ABSTRACT

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BIOCHEMICAL STUDIES ON G_{M1}-GANGLIOSIDOSIS: A. Investigation of Type II, G_{M1}-gangliosidosis. B. Biochemical Investigation of the liver from a patient with Type I, G_{M1}-gangliosidosis. C. Type II, G_{M1}-gangliosidosis: Observations on a fibroblast cell strain.

G_{M1}-gangliosidosis is an inherited disease quite distinct from Tay-Sachs and Hurler's diseases. It exists in two clinical forms.

These studies were initiated on a patient with the Type II form. G_{M1}-ganglioside was accumulated in the brain and β-galactosidase activity of brain, leucocytes and cultured skin fibroblasts was 2-6% of normal. The leucocyte β-galactosidase activity of the parents (carriers) was 50% of normal. Two glycosaminoglycan fractions were isolated from the urine. Both CPC- and non-CPC-precipitable fractions contained large amounts of galactose-glucosamine-containing materials of the keratan sulfate type but they were undersulfated.

Glycosaminoglycans of similar composition were isolated in large amounts from the liver of a Type I patient. They were polydisperse, not CPC-precipitable, had an alkali-labile protein-polysaccharide linkage region and were similar to keratan sulfate.

The cultured fibroblasts of Type II contained two gangliosides not found in normal cell cultures. The glycosaminoglycans, labeled by incubation with ^{14}C-galactose and ^{14}C-glucosamine, accumulated in the Type II cells and were similar to those found in the urine and in the liver.

BIOCHEMICAL STUDIES ON G_{M1}-GANGLIOSIDOSIS

A INVESTIGATION OF TYPE II, G_{M1}-GANGLIOSIDOSIS

B BIOCHEMICAL INVESTIGATION OF THE LIVER FROM A PATIENT WITH TYPE I, G_{M1}-GANGLIOSIDOSIS

C TYPE II, G_{M1}-GANGLIOSIDOSIS: OBSERVATIONS ON A FIBROBLAST CELL STRAIN

by

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The author was Reford Fellow in Neurochemistry of the Montreal Neurological Institute during the latter stages of this work.

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G_{M1}-gangliosidosis is a lipid storage disease of the central nervous system similar in many respects to Tay-Sachs disease and a glycosaminoglycan storage disease of extraneural tissues, with some clinical features of Hurler's disease. Two major types of G_{M1}-gangliosidosis are recognized on the basis of clinical criteria. In the Type I form the most striking clinical characteristics include features usually associated with Hurler's disease while in the Type II form these characteristics are absent. G_{M1}-ganglioside accumulates in the neurons of both types of this disease.

Histochemical studies have shown foam cell histiocytosis in bone marrow, spleen, liver and other extraneural tissues in both types of G_{M1}-gangliosidosis which cannot be accounted for by lipid accumulation alone. The objective of the work reported here was to characterize the visceral storage substances in the two forms of the disease, and to correlate the findings with the same enzyme deficiency in spite of the differences in phenotypic expression of the genetic defect.

This study began with the identification of an accumulation of G_{M1}-ganglioside in the brain of a 3 year old girl (case 1). The patient did not have chondrodystrophy or visceromegaly and was thus a Type II form of G_{M1}-gangliosidosis. It was shown that β-galactosidase activity was deficient in brain, leucocytes and cultured fibroblasts of this patient. The parents showed β-galactosidase activities intermediate between normal and affected humans. Thus the carrier state can be recognized. Similar results were obtained in a child with the Type I form of G_{M1}-gangliosidosis and his parents (case 2).

The Type II patient showed faintly positive metachromasia in the urine,

a finding not previously investigated. Brain neuraminidase activity was low but this is nonspecific. Brain sialomucopolysaccharides were not elevated. Analysis of the urinary glycosaminoglycans showed elevated excretion of CPC- and non-CPC-precipitable glycosaminoglycans of the keratan sulfate type. These glycosaminoglycans were highly undersulfated.

Glycosaminoglycans were isolated from the liver of a Type I patient and were characterized. The results show that they are highly undersulfated, polydisperse polysaccharides containing galactose and glucosamine as their main carbohydrate constituents. These polysaccharides found in very high quantities, are not CPC-precipitable and are similar if not identical to undersulfated keratan sulfates.

Cultured skin fibroblasts derived from the Type II patient (case 1) were examined. Lipid analysis showed the presence of two gangliosides, not found in control cells, which corresponded in chromatographic behaviour to G_{M2}- and G_{M1}-gangliosides. Neutral glycolipid analysis suggested that the structure of the G_{M2}-ganglioside was different from that of brain G_{M2}-ganglioside. Isotopically labeled galactose and glucosamine were incorporated into weakly anionic, non-CPC-precipitable glycosaminoglycans which corresponded in their chromatographic behaviour to the glycosaminoglycans isolated from the liver of a Type I patient.

The results show that both types of G_{M1}-gangliosidosis are biochemically identical. This disease can be characterized as a general β-galactosidase deficiency disease with the accumulation of G_{M1}-ganglioside in the brain and, to a lesser degree, in the viscera. The characteristic foam cell histiocytosis in both types is the result of excessive accumulation in the visceral organs and excessive excretion of undersulfated keratan sulfates.

LIST OF ABBREVIATIONS

Arab	Arabinose
AspN	Aspartamine
Carb	Carbazole
CPC	Cetylpyridinium chloride
C/M	Chloroform : Methanol
CS	Chondroitin sulfate
CSB	Chondroitin sulfate B (dermatan sulfate)
C 4-S	Chondroitin 4-sulfate
Gal	Galactose
Glc	Glucose
GlcUA	Glucuronic acid
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
HS	Heparan sulfate
HexN	Hexosamine
Hex	Hexose
HA	Hyaluronic acid
Hyase	Hyaluronidase
IdUA	Iduronic acid
KS	Keratan sulfate
Mann	Mannose
M.W.	Molecular weight
NANA	N-acetylneuraminic acid
NGNA	N-glycolylneuramic acid
Rf (relative to Neuraminyllactose)	$\frac{\text{Distance traveled by sialyloligosaccharide}}{\text{Distance traveled by neuraminyllactose}}$
Ser	Serine
Thr	Threonine
TLC	Thin layer chromatography
Xyl	Xylose

Chapter I

HISTORICAL REVIEW

G_{M1}-gangliosidosis is an inherited disorder of both glycosphingolipid and glycosaminoglycan metabolism. This disease is characterized by an excessive accumulation of both a specific ganglioside in the brain and, to a lesser extent in the visceral organs, and keratan sulfate-like glycosaminoglycans in the viscera. The objective of the initial two parts of this chapter is to discuss the more salient features of glycosphingolipid and glycosaminoglycan metabolism, with particular emphasis on the gangliosides and the keratan sulfates. Then, to follow clearly the emergence of G_{M1}-gangliosidosis as a distinct disease entity, Tay-Sachs and Hurler's disease will be discussed briefly. G_{M1}-gangliosidosis has clinical and pathological characteristics in common with both Tay-Sachs disease, the prototype ganglioside storage disease, and with Hurler's disease, an inherited disorder of glycosaminoglycan metabolism. It is not the aim of this thesis to enumerate all the clinical, pathological and biochemical findings of these diseases. Only the most important features relevant to the discussion of G_{M1}-gangliosidosis will be included.

A GANGLIOSIDES AND THEIR METABOLISM

1 Structure and Occurrence of Gangliosides

The gangliosides are glycosphingolipids characterized by the presence in their structure of one or other of the sialic acids, a group of 9-carbon amino sugars having a carboxyl group attached to the anomeric carbon (1). The most important sialic acid in human tissues is N-acetyl-

neuraminic acid (NANA). The gangliosides are a heterogeneous class of molecules which, although present in highest concentration in neurons (including the axons and dendritic tree) of the central nervous system(2-5) are now known to be present in many tissues of the body (6-15). Six major gangliosides, varying both in their oligosaccharide moiety and in number of NANA residues, are normally found in human and vertebrate brain. About 90% of the total gangliosides in brain have a basic oligosaccharide structure similar to that of G_{M1}-ganglioside, as named in the classification of the different ganglioside types by Svennerholm(9) (Table 1), with one or more additional sialic acid moieties. The other gangliosides are composed of a mixture of species which are simpler in structure(9). These are G_{M3}-ganglioside or hematoside and G_{M2}-ganglioside or Tay-Sachs ganglioside. In addition a ganglioside, tentatively designated G_{M4}-ganglioside, has been isolated from normal human brain (16). It contains equimolar amounts of sialic acid, galactose and ceramide. The early history of the gangliosides and some views on their function has been recently reviewed by several authors (13,17,18) and in theses from this laboratory (19,20) and will not be discussed here.

In general the carbohydrate moiety of gangliosides contains, in addition to sialic acid, glucose, galactose and N-acetylgalactosamine. The ceramide moiety comprises two distinct components - an aliphatic base and a fatty acid. Four aliphatic bases have been recognized in the brain gangliosides: sphingenine, icosisphingenine and the dihydroderivatives of both (21-24). In adult brain gangliosides, sphingenine comprises about half of the total aliphatic base, while icosisphingenine comprises about 47%. The dihydroderivatives constitute the last 3% (22). The structure of sphingenine (D-erythro-1,3-dihydroxy-2-amino-4-octadecene) has been established chemically (25,26) and biochemically (27). The fatty acids

TABLE 1

The Most Common Gangliosides in Normal Human Brain

Common Name	Abbreviation	Structure
Monosialoganglioside	G_{M4}	NANA → gal → R
Hematoside	G_{M3}	NANA (2→2) gal (β,1→4) glc (1→ 1) R
Disialoganglioside	G_{D3a}	NANA (2→8) NANA (2→3) gal (β,1→4) glc (1→ 1) R
Tay-Sachs ganglioside	G_{M2}	galNAc (β,1→4) gal (β,1→4) glc (1→ 1) R (3←2) NANA
Monosialoganglioside	G_{M1}	gal (β,1→3) galNAc (β,1→4) gal (β.1→4) glc (1→ 1) R (3←2) NANA
Disialoganglioside	G_{D1a}	gal (β,1→3) galNAc (β,1→4) gal (β,1→4) glc (1→1) R (3←2) NANA (3←2) NANA
Disialoganglioside	G_{D1b}	gal (β,1→3) galNAc (β,1→4) gal (β,1→4) glc (1→1) R (3←2) NANA (8←2) NANA
Trisialoganglioside	G_{T1}	gal (β,1→3) galNAc (β,1→4) gal (β,1→4) glc (1→1) R (3←2) NANA (3←2) NANA (8←2) NANA

R is ceramide

present in brain gangliosides are N-acylated to the free amino group at the C_2 position of the aliphatic base. In human and beef brain gangliosides the major fatty acid is stearic acid (18:0) with smaller quantities of myristic (14:0), palmitic (16:0), and arachidic (20:0) acids (28,31).

The elucidation of the structure and stereochemistry of the gangliosides was achieved in the German schools of Klenk and Kuhn (32-38). Purified brain gangliosides were hydrolyzed by weak acid and the oligosaccharides were isolated and permethylated with a mixture of BaO, $Ba(OH)_2$ and methyliodide in dimethylformamide. In addition, periodate oxidation-borohydride reduction and acetolysis studies were performed(32,34,35). As a result they proposed the following structure for major monosialoganglioside (G_{M1}):

gal(β,1→3)galNAc(β,1→4)gal(β,1→4)glc(β,1→1)ceramide
3
↑
2 NANA

These results were confirmed using paper and gas chromatographic analysis of methylated sugars (32,35,36). Subsequently, Klenk and coworkers established the presence of a NANA-NANA dimer in the polysialogangliosides (Table 1), in which the terminal NANA was attached in 2→8 linkage to the NANA linked 2→3 to galactose (37,38). Sialic acid appears to be joined to ceramide oligosaccharide through a ketosidic linkage in the α-configuration (39).

Intact sialyloligosaccharides have been isolated from the gangliosides by ozonolysis in methanol and weak alkaline hydrolysis (1% Na_2CO_3) (40). More recently, the structure of the gangliosides as well as other glycosphingolipids has been confirmed by mass spectrometry(41,42)

and by mass spectrometry-gas chromatographic studies (42).

In addition to brain, gangliosides have been isolated from a variety of other tissues. Hematoside, G_{M3}-ganglioside, is the most common ganglioside in extraneural tissue and in tissues derived embryologically from neural tissue (9,15). Two species of hematoside are recognized each containing a different sialic acid. One species contains NANA and the other N-glycolylneuraminic acid (NGNA) (13). Both of these hematosides isolated from erythrocyte stroma, contain lignoceric acid (24:0) as the major fatty acid and sphingenine as the aliphatic base (44,45). G_{M3}-gangliosides have been isolated from the erythrocytes of horse, cat and dog (6,10), the placenta (11), human and beef spleen (7,8), pig lung (12), the lens of the human eye (14) and bovine adrenal medulla (15). The major gangliosides in the dog intestine and in the lens of the human eye were found to contain ceramide, glucose, galactose and sialic acid in the molar ratio 1:1:2:1. Lignoceric acid was the major fatty acid in both cases.

An unusual, previously unknown, sialic acid-containing sulfolipid has been isolated from horse and beef hooves and identified in human hair, nails, epidermis and kidney (46). This glycolipid named "Ungulic acid" contained sphingenine, galactose, galactosamine and sialic acid in equimolar amounts. Stearic acid was the major fatty acid. Unusually strong acid hydrolysis was required to release the sialic acid from this compound. Perhaps the "S-lipid" found by Ray et al.(47) in subcellular fractions of liver is related to this unusual ganglioside.

Bovine adrenal medulla appears to be the richest source of ganglioside-sialic acid outside of the nervous system (15). The lipid-bound sialic acid (223 µg/g wet weight tissue) is about 50% of the normal

gray matter level. While 92% of the ganglioside-sialic acid is present in the two types of hematoside, the remainder of the sialic acid, both NANA and NGNA, was found mixed in the polysialogangliosides. Puro et al. (48) have also identified the polysialogangliosides in small quantities in extraneural tissues of the pig, rat and rabbit including cardiac muscle, kidney, spleen and intestine.

Thus the predominant gangliosides in extraneural tissues are the hematosides although other types are present. In the brain, the major gangliosides quantitatively are G_{M1}- and the more complex polysialogangliosides. Furthermore, lignoceric acid is the major ganglioside fatty acid in extraneural tissue while in brain gangliosides, stearic acid predominates.

2 Developmental Pattern of Gangliosides in Brain

Gangliosides are found early in human fetal development but the relative distribution of ganglioside types changes continuously until the adult stage is reached at about 20 to 30 years of age (9, 49-54). From the third to fourth fetal month until term all lipids increase (49). Lipid-bound NANA and hexosamine increase twofold over this period. In the brain of the young human fetus, four major gangliosides have been found: G_{M1}, G_{D1a}, G_{D1b} and G_{T1}. G_{T1}-ganglioside contained 29.6% of the total lipid-bound sialic acid while G_{D1b}, G_{D1a} and G_{M1} contained 27.1, 23.1 and 13.1% of the total respectively (49). This pattern of ganglioside-NANA distribution in human fetal brain is quite different from that of premature and newborn babies (9,51,52). In the human newborn brain, the concentration of G_{D1a}-ganglioside undergoes a very substantial increase from 23.1 to 60.1% of the total ganglioside-NANA. On the other hand, G_{M1}-ganglioside increases

about twofold (25.2% of the total), while the other gangliosides G_{D1b} and G_{T1} decrease to 3.3 and 4.8% respectively (9). Two other gangliosides were also found in the newborn brain, G_{M3}- and G_{M2}-gangliosides which contained 0.7 and 5.7% of the total NANA, respectively (9). It should be noted here that the actual increase in G_{M1}-ganglioside during this development period was higher than for G_{D1a} on a total weight basis since G_{D1a}-ganglioside contains twice as much NANA as G_{M1}-ganglioside. The high value of sialic acid in the G_{D1a} fraction decreases slowly during the first decade of life and reaches its adult level at about age 30. Likewise in normal infant brain, the high level of G_{M1}-ganglioside slowly decreases and reaches its adult level at about 20 to 30 years (32). The content of sialic acid in G_{D1b}- and G_{T1}-gangliosides increases in value with time and reaches the adult level at about the same time as G_{D1a}-ganglioside. Thereafter there is little change in the distribution of ganglioside types in the brain even up to senescence (52). Human fetal gangliosides are almost devoid of the C_{20} aliphatic base, icosisphingenine, while in the adult almost equal quantities of sphingenine and icosisphingenine are found (53) as indicated earlier. The pattern of development of gangliosides in the human is essentially identical to that of rat brain (52). In the newborn rat significant quantities of gangliosides are present and the level increases rapidly during the period before myelination to reach the adult level at about 20 days (3). The gangliosides in young rats (less than 10 days old) were found to be concentrated in the crude mitochondrial fraction and from this fraction a ganglioside-rich membrane fraction was isolated (3). It is generally agreed that gangliosides are membrane constituents and in the brain are concentrated in synaptic membranes.

Measurement of the incorporation of subcutaneously injected $U^{14}C$-D-glucose into the gangliosides of 7 day old rats has indicated that none of the major gangliosides present (G_{M1}, G_{D1a}, G_{D1b}, and G_{T1}) are the precursors of the other types (54). While the radioactivity of each of the carbohydrate moieties from each ganglioside varied slightly, the neuraminidase-labile NANA had a lower specific activity than neuraminidase-resistant NANA. The different specific activities of NANA suggest that two pools of sialic acid exist in young rat brain (54). In a recent study, Suzuki (55) demonstrated with isotopically labeled glucose and glucosamine that in the rat the relative rate of formation of gangliosides paralleled the developmental pattern changes. No significant turnover of the gangliosides was observed until each ganglioside reached its normal adult level (52), i.e. 5 to 10 days after the myelination period (55).

3 Ganglioside Metabolism

The biosynthetic pathway of gangliosides has been elucidated in recent years chiefly by the Roseman school (56-59). The embryonic chick brain (56-59), tadpole brain (60) and newborn rat brain particulate fractions (60,61) have been the major tissues which so far, by techniques presently available, exhibit measurable ganglioside synthetic ability.

The first step in ganglioside biosynthesis is the formation of ceramide (62,63). Gatt has isolated an enzyme capable of both the synthesis and hydrolysis of ceramide. This enzyme, ceramidase, initially particle bound, can be rendered soluble by extraction with sodium cholate.

The enzyme, isolated from rat and guinea-pig kidney, liver and brain, synthesized ceramide from free fatty acid and sphingenine at pH 3.8. Ceramide did not inhibit the reaction. Ceramide then acts as the acceptor for glucose transferred from UDP-glucose. The enzyme operative in glucosyltransfer was isolated from 13 day old embryonic chick brain. It was particulate, required detergent for activity and did not require metal ion (58). The subsequent steps in the ganglioside biosynthetic pathway have been established as follows:

1-0-glucosylceramide

	UDP-galactose	→	1-0-galactosylglucosyl-ceramide	(1)
			I	
I	CMP-NANA	→	G_{M3}-ganglioside	(2)
			II	
II	UDP-galNAc	→	G_{M2}-ganglioside	(3)
			III	
III	UDP-gal	→	G_{M1}-ganglioside	(4)

The galactosyltransferase which catalyzes reaction I has been itentified in particles from embryonic chick brain (58) and in aqueous or

sucrose homogenates of several rat tissues (64) including the spleen, liver, kidney and brain. The chick enzyme has a pH optimum of 6.8 and catalyzed the galactose transfer to 1-O-glucosylceramide in the presence of Mg^{++}. Although the enzyme was specific for UDP-galactose, galactosyl transfer was also observed with G_{M2}-ganglioside as acceptor in the presence of Mn^{++} or Mg^{++}. The rat enzyme was found to be most active at pH 6.9 in the presence of Mn^{++}. ATP and UTP were effective inhibitors of the reaction. The N-acetylgalactosaminyltransferase enzyme (reaction 3) of chick brain (57) appears to be specific for the glycolipid acceptor since transferase activity could be detected only with G_{M3}-ganglioside as acceptor but not with lactosylceramide.

The galactosyltransferase of reaction 4 was most active in the presence of Mn^{++}, but was relatively nonspecific with respect to its acceptor (56,57). The chick particulate enzyme transferred galactose to N-acetylglucosamine, as well as to enzymatically hydrolyzed glycoprotein acceptors. Heat inactivation studies suggested the presence of more than one galactosyltransferase in this preparation (56,57).

Another galactosyltransferase has been isolated from adult frog and tadpole brain (60). This enzyme, also present in the brain and kidney of the 8 day old rat (60), catalyzes the transfer of galactose from UDP-galactose to galNAclactosylceramide:

$$\underset{\text{galNAcgalglc ceramide}}{\text{UDP-gal}} \rightarrow \underset{\text{galgalNAcgalglc ceramide}}{} \qquad (5)$$

This galactosyltransferase (reaction 5) was also found to be particulate but could be solubilized by sodium deoxycholate treatment. Magnesium ions were required for activity and galactosyl-galactosyl-glucosyl ceramide was not active as glycolipid acceptor.

The sialyltransferase enzymes catalyze the transfer of NANA from CMP-NANA to a variety of glycolipid acceptors. The activation of NANA requires CTP, Mg^{++} and a specific enzyme which was isolated by Roseman from hog submaxillary gland (65). Other aspects of the biosynthesis of N-acetylneuraminic acid and its enzymatic activation have been reviewed (66,67). At least three enzymes active in the CMP-NANA transferase reaction have been recognized in chick brain particles. As proposed by Roseman and coworkers (57,59), these sialyltransferases have been designated A, B and C. Sialyltransferase A catalyzes reaction 2 while sialyltransferase B is capable of sialylating G_{M1}-ganglioside, lactose and ceramide-N-tetrahexoside, the product of reaction 5. The product formed by the action of sialyltransferase B in the presence of G_{M1}-ganglioside had the chromatographic properties of G_{D1a}-ganglioside while with ceramide-N-tetrahexoside as glycolipid acceptor both G_{M1}- and G_{D1a}-gangliosides were found. The third sialyltransferase, C, catalyzed the transfer of sialic acid to a hematoside containing NGNA. The product of this reaction was identified as:

N-acetylneuraminyl(2→8)N-glycolylneuraminyllactosyl ceramide.

Only the NANA was radioactive and susceptible to periodate oxidation, thus indicating that it was present in the terminal position.

The action of sialyltransferase C provides a tentative mode for the attachment of NANA to form a dimer known to be present in G_{D1b}-, G_{T1}- and the tetrasialogangliosides. The presence of sialyltransferase C activity is in accord with other findings in young rat brain. Arce et al. (61) found that young rat brain deoxycholate disrupted microsomes were capable of transferring sialic acid to lactose, 1-O-lactosylceramide and

asialoganglioside but in the presence of G_{M1}- and disialogangliosides no sialyl transfer could be observed.

The most striking aspect of the ganglioside biosynthetic pathway is its particulate nature. In all cases, detergents, both neutral or anionic, were absolutely required for measurable catalytic activity. In addition, the majority of the enzymes demonstrated to date are highly specific for the glycose donor but less specific in their lipid acceptor requirement. If the synthetic pathway in vivo is at all similar to that in vitro, it can be viewed as a tightly membrane-bound multistage process in which the neutral asialogangliosides play an important role. The in vitro pathway, as outlined above, would allow the independent synthesis of each of the ganglioside types in agreement with the developmental pattern observed in vivo.

In the rat, while ganglioside biosynthesis is an early event, ganglioside degradation does not appear to be a process of major occurrence until about a week after the onset of myelination (55). The first stage of ganglioside degradation requires, in the case of the polysialogangliosides and G_{M3}-ganglioside, the cleavage of sialic acid by an acid hydrolase, neuraminidase. This enzyme has been isolated from calf (68) and human brain (69) and rat liver and kidney (70). In brain preparations the enzyme hydrolyzed gangliosides, G_{T1}-, G_{D1b}-, G_{D1a}- and G_{M3}-, with G_{M1}-ganglioside or 1-O-lactosylceramide as the final products. The calf and human brain neuraminidases were membrane bound, resisted solubilization and required detergent for optimal activity. Although Leibovitz and Gatt (68) could not discount the possibility of multiple forms of neuraminidase in the calf brain preparation, heat inactivation and mixed substrate studies

suggested that only one neuraminidase enzyme was present in the human brain (69). Enzymes having neuraminidase activity have also been identified in tissues from embryonic chick and duck (71). In agreement with the findings in the human brain, neuraminidase activity was low in embryonic chick brain and increased with age (71). The full term human infant was found to possess a brain sialidase activity about 50% of the adult level (69).

