-deoxy-O-trimethylsily Alditols*

| *Compound* | *Relative retention time*[a] |
|---|---|
| 2-Amino-2-deoxy-1,3,4,5,6-penta-*O*-trimethylsilylglucitol[b] | 1.22 |
| 2-Amino-2-deoxy-1,3,4,5,6-penta-*O*-trimethylsilylgalactitol[b] | 1.18 |
| 2-Amino-2-deoxy-1,3,4,5,6-penta-*O*-trimethylsilylmannitol[b] | 1.23 |
| 2-Deoxy-2-trimethylsilylamino-1,3,4,5,6-penta-*O*-trimethylsilylglucitol[c] | 0.92 |
| 2-Deoxy-2-trimethylsilylamino-1,3,4,5,6-penta-*O*-trimethylsilylgalactitol[c] | 0.91 |
| 2-Deoxy-2-trimethylsilylamino-1,3,4,5,6-penta-*O*-trimethylsilylmannitol[c] | 0.92 |

[a] Retention time relative to 1,2,3,4,5,6-hexa-*O*-trimethylsilylmannitol (10.4 min) on 3% SP 2100, Supelcoport 80/100 mesh (Supelco, Inc., Bellefonte, Pa.) at column temperature of 180° isothermal.

[b] Derivatized with trimethylsilylating Reagent I.

[c] Derivatized with trimethylsilylating Reagent II.

TABLE IV

*Retention Times of the Partially O-Methylated 2-N-methylglucosaminitol Acetates*

| *Position of O-$CH_3$ groups* | *Relative retention time*[a] |
|---|---|
| 3, 4, 6 | 1.00 (retention time = 8.3 min) |
| 3, 6 | 1.68 |
| 3, 4 | 2.19 |
| 4, 6 | 2.51 |
| 3 | 2.91 |
| 4 | 3.64 |
| 6 | 4.51 |

[a] Isothermal at 190°, 3% OV-210 on Supelcoport 80/100 mesh (Supelco, Inc., Bellefonte, Pa.).

TABLE V

*Major Fragment Ions Observed in the Mass Spectrum of 2-Acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-D-galactopyranose (MW = 389)*[a]

| *m/e* | *Relative Intensity* |
|---|---|
| 43 | 100.0 $[CH_3CO]^+$ |
| 72 | 10.9 |
| 84 | 10.4 |
| 97 | 6.9 |
| 108 | 3.3 |
| 110 | 0.9 |
| 114 | 48.9 |
| 126 | 9.1 |
| 139 | 13.6 |
| 144 | 1.3 |
| 150 | 4.7 |
| 156 | 19.7 |
| 168 | 6.6 |
| 181 | 7.2 |
| 198 | 5.0 |
| 199 | 6.7 |
| 210 | 1.8 |
| 241 | 14.3 |
| 330 | 2.6 ($M^+$-59) |
| 346 | 1.5 ($M^+$-43) |

[a] References *13* and *14* give detailed descriptions of fragmentation pathways and identifications of ions.

TABLE VI

*Major Fragment Ions Observed in the Mass Spectrum of 2-Acetamido-1,3,4,5,6-penta-O-acetyl-2-deoxy-D-glucitol (MW = 433)*[a]

| *m/e* | *Relative intensity* |
|---|---|
| 43 | 100.0 $[CH_3CO]^+$ |
| 60 | 21.4 |
| 84 | 73.8 |
| 85 | 20.3 |
| 102 | 22.9 |
| 114 | 8.9 |
| 115 | 7.9 |
| 126 | 15.1 |
| 139 | 12.5 |
| 144 | 23.0 ($M^+$-289) |
| 145 | 7.7 |
| 151 | 9.4 |
| 156 | 8.6 |
| 157 | 4.8 |
| 168 | 7.4 |
| 216 | 1.5 |
| 217 | 1.4 |
| 288 | 0.3 |
| 289 | 0.4 |
| 318 | 8.3 ($M^+$-73-42) |
| 360 | 1.1 ($M^+$-73) |
| 374 | 0.2 ($M^+$-59) |
| 390 | 0.1 ($M^+$-43) |

[a] References *15* and *16* give detailed descriptions of fragmentation pathways and identifications of ions.

TABLE VII

*Major Fragment Ions Observed in the Mass Spectra of Partially O-Methylated 2-N-Methylglucosaminitol Acetates*

| | *Position of $CH_3O$- groups* | | | | | | |
|---|---|---|---|---|---|---|---|
| *m/e* | 3,4,6 | 3,6 | 3,4 | 4,6 | 3 | 4 | 6 |
| 43 | + | + | + | + | + | + | + |
| 45 | + | + | + | + | + | + | + |
| 74 | + | + | + | + | + | + | + |
| 87 | + | + | + | + | + | + | + |
| 98 | + | + | + | + | + | + | + |
| 116 | + | + | + | + | + | + | + |
| 124 | | + | | | + | | |
| 128 | | | | | | | + |
| 129 | + | + | + | + | | + | + |
| 142 | + | + | + | + | + | | + |
| 145 | + | | + | | | | |
| 158 | + | + | + | + | + | + | + |
| 161 | + | | + | + | | | |
| 170 | | + | | + | | + | + |
| 173 | | + | | | | | |
| 189 | | | + | | | + | |
| 202 | + | + | + | | + | | |
| 205 | + | | | | | | |
| 230 | | | | + | | | |
| 233 | | + | | | | | |
| 261 | | | | | + | | |
| 274 | | | | + | | | |

[a] References *5*, *9*, *11*, *16*, *17*, and *18* give detailed descriptions of fragmentation pathways and identifications of ions.