The degradation of ganglioside from G_{M1}-ganglioside was visualized by Burton (72) as involving a stepwise, multistage process in which the product of one hydrolytic cleavage served as the substrate for the next enzyme. This interpretation was based on the findings of Korey and Stein (73) who postulated that a "gangliosidase system" existed in the brain.

The complete catabolic sequence of ganglioside metabolism has recently been described by Gatt and associates (74-79). The pathway from G_{M1}-ganglioside follows the course:

G_{M1}-ganglioside	→	G_{M2}-ganglioside	(6)
G_{M2}-ganglioside	→	G_{M3}-ganglioside	(7)
G_{M3}-ganglioside	→	1-O-lactosylceramide	(8)
1-O-lactosylceramide	→	1-O-glucosylceramide	(9)
1-O-glucosylceramide	→	ceramide	(10)
ceramide	→	fatty acid + sphingenine	(11)

The β-galactosidase and β-N-acetylhexosaminidase of reactions 6 and 7 respectively, also hydrolyze the terminal sugars from their asialoderivatives, tetraglycosylceramide and triglycosylceramide, respectively. All enzymes in the above scheme have been localized in the same subcellular particles, i.e. lysosomes, they require detergent for activity with lipid substrates and they have similar pH optima (4.5 - 5.0) except for the β-N-acetylhexosaminidase enzyme which has a pH optimum of 3.2 (74-79). The β-galactosidase, active in reactions 6 and 9, has been isolated from rat and calf brain (75). In the presence of G_{M1}-ganglioside as substrate only G_{M2}-ganglioside was formed. The presence of G_{M2}-ganglioside did not inhibit the reaction even when present at concentrations several fold higher than the substrate concentration. The rat brain β-galactosidase also hydrolyzes 1-O-lactosylceramide at a high rate (reaction 9) (74,76,77). Hydrolysis of this substrate could be inhibited by sphingenine, palmitic acid and ceramide and to a lesser extent by galactose. Action of β-glucosidase on the product of reaction 9 can be inhibited by sphingenine but not by ceramide (76). The ceramidase enzyme of reaction 11 has the capacity both to hydrolyze and synthesize ceramide at the same pH (62,63). Since human brain neuraminidase isolated from lysosomes could not be freed of ganglioside and the action of neuraminidase released latent acid phosphatase and β-glucuronidase from subcellular particles of brain, liver and kidney (80), this enzyme may play an important role in rendering potential substrates available for hydrolysis as well as initiating ganglioside catabolism.

B KERATAN SULFATES AND THEIR METABOLISM

The keratan sulfates are glycosaminoglycans which contain galactose, glucosamine and sulfate. Keratan sulfates occur in cartilage, bone, cornea and in small amounts in the urine. The keratan sulfates in cartilage are constituents of proteoglycans in an intimate association with chondroitin 6-sulfate. Scanty evidence is available on their metabolism but they are likely synthesized in a manner analogous to the formation of chondroitin sulfates.

1 Chemical Structure of Keratan Sulfates

Two types of keratan sulfate are recognized in mammalian tissue, a corneal type, abbreviated KS_1, and a skeletal type, KS_2. Keratan sulfate was first obtained from bovine cornea by Meyer et al. in 1953 (81). This polysaccharide comprised approximately half of the total mucopolysaccharide isolated from this tissue and had the following composition: hexosamine, 33.4%; hexose, 36%; sulfate, 18.4%, and acetyl, 10.4%. It was found to be dextrorotatory (+ 4.5°). The major carbohydrate constituents, 2-amino-2-deoxy-D-glucose (glucosamine) and D-galactose were identified as their N-carbobenzoxy and α-methylphenylhydrazone derivatives, respectively.

Shortly thereafter, the skeletal form of keratan sulfate was isolated from nucleus pulposus (82). Elucidation of the structure of both KS_1 and KS_2 was obtained by the use of chemical methods, including permethylation in aqueous or organic solvents, desulfation and methylation, acid hydrolysis and gas chromatographic analysis (83-86).

The major methylated sugars in the sulfated or desulfated KS_1 were found to be 2, 4, 6-tri-O-methyl-D-galactose and 2, 4-di-O-methyl-D-galactose (83,85). Analysis of the amino sugars methylated in aqueous

solvents showed the major species to be 2-amino-2-deoxy-3-O-methyl-D-glucose (83) while with the methylation of the KS_1 in dimethylformamide after prior acetylation, three species of N-methylglucosamine were found (85). Similar methylation studies with KS_2 in dimethylformamide as solvent gave essentially the same findings as those reported for KS_1 (86). Thus the principal neutral sugar 2, 4, 6-tri-O-methyl-D-galactose is attached to hexosamine in linkages involving the C-1 and C-3 hydroxyls.

These methylation studies established that in KS_1 74% of the N-acetylglucosamine groups were sulfated at C-6 with 40% of the galactose residues also sulfated at C-6 (85). In skeletal keratan sulfate, however, a slightly different distribution of sulfate was found. About 60% of the glucosamine and 50% of the galactose was sulfated at C-6 in KS_2 (86). The desulfated polymer of KS_1 and KS_2 appears to be a linear polymer of 3-linked galactose and 4-linked glucosamine in an alternating sequence (86). The β-configuration of both glycosidic linkages was established by enzymatic hydrolysis of KS_1 and KS_2 with Coccobacillus extracts (83,86). The basic repeating unit of both KS_1 and KS_2 has been established as: (1→3)-O-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside. Larger amounts of 2,3,4,6-tetramethyl- and 2,3,4-trimethyl-D-galactose and 2,3,4-trimethyl-L-fucose were found in acid digests of methylated KS_2 than of KS_1, indicating the presence of more branch chains in KS_2 (85,86). A high degree of branching in KS_2 was also indicated by the specific loss of sialic acid, fucose and galactose with the loss of minor quantities of glucosamine during desulfation and weak acid hydrolysis. The size and number of these branches has not been determined but the results indicated that sialic acid, fucose and galactose were present individually or in

small groups. Fucose was found to be attached at acid labile non-reducing terminal points while the galactose appeared to be substituted at the C-6 position of the nonsulfated sugars in the polysaccharide chain. Small amounts of mannose have also been found in keratan sulfates from several sources (86). Another difference between KS_1 and KS_2 is in their content of galactosamine. The glucosamine to galactosamine molar ratio in corneal keratan sulfate is in the order of 50:1 while in the skeletal form this ratio is decreased to about 11 or 12:1 (87,88).

Keratan sulfates are known to vary in their degree of sulfation, optical rotation and amino acid, methylpentose and sialic acid contents (87,89). Skeletal keratan sulfate isolated from old human rib cartilage is polydisperse on molecular sieve chromatography, 21 to 38% of the population having an apparent molecular weight of 4500 to 10,000 while the remainder is in the range of 3600 to 4500 (90). Corneal KS however has an apparent molecular weight of 10,000 to 20,000 (87,88).

Each of the keratan sulfates has a small peptide fragment attached to its polysaccharide chain. The protein components of both KS_1 and KS_2 are more resistant to papain digestion than those of the chondroitin sulfates (87). The peptide fragments of the 4500 to 10,000 molecular weight species of KS_2 after papain digestion contained larger amounts of serine, threonine, glutamic acid and proline and smaller quantities of aspartic acid (90). Further exhaustive pronase digestion reduced the amino acid content of KS_2 but did not alter the relative proportions of the amino acids (90). However, the amino acid content of KS_1 can be reduced from 5 to 10% to 1.5% by exhaustive digestion (87). In the peptides still linked to KS_1 after pronase treatment, the aspartic acid content

remains high while the valine and glutamic acid contents are reduced to one half and one third of their predigestion levels respectively. These differences suggested that KS_1 and KS_2 may have different carbohydrate linkages to protein. Mathews and Cifonelli (88) confirmed that in bovine corneal KS, aspartic acid was the principal amino acid involved in the protein-polysaccharide linkage while in KS_2 the aspartate content was insufficient for it to play an important role in the glycosidic linkage.

Another major difference between corneal and skeletal keratan sulfates resides in the stability of the protein-polysaccharide linkage when exposed to 0.5 M NaOH. Exposure of old human rib cartilage KS_2 to 0.5 N NaOH reduced the amino acids from 7.1 to 3.3%, and destroyed some of the serine and threonine present in the peptide fragment (87,90). This destruction of hydroxyamino acids was reflected in the appearance of dehydroalanine and α-aminocrotonic acid which presumably arose from serine and threonine through an alkali mediated β-elimination reaction (91). In the case of corneal keratan sulfate this effect was not seen. This again suggested that the protein-polysaccharide linkage region is not the same for both keratan sulfates. In addition to the preferential destruction of galactosamine, serine and threonine, treatment of the KS_2 complexes with weak alkali resulted in the production of polysaccharide species having a slightly lower molecular weight on Bio-Gel chromatography. However, some of the galactosamine, which gave a Morgan-Elson reaction after alkali treatment, was found attached to a molecular species having a mobility on Bio-Gel sieve chromatography identical to the starting material. Thus, although KS_2 has an alkali-labile protein-polysaccharide linkage region, such treatment does not always split KS_2 into peptide and polysaccharide

fragments. Fragmentation of KS_2 into polysaccharide and peptide moieties occurs when the galactosamine is linked through its C-3 hydroxyl to the hydroxyl group of serine and threonine but galactosamine linkage through its C-6 hydroxyl appears to produce a Morgan-Elson chromogen without fragmentation. This evidence strongly implicates hydroxyamino acids and galactosamine in the linkage region of KS_2 (90).

Mannose now appears also to be a component of the linkage region in keratan sulfates. Baker et al. (92) isolated two glycopeptides from corneal keratan sulfate after chromatography on Dowex-50 and Sephadex G-50. One of these glycopeptides contained mannose, galactose, glucosamine and aspartic acid in the molar ratio 2:1:2:1. Only traces of other amino acids were found. Subsequently Baker et al. (93) found that the protein-polysaccharide linkage in KS_1 includes an aspartamine linked to N-acetyl-glucosamine. The exact location of mannose and galactose in the linkage oligosaccharide is unclear. Glycopeptides rich in mannose were also isolated from cartilage keratan sulfate (93) but their structure has not been reported.

The linkage region of KS_1 thus is alkali-stable and contains aspartic acid, galactose, mannose and glucosamine while for KS_2 it is alkali-labile and contains serine and/or threonine, with mannose and galactosamine. An abbreviated structure of keratan sulfates is compared to the other glycosaminoglycans in Table 2.

TABLE 2

Abbreviated Structures of the Mammalian Glycosaminoglycans

Glycosaminoglycans			
New Name	Old Name	Linkage Region to Protein	Repeating Unit
Keratan Sulfate	Keratosulfate	-	
1. Corneal		aspN-glcN(mann,gal)	(β,1→3)gal(β,1→4)glcNAc-6-0-sulfate*
2. Skeletal		ser-thr-galN(mann,gal)	(β,1→3)gal(β,1→4)glcNAc-6-0-sulfate*
Chondroitin 4-Sulfate	Chondroitin Sulfate A	ser-xyl(4←1,β)gal(3←1,β)gal	(β,1→3)glcUA(β,1→4)galNAc-4-0-sulfate
Chondroitin 6-Sulfate	Chondroitin Sulfate C	ser-xyl(4←1,β)gal(3←1,β)gal	(β,1→3)glcUA(β,1→4)galNAc-4-0-sulfate
Dermatan Sulfate	Chondroitin Sulfate B	ser-xyl(4←1,β)gal(3←1,β)gal<	(α,1→3)IdUA(β,1→4)galNAc-4-0-sulfate
Heparan Sulfate	Heparitin Sulfate	ser-xyl(4←1,β)gal(3←1,β)gal	(α,1→4)glcUA(α,1→4)glcN-sulfamido-6-0-sulfate†
			(α,1→4)glcUA(α,1→4)glcNAc-6-0-sulfate†
Hyaluronic Acid**		protein? (glc,arab)	(β,1→3)glcUA(β,1→4)glcNAc

**The linkage to protein is not clear - glucose and arabinose have been implicated.

<Glucuronic acid is also found between linkage region and polysaccharide chain, see text.

*40-50% of galactose is 6-sulfated (see text)

†The repeating unit is not firmly established.

2 Occurrence of Keratan Sulfates in Nature

Keratan sulfates of vertebrates can in general be isolated from cartilage, bone and cornea. In cornea, the keratan sulfate is found along with chondroitin 4-sulfate but can be readily separated from it by fractional precipitation with calcium salts and ethanol. In cartilage tissue, on the other hand, skeletal keratan sulfate is generally found in association with chondroitin 6-sulfate.

Mathews (94) isolated mucopolysaccharide-protein complexes from shark cartilage which contained oversulfated chondroitin 6-sulfate (chondroitin sulfate D), keratan sulfate and noncollagenous protein. The keratan sulfate protein complexes were heterogeneous. One species had a sulfate to hexosamine ratio of 1:0 while another species had a molar ratio of these components of 1.3:1. In the nucleus pulposus of tiger shark, a mixture of polysaccharide complexes was isolated containing primarily chondroitin sulfate with a keratan sulfate content of 25%.

Lloyd, Dodgson and Price (95)studied the cartilage polysaccharides of Elasmobranch fishes. Chondroitin and keratan sulfates were identified in adult specimens from the blue shark, skate and dogfish while in the fin whale chondroitin 4-sulfate was the major aminopolysaccharide, with chondroitin 6-sulfate and keratan sulfate found in very small amounts. The keratan sulfate found in the shark, skate and dogfish was heterogeneous with respect to sulfate content. Polysaccharides similar to keratan sulfate have been isolated from the organs of Lorenzini of the dogfish (96). These compounds had a high galactose and glucosamine content, were highly sulfated and on electrophoresis migrated as a distinct species. Mixtures of these "Lorenzan sulfates" with chondroitin sulfates could not be resolved

into separate species, suggesting the formation of hybrid complexes between the two polysaccharides. This hybrid formation has been observed by other authors (88).

Keratan sulfate has also been identified in rabbit nucleus pulposus (97), the rib cartilage (98) and isolated in a pure form from chick allantoic fluid (99). The chick allantoic fluid keratan sulfate has many properties of skeletal keratan sulfate (KS_2) such as: a negative optical rotation, polydispersity, alkali-labile protein-polysaccharide linkage and high levels of threonine and serine in the peptide fragment. However, it contained less sulfate and more galactosamine and sialic acid than keratan sulfate (KS_2) and was immunologically active.

The presence of keratan sulfate as an integral part of protein-polysaccharide compleses (PP) containing chondroitin sulfates has been observed by many authors (100-109) The protein-polysaccharide complexes are usually extracted with water. Sliced, fresh or powdered, dried cartilage is homogenized with water in a high speed homogenizer. The aqueous extracts of PP can be separated into two major components by centrifugation, one of which sediments as heavy particles (PP-H) and the other remains in the supernatant fluid (PP-L), The PP-L complexes can then be separated into several species by differential centrifugation in salt solutions. This technique has been used to isolate PP from various tissues including bovine nasal cartilage and human intervertebral discs and costal cartilage (109-113). The content of keratan sulfate in these complexes is variable from tissue to tissue as well as within the heterogeneous population of PP isolated from one tissue. The presence of keratan and chondroitin sulfates in these complexes does not appear to be due to simple aggregation of two distinct complexes (97,103,104,107,108,114). Digestion of PP from human

nucleus pulposus with testicular hyaluronidase destroyed a large amount of the chondroitin sulfate, but this treatment had little effect on the ratio of keratan sulfate to protein in the complex (107). Mild alkali was sufficient to release some chondroitin sulfate from the complex isolated from bovine nasal septa but the remainder was found associated with keratan sulfate and protein (115,116). Even extraction of protein-polysaccharides from cartilage with hot acetic acid and refluxing with hot water was not sufficient to completely separate chondroitin sulfate from keratan sulfate and protein (103,104). Fractions of the PP containing high quantities of keratan sulfate relative to the chondroitin sulfate content generally have high molecular weights. Fractions rich in chondroitin sulfate but having a low content of keratan sulfate generally have low molecular weights and a small protein fragment (103,104,107,108). The heterogeneous nature of the keratan and chondroitin sulfate-containing protein complexes does not appear to arise during isolation, nor can it be ascribed to the action of proteolytic enzymes (103,104,107,108). Papain digestion of the intact protein-polysaccharide complexes of bovine nasal septa afforded the separation of chondroitin sulfate-rich fragments and keratan sulfate-rich fragments. The high molecular weight fragments contained high quantities of keratan sulfate and most of the amino acids with a somewhat increased level of glutamic acid and proline. The chondroitin sulfate chains were concentrated either in a fraction of molecular weight of 100,000 or more, or in individual chains of about 20,000 molecular weight with a small peptide fragment. Hoffman et al. (103,104) concluded that both chondroitin sulfate and keratan sulfate were attached to the same protein core through alkali-labile serine residues.

Eyring and Yang (117) in their studies on the PP complex from bovine

nasal cartilage concluded that the complex consisted of a highly disordered protein with polysaccharide side chains attached to it. The complex behaved as a polyelectrolyte but was not an aggregate, had no labile disulfide linkages and was found to contract in the presence of salts, urea and guanidine hydrochloride. They estimated that no more than 30 points of protein-polysaccharide covalent linkage were present. Hoffman et al. (103) held out the possibility that the polysaccharide chains were attached to a short peptide side chain which was in turn linked to a common protein core. Tsiganos and Muir (108), on the other hand, found a number of N-terminal amino acids in pig laryngeal cartilage complexes suggesting a series of protein chains in the complex, in which larger molecular weight species had a greater number of carbohydrate chains but a lower proportion of chondroitin sulfate relative to keratan sulfate (108). The attachment of polysaccharide to protein in these complexes therefore does not appear to be random but ordered (103).

Brandt and Muir (118) compared the mucopolysaccharide profile of immature and adult pig articular cartilage. They isolated intact protein-polysaccharide complexes from 5 week old and 9 month old cartilage. The PP complexes were more difficult to isolate from the older cartilage, contained a higher keratan sulfate content and less chondroitin sulfate than immature cartilage. The complexes from both age groups were heterogeneous on agarose columns. The yield of PP from immature cartilage was less than for the adult, with the keratan sulfate present exclusively in the larger molecular weight fractions. Some glucosamine-containing material was not extracted from the cartilage even in the immature animal. This may be related either to the carbohydrate content of collagen or to the presence of sialoprotein, a normal component of bone and cartilage. Both these macromolecules are

known to contain relatively high contents of galactose, glucosamine and sialic acid as well as smaller amounts of glucose, fucose, mannose and galactosamine (119-121).

Thus the keratan sulfate proteoglycans contain variable amounts of chondroitin sulfate attached to its core protein but there is as yet no agreement as to the presence of one or more core protein. Detailed reviews on the occurrence and composition of keratan and chondroitin sulfate proteoglycans have appeared (113,122-124).

3 Keratan Sulfates and Aging

The content of keratan sulfate is lowest in cartilage from newborn animals and increases with age (125,126). Kaplan and Meyer (125) measured the relative amounts of mucopolysaccharides in human rib cartilage at a range of ages from the newborn to the very old (74 years). In the human newborn, the major mucopolysaccharide in rib cartilage is chondroitin 4-sulfate but during skeletal development this is replaced by chondroitin 6-sulfate with a simultaneous increase in keratan sulfate. Chondroitin 4-sulfate comprised about 6% of the dry weight of the cartilage in the newborn but was reduced to about 1% in aged cartilage. The keratan sulfate content was about 3 to 5% of the total mucopolysaccharides at the age of 5 and continued to rise up to the age of 20 or 30 years at which time it reached a plateau level maintained thereafter until senescence. In old cartilage, keratan sulfate comprised about 50% of the total mucopolysaccharides, with a substantial amount of chondroitin 6-sulfate. Costal cartilage from a young cow and a young pig and calf tracheal cartilage showed ratios of keratan sulfate to total mucopolysaccharides similar to those seen in immature humans.

A study of keratan sulfate metabolism in rabbit nucleus pulposus fortifies the above findings. Davidson and Small (97) measured the incorporation of ^{14}C-D-glucose into the hexosamine moieties of the polysaccharides of this tissue in vivo. They found that the ratio of keratan sulfate to chondroitin 6-sulfate (reflected in the molar ratio of glucosamine to galactosamine) increased with age. At one month of age, the molar ratio was about 0.5; it rose to 2.0 at one year and to 2.7 at 18 months. Treatment of the older rabbits with various hormones such as testosterone, estrogen and growth hormone effected a reversal of the keratan to chondroitin sulfate ratio from that of older to immature cartilage. After termination of hormone treatments the ratio reverted to that of pretreatment, a process requiring about 8 months. The rate of appearance of radioactive carbon in keratan sulfate in adult animals was very slow but followed a generally linear increase throughout the life of the animal. Once labeled, the keratan sulfate lost radioactivity at a very slow rate with a metabolic half-life of about 120 days. Davidson and Small (97) also noted the close correlation between the low metabolic activity of keratan sulfate and of collagen and the net increase in their concentration with time.

Immature cartilage can thus be characterized as containing primarily chondroitin sulfates with a low keratan sulfate content. Deviation from this pattern can be considered grossly abnormal. In this regard Meyer et al.(127) measured the mucopolysaccharide content of costal cartilage from two normal young adults and two cases of Marfan's syndrome, a mucopolysaccharidosis related to Hurler's disease. The total mucopolysaccharides from normal cartilage constituted 16 to 18% of the dry weight while in the cases of Marfan's syndrome the values were 11 and 12% of the dry weight. The

ratio of keratan to chondroitin sulfate was 1:1 in both Marfan's cases, significantly different from the normal ratio of 1:2.5.

4 Metabolism of Keratan Sulfates

Very little is known about keratan sulfate metabolism. The available data refer mainly to glycosaminoglycans other than the keratan sulfates. Some aspects of keratan sulfate metabolism in rabbit nucleus pulposus (97) and human rib cartilage (125) have been mentioned above (Chapter I,B,3). The biosynthetic pathway of the formation of glycosaminoglycan, chiefly the chondroitin sulfates, has been elucidated by Dorfman, Silbert, Rodén and their associates (128-137).

Biosynthesis of the chondroitin sulfates has been achieved *in vitro* with chick embryonic cartilage microsomal preparations in the presence of UDP-glucuronic acid, UDP-N-acetylgalactosamine and Mg^{++} (128,129,131,133). The newly synthesized glycosaminoglycan is tightly membrane bound, of high molecular weight, has a 1:1 molar ratio of glucuronic acid to N-acetylgalactosamine as well as other properties of an undersulfated chondroitin sulfate. The synthesis of this macromolecule in the presence of nucleotide-sugar is inhibited by puromycin (130).

The stepwise biosynthesis of chondroitin sulfate requires the transfer of xylose from UDP-xylose to noncollagenous protein acceptor (132). The xylosyltransferase enzyme is loosely bound to the particles and is stimulated by magnesium or manganese ions. The initial phase of chondroitin sulfate synthesis involves the formation of the linkage region to protein

(reactions 12-14) and subsequently chain elongation by alternate addition of glucuronic acid and N-acetylgalactosamine (reactions 15,16). The product formed from this multistage process, chondroitin, can then be sulfated to form either chondroitin 4- or chondroitin 6-sulfate. In the pathway outlined below R-OH represents the endogenous microsomal glycose acceptor containing a hydroxyamino acid:

R-OH + UDP-xyl → R-O-xylose (I) (12)

I + UDP-gal → R-O-xyl-4-gal (II) (13)

II + UDP-gal → R-O-xyl-4-gal-3-gal (III) (14)

III + UDP-glcUA → R-O-xyl-4-gal-3-gal-glcUA (IV) (15)

IV + UDP-galNAc + UDPglcUA $\xrightarrow{n}$ chondroitin (16)

n = 24-80 (approx.)

The transfer of xylose from UDP-xylose to endogenous acceptor (reaction 12) was first shown by Robinson, Telser and Dorfman (132). The product of this reaction, xylosyl-serine, was isolated by proteolytic digestion of the microsomes. The linkage of xylose to serine was alkali-labine and therefore identical to that of chondroitin sulfate. The same particulate fraction was also capable of transferring galactose from UDP-galactose to the endogenous acceptor (reaction 13). The β-configuration of the galactose-xylose linkage was established by the action of β-galactosidase which quantitatively released the labeled galactose (132). Transfer of the next galactose to the growing oligosaccharide chain (reaction 14) is some-

what sluggish with the endogenous acceptor. Helting and Rodén (136), however, obtained significant glycosyl-transfer in the presence of exogenous acceptors such as galactosyl-4-xylose and galactosyl-4-xylosyl-serine. They proposed that either two separate galactosyltransferases or two separate active centres on one enzyme were active in reactions 13 and 14. Reaction 15 has been demonstrated with both endogenous and exogenous glycose acceptors (137). There does not appear to be a high degree of acceptor specificity in reaction 15 since exogenous gal-6-gal was as active as gal-4-gal-3-xylose when incubated in the presence of the particulate enzyme. After the formation of the linkage region (reactions 12-14), alternating glucuronic acid with N-acetylgalactosamine monosaccharide units are added to the nascent chondroitin sulfate in a stepwise manner. The glucuronosyltransferase active in reaction 15 does not appear to be the same as that active in the subsequent glucuronic acid transfer reactions (reaction 16) (131) since the presence of a pentasaccharide derived from chondroitin sulfate had no effect on the transfer of the endogenous acceptor (reaction 15) but did inhibit the transfer of glucuronic acid to a trisaccharide containing nonreducing terminal N-acetylgalactosamine (137).

"Chondroitin sulfate synthetase" (a series of reactions summarized in reaction 16) appears to have a high degree of specificity toward the glycose unit accepted at the nonreducing terminal position of the nascent chondroitin sulfate. It appears to be a highly ordered, tightly membrane bound "synthetase" holding the growing polysaccharide chain in close proximity for maximal synthetic efficiency (137). In addition, fully sulfated chondroitin sulfate present in the membrane may act as a primer for either polysaccharide synthesis or sulfation or both (133).

Sulfation of the newly synthesized glycosaminoglycan occurs after the polysaccharide chain is completed (128,129,133,135). The sulfotransferases are only partially membrane bound (129,135). The sequence of addition of sulfate to the polysaccharide from 3'-phosphoadenosine-5'-phosphosulfate is as yet unclear but it occurs while the glycosaminoglycan is membrane bound (135).

Dorfman and coworkers recently showed that the pattern of sulfation in embryonic chick cartilage chondroitin sulfate changes with age (134). By measuring the incorporation of acetate-^{14}C into the polysaccharide chain and of ^{35}S-sulfate, they found a high proportion of chondroitin 6-sulfate in young cartilage and a lower content in older cartilage. Thus embryonic chick cartilage has the capacity for 6-sulfation and 4-sulfation simultaneously but on separate polysaccharide species.

It is conceivable that keratan sulfate biosynthesis follows a course of stepwise addition of monosaccharide units to a particulate endogenous acceptor in a fashion similar to that outlined above. However the linkage of keratan sulfate to protein involves a hexosamine instead of xylose. In this regard, studies on glycoprotein synthesis in Ehrlich ascites cells (138), calf thyroid (139) and rat liver (140) have shown the incorporation of N-acetylglucosamine into membrane bound protein both <u>in vivo</u> and <u>in vitro</u>. More recently Spiro and Spiro (141,142) have purified a galactosyl- and sialyltransferase from calf thyroid tissue active in the stepwise addition of galactose and sialic acid into glycopeptides of fetuin and Unit B of thyroglobulin.

There is good evidence that the degradation of the acid mucopolysaccharides is, at least in part, a lysosomal event. Aronson and Davidson

(143,144) have purified a hyaluronidase from isolated rat liver lysosomes which degrades hyaluronic acid to octasaccharides. Chondroitin 4- and 6-sulfates are also substrates for this enzyme in the presence of salt. Chondroitin sulfate B, heparin and heparan and keratan sulfates reversibly inhibit the enzyme in the absence of salt (144). In a similar study, isolated lysosomes derived from leucocytes or rabbit liver degraded protein-polysaccharide complexes (145). The degradation of PP with the leucocyte particulate and crude extracts occurred at either alkaline or acid pH while the rabbit liver lysosomes degraded the complexes only at an acid pH. It was suggested that lysosomal digestion of PP in leucocytes primarily involved proteolysis at either acid or alkaline pH while in the rabbit liver lysosomes "polysaccharidase" activity similar to that of rat liver lysosomes predominated. More recently, Aronson and Davidson (146) found that isolated rat liver lysosomes were capable of the degradation of several glycoproteins and protein-polysaccharides but at different rates. Furthermore, the course of the hydrolysis varied with the glycoprotein substrate suggesting that several factors control the digestive processes including the type and structure of the protein and of the polysaccharide and the degree of polysaccharide branching. Intravenously injected chondroitin sulfate B does appear in measurable quantities in lysosomes (147). A partial degradation of dermatan sulfate likely occurs at this point since recent studies on the structure of umbilical cord and skin dermatan sulfate have shown the presence of appreciable quantities of hyaluronidase-susceptible glucuronic acid groups in the polysaccharide chain adjacent to the linkage region (148-151). Enzymes active in the hydrolysis of heparin and of heparan and dermatan sulfates can be induced in certain micro-organisms such as Proteus vulgaris and Flavobacterium heparinum but similar enzymes

have not been observed in mammalian tissue (152-155).

The larger oligosaccharide products from hyaluronidase action on the acid mucopolysaccharides can be further degraded by oligosaccharidases (156) and the exoglycosidases of lysosomes such as β-glucuronidase, β-N-acetylglucosaminidase, β-N-acetylgalactosaminidase, α-fucosidase and β-D-xylosidase (146,157). Beta-galactosidase would be expected to hydrolyze keratan sulfate at acid pH since β-galactosidases present in Coccobacillus extracts have been used to confirm the β-configuration of keratan sulfate (83,86). Thus many enzymes having protease exo- and endoglycosidase, and transglycosidase activity have been implicated in the metabolism of some of the glycosaminoglycans. The exact sequence of events, however, is still in doubt. Several reviews have discussed, in addition to the above, the fate of intraperitoneally administered glycosaminoglycans and papain, and the effect of steroids on proteoglycan catabolism in cartilage (122,124,158).

C G_{M1}-GANGLIOSIDOSIS AND ITS RELATION TO TAY-SACHS AND HURLER'S DISEASES

G_{M1}-gangliosidosis is now clearly recognized as an inborn error of glycosphingolipid metabolism quite distinct from Tay-Sachs disease (G_{M2}-gangliosidosis) and other forms of neurolipidosis. Many other diseases are known which involve storage of lipids in the central nervous system. It is not the aim of this thesis to enumerate each one in detail nor is it intended to examine all the known findings available which have been drawn from several areas of medicine. Only the more salient features of those diseases which have relevance to G_{M1}-gangliosidosis and the biochemical studies which are the subject of this thesis will be discussed.

G_{M1}-gangliosidosis has clinical and pathological characteristics

shared by Tay-Sachs and by Hurler's diseass. To follow its emergence as a separate disease, or maybe even more than one disease entity, Tay-Sachs and Hurler's diseases will first be discussed briefly.

1 G_{M2}-gangliosidosis: Tay-Sachs Disease

G_{M2}-gangliosidosis is the prototype of ganglioside storage diseases. It was first described independently in Britain and America. Tay (160), a British ophthalmologist, described an infant who had a bilateral cherry red spot in the fundus of the eye, while later Sachs (161), an American neurologist, described the same condition as a familial form of amaurotic idiocy and provided the first clinical and pathological accounts of the disease. Several reviews are available on the clinical and pathological signs characteristic of this disease (162-164). The most common clinical course is as follows: at about one month after birth affected infants show an excessive startle response to sound, at two months of age the cherry red spot may be visible and the infants are unable to hold their heads upright (165). Subsequently, there is progressive mental and motor retardation with seizures developing as a prominent feature at one year. Enlargement of the head is prominent at two years, and death generally occurs before the age of three (165). In the brain, the neurons are swollen or "ballooned" with the nucleus displaced to the periphery. Even in the early work the nuclear displacement was attributed to the accumulation of unknown substances shown histochemically to be predominantly lipid in nature.

Although many more cases of the disease were discovered and reported in the intervening years (1900-1939, see Rothstein and Welt (162)), it was not until 1939 that any biochemical identification of the storage substances was reported. In that year, Klenk (166) isolated a lipid

"substance-X" from the brain of a patient with infantile amaurotic idiocy. Histochemically this lipid corresponded exactly to that known to be present in the cortex and white matter of Tay-Sachs disease patients. Klenk (166) suggested that this material was a cerebroside-like lipid containing N-acetylneuraminic acid. He observed that this material was water soluble, decomposed on hydrolysis and formed a red color on heating with orcinol and acid. He also noted that a small amount of this material was present in normal brain. Since these lipids were located in the central nervous system, particularly in ganglion cells, and because of their glycosidic nature, he suggested the name "ganglioside"(167,168). The early determinations of N-acetylneuraminic acid in Tay-Sachs cerebral gray matter showed levels which were 10 to 20 times normal. Klenk (169) later showed that gangliosides were also increased in white matter in this disease (170,171).

The chemical structure of the stored ganglioside in brain, both in gray and white matter and, to a lesser degree, in extraneural tissue particularly the spleen and liver, is that of G_{M2}-ganglioside (Table 1) which differs from G_{M1}-ganglioside only in the absence of a terminal galactose (172,173). The asialo-derivative of this ganglioside also accumulates in abnormal amounts (31,174,175). The studies of Korey, Terry and coworkers (171,176,177) have conclusively shown that in neurons the G_{M2}-ganglioside is present associated with other lipids and protein in characteristic cytoplasmic organelles termed membranous cytoplasmic bodies.

From examination of the structure of G_{M2}-ganglioside in relation to other gangliosides, one obvious explanation for its accumulation was the absence or deficiency in Tay-Sachs disease of a β-N-acetylgalactosaminidase which would thus curtail the normal ganglioside metabolism. However initial studies revealed that hexosaminidase activity was not decreased but was normal or elevated (178). This presented a dilemma which has only recently

been resolved. Frohwein and Gatt (79,179-182) showed that in calf brain there are three distinct enzymes having β-N-acetylhexosaminidase activity. Two of these enzymes were soluble, one a β-N-acetylglucosaminidase and the other a β-N-acetylgalactosaminidase. Neither of these enzymes cleaved the terminal N-acetylgalactosamine from G_{M2}-ganglioside. The particulate enzyme, on the other hand, which was resistant to detergent solubilization hydrolyzed G_{M2}-ganglioside, while with synthetic substrates it exhibited both N-acetylglucosaminidase and N-acetylgalactosaminidase activity (79). Recently O'Brien and associates (183) demonstrated that β-N-acetylhexosaminidase exists in two "isozymic" forms, A and B in humans. In Tay-Sachs disease they showed that the A form was missing in cerebral cortex, liver, kidney, leucocytes and cultured fibroblasts. The two enzyme forms were located in both soluble and particulate fractions and are likely similar to the enzymes reported by Frohwein and Gatt (179-182). Kolodny in Brady's laboratory (184) and Hultberg in Sweden (185) have confirmed O'Brien's results and it is now possible to specifically diagnose Tay-Sachs disease by β-N-acetylhexosaminidase enzyme assays in muscle or skin biopsies and leucocytes.

2 Hurler's Disease (Gargoylism)

The prototype of inherited mucopolysaccharide storage disease is Hurler's disease. Gargoylism was first recognized as a disease entity by a Scottish physician, Thompson in 1900 (see Henderson (186)). Hunter (187) was the first to provide an adequate description of gargoylism but it was Hurler who first described the disease in its fully developed form (188).

The name "gargoylism" was adopted since the disease is manifested by grotesque appearances of facial structures. The most common characteristic of patients with Hurler's disease besides the facial abnormalities are marked bony changes affecting the long bones and the spine. The cornea is frequently cloudy and many patients are severely mentally retarded. Hepatomegaly and splenomegaly are commonly present (see Dorfman,189).

In 1948 Green (190) reported a case of Hurler's disease in which he described in great detail the central nervous system involvement in this disease. In the brain, using histochemical techniques, Green observed the presence of abnormal cell bodies. These cells were highly swollen, there was a reduction of Nissl substance with displacement of the nucleus. Associated with these features there was excessive accumulation of materials identified as being primarily lipid in nature. The author noted that the lipid deposits in the neurons of this Hurler patient appeared to be very similar to those found in the infantile form of amaurotic idiocy (Tay-Sachs disease). Green suggested that although Hurler's disease and Tay-Sachs disease were clinically distinct entities, the lipid deposits demonstrated histochemically were quite similar and thus these two diseases had a common causative factor. The similarity of the changes in this disease to those of Tay-Sachs disease suggested to Green that the alterations in the nervous system in these diseases were due to an inherent disturbance in lipid metabolism.

Later, Jervis (191) confirmed the findings of Green but noted that although over 100 cases of the disease had been reported by 1950, less than 15 had been examined pathologically and only 7 reports had included an examination of the central nervous system. As a result he reviewed the major pathological findings in this disease. He noted that histochemically in the liver, the staining reactions were such as to indicate the presence of polysaccharides but not glycogen. The view prevalent at that time was

that the massive involvement of visceral organs in Hurler's disease could be attributed to lipid deposition although in many instances lipid storage could not be demonstrated in the viscera.

Brante (192) was the first to obtain direct chemical information about the deposits in Hurler's disease. He noted large depositions of metachromatic material in both hepatic and Kupffer cells. He isolated a sulfated polysaccharide from dried liver and reported the composition of this material to be 3.9% sulfur, 27% hexosamine and 26% uronic acid. This substance represented approximately 10% of the dry weight of liver and was highly water soluble. Uzman (193) isolated two different types of substances from the liver and spleen of patients with Hurler's disease. One type was a complex polysaccharide containing hexoses (glucose, galactose), hexosamines (glucosamine, galactosamine) and sulfate. The other fraction, a crude glycolipid complex mixture, contained fatty acid, sphingenine, sialic acid, hexuronic acids, hexosamine, glucose and galactose. Stacey and Barker (194) isolated a sulfated polysaccharide from Hurler's disease liver which had a small degree of anticoagulant character. They believed this material to be similar to heparin since on hydrolysis the disaccharide, heparosine sulfate, was obtained. This polysaccharide, which was isolated in high yield, was recovered from the lipid free liver residue by hot water extraction. It precipitated with cetyl trimethylammonium bromide and had an optical rotation of $+18.1^{o}$. These heparin-like polysaccharides were stored in large quantities at the expense of glycogen since only a minor fraction of the glycogen material usually present was isolated. The liver specimen examined by Stacey and Barker was obtained from the patient described by Bishton et al. (195) who were unable to confirm the earlier

findings of Brante. Brante (192) had reported an elevation in brain gangliosides in his cases of Hurler's disease but Bishton et al.(195) found no significant deviation from normal in these components.

Dextro-rotatory oligosaccharides comprising 0.4 to 1.4% of the wet weight were isolated from Hurler's disease liver by Brown (196). These materials were not found in normal liver. The oligosaccharides were composed of glucosamine and uronic acid with about two thirds of the hexosamine amino group N-acetylated and the rest sulfated. The isolated oligosaccharides had a molecular weight in the range of 1200-2000 and were heterogeneous, having sulfur to nitrogen ratios from 0.66 to 2:0. These oligosaccharides were likely derived from the heparan sulfate described by Jorpes and Gardell (197).

Meyer and coworkers (198,199) utilized cetyl trimethylammonium bromide to isolate large quantities of acid mucopolysaccharides from the urine of patients with Hurler's disease. These mucopolysaccharides were recognized as chondroitin sulfate B (dermatan sulfate) and heparitin sulfate (heparan sulfate) while only the latter was found in normal liver. Chondroitin sulfate B normally occurs in all connective tissue with the exception of cartilage, bone and cornea while heparan sulfate also occurs in lung, aorta and in liver (148-152,200-208). In the brain of Hurler's disease only a moderate increase in the amounts of chondroitin sulfate B and heparan sulfate have been observed, while in normal brain hyaluronic acid and heparan sulfate are the predominant glycosaminoglycans (198,199). Landing and Freiman (209) summarized in 1959 the prevailing views on these diseases when they stated that a number of conditions having the common property of swollen nerve cell bodies but varied clinical symptomatology

could be grouped either with the amaurotic idiocies or with other diseases related to Hurler's disease. The chemical nature of the lipid storage substances in the brain of Hurler's disease was unknown except for histochemical characteristics which were insufficient to accurately define the type and quantity of stored materials.

The lipid storage materials in Hurler's disease were identified for the first time in a preliminary communication in 1964 (210). Three gangliosides in the brain of Hurler's disease were present in increased amounts compared with normal brain, namely G_{M3}-, G_{M2}- and G_{M1}-gangliosides(210,211). Associated with the increase in all monosialogangliosides there was a corresponding reduction in the amounts of the G_{D1a} disialoganglioside, while the other polysialogangliosides were present in normal amounts (52). An unusual ganglioside containing 2 moles of galactose and one mole of sialic acid but no hexosamine has also been found in Hurler's disease brain (212). The ganglioside accumulations in neurons in Hurler's disease are concentrated in cytoplasmic organelles called "zebra bodies" which are of similar structure and origin to the membranous cytoplasmic bodies of Tay-Sachs disease (213,214).

The basic biochemical defect in Hurler's disease has been obscure for many years and only recently has significant progress been achieved. Dorfman and his colleagues clearly showed that fibroblast cell cultures derived from Hurler's patients accumulated intracellularly very high quantities of dermatan sulfate and small amounts of hyaluronic acid and a chondroitin sulfate (215). The dermatan sulfate was synthesized in a manner similar to that of normal tissue, had a high molecular weight and contained serine and aspartic acid in its protein core (216). In addition, the major gangliosides in the fibroblasts were similar to those found to be elevated

in Hurler's brain (217). In Dorfman's view, the accumulation of intracellular dermatan sulfate was the result of either overproduction or faulty degradation (215,216). The latter interpretation now appears to be the correct one. Neufeld and coworkers, with cell culture studies similar to those of Dorfman, were able to distinguish between two intracellular pools of mucopolysaccharide, (1) a secretory pool and (2) a pool for degradation (218). They showed that Hurler's fibroblasts were capable of secretion of large molecular weight, newly-synthesized mucopolysaccharides in a manner similar to normal fibroblasts, but in the pool of mucopolysaccharides destined for degratation, factors required for the normal catabolism were lacking (218,219). Consequently this second pool of mucopolysaccharide continually increased. The complicated picture was somewhat clarified when these workers identified a corrective factor capable of facilitating the removal of the stored mucopolysaccharide as smaller fragments from the cell to the medium. This factor, present in normal cells but not membrane bound, was found to be insensitive to nucleases, heat labile and dialysable (220). It was associated with the serum proteins of the culture medium. This "corrective factor" was discovered in co-cultures of fibroblasts from Hurler's and Hunter's diseases (219). Intracellular accumulation of mucopolysaccharides in these cell strains was almost abolished when cells from each disease were cultured together or in medium preconditioned with normal cells. The co-culture experiment suggests that more than one of these factors exists although their specific chemical nature is as yet unclear (220).

Another recent development in this field has been the recognition of a specific loss of a β-galactosidase "isozyme" in Hurler's and in Hunter's

diseases (221-225). Total β-galactosidase activities are low in skin and liver tissues, almost normal in kidney and slightly elevated in plasma. This variability has been attributed to the loss of a specific pH 4.3-4.7 β-galactosidase "isozyme." The loss of this isozyme in Hurler's disease has been shown also by Sephadex G-150 chromatography and starch gel electrophoresis (224,225). Thus at present the genetic defect of Hurler's disease appears related to an absence or defective activity of a low molecular weight component required for the normal degradation of the dermatan sulfates together with a loss or decrease of one or more isozymes of β-galactosidase.

3 G_{M1}-gangliosidosis

In 1959 the first report appeared on a condition combining the features of Hurler's disease in the viscera and of Tay-Sachs disease in the brain. Norman and associates (226) described an infant having a cherry red spot in the fundus of the eye, with grotesque facial features reminiscent of the Hurler's facies but with no skeletal X-ray abnormality. This child had an enlarged liver but the spleen was not palpable. Histochemically in the cerebral cortex, the nerve cell bodies were ballooned and the nuclei displaced. In the cortex, as well as in other parts of the brain, the swollen cells contained PAS-positive granules. In all the visceral organs examined (liver, spleen, lymph gland, thymus, bone marrow, lung, pancreas, intestine, kidney, adrenal medulla and thyroid) there were foam cells containing a highly vacuolated cytoplasm. Chemical analysis was performed on samples of brain, liver and spleen. In the brain there was a three-fold increase in NANA, a two-fold increase in the content of galactose

and an increase in ganglioside content of the gray and white matter. The characterization of this accumulated ganglioside was not reported. In the liver and spleen, an increased content of galactose and hexosamine was observed but there was no abnormality in the content of NANA. The authors noted that although the nonlipid hexosamine was much greater than the lipid hexosamine, only a small percentage of the total was water soluble hexosamine. The authors believed that the infant had Tay-Sachs disease with visceral involvement and cited previous cases of this disease in which some degree of foam cell histiocytosis was found (6,227). This infant was from a family which included two previous siblings who had died from a similar condition and five normal children. This was the first documented case of the disease now recognized as G_{M1}-gangliosidosis. Craig and coworkers (228) in the same year described an infant with "foam cell" histiocytosis in the viscera, pronounced ballooning of the renal glomerular epithelium and clinical and radiological features suggestive of Hurler's disease. They called their disease a variant of Hurler's disease in which no chondroitin sulfaturia was observed (228,229).

In 1964, Landing et al. (230) described a series of eight patients with a condition characterized by severe progressive mental deterioration, accumulation in the brain and viscera of acidic glycolipids similar to gangliosides, and the presence of skeletal abnormalities. Urinary spot tests revealed no acid mucopolysaccharidurıa. In one case, demineralization of the long bones was observed as well as features seen in the cartilaginous skeletal dysplasias. The authors preferred to call this condition "familial neurovisceral lipidosis".

Although Norman et al. (226) had demonstrated increased ganglioside-NANA in the brains of patients with this disease, it was not until 1965 that the nature of the lipid storage substances was defined. O'Brien and associates (29) reported chemical investigations on a ninth patient of the group reported by Landing et al. (230). They isolated a mixture of gangliosides from the brain, liver and spleen of this patient utilizing methods which minimize the acid hydrolysis of glycosidic linkages. On thin layer chromatography (231) the pattern of gangliosides obtained was grossly different from that obtained with normal and Tay-Sachs disease brain gangliosides. The gangliosides accumulated in this disease migrated like the major monosialoganglioside (G_{M1}-ganglioside) of normal brain. This acidic glycolipid, comprising 70 to 90% of the total gangliosides, contained ceramide, hexose, hexosamine and sialic acid. Glucose and galactose were present in the molar ratio of 1:2 respectively. Stearic acid (18:0) was the major fatty acid with small amounts of palmitic (16:0), myristic (14:0) and arachidic (20:0) acids. The gangliosides isolated from liver and spleen were shown to migrate on TLC with Rf values identical to the major brain ganglioside in this disease. The presence of G_{M1}-ganglioside in the liver and spleen was unusual since it had been shown (231,232) that the major ganglioside normally present in these tissues was hematoside (G_{M3}-ganglioside, see Table 1 and Chapter 1, part A,2). The finding of G_{M1}-ganglioside in the brain and in the viscera led these authors to propose the designation of this disease as "generalized gangliosidosis". The authors did suggest, however, that the accumulation of G_{M1}-ganglioside in the liver and spleen could not, in itself, be responsible for the very large number of foam cells found in these organs.

Jatzkewitz and Sandhoff(233) reported G_{M1}-ganglioside storage in the brain of a patient with amaurotic idiocy. Although the brain had been stored in formalin for 26 years, comparison with a normal brain sample stored for the same length of time indicated that ganglioside destruction due to the action of formalin could not account for the excessive accumulation of the G_{M1}-ganglioside. Formalin fixation of tissues has been shown to degrade gangliosides to give mainly G_{M1}-ganglioside in a short period of time (234,235).

Gonatas and Gonatas (214) in 1965 described a patient with cerebral G_{M1}-gangliosidosis as well as a large degree of visceral involvement. No bony abnormalities were noted on X-ray examination but large populations of foam cells were found in the liver, spleen and bone marrow. They designated this disease "systemic late infantile lipidosis" since the signs were sufficiently different from those observed in other reports of the disease. The ultrastructure of the brain in this case, as well as a case of Hurler's disease, revealed large populations of membranous cytosomes similar to those found in Tay-Sachs disease. G_{M1}-ganglioside was stored in the brain of this patient, a confirmation of the findings of O'Brien and associates (29). G_{M1}-gangliosidosis was by now firmly established as a disease entity distinct from Tay-Sachs and Hurler's diseases. In another report (211) the G_{M1}-ganglioside isolated from the brain of a case of systemic late infantile lipidosis and of Hurler's disease was shown to be identical with G_{M1}-ganglioside of normal human brain (32,231,236). In addition to the fatty acid and carbohydrate findings previously enumerated, this ganglioside was found to contain four types of long chain aliphatic bases as in normal brain gangliosides.