TABLE VIII

*Major Fragment Ions Observed in the Mass Spectrum of 2-Acetamido-2-deoxy-1,3,4,5,6-penta-O-trimethylsilylmannitol (MW = 583)*[a]

| *m/e* | *Relative intensity* |
|---|---|
| 73 | 100.0 $[(CH_3)_3Si]^+$ |
| 103 | 18.5 |
| 132 | 23.1 |
| 147 | 23.8 |
| 157 | 16.9 |
| 174 | 14.9 |
| 186 | 29.4 |
| 205 | 18.2 |
| 217 | 25.1 |
| 247 | 13.2 |
| 276 | 12.4 |
| 319 | 18.4 |
| 378 | 7.6 ($M^+$-205) |
| 390 | 4.0 ($M^+$-90-103) |
| 478 | 1.5 ($M^+$-15-90) |
| 480 | 1.9 ($M^+$-103) |
| 568 | 6.1 ($M^+$-15) |

[a] References *8*, *16*, and *19* give detailed descriptions of fragmentation pathways and identifications of ions.

TABLE X

*Major Fragment Ions Observed in the Mass Spectrum of 2-Deoxy-2-trimethylsilylamino-1,3,4,5,6-penta-O-trimethylsilyl-D-mannitol (MW = 613)*[a]

| *m/e* | *Relative intensity* |
|---|---|
| 73 | 46.9 $[(CH_3)_3Si]^+$ |
| 103 | 6.5 |
| 204 | 100.0 |
| 205 | 21.1 |
| 217 | 5.0 |
| 307 | 1.4 |
| 420 | 2.0 ($M^+$-103-90) |
| 510 | 1.8 ($M^+$-103) |
| 598 | 0.5 ($M^+$-15) |

[a] References *8*, *16*, and *19* give detailed descriptions of fragmentation pathways and identifications of ions.

TABLE IX

*Major Fragment Ions Observed in the Mass Spectrum of 2-Amino-2-deoxy-1,3,4,5,6-penta-O-trimethylsilyl-D-glucitol (MW = 541)*[a]

| *m/e* | *Relative intensity* |
|---|---|
| 73 | 83.2 $[(CH_3)_3Si]^+$ |
| 103 | 20.2 |
| 132 | 32.1 |
| 147 | 17.5 |
| 204 | 25.7 |
| 205 | 10.9 |
| 217 | 100.0 |
| 258 | 8.8 |
| 348 | 7.2 ($M^+$-103-90) |
| 438 | 6.6 ($M^+$-103) |
| 451 | 0.3 ($M^+$-90) |
| 526 | 5.7 ($M^+$-15) |

[a] References *8*, *16*, and *19* give detailed descriptions of fragmentation pathways and identifications of ions.

TABLE XI

*Major Fragment Ions Observed in the Mass Spectrum of Methyl 2-Acetamido-2-deoxy-3,4,6-tri-O-trimethylsilyl-D-galactopyranoside (MW = 451)*[a]

| *m/e* | *Relative intensity* |
|---|---|
| 73 | 91.6 $[(CH_3)_3Si]^+$ |
| 75 | 14.4 |
| 131 | 26.5 |
| 147 | 20.9 |
| 173 | 100.0 |
| 204 | 10.9 |
| 217 | 7.6 |
| 218 | 10.9 |
| 226 | 0.8 |
| 247 | 11.2 |
| 259 | 3.9 |
| 314 | 2.8 |
| 330 | 0.5 ($M^+$-31-90) |
| 346 | 0.4 ($M^+$-15-90) |

[a] References *8*, *16*, *20*, and *21* give detailed descriptions of fragmentation pathways and identifications of ions.

TABLE XII

*Major Fragment Ions Observed in the Mass Spectrum of 2-Acetamido-2-deoxy-1,3,4,6-tetra-O-trimethylsilyl-D-galactopyranoside (MW = 509)*[a]

| *m/e* | *Relative intensity* |
|---|---|
| 73 | 73.0 $[(CH_3)_3Si]^+$ |
| 103 | 5.7 |
| 117 | 5.8 |
| 131 | 21.7 |
| 147 | 13.8 |
| 173 | 100.0 |
| 204 | 10.4 |
| 217 | 9.5 |
| 233 | 3.9 |
| 305 | 1.5 |
| 314 | 3.2 ($M^+$-15-90-90) |
| 404 | 0.8 ($M^+$-15-90) |
| 494 | 1.6 ($M^+$-15) |

[a] References *8*, *16*, *20*, and *21* give detailed descriptions of fragmentation pathways and identifications of ions.

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