Suzuki and coworkers (175,237) described a patient similar to that

of Gonatas and Gonatas (214) in which no detectable bony abnormalities were visible on X-ray examination and the typical Hurler's disease characteristics were missing. Unlike previous cases where an early onset of clinical symptoms was noticed (see O'Brien, 238) this child developed normally up to the age of 5 to 7 months. Thereafter the child progressively deteriorated and died at the age of 37 months. The age at death was unusual since all previous cases of the disease having Hurler's characteristics died at or before two years of age (238). Notwithstanding these differences from previously reported cases, the storage of G_{M1}-ganglioside was demonstrated in the nerve cell bodies but not in the liver and spleen, although foam cells were observed in these visceral organs.

Thus as recently as early 1968, there was considerable confusion about this disease, identified as a G_{M1}-gangliosidosis on the basis of the nature of lipid storage. Several aspects of this disease contributed to the confusion. First, the nomenclature of this disease was as abundant as the authors studying the condition (summarized by O'Brien, 238). Secondly, the storage of G_{M1}-ganglioside was found in patients who had clinical signs of Tay-Sachs and Hurler's diseases combined, some had the Hurler's facies as a prominent feature and others did not have these characteristics. Some patients had an early onset of neurological deterioration and still others were apparently normal until the latter half of the first year. Furthermore the time course of the disease was different. Patients having the gargoylism characteristics died before two years of age while patients not having these features lived longer.

This variability of clinical expression was recognized by Derry, Wolfe and coworkers (239) who proposed that G_{M1}-gangliosidosis existed as two types based on phenotypic expression. In Type I (now commonly termed "generalized gangliosidosis") the onset of clinical signs occurs from birth

to 5 months with hepatosplenomegaly and skeletal X-ray changes similar to those of Hurler's disease. In Type II, the onset of clinical signs is from 5 to 14 months of age, there is no visceromegaly and no characteristics of gargoylism. Derry et al. suggested that another point of distinction between the two types of this disease was the presence or absence of increased amounts of G_{M1}-ganglioside in the liver and spleen, although this finding may be related to variability between cases (see Suzuki and associates, 31 and 240). In both types of this disease, G_{M1}-ganglioside is stored in the neurons of the central and peripheral nervous system and there are large populations of foam cells in the liver, spleen and bone marrow together with a characteristic vacuolation of the glomeruli and proximal convoluted tubules.

The first reports on an enzymatic deficiency in G_{M1}-gangliosidosis appeared in 1967. A markedly deficient β-galactosidase activity, measured at pH 3.6, was reported in liver biopsy specimens of two cases of Type I G_{M1}-gangliosidosis (241,242). These measurements were made in the laboratory of Van Hoof and Hers who later reported an extensive study on β-galactosidase and other lysosomal hydrolases in Type I, G_{M1}-gangliosidosis and other mucopolysaccharidoses (157). Subsequently Okada and O'Brien (243) found that brain, liver and spleen tissues of patients with generalized gangliosidosis showed greatly reduced capacity to release the terminal galactose from G_{M1}-ganglioside.

The studies reported in this thesis were initiated in early March 1968. A case of the Type II form of G_{M1}-gangliosidosis was diagnosed on the basis of increased amounts of G_{M1}-ganglioside isolated from a cerebral biopsy specimen. This patient is still alive at the age of 5. At the time, no reports had appeared on the enzyme deficiency in this form of G_{M1}-

gangliosidosis, nor had the non-lipid storage substances been identified in either type of this disease. We therefore initially embarked on biochemical studies on the Type II form of G_{M1}-gangliosidosis to identify the genetic defect in this patient, to attempt detection of the heterozygous condition and to characterize the non-lipid visceral storage substances. The urine of this patient showed positive toluidine blue screen test for mucopolysaccharides. In the Type I form of the disease, no elevation in urinary uronic acid has been observed (228-230,242). However, Derry et al. (239) observed faintly positive metachromasia in the urine of their Type II patient. Microscopic examination of the liver and spleen showed the presence of foam cells and vacuolated cyroplasm similar to that of Type I patients (237). Norman and coworkers (226) had previously observed excessively high levels of non-lipid bound galactose and hexosamine in the liver of their Type I patient but did not characterize these compounds. In Hurler's disease, there is a close correlation between the accumulation of acid mucopolysaccharides in the liver and the elevated level of urinary uronic acid-containing materials. This suggested that an elevated excretion of galactose-containing polysaccharides may be responsible for the positive toluidine blue screen tests in this patient. We examined this possibility. The results of the urinary studies as well as recognition of the enzyme defect in the patient and her family have been reported (244, 245).

Subsequently we isolated and characterized the polysaccharides accumulated in the liver in the Type I form. While this work was in progress Suzuki and coworkers (31,240) tentatively identified the materials accumulated in the liver and spleen of G_{M1}-gangliosidosis as keratan sulfates on

the basis of their carbohydrate composition and electrophoretic mobility. Their results are substantially in agreement with the results reported in this thesis. In addition, we established fibroblasts cell strains from skin biopsies of patients with both types of G_{M1}-gangliosidosis. The aim of the cell culture studies was to investigate the metabolism of the glycolipids and glycosaminoglycans in this disease. Such studies should provide valuable information regarding the etiology of the two types of G_{M1}-gangliosidosis.

Chapter II

MATERIALS AND METHODS

A CLINICAL MATERIAL

1 G_{M1}-gangliosidosis

Case 1. A young girl of French Canadian and Mohawk Indian ancestry was referred to us by Dr. C.R. Scriver, Montreal Children's Hospital. She was the second child in the family to be affected with a progressive degenerative neurological illness. She was considered normal at birth but her condition deteriorated after 10 months of age. Seizures developed at the age of two and a half years and she was admitted to the Montreal Children's Hospital early in 1968 at the age of 33 months. Neurological and electroencephalographic tests were carried out. The spleen and liver were not palpable and a skeletal survey was normal. Alcian and Toluidine blue tests on the urine were positive. A right posterior cerebral biopsy was carried out. A fibroblast cell strain was developed from a punch biopsy of skin taken from the deltoid area. This patient was diagnosed as type II, G_{M1}-gangliosidosis on the basis of clinical, pathological and biochemical findings. The studies on the brain leucocytes, urine and cultured fibroblasts obtained from this patient form a large part of this thesis. A complete clinical history and the pathological and biochemical findings have been published (244,245).

Case 2. This child came to our attention in August 1969. He displayed the clinical characteristics of the type I form of G_{M1}-gangliosidosis confirmed initially by leucocyte β-galactosidase assay and subsequently by analysis of gangliosides isolated from brain, liver, spleen and appendix. The biochemical findings available on this patient and the family will be included here. Fibroblast cell strains were also developed from the skin of this patient but only preliminary experiments on these cell strains have been carried out and any results have not been included here.

Case 3. This child was reported by Derry, Wolfe et al. in 1968 (239). The gangliosides isolated from the brain of this patient served initially as markers for recognition of G_{M1}-ganglioside accumulation in the brain of other patients.

Case 4. Samples of brain and liver from this patient who had type I G_{M1}-gangliosidosis were generously supplied by Dr. J.A. Lowden of the Hospital for Sick Children, Toronto, Ontario. Diagnosis was made in Toronto on the basis of clinical, pathological and biochemical examination. The liver specimen was used for the studies on the isolation and characterization of undersulfated keratan sulfate glycosaminoglycans reported in this thesis.

All specimens were frozen in liquid nitrogen or obtained frozen and stored at -20^{o} C.

2 G_{M2}-gangliosidosis and Other Neurolipidoses

For the sake of comparison, several biopsy and autopsy specimens have been included in this work. Samples of brain were obtained at autopsy from a case of G_{M2}-gangliosidosis. Biopsy of specimens of brain were obtained from two patients (cases 1 and 2) diagnosed as the Jansky-Bielschowsky form of late infantile amaurotic family idiocy (curvilinear body disease). Liver specimens were obtained at autopsy from two additional cases (3 and 4) of late infantile amaurotic family idiocy and from a case of Niemann-Pick disease. Late infantile family idiocy is not recognized as a ganglioside storage disease while Niemann-Pick disease is a sphingomyelin lipidosis (246-248).

B LIPID ANALYSIS

1 Ganglioside Extraction

Gangliosides from 1 g of frozen human cerebral cortex were extracted with 19 volumes chloroform-methanol (2:1, v/v) by the method of Suzuki (235). The extract was filtered through preweighed sintered glass funnels. The residue remaining was dried and used for extraction of sialomucopolysaccharides (p. 56). The chloroform-methanol extract was partitioned by the method of Folch, Lees and Sloane-Stanley (249). The upper phases were pooled, concentrated, and dialyzed for 36 hrs with several changes of distilled water. The sphingolipids in the chloroform-methanol lower phase were isolated as outlined below. Aliquots of the dialysed upper phases were taken for assay of N-acetylneuraminic acid (NANA). The remainder of the dialysed upper phases were freeze dried, weighed and the NANA content

of the dried material was again determined. Individual gangliosides were separated on thin layer chromatograms, as described.

Ganglioside standards used for structural analysis and as substrates for enzymes were obtained from ox brain. Fresh ox brain was chilled, the meninges removed and the cortex was recovered by scraping. The fresh cortex was then weighed and homogenized successively with 20 volumes of acetone (twice) and 20 volumes of ether. The dried cortex powder was stored in a desiccator at -20°C until used. Gangliosides were isolated from 100 g samples of acetone-ether powder (100 g). To the powder 100 ml of 1 M KCl was added (see 250) and the paste was homogenized in 19 volumes of 2:1 chloroform-methanol, as above.

Liver gangliosides were isolated as above except that 10 volumes of chloroform-methanol (2:1) was used. The lipids present in the lower phase after the Folch procedure were not analyzed. The gangliosides of cultured skin fibroblasts were isolated from lyophilized cells as for liver. The lipid-free fibroblast cell residue was retained for glycosaminoglycan analysis.

2 Isolation of Lower Phase Sphingolipids

Lipids in the lower phase of the Folch partition were dried under nitrogen and stored in a small volume of 2:1 C/M at -20°C until used. Total lipid weight was determined after the removal of proteolipid protein (see Suzuki and Chen, 175) and lipid phosphorus was measured by the Martin and Doty procedure (251). The sphingolipid fraction was isolated after removal of phospholipids and neutral lipids during the mercuric chloride saponification procedure of Abramson, Norton and Katzman (252). Fractionation of

the sphingolipids on thin layers or by column chromatography will be outlined later.

C ISOLATION OF BRAIN SIALOMUCOPOLYSACCHARIDES

The residue after the removal of lipids (p.54) was digested with papain as described for the urinary glycosaminoglycans, at a concentration of 1 mg residue/ml buffer, and the sialomucopolysaccharides isolated by the method of Brunngraber and Brown (253).

D ENZYME DETERMINATIONS

1 Assay of β-galactosidase

Brain. Beta-galactosidase was extracted from 300 mg of cerebral cortex by homogenizing in 90 volumes of 0.25M sucrose- 1 mM EDTA at pH 7.0 for 1.5 min, as reported by Gatt and Rapport (75) and O'kada and O'Brien (243). Enzyme activity was measured in the crude homogenate fluorimetrically with 4-methylumbelliferyl β-D-galactopyranoside (Koch-Light, Colnbrook, U.K.) as substrate or spectrophotometrically with O-nitro-phenyl-β-D-galactopyranoside (CalBiochem, Calif.) as substrate by the method of Gatt and Rapport (75).

Leucocytes. Leucocytes were isolated from blood by the method of O'kada and O'Brien (personal communication). 5 ml heparinized venous blood was added to 10 ml of 3% Dextran-75 in saline, the red blood cells allowed to settle for 1 hr at room temperature and the plasma decanted. The leucocytes were collected from the plasma by centrifuging and suspended in 1 ml of 0.1M acetate buffer pH 5.0 and aliquots withdrawn for

leucocyte counts and for spectrophotometric enzyme assay by the Gatt and Rapport procedure (75). In general, the assay mixture included 65 µmoles of buffer, 50 µg of sodium taurocholate and 1 µmole of substrate in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37°C in a shaker bath. The reaction was terminated by the addition of 1.5ml of 2.8% TCA. The precipitate was removed by centrifugation and the supernatant fluid was withdrawn to another tube containing 0.9ml of 0.5M NaOH. Glycine-carbonate buffer (0.25mµ,pH 10) 1.5 ml was then added and the optical density was read at 420 λ in a Zeiss PMQ-2 spectrophotometer.

Fibroblasts. Skin fibroblast cultures were established and grown in monolayers (p.61). After removal of the fibroblasts from the monolayer by EDTA-Trypsin treatments (254), the cells were collected by centrifuging and washed with normal saline. The cells were suspended in normal saline and aliquots withdrawn for cell counting and enzyme assay as described for leucocytes. Beta-galactosidase activity was measured by both the fluorometric and spectrophotometric methods.

2 Assay of Brain Neuraminidase

Acetone powders of pieces of cerebral cortex were prepared and neuraminidase activity assayed by the method of Leibovitz and Gatt (68). Free NANA was measured by the thiobarbituric acid method of Warren (255). The following substrates were used: G_{M1}- and G_{D1a}-gangliosides isolated in this laboratory from ox brain by silica gel HR column chromatography (p.64), sialyl-lactose (Sigma Chemical Co., St Louis, Mo); and the sialyl-oligosaccharide from G_{D1a} prepared by ozonolysis, base hydrolysis and column chromatography (pp.65,69,70).

3 Assay of Liver Enzyme Activities

Liver samples from normal, G_{M1}-gangliosidosis and other lipidoses were homogenized in 9 volumes of 0.25 sucrose-0.001 M EDTA, pH 7.0. Beta-galactosidase, β-N-acetylglucosaminidase, β-glucosidase and fucosidase were measured according to the methods of Van Hoof and Hers (157).

E ISOLATION OF URINARY GLYCOSAMINOGLYCANS

Consecutive and intermittent complete 24 hour urine collections were obtained from Case 1 and several normal subjects. Creatinine was routinely determined. The urine was dialysed exhaustively at 4°C, filtered and the sac contents were concentrated to dryness in a continuous flow flash evaporator. The residues were dissolved in approximately 150 ml of 0.1 M acetate buffer at pH 5.5 containing 5 mM cysteine and 0.5 mM EDTA. Papain (Sigma Chemical Co., St. Louis, Mo.) was added at a concentration of 35 µg enzyme protein/ml buffer and the mixture incubated for 24 hours at 60°C in a shaker bath. The sediment was removed and the supernatant fluid dialysed for 48 hours against distilled water. The dialysed digest was separated into two crude fractions on the basis of their precipitability with cetylpyridinium chloride (CPC). Two percent CPC was added to the digest dropwise with stirring until no further precipitate formed. The precipitate was allowed to settle overnight at 4°C, collected by centrifuging. The supernatant fluid was kept and treated as described below. The precipitate was dissolved in propanol and the crude glycosaminoglycans re-precipitated as their sodium salts by the addition of ethanol and saturated sodium acetate by the procedure of Antonopoulos et al. (256). The precipitate was washed consecutively with 95% ethanol, 100% ethanol and dry ether.

The dried precipitate was dissolved in water, made to 10% with trichloroacetic acid and centrifuged. The supernatant fluid was dialysed exhaustively and freeze dried. This fraction is called the "crude CPC-precipitable glycosaminoglycans".

After removal of the CPC precipitate, 3 volumes of 95% ethanol and saturated sodium acetate (13 ml/100 ml of solution) were added to the supernatant fluid. The solution was allowed to stand at 4°C overnight and the precipitate collected by centrifuging. This fraction is called the "crude non-CPC precipitable glycosaminoglycans". In some preparations a further proteolytic digestion with Pronase (CalBiochem., Los Angeles) (257) was carried out and the glycosaminoglycans recovered as described. Subsequently both glycosaminoglycan fractions were purified by fractionation on column chromatography. The crude and purified glycosaminoglycan fractions were analyzed for a variety of constituents and subjected to hyaluronidase treatment and electrophoresis.

F ISOLATION OF LIVER GLYCOSAMINOGLYCANS

Two frozen liver samples from Case 4 (22.5 g, preparation No.1, and 15.0 g, preparation No.2) were homogenized in chloroform-methanol essentially according to the procedure of Suzuki (235), except that 10 volumes of solvent per gram wet weight of liver was used. The extract was filtered on Buchner funnels. The residue remaining was air dried and used for extraction as described below. The chloroform-methanol extract was partitioned by the method of Folch *et al*. (249). The upper phases were pooled, concentrated and dialysed for 36 hours against distilled water. The lyophilized sac contents after dialysis constitute the "*ganglioside fraction*".

The lipid free liver residue obtained from preparations No.1 and No.2 were separately digested, in successive steps, with papain (2X recrystallized, Sigma) and α-amylase (B.Subtilis-Type IIA-Sigma). Papain digestion was carried out as before at a concentration of 45 µg of enzyme protein/ml buffer and the mixture incubated for 24 hours at 60°C in a shaker bath. The sediment was removed by centrifugation and the supernatant fluid was dialysed for 48 hours against distilled water and lyophilized. The αamylase digestion was performed with dialysis for 20 hours at room temperature, and at a concentration of 5 mg dried solid/ml buffer. The buffer used was 0.01 M potassium phosphate, pH 7.3 containing 0.9% sodium chloride and 10 mg α-amylase per 100 ml of buffer. At the end of the dialysis period, the sac contents were brought to 5% in TCA in an ice bath. The supernatant fluid obtained was then dialysed for 24 hours against distilled water. The sac contents were then collected, centrifuged and concentrated to reduced volume. This fraction constitutes the total "residue fraction". This fraction (T) was subjected to ethanol fractionation.

Ethanol fractionation

The glycosaminoglycans in the "ganglioside fraction" were subjected to ethanol fractionation after chromatography on DEAE-Sephadex A-50. The effluents from appropriate fractions were pooled and concentrated. To this solution 3 volumes of 100% ethanol was added and the precipitate (P) was allowed to settle overnight at 4°C. The precipitate was washed successively with 100% ethanol and diethyl ether, then dissolved in water and lyophilized. The ethanolic supernatant fluid recovered from the initial step was concentrated and dialysed for 24 hours at 4°C against

distilled water to yield two fractions, the sac contents (SN) and the dialysate (D).

The glycosaminoglycans in the total "residue fraction" (T) were subjected to ethanol fractionation as above, without prior chromatography on DEAE-Sephadex A-50, to yield two major fractions, an ethanol precipitate (P) and a supernatant fluid fraction (SN) as described above. The fraction (P) from the residue fraction was divided into two subfractions by precipitation with cetylpyridinium chloride(CPC). The uronic acid-containing mucopolysaccharides were obtained by precipitation with 2% CPC. The CPC precipitate was dissolved in propanol and the acid mucopolysaccharides were freed from CPC by re-precipitation of the glycosaminoglycans with ethanol and saturated sodium acetate (256). This fraction was called P_2. This procedure gave a recovery of 78% by weight.

G CELL CULTURE STUDIES

1 Culture Conditions

Skin fibroblast cultures were established and maintained by Dr. L. Pinsky, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal. The culture experiments were performed in Dr. Pinsky's laboratory. All cell strains were developed according to Krooth (258). The mutant strain was started from a 4 millimeter full thickness punch biopsy of skin in the deltoid area when the patient was 3 years old (case 1). The control strains were derived either from newborn foreskin of from the deltoid area of healthy adults. The culture medium was Eagle's MEM (259) supplemented with non-essential amino acids, pyruvate,

penicillin G, streptomycin, oxytetracycline and 12% fetal calf serum.

2 Radioactivity Incorporation Experiments

One million cells were planted in 100 mm Falcon dishes with 10 ml of medium. One day later, 10 ml of medium, with one of the following labeled components, was substituted: 1-^{14}C-glucosamine, 0.4 µC; 1-^{14}C-D-galactose, 2 µC; ^{14}C-reconstituted protein hydrolysate (R.P.H.), 2 µC; 1-^{14}C-sodium acetate, 2 µC; $H_2{}^{35}SO_4$, 5 µC. The labeled medium was removed at intervals and the cells were detached from the plates by successive incubation in EDTA and Trypsin (254). They were washed three times in saline, and the cell pellets were stored at -20°C until thawed, resuspended in water and lyophilized. Weighed portions of the dried cell residue were digested in 1 ml of 0.1 M NaOH for 4 hours and aliquots were removed for measurement of protein and radioactivity. The remainder of the dried cells were retained for lipid and glycosaminoglycan analysis.

To investigate the rate of loss of radioactivity, replicate cultures were labeled for 24 hours with 0.4 µC of 1-^{14}C-D-glucosamine, then the regular medium was replaced and cells were harvested at intervals. Aliquots of the medium and washing solutions were digested with Hyamine as described below and counted. The rest of the media were subjected to Pronase digestion by the method of Danes and Bearn (257).

3 Co-culture Experiments

Equal numbers of cells from control and patient strains were plated separately ($1x10^6$ cells total) and together ($1x10^6$ cells total) with 10 ml of medium. The cells were then exposed to medium containing

1-^{14}C-D-glucosamine (0.4 μC). After 24 hours, the cells were harvested as above except that the cells were finally suspended in saline for β-galactosidase enzyme activity, counting and protein measurements.

4 Isolation of Fibroblast Glycosaminoglycans

The lipid-free cell residue,recovered after the extraction of gangliosides, was digested by papain in 1 ml of sodium acetate buffer as described above.

The digests were centrifuged and small aliquots of the supernatant fluid were removed for counting. The remainder was dialysed for 24 hours at 4°C against distilled water. Dialysed extracts were centrifuged. The precipitate was washed twice with 0.5 ml water and the washes and supernatant fluid pooled. For fibroblasts labeled with 1-^{14}C-D-glucosamine, the dialysed papain digests were not subfractionated further. The extracts were lyophilized and small amounts were taken for determination of hexosamine, radioactivity, electrophoresis and Bio-Gel chromatography.

The dialysed papain digests obtained from fibroblasts labeled with 1-^{14}C-D-galactose were subfractionated as follows: To 1 ml of aqueous solution, 25 μl of 2% cetylpyridinium chloride (CPC) was added. The extract was stored overnight at 4°C. The CPC-precipitable glycosaminoglycans were collected, converted to their sodium salts and washed as before. This fraction is called the crude "CPC-precipitable glycosaminoglycans". The supernatant fluid, checked for complete CPC precipitation, was subfractionated by the addition of 4 ml of 100% ethanol followed by 0.3 ml of saturated sodium acetate. After standing overnight at 4°C the precipitate was collect-

ed by centrifugation for 5 min at room temperature and washed with ethanol and ether. The precipitate obtained at this stage was called "non-CPC-precipitable glycosaminoglycans". The supernatant fluid recovered after collection of the non-CPC-precipitable glycosaminoglycans was concentrated and dialysed overnight against distilled water. This extract is called the supernatant fluid (SN). These fractions (except the supernatant fluid, SN) were examined by Bio-Gel chromatography, electrophoresis and were subjected to hyaluronidase digestion.

H GENERAL CHROMATOGRAPHIC PROCEDURES

1 Column Chromatography

Gangliosides and Sialyloligosaccharides

Ox brain gangliosides were fractionated on columns of silica gel H.R. The silica gel (80 g) was preconditioned according to Penick et al. (260). Three solvent mixtures were employed: 500 ml of chloroform-methanol-water (65:30:5); 1 liter total volume with a gradient between C/M/H_2O (60:37:8) and C/M/10%NH_4OH (60:37:8); and 1 liter total volume with a gradient between C/M/H_2O (60:40:10) and C/M/10%NH_4OH (60:40:10). The column dimensions were 100 x 2.2 cm. Fifteen ml fractions were collected at a flow rate of 1.5 ml per minute regulated by nitrogen pressure. Generally 4.5 mg of ganglioside mixture were chromatographed per g. of silica gel H.R. Aliquots of the fractions were dried with nitrogen, and NANA determined. The appropriate fractions were pooled, concentrated to dryness by flash evaporation, dissolved in water and freeze dried. Good recovery of NANA could be achieved by this method (80%) while the total weight recovered was generally 75-80%. Individual

ganglioside species isolated from several such fractionations were pooled and rechromatographed.

Sialyloligosaccharides were derived from individual ganglioside species or from mixtures of gangliosides by ozonolysis and base hydrolysis as described (p. 69, 70). The products from this treatment were chromatographed on Dowex-1 X-2 columns in the acetate cycle by a modification of the procedure described by Schneir and Rafelson (261). The resin was prepared from Dowex-1 X-2 chloride by conversion to the hydroxyl form with several cycles of 1 M NaOH followed by water to neutrality. The resin was then converted to the acetate form by several treatments with 1 N acetic acid and water until no base or chloride could be detected. Immediately prior to use, the resin was treated with 1 M sodium acetate and washed with water to neutrality. For chromatography of the sialyloligosaccharides the column (28 x 1 cm) was eluted with a linear gradient between water (200 ml) and 0.8 N pyridine-acetic acid (200 ml). The flow rate was 25 ml per hour and 10 ml fractions were collected. Aliquots were taken for determination of NANA. The recovery of NANA obtained in this procedure was about 75%. Sialic acid-containing fractions were recovered by lyophilization.

Sphingolipids

Sphingolipids isolated from brain or from fibroblasts were chromatographed on silica gel H.R. according to the method of Svennerholm (49). The following chloroform-methanol mixtures were used as eluants: 19:1, 9:1, 4:1 and 1:4. The columns were finally washed with redistilled dry methanol. Each fraction was dried under a stream of nitrogen and the dried lipids were dissolved in the appropriate eluant mixture. The fibroblast sphingolipids were separated on 15 x 0.7 cm columns with 15 ml of eluant mixtures.

The lipids were subsequently dissolved in 20 µl aliquots of eluant mixture and stored under nitrogen at -20°C until used.

Glycosaminoglycans

Urinary glycosaminoglycans were fractionated by column chromatography on ECTEOLA-cellulose by the method of Antonopoulos et al.(262) and on DEAE-Sephadex A-50 by the method of Schmidt (263). Briefly, the ECTEOLA-cellulose powder is slurried with water and allowed to settle for 5 minutes. The fine particles are decanted and the process repeated. The heavy particles are then treated successively with 1 M NaOH, water to neutrality and then 1 M HCl. This process is repeated several times. The powder recovered by filtration is then washed with water to neutrality. Columns of 14 x 0.7 cm were routinely used. Stepwise elution of the column was employed with 50 ml portions of water, 0.02 N HCl and 1 M, 1.5 M, 2.0 M and 2.75 M ammonium formate dissolved in a 1/10 dilution of concentrated ammonium hydroxide (1.3 M in NH_4OH). The DEAE-Sephadex A-50 (Pharmacia) was pre-washed successively with 0.5 N NaOH, water and 0.5 N HCl. The resin was washed with water and slurried with 0.1 M NaCl. The glycosaminoglycans were eluted with a linear gradient between 0.1 M and 1 M NaCl. Column fractions were screened for hexose and uronic acid as described. Experimental details are given in the legends to the Tables and Figures in the Results section.

Glycosaminoglycan fractions isolated from liver were chromatographed on DEAE-Sephadex A-50 as described above and screened for hexose. Bio-Gel chromatography was utilized to estimate the approximate molecular weight of glycosaminoglycan fractions from both the liver and the fibro-

blasts. Bio-Gel resin P_2, P_4, P_{10} (Bio-Rad Laboratories, Richmond, Calif.) were swelled overnight in water, with stirring. Prior to use, the resins were de-aerated for 2 hours in water. The resins were used in non-siliconized tubes, were developed with water and the fractions were screened for hexose. The approximate molecular weight of the fractions was calculated from estimation of the bed volume, the elution volume to peak height and the molecular weight exclusion of the resin (see Bio-Rad Technical Bulletin). Blue Dextran 2000 (B.D.) was obtained from Pharmacia.

2 Thin Layer and Paper Chromatography

Thin Layer Chromatography

Individual gangliosides were separated on thin layer chromatograms of silica gel G using the following solvent systems ascending: n-propanol-2.5N ammonia (7:3 v/v) or chloroform-methanol-2.5N ammonia (60:35:8 v/v/v). The best separations were achieved with the chloroform-methanol-ammonia system run at 10-12° in a constant temperature room. Running time was 90 minutes. The gangliosides were visualized by spraying with the resorcinol reagent (264) or were quantitated by the method of Suzuki (50). For the determination of radioactivity, ganglioside zones identified by visualizing marker lanes with iodine vapor, were scraped into scintillation vials. One ml of water was added to the scrapings followed after one hour by 15 ml of scintillant mixture. The samples were then counted. Neutral glycosphingolipids were separated on silica gel G developed with chloroform-methanol-water (70:30:4 v/v/v) and visualized by charring with 10 N sulfuric acid at 125°C. Radioactive sphingolipid zones, identified by visualizing

marker lanes with iodine vapor, were scraped into scintillation vials. One ml of dry methanol was added and the samples were counted after the addition of toluene scintillant.

Paper Chromatography

Monosaccharides were released from glycosaminoglycans by hydrolysis at 100°C for 10 hours in 1 N HCl. The hydrolysates were dried under vacuum over NaOH pellets. The solvent used for chromatography on Whatnam 4 MM paper was: pyridine-ethyl acetate-glacial acetic acid-H_2O (5:5:1:3 v/v/v/v). Running time was 6 hours. Sugars were visualized by $AgNO_3$-NaOH spray reagents. Standards chromatographed simultaneously were: galactose, glucose and glucosamine (Fisher) and galactosamine and fucose (Mann Res).

Sialyloligosaccharides, derived chemically from ox brain gangliosides, were chromatographed on Whatnam 4 paper in a descending manner. The solvent used was: pyridine-ethyl acetate-glacial acetic acid-water (5:5:1:4 v/v/v/v). Running time was about 18 hours. The chromatograms were dried and the reducing oligosaccharides were visualized by heating at 95°C for 2-3 minutes after the papers were dipped in an aniline diphenylamine-phosphate reagent in acetone. Standard monosialooligosaccharide, derived from G_{M1}-ganglioside, was generously supplied by Dr. H. Wiegandt, of the University of Marburg.

3 Electrophoresis

Electrophoresis of the glycosaminoglycans was carried out on Sepraphore III strips (Gelman Inst.Co., Ann Arbor, Mich.) for one hour

in 0.05 M lithium acetate, pH 5.0 or 0.1 M sodium phosphate pH 7.4. The spots were detected by 0.5% Alcian Blue in 2% acetic acid or in 0.1 M citrate buffer pH 3.0 in 50% ethanol. For measurement of radioactivity, the strips were cut into small (1.0 or 0.5 cm wide) slices placed into scintillation vials and scintillant added. Pure standard skeletal keratan sulfate was kindly provided by Dr. K. Meyer, Columbia University, N.Y. and other glycosaminoglycan standards were generously supplied by Dr. M.B. Mathews, Dept. of Pediatrics, University of Chicago, Ill.

J CHEMICAL PROCEDURES

1 Ozonolysis

Sialyloligosaccharides were prepared from gangliosides by ozonolysis and base hydrolysis according to the method of Wiegandt and Baschang (40). Individual gangliosides purified by column chromatography (see p64) were dissolved in freshly distilled dry methanol, jacketed with a dry ice-acetone mixture (-65°C) or on ice water bath (0°C). Comparable results were obtained at either temperature. Ozone was generated by a Welsbach ozonizer held at 115 volts. The oxygen flow rate was 1.3 l per minute at a pressure of 10 lb per square inch. Ozone (5% ozone in oxygen) was bubbled through the reaction mixture at a flow rate of 0.3 l per minute. The end-point was detected by the formation of yellow color imparted to a 2% solution of potassium iodide. Total reaction time was 1-2 minutes. The ozonolyzed ganglioside was recovered from the methanolic solution by flash evaporation. The product was then dissolved in an aqueous solution of sodium carbonate (10%) to a final

concentration of 3 mg/ml. The solution was stirred overnight at room temperature. In some cases, the ozonolyzed ganglioside in methanol was exposed to hydrogen (activated by platinum oxide) in a closed vessel for 4 hours, followed by sodium carbonate hydrolysis. The basic hydrolysate was then deionized by stirring in the presence of Dowex 50 X-8 in the H^+ cycle, to a final pH of 2.7. The crude sialyloligosaccharides were then recovered from the aqueous solution by lyophilization and purified by column chromatography on Dowex-1-acetate (p 65).

In a representative experiment 35.3 mg of unfractionated ox brain gangliosides was ozonolyzed and hydrolyzed with sodium carbonate as above. The product (yield - 30.6 mg., 86% recovery) was then chromatographed on a Dowex-1 X-2 acetate column (Fig. 1). Thin layer chromatography of the ozonolyzed-base hydrolyzed material indicated that the majority of the ganglioside had been converted to more polar, resorcinol-positive material. Four pooled fractions were recovered from the Dowex-1-acetate column. Pool I (6.6 mg) contained unreactive ganglioside and lipid fragments as determined by thin layer chromatography and a negative reducing sugar reaction on paper chromatography. Pool II (3.4 mg) consisted of monosialoligosaccharide (Rf, relative to neuraminyllactose, 0.61) while pool III (9.7 mg) consisted of two disialyloligosaccharides, one derived from G_{D1a}-ganglioside (Rf 0.46) and the other derived from G_{D1b}-ganglioside (Rf 0.35). Pool IV (7.5 mg) was a trisialyloligosaccharide (from G_{T1}-ganglioside) having an Rf 0.28. Total yield of sialyloligosaccharides was 67% by weight.

In another experiment, 144.3 mg of ozonolyzed base hydrolyzed gangliosides were chromatographed on a Dowex-1-acetate column, as above. Seven fractions were collected (yield of reducing oligosaccharides, 55%)

Figure 1

Chromatography of Crude Sialyloligosaccharides on Dowex-1-Acetate

The column (28x1 cm) was eluted by a gradient between 200 ml of water and 200 ml of 0.8 N pyridine-acetic acid, pH 5.2, at a flow rate of 20 ml per hour. The volume of the fractions was 11.5 ml. Aliquots were removed for the determination of NANA by the Bial's orcinol reaction. Four pooled fractions were collected. Pool I is fraction 1, Pool II, fractions 5 - 9, Pool III, fractions 10 - 16, and Pool IV, fractions 18 - 24. Total recovery of NANA was 88.6%.

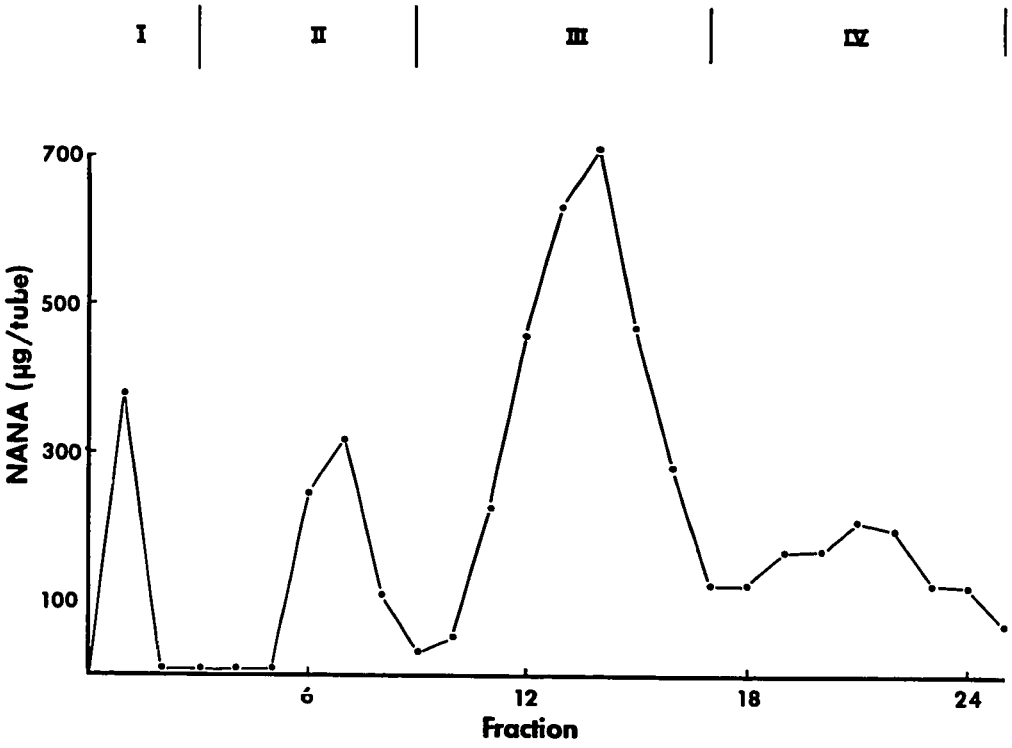

I
II
III
IV
700
500
300
100
NANA (µg/tube)
6
12
18
24
Fraction

FIGURE 2

Paper Chromatography of Sialyloligosaccharides

Six fractions (I, III-VII) from a Dowex-1-acetate column of ozonolyzed-base hydrolyzed gangliosides were chromatographed on Whatnam 4MM paper. The solvent was pyridine-ethylacetate-glacial acetic acid-water (5:5:1:4 v/v/v/v). Running time descending) 18 hours. Approximately equal amounts (200 μg) of the fractions were spotted. Reducing oligosaccharides were visualized with aniline-diphenylamine-phosphate. The standards were neuraminyllactose and lactose. Lactose runs to the top of the paper while neuraminyllactose has an Rf relative to lactose of 0.7. Fraction I contained unreacted ganglioside and was not detected. Fractions III and IV consisted of monosialyloligosaccharide (Rf relative to neuraminyllactose, 0.7-0.8). Fraction V was disialyloligosaccharide from G_{D1a}-ganglioside (Rf 0.49). Fractions VI and VII were trisialyloligosaccharides (Rf 0.25-0.27). The standard monosialyloligosaccharide (from G_{M1}-ganglioside) provided by Dr. H. Wiegandt had an Rf (relative to neuraminyllactose) of 0.71 in this system.

Origin Std. IV III I Std. Std. VII VI V Std.

and six were chromatographed on paper as described (Fig. 2). Fraction V (25.3 mg) had an Rf of 0.49 and corresponded to the disialyloligosaccharide of G_{D1a}-ganglioside. This fraction contained 45% NANA (theoretical NANA for pure disialyloligosaccharide 45.7% NANA) and tried as a substrate for brain neuraminidase (p.57).

2 Periodate Oxidation

The glycosaminoglycans, isolated from the G_{M_1}-gangliosidosis liver (case 4), were obtained in quantities sufficient to allow a partial chemical characterization of their structure. Therefore, periodate oxidation, mild alkali and β-galactosidase hydrolysis was carried out with a purified, single molecular weight species. A portion of a liver glycosaminoglycan fraction (p.60) purified after column chromatography on DEAE-Sephadex A-50 and Bio-Gel P_{10} and P_4 was treated with periodate according to the procedure of Rothfus and Smith (265) see pp.69 and 70. The polysaccharide was allowed to react with 0.06 M sodium metaperiodate at a concentration of 6 mg polysaccharide/ml of periodate. Half of the sample was removed immediately after the addition of the periodate solution and the periodate was destroyed with 0.5 ml of ethylene glycol. The rest of the sample was allowed to stand at room temperature (23°C) for 24 hours, in the dark, after which time the remaining periodate was destroyed by the addition of 0.5 ml of ethylene glycol. Both the experimental and control samples were concentrated to 0.2-0.4 ml with a N_2 stream, and were chromatographed separately on the same Bio-Gel P_4 column. The fractions were screened for hexose. The hexose-containing fractions were pooled and concentrated to

dryness with nitrogen. Both control and experimental samples were then re-dissolved in water and sodium borohydride was added (at 10 mg sodium borohydride for each mg of original polysaccharide). This solution was stirred for 2 hours in an ice bath. The solution was then neutralized with dilute HCl. Each sample was desalted by percolating the solution through small Bio-Gel P_4 columns. The effluents were collected, concentrated and aliquots were removed for hexose, hexosamine and sialic acid measurements.

3 Alkali Treatment

A portion of the liver glycosaminoglycan fraction used in the periodate oxidation study was allowed to react with 0.5 M NaOH at a concentration of 10 mg/ml according to the method of Bray et al. (90). Control and experimental samples were subjected separately to Bio-Gel P_2 chromatography. The fractions were screened for hexose by the anthrone reaction.

Another portion of the same material was allowed to react with 0.5 N NaOH in a quartz cuvette with 1.0 cm light path and the O.D. was measured with time at 241 mμ (91). The liver polysaccharide fraction was measured at 200 μg and 600 μg per ml of .5 N NaOH, simultaneously with 500 μg of a sample of old human rib cartilage keratan sulfate (MVII 190V) kindly provided by Dr. K. Meyer. As control, 70 μg of N-acetylgalactosamine per ml of base was also measured as described.

4 The Action of β-galactosidase from Beef Liver on a Glycosaminoglycan Fraction from Human Liver

Beef liver β-galactosidase was prepared by a procedure described by Jungalwala and Robins (266) for brain, except for the omission of the acetone powder step. Final specific activity of the partially purified preparation was 4.23 μmoles of substrate p-nitrophenyl-β-D-galacto-pyranoside (PNPG) cleaved per hour per mg protein.

A portion of the purified liver glycosaminoglycan fraction was incubated at a concentration of 11 mg/ml in the presence of 50 μg sodium taurocholate, 60 μmoles of sodium acetate buffer, pH 5.0 and 54 units of β-galactosidase (1 unit is one μmole of PNPG substrate cleaved per hour at 37°C). Final volume was 1 ml. Half of the sample was removed after 5 hour incubation and the rest after 23 hours at 37°C. Both samples were heated two minutes in a boiling water bath, centrifuged and the clear supernatant was collected. The precipitate was washed twice with 0.5 ml of water. The supernatant fluids and washings were pooled and concentrated to 0.4 ml. The 5 hour and 23 hour samples were chromatographed separately on the same Bio-Gel P_4 column. The fractions were screened for hexose. Untreated polymer was chromatographed on the same column. A control system containing enzyme, sodium taurocholate and buffer was incubated simultaneously to measure the extent of enzyme destruction due to incubation alone. 66.4% of the initial activity remained after 23 hours of incubation at 37°C.

In the preliminary experiments beef liver β-galactosidase, isolated as stated above, was incubated (as outlined on p. 56) with monosialoganglioside, lactose and glycosaminoglycan isolated from G_{M1}-gangliosidosis liver (1 mg, 180 μg, and 1.5 mg per ml of assay mixture respectively).

Galactose released was measured by the Galactostat method (Worthington). Subsequently, the Bio-Gel column method for determination of the action of β-galactosidase on the liver glycosaminoglycan was adopted because the polysaccharide interfered with the estimation of galactose by the Galactostat method.

K ANALYTICAL PROCEDURES

Ganglioside-bound sialic acid was estimated by the Miettinen and Takki-Luukkainen modification (267) of the Svennerholm resorcinol method (264) or by Bial's orcinol reaction as described by Gottschalk (268). Crystalline N-acetylneuraminic acid (Sigma) was used as standard. Glycolipid hexose was measured according to Svennerholm and expressed in terms of galactose (269).

Constituents of the crude and purified glycosaminoglycan fractions were analyzed for a variety of constituents. Hexuronic acids were estimated by the Dische carbazole method (270) and the orcinol method of Mejbaum as cited by Davidson (271) with glucuronolactone as standard. Carbazole-orcinol ratios were used to estimate the dermatan sulfate content. The values obtained were compared to mixtures of chondroitin 4-sulfate and dermatan sulfate.

Hexosamine was determined by the Elson-Morgan reaction (271) after hydrolysis with 4 N HCl at 100°C for 4 hours in sealed tubes. Glucosamine-galactosamine ratios were determined colorimetrically by the sodium tetraborate method of Tracey (272) or by separation of the hexosamines on Dowex-50 X-8 by the procedure of Gardell (273) and their estimation by

the Elson-Morgan reaction. The hexosamines were eluted with 0.3 N HCl. Fractions (0.4 ml) were collected and diluted to 3 ml with water prior to assay for hexosamine. Separation of glucosamine and galactosamine was achieved with columns of 29 x 0.7 cm at a flow rate of 10 ml per hour. N-acetylhexosamines were determined by the method of Reissig et al. (274) after testicular hyaluronidase digestion (275) with N-acetylglucosamine and N-acetylgalactosamine (Mann) as standards. Total hexose was determined on unhydrolyzed material by the anthrone reaction of Roe (276). Glucose was measured by Glucostat and galactose by Galactostat methods (Worthington Biochem.Corp., Freehold, N.Y.) after hydrolysis in 1 N HCl, 100°C, 10 hours. Protein was measured by the method of Lowry et al. (279). Sialic acid was determined by the TBA reaction of Warren (255) after hydrolysis in 0.1 N HCl for 90 min. Sulfate was measured by the benzidine method of Antonopoulos (277). Heparin and heparan sulfates were identified by the estimation of N-sulfation by the Lagunoff and Warren method (278).

Various glycosaminoglycan fractions were digested with bovine testicular hyaluronidase (275) (Type IV Sigma Chem. Co., St.Louis, Mo.) in 0.15 M sodium chloride and 0.1 M acetate buffer, pH 5.0. The reaction mixtures contained 0.6 mg of hyaluronidase for each 1.0 mg of polysaccharide and were incubated at 37°C in a shaker bath for 18 hours.

L MEASUREMENT OF RADIOACTIVITY

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Aqueous samples were counted in a scintillant containing equal volumes of ethanol, dioxane and toluene with 80 g

naphthalene, 5 g PPO and 50 mg POPOP per liter. Chloroform-methanol soluble material was counted in a scintillant containing 5 g POP and 0.3 g POPOP per liter of toluene. Hyamine digestion was carried out in scintillation vials as follows: 0.5 ml of the appropriate solution was mixed with 0.5 ml of methanol and 0.5 ml of 1 M Hyamine in methanol. After drying at 62°C, 1 ml methanol was added and the solution was again dried at 62°C. The residue was finally dried after a further addition of methanol and Hyamine and redissolved in 0.5 ml of methanol. Fifteen milliliters of naphthalene scintillant was then added and the solution counted. For measurement of radioactivity on electrophoresis strips, the strips were cut into small (1.0 or 0.5 cm) slices, placed into scintillation vials and naphthalene scintillant added. The strips dissolved completely in this solution and a constant degree of quenching was observed. Conversion of cpm to dpm was made by the method of Channel ratio. Quench curves were prepared for each experimental condition.

Chapter III

BIOCHEMICAL STUDIES ON G_{M1}-GANGLIOSIDOSIS

A. INVESTIGATION OF TYPE II, G_{M1}-GANGLIOSIDOSIS

The present study was begun in March, 1968 and was to our knowledge the first study of the Type II form of G_{M1}-gangliosidosis in a living patient (case 1) and of the immediate family. Some of the results presented here have already been published (244,245).

The patient (case 1) was diagnosed as an example of the Type II form of G_{M1}-gangliosidosis by analysis of the gangliosides from a cerebral biopsy specimen. Subsequently, a patient (case 2) with Type I, G_{M1}-gangliosidosis was also diagnosed and the gangliosides measured. Another patient with Type II, G_{M1}-gangliosidosis (case 3) has previously been reported (239). An autopsy specimen of cerebral cortex from a case of Tay-Sachs disease (G_{M2}-gangliosidosis), and two brain biopsy specimens (cases 1 and 2) and two brain autopsy specimens (cases 3 and 4) from patients with Late Infantile Amaurotic Family Idiocy (LIAFI) were included for comparison.

1 Brain Lipid Analysis

The size of the biopsy tissue obtained for chemical analysis did not enable clean white matter samples to be examined separately. The analyses given here are predominantly from gray matter. The level of total ganglioside N-acetylneuraminic acid

TABLE 3

GANGLIOSIDE NANA OF CEREBRAL CORTEX

Subject	μmoles NANA/g wet wt.
Normal	1.79 ± 0.18 (10)
G_{M1}-gangliosidosis	
case 1	7.73
case 2	3.90
case 3	3.79
G_{M2}-gangliosidosis	10.89
LIAFI*	
case 1	2.85
case 2	2.38
case 3	1.57
case 4	0.94

*Late infantile amaurotic family idiocy (Jansky-Bielschowsky type). Cases 1 and 2 are biopsy specimens while cases 3 and 4 are autopsy specimens.

(NANA) was greatly increased over normal values in G_{M1}-gangliosidosis, case 1 but less so for cases 2 and 3 (Table 3). The ganglioside-NANA content of the brain of the G_{M2}-gangliosidosis patient (initials--K.B.) was also greatly increased over normal. The high values of ganglioside-NANA for both the three G_{M1}-gangliosidosis patients and the case of G_{M2}-gangliosidosis reported here are in agreement with the values of others (31,52,237). The total ganglioside content of the two specimens obtained by biopsy from patients with LIAFI (cases 1 and 2) was slightly greater than normal while in the autopsy specimens from two other patients with this disease (case 3 and 4), the ganglioside-NANA content was less than normal, a reflection of the degenerative nature of this disease (163,246).

Identification of the type of neuronal gangliosidosis in case 1 was made by thin layer chromatography of the gangliosides on silica gel and then by quantitation of NANA in the appropriate zones (Figure 3 and Table 4). One major component migrating like G_{M1}-ganglioside can be seen G_{M1}-gangliosidosis case 1 and 3 (lanes 3 and 4, Figure 3) while G_{M2}-ganglioside is the major component in Tay-Sachs disease brain (K.B. lane 2). Seventy-six and 72% of the total ganglioside-NANA in cases 1 and 3 respectively corresponded to G_{M1}-ganglioside while 77% of the total gangliosides migrated like G_{M2}-ganglioside in the case of Tay-Sachs disease (G_{M2}-gangliosidosis, Table 4). Percentage distribution of NANA among the ganglioside types in normal brain tissue reported here agrees well with the values reported by Suzuki et al. (31,52). G_{M1}-ganglioside was also elevated in

FIGURE 3

ASCENDING THIN LAYER CHROMATOGRAM OF BRAIN GANGLIOSIDES

Solvent system, chloroform: methanol: 2.5 N ammonium hydroxide (60:35:8, v/v/v). Gangliosides were detected with resorcinol. Twenty-five micrograms of glycolipid-NANA was applied in each column. The extreme right and left columns are normal adult; K.B. is a case of Tay-Sachs disease; W.J., case 1 and F.J., case 3 (previously reported, see 239) are G_{M1}-gangliosidosis, Type II cases. G_{M3}, G_{M2} and G_{M1} are different types of monosialogangliosides, G_{D1a} and G_{D1b} are two types of disialogangliosides and G_{T1} is a trisialoganglioside.

TLC of Brain Gangliosides

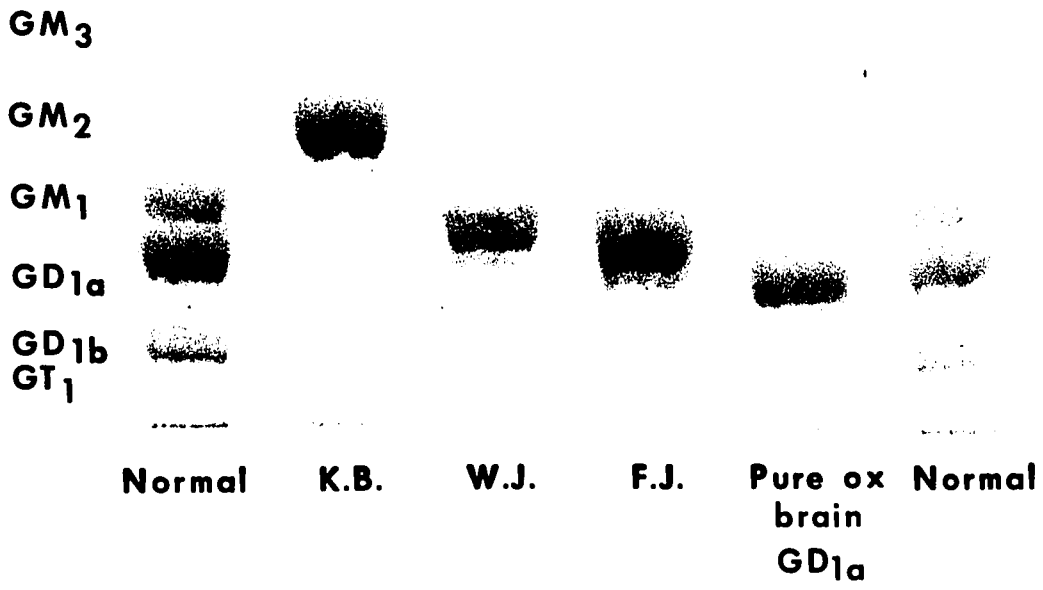


TABLE 4

DISTRIBUTION OF GANGLIOSIDE TYPES IN NORMAL AND ABNORMAL CEREBRAL CORTEX BIOPSIES

Ganglioside Type	Distribution of Types* (mean % lipid NANA)			
	Normal	Gangliosidoses		
	Adult**	Case 1 G_{M1}-	Case 3	G_{M2}-
G_{M3}	3.4	2.8	3.4	6.3
G_{M2}	2.2	4.4	4.5	76.8
G_{M1}	22.5	76.3	71.8	7.6
G_{D1a}	25.6	7.5	10.0	5.8
G_{D1b}	24.1	4.6	4.9	2.6
G_{T1}	22.2	4.6	5.3	0.8

*Approximately equal quantities of gangliosides (200 µg) were spotted in each case. Recoveries of NANA of 85-95 percent were obtained.

**The age of the adult was 43 years.

the brain of the type 1 form of G_{M1}-gangliosidosis (case 2) when chromatographed on Silica gel thin layers (data not shown). Associated with the specific increases of G_{M1}-ganglioside in the brain (Figure 3 and Table 4), there is an apparent reduction in the polysialogangliosides when expressed as a percentage of the total. Suzuki and Chen (175) have indicated however that the absolute amounts of these gangliosides are, in reality, found at normal levels.

The G_{M1}-ganglioside isolated from the brain of case 3 was purified by column chromatography and ozonolysed and base hydrolysed as described (pp. 69,70). The sialyloligosaccharide obtained has an Rf on paper chromatography (0.68) which correspond well to the standard monosialyloligosaccharide of Wiegandt (Rf 0.70).

Thin layer chromatography of the ceramide hexosides from the brain of case 1 (see Methods, p. 67) confirmed the results of Suzuki and Chen (175) that there is an increase in the ceramide tetrahexoside fraction (asialogangliosides) (Figure 4, lane 4).

The total brain phospholipid in G_{M1}-gangliosidosis was reduced from normal (case 1 - 12.1 μmoles lipid Phosphorus/g wet weight, case 3 - 15.77 and normal - 25.9).

These results clearly show the specific accumulation of G_{M1}-ganglioside in the brain of case 1, case 2, and case 3 (as reported earlier, 239). The asialoderivative of this ganglioside is also increased in case 1 (not examined for case 2). The G_{M1}-ganglioside in the brain of G_{M1}-gangliosidosis patients

FIGURE 4

ASCENDING THIN LAYER CHROMATOGRAM OF BRAIN SPHINGOLIPIDS

The brain sphingolipids were chromatographed on Silica Gel G with the solvent system: Chloroform-Methanol-Water (70/30/4, v/v/v).

Lanes 1 and 5: standard-cerebroside (120 µg) and sulfatide (120 µg).

Lane 2: glycosphingolipids from normal brain (400 µg).

Lane 3: glycosphingolipids from Tay-Sachs brain (400 µg).

Lane 4: glycosphingolipids from the brain of case 1 (400 µg).

Identification of the various components is as follows:

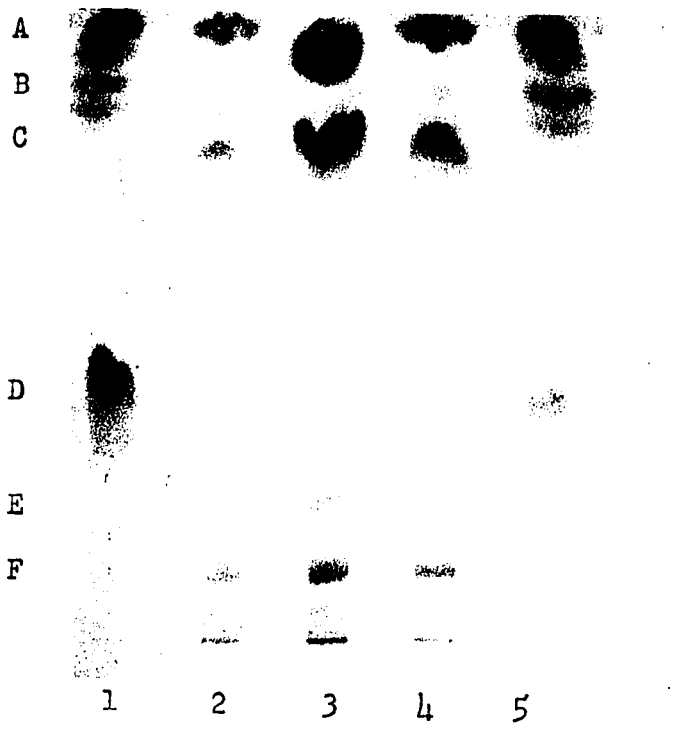
zone A - ceramide and neutral lipid; zone B - cerebrosides;

zone C - ceramide monohexoside; zone D - sulfatide;

zone E - trihexosylceramide (asialo- G_{M2}-ganglioside);

zone F - tetrahexosylceramide (asialo- G_{M1}-ganglioside).

Zones were visualized by charring at 120°C after spraying the plate with 10 N sulfuric acid. The cerebroside standard was contaminated with ceramide (zone A, lanes 1 and 5).


A
B
C
D
E
F
1
2
3
4
5

is identical to that from normal human brain.

2 Deficiency of β-Galactosidase in Type II, G_{M1}-Gangliosidosis

The accumulation of G_{M1}-ganglioside in the brain of case 1 suggested that a deficiency of β-galactosidase activity may be responsible for this gangliosidosis. Previously, Ōkada and O'Brien (243) and others (241,242) had demonstrated a deficient β-galactosidase in the Type I form of this disease. To test this possibility, β-galactosidase activity was measured in peripheral blood leucocytes and crude homogenates of brain.

The β-galactosidase activity of blood leucocytes isolated from case 1 was about 5% of the mean of normal values obtained (Table 5), while no enzyme activity was observed on one occasion in leucocytes from the Type I form (case 2). The activity of this enzyme was then measured in the other members of the two families. In both instances determinations made on the mother and father showed values significantly lower than normal. However, the values from three unaffected siblings of case 1 were normal while the one sibling of case 2 showed an intermediate level of activity (Table 5). It has not been possible to repeat the measurements of the family of case 2. The normal values obtained were independent of the age or sex of the subject. The intermediate values of β-galactosidase activity found in both sets of parents and one sibling (of case 2) were outside the range of normals tested.

Thus it seems likely that the heterozygous state can be detected by β-galactosidase analyses in leucocytes from both

TABLE 5

BETA-GALACTOSIDASE ACTIVITY OF LEUCOCYTES

Subject	mμmoles substrate cleaved/10^7 cells/hr.* (mean ± S.D.)	
Normals (age 13 mos. - 42 yrs.)**	200 ± 43	(18)
G_{M1}-gangliosidosis		
case 1	10.8 ± 7.2	(4)
case 2	0.0	(1)
Family (case 1)		
Mother	101 ± 5	(3)
Father	71,85	
Sibling 1	145	
2	154	
3	187	
Family (case 2)		
Mother	91	
Father	78	
Sibling 1	85	

*Substrate, O-nitrophenyl-β-D-galactopyranoside, pH 5.0, 37 C. The enzyme activity of each leucocyte isolation was determined in triplicate. The values for the patient and parents are from blood leucocytes isolated at different times.

**Six females and six males were used. One male was tested on five different occasions and one female three times.

TABLE 6

β-GALACTOSIDASE ACTIVITIES OF CEREBRAL GRAY MATTER*

Subject		mμmoles substrate cleaved per g. wet wt. tissue/hr	mμmoles substrate cleaved per mg protein/hr
Normal (autopsy)	1	757.0	13.5
	2	608.7	11.0
	3	1088.0	-
G_{M1}-gangliosidosis case 1		54.8	0.7
G_{M2}-gangliosidosis		2577.0	43.4

Assays were made from approximately 300 mg fresh weight of tissue. Each value is the mean of 3 separate determinations.

*β-galactosidase activity was measured spectrofluorimetrically at pH 5.0, 37°C. with 4-methylumbelliferyl-β-D-galactopyranoside (Koch-Light, Colnbrook, U.K.) as substrate.

types of this disease.

The β-galactosidase activity of crude homogenates of gray matter of case 1 was diminished to 6-8% of normal levels (Table 6). On the other hand, in crude homogenates of Tay-Sachs brain the activity of this enzyme was greater than normal. The decrease in β-galactosidase activity was not due to the presence of endogenous inhibitors since mixtures of crude homogenates of brain from normal and case 1 gave 96% of the activity expected when the enzyme was assayed separately.

The activity of the enzyme in normal and case 1 brain homogenates was determined at various pH values (Figure 5). Beta-galactosidase from normal gray matter exhibited a sharp optimal activity at pH 5.0 and was inactive at pH values below 4.0. The residual brain β-galactosidase activity of case 1 showed no distinct pH optimum and was more active than the normal enzyme at pH 3.5. Several pH optima of lysosomal β-galactosidase activity have been demonstrated in the pH range tested (223, 224,266,280, 281).

Neuraminidase activity was measured in acetone powders of brain from Type II, G_{M1}-gangliosidosis (case 1), Tay-Sachs and Niemann-Pick disease. A marked decrease in neuraminidase activity from normal values was observed for case 1 but this was not specific since a comparably low value was also found in Tay-Sachs brain (Table 7). The neuraminidase activity in a case of Niemann-Pick disease was normal. The neuraminidase measured was similar in its substrate specificity to the enzyme reported by Leibovitz and Gatt (68). The G_{M1}-ganglioside tested as a

FIGURE 5

CEREBRAL CORTEX β-GALACTOSIDASE ACTIVITIES IN HOMOGENATES OF NORMAL ADULT AND CASE 1 MEASURED AT DIFFERENT pH VALUES

Enzyme activity was measured in the crude homogenates with 4-methylumbelliferyl-β-D-galactopyranoside as substrate. Patient, W.J., is case 1. Glycine-HCl buffer, 0.1M (65 μmoles per ml assay mixture) was used at pH values, 2.95 - 3.60 and sodium acetate, 0.1M, (65 μmoles per ml assay misture) at pH values 3.60 - 5.95.

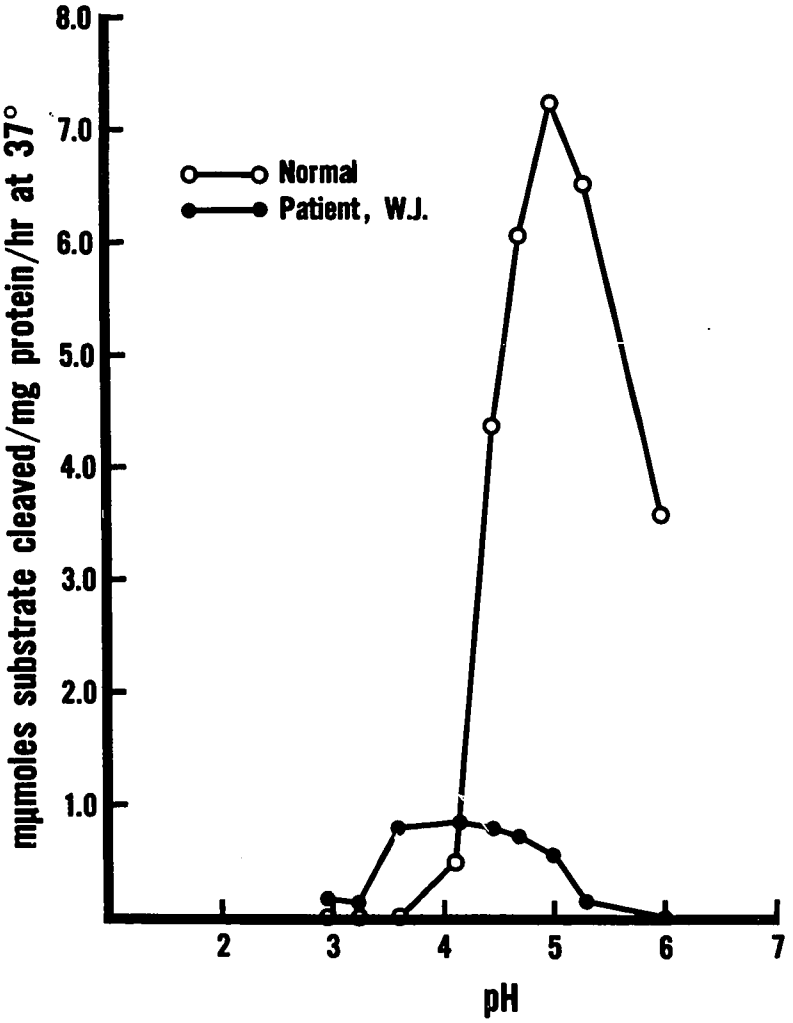

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
mμmoles substrate cleaved/mg protein/hr at 37°
Normal
Patient, W.J.
2
3
4
5
6
7
pH

TABLE 7

NEURAMINIDASE ACTIVITIES IN ACETONE POWDERS OF CEREBRAL GRAY MATTER

Subject	Substrates*	mμmoles NANA released/mg protein/hr	
Normal, biopsy	G_{D1a}	15.9 ± 1.3	(13)
	G_{M1}	3.8	(2)
	sialyl-lactose	0	(2)
	disialyloligosaccharide from G_{D1a}	0	(2)
Normal, autopsy	G_{D1a}	23.6 ± 2.0	(16)
G_{M1}-gangliosidosis case 1	G_{D1a}	9.5 ± 2.6	(5)
G_{M2}-gangliosidosis	G_{D1a}	9.3 ± 1.8	(15)
Niemann-Pick, autopsy	G_{D1a}	20.3 ± 2.3	(14)

*G_{M1}- and G_{D1a}-gangliosides were isolated from ox brain (see p. 64). The disialyloligosaccharide was prepared from G_{D1a} by ozonolysis followed by base hydrolysis and column chromatography (pp.65,69,70).

Values represent mean ± S.D. with number of determinations in parentheses.

substrate in these experiments contained a small amount of G_{D1a}-ganglioside as a contaminant. The low levels of neuraminidase activity in both G_{M1}- and G_{M2}-gangliosidosis brain but not in Niemann-Pick brain may reflect a disturbance of lysosomal enzyme activities related to the accumulation of gangliosides in general.

These results show a marked deficiency of β-galactosidase activity in leucocytes and brain of a case of Type II, G_{M1}-gangliosidosis. The accumulation of G_{M1}-ganglioside in the brain of case 1 is likely a direct result of the deficiency of this enzyme. A β-galactosidase deficiency appears to be closely associated with the homozygous phenotype since it is partially expressed in obligate heterozygotes. It is suggested that the leucocyte assay for β-galactosidase activity can be readily used for early case finding and for counselling of families as to carrier status.

3. Brain Sialomucopolysaccharides

Sialomucopolysaccharide-NANA was reduced in the gray matter of case 1 (Table 8). The significance of this change is unclear but it does not appear to be specific for G_{M1}-gangliosidosis as it was also seen in Tay-Sachs disease (Table 8). Further, there was no increase in the amounts of hexose or hexosamine in the unfractionated sialomucopolysaccharides in case 1 (results not presented). Thus it is unlikely that in the brain accumulation of mucopolysaccharides of the keratan sulfate type occurs as has been found in the urine (see below) and in the viscera as

TABLE 8

SIALOMUCOPOLYSACCHARIDES FROM CEREBRAL GRAY MATTER

Subject	sialomucopolysaccharide µmoles NANA/g. wet wt.
Normal	0.52 (mean of 2 detm.)
G_{M1}-gangliosidosis case 1	0.32
G_{M2}-gangliosidosis	0.27
LIAFI*, case 1	0.48

*Late infantile amaurotic family idiocy.

reported by Suzuki (240).

4 Urinary Glycosaminoglycans

The alcian blue and toluidine blue clinical screening test for urinary acid mucopolysaccharides were faintly positive on several occasions in case 1 as well as in a previously reported case of G_{M1}-gangliosidosis (case 3, this thesis see reference 239). Studies published very recently (31,240) have shown that in the viscera but not in the brain there is an abnormal accumulation of highly water-soluble mucopolysaccharides of the keratan sulfate type as well as a less soluble sialomucopolysaccharide related in chemical composition to a keratan sulfate. At the time at which the present investigation was in progress these facts were not known to us. Our objective was to determine if there were abnormalities in the urinary excretion of glycosaminoglycans of case 1 which might account for the faintly positive screening tests.

Measurement of the 24-hour excretion of uronic acid/g. creatinine can be used to exclude the Hurler syndrome in which there is an excessive excretion of uronic acid-containing glycosaminoglycans. No increase above the normal range in the excretion of these compounds was found (Table 9). Thus the faintly positive toluidine blue reactions of the urine were unlikely to be due to increased dermatan and heparan sulfates characteristic of the Hurler syndrome.

Two glycosaminoglycan fractions were isolated from the urine of case 1 which differed in their precipitability by

TABLE 9

URINARY EXCRETION OF GLYCOSAMINOGLYCANS CONTAINING URONIC ACID

Subject	Age	Uronic acid (mg/24 hr.)	Uronic acid / creatinine (mg/g/24 hr)	Fit to normal 95% probability range*
Normals 1	6	5.3	16.5	within
2	15	2.4	4.4	within
3	18	1.2	1.4	within
4	26	1.2	0.9	within
case 1**	3	3.2 ± 0.5	14.3 ± 3.4	within
Normal range*	3	0.5-6	8-29	-
Hurler's syndrome*	4-14	6.7-24.5	21-118	outside

*See Teller et al. (282).

**Mean ± S.D. of 3 separate 24-hour urine collections.

cetylpyridinium chloride (CPC) (see p. 58). Both these fractions were obtained as highly water-soluble white powders. The amount of the total CPC-precipitable glycosaminoglycan fractions in mg/24 hours was not significantly greater than normal (see Dorfman, 189 and Teller et al., 282). However considerable amounts of non-CPC-precipitable glycosaminoglycans were obtained in the patient's urine, in marked contrast to normal urine where only very small quantities of this fraction were found. The composition of these two fractions is given in Table 10. Noteworthy features of these results are: (1) the low uronic acid content in the non-CPC-precipitable fraction, (2) the high glucosamine to galactosamine molar ratio in the non-CPC-precipitable fraction, (3) the high hexose values in the CPC and particularly in the non-CPC fractions and (4) the low total sulfate and negligible N-sulfate content of the non-CPC-precipitable fractions.

Separation of the two crude glycosaminoglycan fractions (urine pool 2, Table 10) from the urine of case 1 was carried out on ECTEOLA-cellulose columns developed with increasing concentrations of ammonium formate in 1.3 M ammonia by the method of Antonopoulos et al. (262). In the CPC-precipitable fraction the largest amounts of material were recovered in the 1.0-1.5 M ammonium formate eluates with smaller amounts in all the other eluates (Table 11). All the column eluates were mixtures of uronic acid- and hexose-containing components. The analysis of the 0.02 N HCl eluate was unsatisfactory and was not repeated due to the small amount of material obtained but

TABLE 10

COMPOSITION OF THE CRUDE GLYCOSAMINOGLYCAN FRACTIONS FROM THE URINE OF CASE 1*

CONSTITUENTS	% by weight			
	CPC fraction**		non-CPC-fraction**	
	Urine Pool 1	Urine Pool 2	Urine Pool 1	Urine Pool 2
Protein (Lowry)	13.3	7.3	15.5	19.4
Uronic acid (carbazole)	15.4	18.0	3.4	6.6
Hexosamine (Elson-Morgan)	22.6	21.5	29.7	24.8
glcN:galN molar ratio	(1.63)	-	(7.0)	-
Hexose (anthrone as gal)	17.2	18.1	32.4	32.2
Total sulfate (benzidine)	7.5	8.1	2.5	1.1
% of sulfate as N-sulfate***	(21.8)	-	(<0.1)	-
Sialic acid (Warren)	10.0	2.7	7.4	3.7
Total	86.0	75.7	90.9	87.8
mg of material obtained/24 hr	20	22	7	10

*Results obtained from 2 pools each of 3 complete 24-hour collections. Urine volumes: pool 1, 1550 ml; pool 2, 1163 ml.
**See p. 58 for details of isolation. The analytical methods used are given in the brackets.
***Heparan sulfate standard had a molar N-sulfate to hexosamine ratio of 0.51.

TABLE 11

ECTEOLA COLUMN FRACTIONATION OF CPC-PRECIPITABLE GLYCOSAMINOGLYCANS OF CASE 1. (URINE POOL 2)

Crude glycosaminoglycans (55.2 mg) were chromatographed in two lots (22.4 and 32.8 mg) on two separate columns (14x0.7 cm). A stepwise gradient of 25 ml of each eluant was employed. The sample was initially dissolved in water (1 ml) and the column washed with water. No uronic acid or hexose was detected in the water eluate. Fractions (2.5 ml) from both columns were analyzed for hexose and uronic acid and then pooled according to eluant. Flow rate 25 ml/hour.

Fraction	% recovered material	Mole ratios					Electrophoretic Behaviour
		Uronic acid	Carb. Orcinol	Hexose	Hexosamine	Sulfate	
.02N HCl	8.8						HS, KS
1.0M AF*	63.5	0.80	1.01	1.29	1.00	1.10	CS, KS, HS
1.5M AF	17.3	0.70	0.82	0.78	1.00	0.53	CSB, KS
2.0M AF	5.2	0.79	-	1.06	1.00	-	CS, KS
2.75 AF	2.6	0.15	-	0.72	1.00	-	CS, KS
2.0M NaCl	2.6	-	-	-	-	-	CS

Recoveries from column: 70.4% (wt), 60% (uronic), 63% (hexosamine), 79% (hexose).

*Ammonium formate solution contained 1.3M ammonia.

it did contain uronic acid and hexose and a large amount of protein. The 1.0 M ammonium formate fraction had a high carbazole-orcinol ratio, a high sulfate content and contained some N-sulfated material (HS) while the 1.5 M fraction had a lower carbazole-orcinol mole ratio and no N-sulfate.

Standard corneal keratan sulfate (KS_1, courtesy of Dr. Karl Meyer) chromatographed on these columns was eluted mainly by 1.0-1.5 M ammonium formate but with tailing up to 2.75 M ammonium formate.

All of the urinary glycosaminoglycan fractions were subjected to electrophoresis on Sepraphore III before and after hyaluronidase digestion. Bovine Nasal Septa chondroitin sulfate was almost completely digested under the conditions used (360 µg in 150 µl of 0.1 M sodium acetate buffer, pH 5.0, fortified with NaCl as described and containing 100 µg hyaluronidase). Each fraction (250-400 µg) was incubated for 24 hours at 37° C, and the Hyase digest was concentrated and re-run on Sepraphore III. In the 0.02 N HCl eluate material two bands were seen before and after Hyase digestion, one migrating to the chondroitin sulfate zone and the other less anodic. In all other ammonium formate fractions alcian blue-staining material was observed migrating like a chondroitin sulfate-keratan sulfate mixture which could not be resolved under the electrophoretic conditions used. No alcian blue-staining material could be detected in the 2.0 M NaCl eluate after Hyase digestion. The analytical and electrophoretic results suggested that significant amounts of keratan sulfate

material were present in the ammonium formate eluted fractions.

In an attempt to characterize the hexose-containing material more fully, 5 mg of the 1.0 M ammonium formate column fraction was incubated for 38 hours with 2.6 mg Hyase in a final volume of 1.5 ml citrate phosphate buffer containing sodium chloride, pH 4.9. As control, a similar digest mixture containing Bovine Nasal Septa chondroitin sulfate (5.2 mg per 1.5 ml final volume) was included. At the end of the incubation period the digests were boiled for 1 minute and centrifuged. The polysaccharides remaining were precipitated by the addition of ethanol. The precipitates were then dissolved in water, dialysed and lyophilized. After correction for losses during isolation, only 12.6% of the Nasal Septa chondroitin sulfate was resistant to Hyase digestion while 42.5% of the 1.0 ammonium formate fraction was obtained. The latter sample was analyzed and found to contain a high content of galactose (measured by Galactostat after removal of hexosamine by Dowex 50) and hexosamine but reduced amounts of uronic acid and sulfate (Table 12). On electrophoresis, the Hyase-resistant material gave an intensely staining band migrating similar to KS_2 with a slightly more anodic diffuse band. These results indicate the presence of keratan sulfate-like material in significant quantities in the CPC-precipitable fraction of the urine of case 1.

The non-CPC-precipitable material (urine pool 2, Table 10) yielded, after ECTEOLA-cellulose column fractionation, two major and one minor fractions (Table 13). One fraction was eluted from the column by 0.02N HCl and was a protein-

TABLE 12

CPC-PRECIPITABLE GLYCOSAMINOGLYCANS OF CASE 1
1.0M AMMONIUM FORMATE ECTEOLA COLUMN FRACTION

Treatment	Mole ratio				$\frac{GlcN}{GalN}$	Electrophoresis
	Uronic	Hexose	Hexosamine	Sulfate**		
Before Hyase*	0.80	1.29	1.00	1.10	0.69	1 broad band in CS-KS region
After Hyase	0.40	1.04	1.00	0.40	-	1 diffuse band. 1 intense band with mobility of KS_2

*Testicular hyaluronidase digestion. 42.5% starting weight recovered.
**N-sulfate determination gave values equivalent to 90 µg H.S. per mg starting material.

TABLE 13

ECTEOLA COLUMN FRACTIONATION OF NON-CPC-PRECIPITABLE GLYCOSAMINOGLYCANS OF CASE 1. (URINE POOL 2)

Crude glycosaminoglycans (34 mg) were chromatographed as before (see legend to Table 11) except 3 ml fractions were collected up to the end of the first ammonium formate eluant (1.0M) and thereafter 5 ml Fractions.

Fraction	% by wt recovered	Mole ratios				
		Uronic acid	Hexose	Hexosamine	Sulfate	% Protein
0.02N HCl	47	0.17	0.93	1.00	0.08	34
1.0M AF*	37					
before Hyase**		0.47	0.90	1.00	0.29	20
after Hyase**		0.20	0.87	1.00	0.31	17
1.5M AF	8	0.65	1.13	1.00	0.77	-

Recoveries from column: 75% (wt), 100% (uronic acid), 90% (hexose).

*Ammonium formate solutions contained 1.3M ammonia.
**Testicular hyaluronidase digestion. 82% not digested. The 1.0M AF eluate gave two components on electrophoresis before digestion and the mole ratio of glucosamine to galactosamine was 11.5/1.0. After Hyase, one band was observed on electrophoresis with the mobility of standard KS_2.

polysaccharide complex which contained little uronic acid or sulfate and the hexose-hexosamine mole ratio was close to 1:1. The 1.0 M ammonium formate fraction was characterized by electrophoresis as a mixture of chondroitin sulfate and keratan sulfate but with the latter predominating. Only 18% of this fraction was digested by hyaluronidase and in the material recovered, the hexosamine was predominantly glucosamine and the hexose was galactose. The uronic acid was reduced by more than 50% with Hyase treatment but there was no change in the sulfate to hexosamine ratio. The Hyase-resistant material migrated like KS_2 on electrophoresis. The results suggest that the keratan sulfate-like material in the non-CPC-precipitable urinary glycosaminoglycans has a very low content of sulfate.

The CPC- and non-CPC-precipitable glycosaminoglycan fractions from urine pool 1 (see Table 10) were separated on DEAE-Sephadex A-50 columns by the method of Schmidt (263). The columns were eluted with a gradient between 0.1 and 2.0 M NaCl and the elution profile followed with uronic acid and hexose analyses. The chemical analysis of the major fractions obtained from the column for the CPC fraction is given in Table 14. The individual glycosaminoglycans were not cleanly separated on the DEAE-Sephadex column. However, fraction I was poor in uronic acid and sulfate, rich in hexose and hexosamine with molar ratios close to 1:1 and the predominant hexosamine was glucosamine. In fractions II and III there is progressively more uronic acid and sulfate and less hexose. Electrophoresis on cellulose polyacetate (Sepraphore III) in 0.1 M phosphate

TABLE 14

SEPARATION OF THE CPC-PRECIPITABLE GLYCOSAMINOGLYCANS OF THE URINE OF CASE 1 ON DEAE-SEPHADEX A-50

22.4 mg was applied to 1.5x29 cm column. Void volume 15 ml. Column eluted with a linear gradient from 0.1 to 2.0 M NaCl (total volume 300 ml). 2 ml fractions collected. Alternate tubes were analysed for uronic acid and hexose.

Pooled column fractions	% of recovered wt*	Molar ratios					
		Uronic acid	HexN	Hex	SO_4	Carb. / orcinol	GlcN / GalN
I (24-60 ml)**	36	0.24	1.00	0.86	0.10	-	5.7
II (61-116 ml)	15	0.46	1.00	0.81	0.52	0.92	4.0
III (117-180 ml)	30	0.64	1.00	0.47	0.87	1.05	0.6

*total recovery by weight 71.0%
**volume after the void volume

buffer, pH 7.4 showed that the material in fraction I migrated with the mobility of a standard skeletal keratan sulfate (KS-2). Fractions II and III were mixtures of keratan sulfate and chondroitin sulfates A and C. The composition of the CPC-precipitable glycosaminoglycan fraction from the urine of case 1 is quite unlike that in normal urine which contains small amounts of a highly sulfated keratan sulfate (283).

Column fractionation of the non-CPC-precipitable fractions on DEAE-Sephadex showed that the major constituent was an undersulfated keratan sulfate which also contained a considerable amount of a sialic acid (Figure 6 and Table 15). The major peak from the column (pool 2) was dialysed exhaustively and freeze dried. The composition of this undersulfated keratan sulfate is given in Table 16. The polysaccharide isolated from the non-CPC-precipitable fraction of urine pool 1 (Table 10) by DEAE-Sephadex A-50 chromatography (Figure 6, Table 15) is similar to the hyaluronidase resistant material isolated by ECTEOLA-cellulose column chromatography (Table 13).

It seems clear that the non-CPC-precipitable fraction from the urine of case 1 consists mainly of undersulfated keratan sulfate-protein complexes which differ in their protein and sulfate content. The CPC-precipitable fraction also contains keratan sulfate complexes closely associated with the chondroitin sulfates. Based on all the analytical data available to date, the amounts of the various types of glycosaminoglycans in the CPC-precipitable fraction can be estimated (Table 17). The most conspicuous feature is the greatly elevated amount of

TABLE 15

SEPARATION OF THE NON-CPC-PRECIPITABLE GLYCOSAMINOGLYCANS OF THE URINE OF CASE 1 ON DEAE-SEPHADEX A-50

Conditions as in Table 14. 10.5 mg of material applied to the column.

Pool column fractions	% of recovered wt*	Molar ratios				
		Uronic acid	GlcN**	Gal**	SO_4	Sialic acid
I (20-52 ml)	24	-	1.00	1.81	-	-
II (53-94 ml)	68	0.06	1.00	1.30	0.49	0.33

*total recovery by weight 68%
**About 95% of the hexosamine was glucosamine and all the hexose was accounted for as galactose by Galactostat.

FIGURE 6

DEAE-SEPHADEX A-50 COLUMN CHROMATOGRAPHY OF THE NON-CPC-PRECIPITABLE GLYCOSAMINOGLYCANS FROM THE URINE OF CASE 1

Elution profile from DEAE-Sephadex A-50 of the non-CPC-precipitable glycosaminoglycans from urine pool 1 of case 1 (see Table 10). Volume of each fraction 2 ml. A linear gradient between 0.1 and 2.0 M sodium chloride was employed. Composition of fractions I and II are given in Table 15. Hexose was measured by the anthrone reaction.

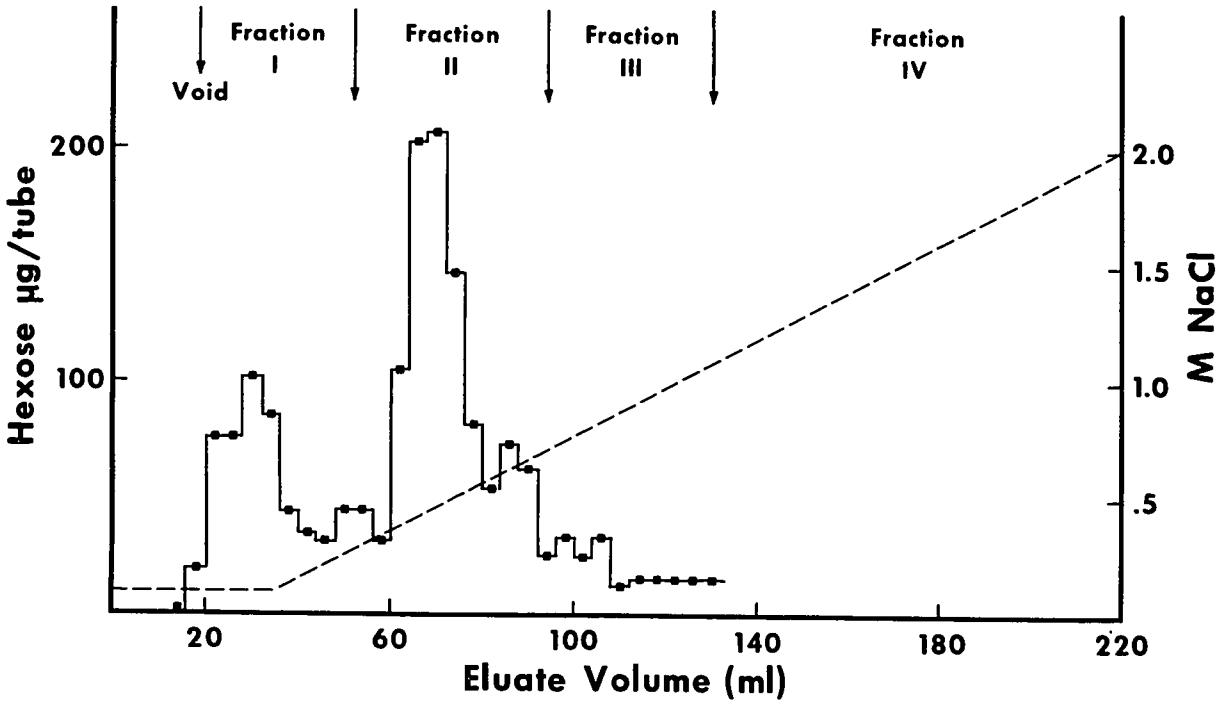

Void
Fraction I
Fraction II
Fraction III
Fraction IV
Hexose µg/tube
200
100
2.0
1.5
1.0
.5
M NaCl
20
60
100
140
180
220
Eluate Volume (ml)

TABLE 16

COMPOSITION OF AN UNDERSULFATED KERATAN SULFATE-PROTEIN COMPLEX ISOLATED FROM THE URINE OF CASE 1

Constituent	% by wt.
Protein	28.2
Uronic acid	1.1
Galactose	21.9
Glucosamine	18.2
Sialic acid	9.5
Sulfate	4.4
	83.3

TABLE 17

URINARY CPC-PRECIPITABLE GLYCOSAMINOGLYCANS

Constituent	% of total glycosaminoglycans	
	Normal*	Case 1
Chondroitin 6-SO_4 Chondroitin Hyaluronic acid	60	42-48
Chondroitin 4-SO_4	31	27
Heparan sulfate	8	6
Dermatan sulfate	1	2
Keratan sulfate	1 (highly sulfated)	17-23 (undersulfated)

*from Varadi et al. (283).

undersulfated keratan sulfates present in the urine of the Type II, G_{M1}-gangliosidosis patient (case 1).

In summary, there is an elevated excretion of keratan sulfate-like glycosaminoglycans in this patient (case 1) with Type II, G_{M1}-gangliosidosis. Two species were isolated on the basis of precipitability with CPC. The keratan sulfate-like glycosaminoglycans precipitated with the uronic acid-containing glycosaminoglycans were not isolated free of uronic acid even after Hyase digestion. Only in the glycosaminoglycan fraction not precipitated with CPC could the keratan sulfate-like polysaccharides be obtained free of uronic acid. Our results suggest that in the urine of case 1 the faintly positive screen tests for metachromasia results from the presence of excessive quantities of these undersulfated polysaccharides. Thus, it is likely that the visceral storage substances in the Type II form (reflected by the elevated excretion of undersulfated keratan sulfate-like glycosaminoglycans) are similar to those identified in the liver and spleen of patients with the Type I form of G_{M1}-gangliosidosis (31,240).

B BIOCHEMICAL INVESTIGATION OF THE LIVER FROM A PATIENT WITH TYPE I, G_{M1}-GANGLIOSIDOSIS

The only published attempts to characterize the compounds accumulated in the liver of Type I, G_{M1}-gangliosidosis were carried out by Suzuki and co-workers (31,240). They reported preliminary experiments which showed that the major carbohydrate constituents of the polysaccharides were galactose and glucosamine. Further, these compounds migrated like keratan sulfate on electrophoresis and therefore, they proposed that the compounds were keratan sulfates.

The quantities of urinary glycosaminoglycans of the keratan sulfate-type remaining after analysis were insufficient to allow the study of the chemical structure of these materials. The following study was initiated to isolate and characterize the galactose and glucosamine-containing polysaccharides accumulated in the liver of G_{M1}-gangliosidosis patients. At the time, the only material available for study was a liver specimen of one patient with Type I, G_{M1}-gangliosidosis (case 4). Since this specimen was obtained from an outside source, it was of interest to confirm their diagnosis of Type I, G_{M1}-gangliosidosis. It is for this reason that we initially measured the level of β-galactosidase and three other acid hydrolases in crude homogenates of the liver and demonstrated the presence of G_{M1}-ganglioside in lipid extracts of this liver specimen.

In the later stages of this work, another Type I, G_{M1}-

TABLE 18

ACTIVITIES OF SOME HUMAN LIVER ACID HYDROLASES

Liver specimens were homogenized in 9 volumes of 0.25 M sucrose-1 mM EDTA, pH 7.0. Each enzyme was measured in the crude homogenate as described by Van Hoof and Hers (157).

Cases	Enzyme activities*			
	β-galactosidase	β-glucosidase	β-N-acetyl glucosaminidase	α-L-fucosidase
Normal**				
Mean	0.630	0.108	2.39	0.266
Range	(0.28-1.22)	(0.037-0.195)	(1.17-5.28)	(0.104-0.572)
G_{M1}-gangliosidosis				
Type I				
Case 2	0.005	0.419	16.8	1.50
Case 4	0.011	0.412	17.7	5.85
LIAFI***				
Case 3	0.266	0.199	5.38	1.49
Case 4	0.215	0.122	6.59	0.689
Niemann-Pick				
Case 1	0.110	0.031	3.70	-

*Activities are the μmoles substrate cleaved per min. per g wet weight at 37°C. Each figure is the mean of triplicate determinations.
**See Van Hoof and Hers (157).
***LIAFI refers to late infantile amaurotic family idiocy, Jansky-Bielschowsky type, not a ganglioside storage disease.

gangliosidosis patient was diagnosed in our laboratory (case 2). The results obtained on measurements of four acid hydrolases and on ganglioside analysis of the liver of the patient are included for comparison. Any polysaccharides, accumulated in this liver specimen have not been characterized.

1 G_{M1}-Gangliosidosis Liver Acid Hydrolases

The activities of four acid hydrolases were measured in crude homogenates of liver specimens from two cases of Type I, G_{M1}-gangliosidosis (Table 18), two cases of Late Infantile Amaurotic Family Idiocy (cases 3 and 4) and one case of Niemann-Pick Disease. Beta-galactosidase activity was greatly reduced in the liver tissue from both G_{M1}-gangliosidosis cases (cases 2 and 4) while the activities of the three other acid hydrolases tested were increased many fold over normal in accordance with the findings of Van Hoof and Hers (157). Beta-galactosidase activity was reduced slightly in both cases of Late Infantile Amaurotic Family Idiocy and in the one case of Niemann-Pick Disease while the activities of β-glucosidase and β-N-acetylglucosaminidase were within the normal range established by Van Hoof and Hers (157). Fucosidase activity was elevated over normal in all specimens examined.

2 The Identification of G_{M1}-Ganglioside from the Liver

According to the procedure proposed by Suzuki and co-workers (31,240), the first step in the isolation of liver

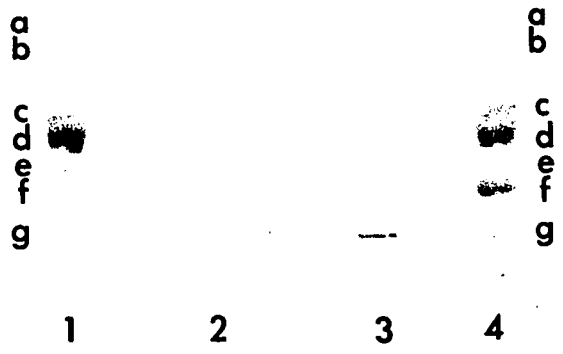
glycosaminoglycans involved extraction of the liver with chloroform-methanol solvent mixtures to remove the lipids and subsequently, digestion of the lipid-free liver residue with papain and α-amylase. We extracted the lipids from the liver according to the method of Suzuki (240), and isolated the gangliosides by Folch partition of the chloroform-methanol extract. In one such extraction (Preparation 1, case 4) we obtained an extremely large amount of polysaccharide in the "ganglioside fraction" while in two other extractions (Preparation 2, case 4; and case 2) only small amounts of these polysaccharides were found in this fraction. The isolation of the liver polysaccharides will be discussed later.

The NANA content of the ganglioside fractions expressed as μmoles NANA/g wet weight liver were 2.25 and 0.27 for preparations 1 and 2 from the liver of case 4 and 0.35 for one sample from the liver of case 2. These values are higher than those reported by Suzuki et al. (31) likely a reflection of the presence, in varying quantities, of sialic acid-containing polysaccharides as contaminants of the ganglioside fraction. The ganglioside fractions were then examined on TLC (Figure 7). The major gangliosides, in the upper phase extracts of the G_{M1}-gangliosidosis liver samples studied, were G_{M3}-(hematoside) and G_{M1}-ganglioside (Figure 7, lanes 2 and 3). Disialogangliosides were not present. For comparison, gangliosides from the brain of G_{M1}-gangliosidosis, case 2 (Figure 7, lane 1) and from normal human brain (lane 4) are included. It may be seen in lane 3

FIGURE 7

ASCENDING THIN LAYER CHROMATOGRAM OF GANGLIOSIDES

Solvent system, chloroform: methanol: 2.5 N ammonium hydroxide (60:35:8, v/v/v). Gangliosides were visualized with resorcinol. Lane 1: 150 µg of brain ganglioside from G_{M1}-gangliosidosis Type I (case 2). Lane 2: 200 µg of liver gangliosides isolated from case 2. Lane 3: 200 µg of liver gangliosides isolated from liver of case 4. Lane 4: 150 µg of normal human brain gangliosides. a, G_{M3}; b, G_{M2}; c, G_{M1}; d, G_{D1a}; e, G_{D1b}; f, G_{T1} ganglioside types. G_O (g) is the material remaining at the origin. In lane 1, the ganglioside (G_{M1}) is slightly retarded (d) in its chromatographic mobility due only to the large amount present (see 239).


a
b
c
d
e
f
g
1
2
3
4

of Figure 7 that there is a very intense resorcinol positive band at the origin and smaller amounts of similar material were found in liver extracts from another case (lane 2). The material remaining at the origin in lane 3 consists of the glycosaminoglycans found in the "ganglioside fraction" of the present investigation.

Thus, in the liver of Type I, G_{M1}-gangliosidosis patients, there is a marked decrease of β-galactosidase activity, increased activity of other acid hydrolases and the presence of G_{M1}-ganglioside. These results are in agreement with those of others (31,157,240).

3 Isolation and Characterization of G_{M1}-Gangliosidosis Liver Glycosaminoglycans

a Isolation of Glycosaminoglycans from Liver

The isolation of the glycosaminoglycans from two separate samples of liver from Type I, G_{M1}-gangliosidosis patient (case 4) (see pp. 59-61) showed that variable amounts were obtained in chloroform-methanol extracts. In the first preparation, the majority of the glycosaminoglycans (25.6 mg/g wet wt) were recovered in the "ganglioside fraction"; that is, they were extracted into chloroform-methanol and partitioned into the aqueous phase in the Folch procedure (249) while much smaller amounts were recovered in the "residue fraction" (1.8 mg/g wet wt after α-amylase digestion). On the other hand, in the second preparation only very small amounts of material were

recovered in the "ganglioside fraction" (1.8 mg/g wet wt) and the bulk of the glycosaminoglycans remained in the lipid-free liver residue. This residue was digested with papain and α-amylase to solubilize the glycosaminoglycans (yield: 15.4 mg/g wet wt). The difference in the amounts of glycosaminoglycans extracted by chloroform-methanol in the two preparations appears to be due to variation in the water content in the extracting solvents which were not specifically dried before use. Total yields were also different in the two liver preparations. Preparation No. 1 yielded 27.4 mg of partially purified gylcosaminoglycans per gm wet weight of liver while the yield in preparation No. 2 was 17.2 mg per gm wet weight liver. Values for the amount of hexosamine per gm wet weight of liver (9.74 and 6.20 mg hexosamine/g wet wt for preparations 1 and 2, respectively) were similar to those of previous reports (31,240). The lower yield of glycosaminoglycans in preparation 2 is probably due to dialysis losses during the enzymic digestion procedure and not to decreased extractability as has been suggested (31).

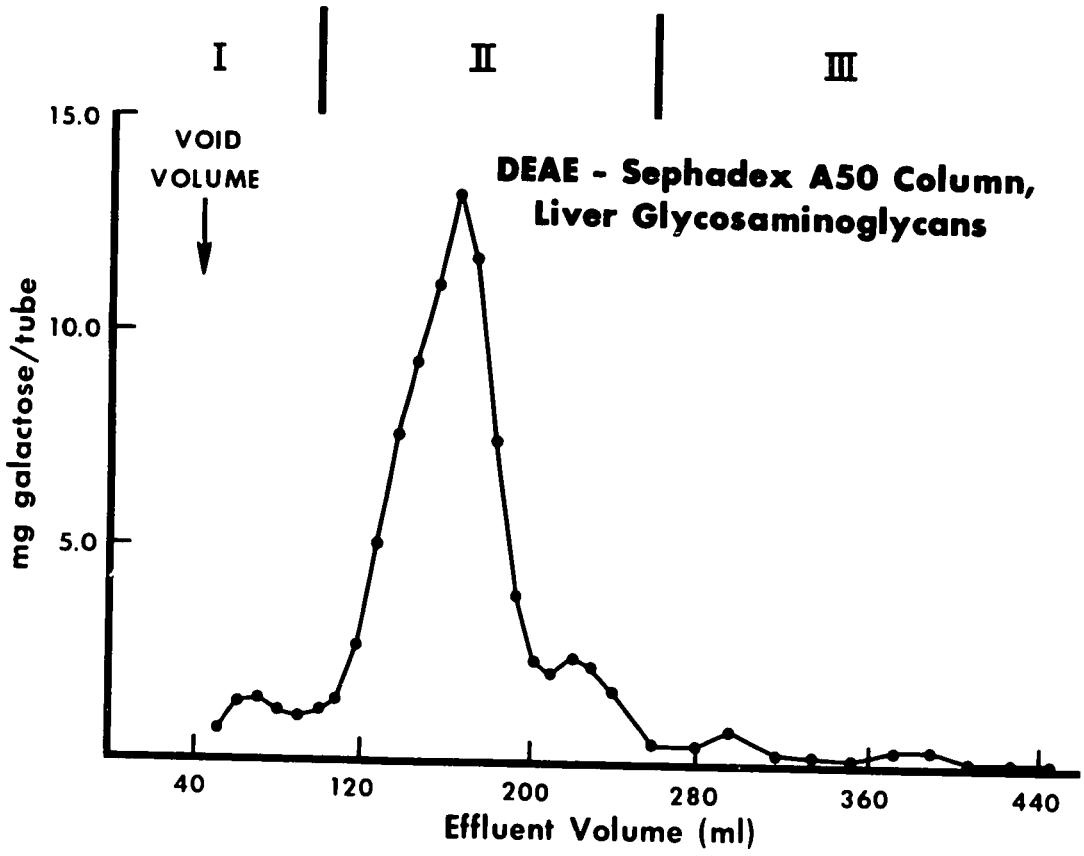
b Characterization of G_{M1}-Liver Glycosaminoglycans

DEAE-Sephadex chromatography of liver glycosaminoglycans isolated in the "ganglioside fraction" (Figure 8) gave two major fractions (I and II), each of which were then subfractionated by the addition of ethanol, and their composition determined (Table 19). The major carbohydrate constituents of the liver glycosaminoglycans in all fractions were galactose

FIGURE 8

DEAE-SEPHADEX A-50 COLUMN CHROMATOGRAPHY OF "GANGLIOSIDE FRACTION" GLYCOSAMINOGLYCANS

The DEAE-Sephadex A-50 was prepared as described in the Methods section (p. 66). Column 45x2 cm., gradient between 0.1 M and 1 M NaCl. Fractions, 9.5 ml collected at a flow rate of 25 ml/hr. Fractions were analysed for hexose by the Anthrone reaction. Analysis of the subfractions obtained after the addition of ethanol to fractions I and II are shown in Table 19.


I
II
III
15.0
10.0
5.0
VOID
VOLUME
DEAE - Sephadex A50 Column,
Liver Glycosaminoglycans
mg galactose/tube
40
120
200
280
360
440
Effluent Volume (ml)

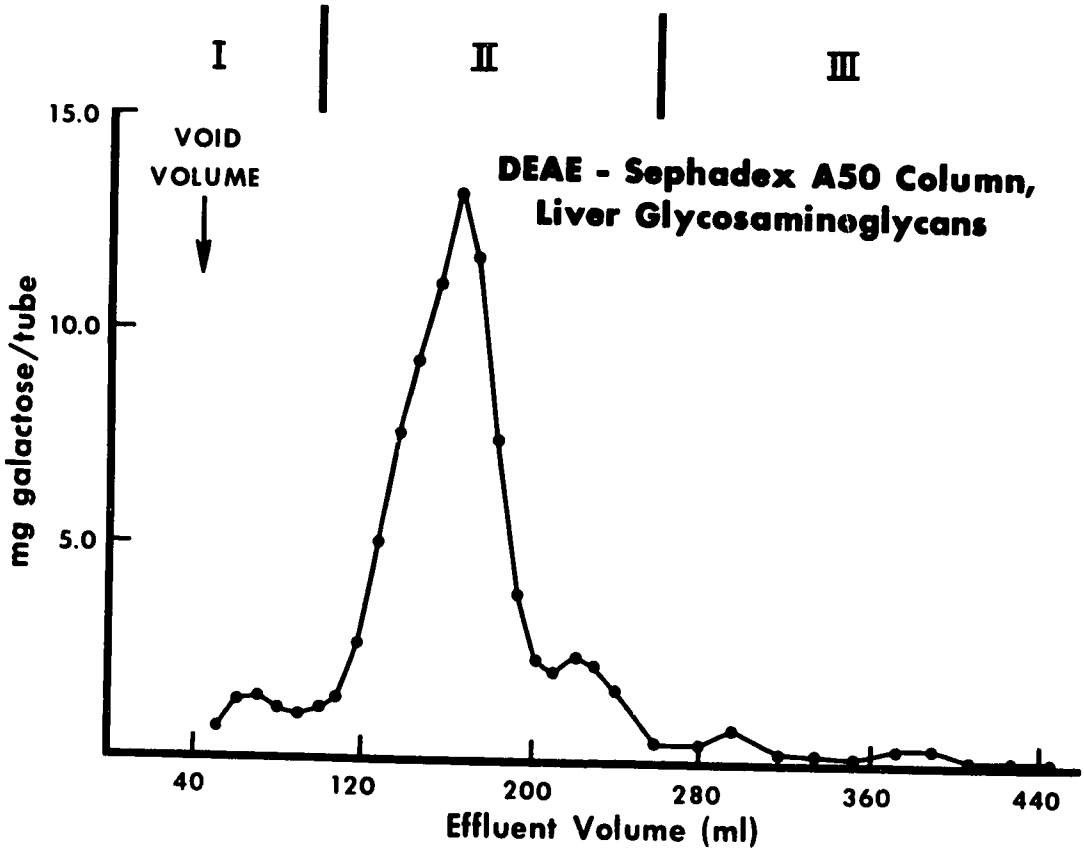

I
II
III
15.0
10.0
5.0
VOID
VOLUME
DEAE - Sephadex A50 Column,
Liver Glycosaminoglycans
mg galactose/tube
40
120
200
280
360
440
Effluent Volume (ml)

TABLE 19

COMPOSITION OF LIVER GLYCOSAMINOGLYCANS AFTER DEAE-SEPHADEX A-50 CHROMATOGRAPHY AND ETHANOL FRACTIONATION

Experimental conditions used were as described in the legend for Figure 8. Fractions I and II are the major fractions seen on DEAE-Sephadex A-50 and the subfractions are obtained with ethanol fractionation as described (p. 60). Results are for the "ganglioside fraction."

Fraction	Wt. Recovered (mg)	Molar Ratios				% by weight	
		Galactose	Glucos-amine	Sialic Acid	Sulfate	Protein	Galactose
I P	12.1	0.785	1.00	0.02	0.05	9.8	35.6
SN	3.6	1.42	1.00	0.03	0.03	5.9	20.4
II P	40.0	1.16	1.00	0.06	0.07	9.7	36.5
SN	64.6	1.16	1.00	0.02	0.03	4.2	50.8
D	30.0	1.03	1.00	0.01	-	2.3	33.3

Recovery by weight 78%

and glucosamine, with small amounts of sialic acid and galactosamine. The galactosamine content was variable but was about 5-10% of the total hexosamine for fractions II P and II SN (Table 19), as determined colorimetrically and by Dowex 50 column chromatography. No fucose or mannose was found on paper chromatography and no uronic acid could be demonstrated by the carbazole reaction. One of the most striking features was a ratio of galactose to glucosamine consistently greater than unity as would be expected for a skeletal keratan sulfate (86). Further, only small amounts of sulfate were detected. The protein content of the "ganglioside fraction" glycosaminoglycans was also low. Human skeletal and corneal keratan sulfates have galactose and glucosamine as the major carbohydrate components but are highly sulfated (85,86,90).

The presence of several subfractions of glycosaminoglycans of similar chemical composition found after ethanol fractionation (Table 19) suggested that the differences may be due to chemically similar species of different molecular weight. This was confirmed by Bio-Gel chromatography (Figure 9 and Table 20). The largest amount of material was obtained in fractions with a molecular weight of 3000-4000 with smaller amounts in the 6000-8000 and 10,000 M.W. range. The dialysable fraction (IID, see Tables 19 and 20 and Methods, p. 60) contained species of M.W. less than 2000 (approximately 1600 by calculation from exclusion volume of Bio-Gel P_2).

The composition of fractions derived from crude glycosaminoglycans of the delipidized liver "residue fraction"

FIGURE 9

BIO-GEL P_{10} COLUMN CHROMATOGRAPHY OF THE LIVER GLYCOSAMINOGLYCANS

Chromatography of liver glycosaminoglycan fractions (see Table 19) from the "ganglioside fraction" was carried out on a 38x2 cm. column of Bio-Gel P_{10} eluted with water at a flow rate of 18 ml/hr. Fraction volumes 2-4 ml. Each fraction was analysed for hexose. Fraction I P, dotted line; fraction II P, broken line; and fraction II SN, solid line. Each fraction was chromatographed separately on the same column.

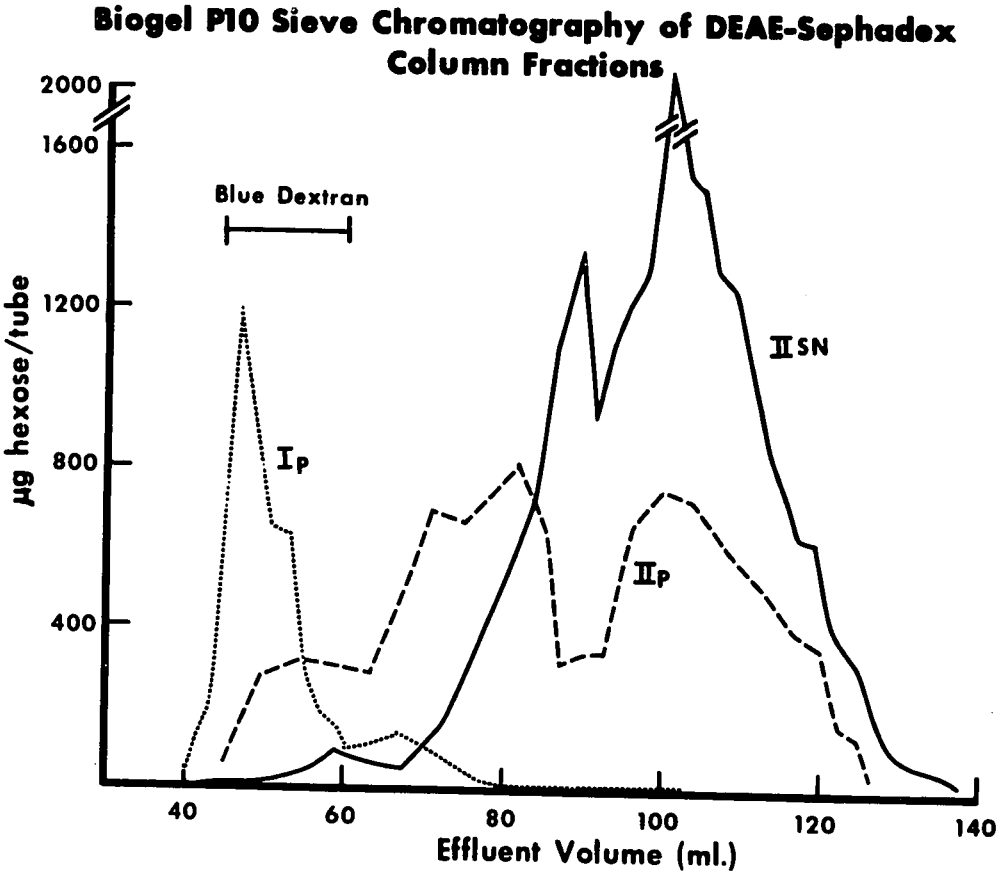

Biogel P10 Sieve Chromatography of DEAE-Sephadex Column Fractions
2000
1600
1200
800
400
µg hexose/tube
Blue Dextran
IP
IIP
IISN
40
60
80
100
120
140
Effluent Volume (ml.)

TABLE 20

BIO-GEL CHROMATOGRAPHY OF LIVER GLYCOSAMINOGLYCAN FRACTIONS ELUTED FROM DEAE-SEPHADEX A-50

Data given in this table were derived from the analysis of the fractions obtained from Bio-Gel columns as described in Figure 9 and Table 19.

Fraction	Weight total (mg)	Gel Resin Used	Per cent Distribution in each fraction*			
			>10,000 M.W.	6-8,000 M.W.	3-4,000 M.W.	<2,000 M.W.
I P	12.1	P_{10}	100	-	-	-
II P	40.0	P_{10}	15	41	44	-
SN**	64.6	P_{10}	2	26	71	
D	30.0	P_2	-	-	-	100
Total % of each species			14	22	42	20

*Based on hexose determinations of column eluates.
**Fraction I SN from Table 19 was not examined.

(preparation 2, case 4) after papain and α-amylase digestion followed by ethanol and CPC precipitation are shown in Table 21. The galactose to glucosamine molar ratios were generally slightly less than 1:1 and contained more protein than the glycosaminoglycans isolated from the "ganglioside fraction" (see Table 19).

The differences between the two preparations appear due to impurities since the galactose to glucosamine ratio increased to more than unity after Bio-Gel chromatography (Figure 10 and Table 22).

The precipitate (P) obtained from the "residue fraction" by ethanol precipitation (Table 21) contained all of the uronic acid-containing mucopolysaccharides which were separated from the other glycosaminoglycans by precipitation with cetylpyridinium chloride. The uronic acid enriched fraction so obtained (P_1) representing only 2.9 per cent of the total recovered glycosaminoglycans was found to contain high amounts of galactose and glucosamine (Table 21). The sulfate to uronic acid ratio was 2.53. The major uronic acid-containing mucopolysaccharides in liver have been shown to be Heparin and Heparan sulfates (197,285) and the presence of these compounds likely accounts for the high uronic acid and sulfate contents of this fraction. The bulk of the glycosaminoglycans were not precipitable by CPC but could be recovered by the addition of ethanol. However, a subfraction (SN in Table 21) was obtained in the initial ethanol treatment of the total unfractionated α-amylase product which

TABLE 21

COMPOSITION OF LIVER GLYCOSAMINOGLYCANS ISOLATED FROM "RESIDUE FRACTION" AFTER ETHANOL AND CPC FRACTIONATIONS

The isolation procedure is given in the Methods section (pp.60,61). The product of papain digestion of the liver residue (Prep. 2) totalled 442 mg. This material was digested with α-amylase to yield fraction T which was then subjected to ethanol and CPC fractionations.

Residue Fraction*	Weight Recovered (mg)	Molar Ratios					% by weight	
		Gal.	Glucos-amine	Uronic Acid	Sialic Acid	SO_4	Protein	Gal.
T	231	0.86	1.00	0.03	0.06	0.03	11.1	34.5
P Subfraction P_1	7.7	0.98	1.00	0.34	0.07	0.85	9.4	21.3
P_2	126.3	0.76	1.00	0.04	0.06	0.05	11.0	33.7
SN	58.2	1.16	1.00	0.04	0.11	0.09	18.3	33.5

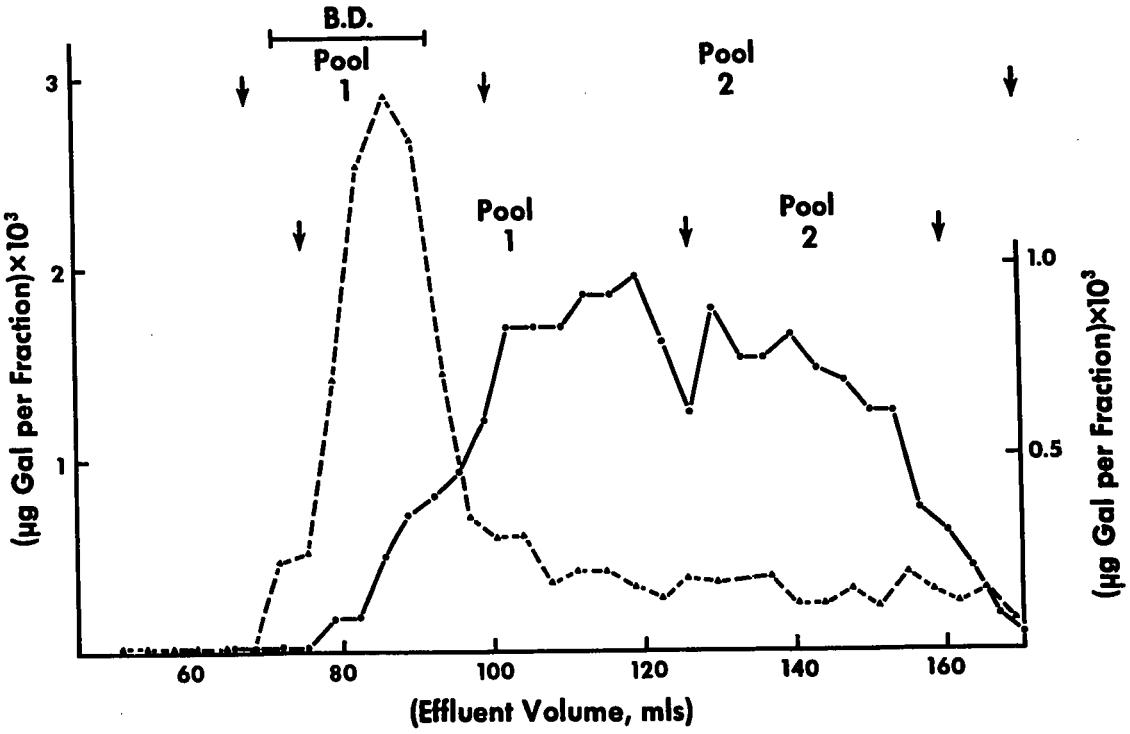
Recovery by weight 83.2%

*T refers to the total unfractionated α-amylase product. P is the precipitate obtained on addition of ethanol to fraction T and SN is the supernatant fluid. Subfractions P_1 and P_2 are the CPC-precipitable and non-CPC-precipitable glycosaminoglycans, respectively, derived from the ethanol precipitate P.

FIGURE 10

BIO-GEL P_{10} SIEVE CHROMATOGRAPHY OF THE LIVER GLYCOSAMINOGLYCANS

Chromatography of liver "residue fraction" glycosaminoglycans was carried out on a 49x2 cm. column of Bio-Gel P_{10} eluted with water. 3.4-3.6 ml fractions were collected, at a flow rate of 18 ml/hr, and aliquots were analysed for hexose. B.D. indicates a Blue Dextran 2000 peak. Broken line is subfraction P_2 (Table 21) which was divided into two pools (upper arrows). Solid line is SN (Table 21) which was also divided into two pools (lower arrows). Left ordinate refers to subfraction P_2 and right ordinate refers to SN. Each fraction was chromatographed separately on the same column. The analysis of each of the pooled fractions is presented in Table 22.


B.D.
Pool 1
Pool 2
Pool 1
Pool 2
(µg Gal per Fraction)×10³
(µg Gal per Fraction)×10³
3
2
1
1.0
0.5
60
80
100
120
140
160
(Effluent Volume, mls)

was not precipitated by ethanol and represents 30 per cent of the recovered glycosaminoglycans. This fraction as well as the ethanol but not CPC-precipitable fraction (P_2, Table 21) were subjected to Bio-Gel P_{10} sieve chromatography (Figure 10). The composition and approximate molecular weights of the fractions obtained are shown in Table 22. It is clear that all of these fractions are keratan sulfate-like polymers with small contents of sialic acid. Each of the pooled fractions (see Figure 10) were subjected to paper chromatography after acid hydrolysis (see Methods p. 68). Galactose and glucosamine were by far the predominating sugars with a small quantity of galactosamine in all fractions recovered after Bio-Gel chromatography (Figure 10). A typical chromatogram is shown in Figure 11. The paper chromatography was repeated with acid hydrolysed polysaccharides spotted to give the equivalent of 100 µg of galactose. Even under these conditions, no fucose could be observed, while mannose would be obscured by the excess quantity of galactose spotted. The results obtained after Bio-Gel chromatography of the polysaccharides in the non-CPC-precipitable material (Table 22) from the "residue fraction" show similar molecular weight polydispersity to those found in the "ganglioside fraction" (Figure 9 and Table 20). Normal human skeletal keratan sulfates have also been shown to be polydisperse after Bio-Gel chromatography with a molecular weight range of 3,600-10,000 (90).

The glycosaminoglycans, isolated from two separate samples

TABLE 22

COMPOSITION OF NON-CPC-PRECIPITABLE LIVER GLYCOSAMINOGLYCANS FROM THE "RESIDUE FRACTION" AFTER MOLECULAR SIEVE CHROMATOGRAPHY ON BIO-GEL P_{10}

Data presented in this table were derived from the chromatography of the P_2 and SN fractions shown in Table 21 and Figure 10.

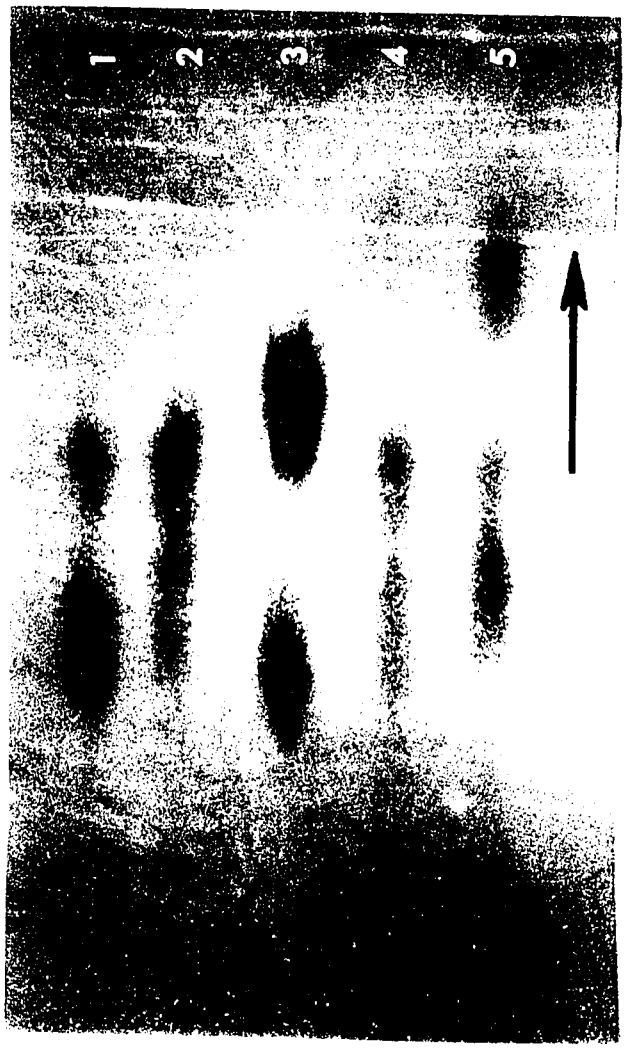
Fractions	Weight* Recovered (mg)	Galactose	Glucos-amine	Sialic Acid	Approximate Molecular Weight
Subfraction P_2**					
Pool 1 (68-100 ml)	33.0	1.01	1.00	0.04	>10,000
Pool 2 (101-170 ml)	8.9	1.04	1.00	0.04	<10,000
Subfraction SN					
Pool 1 (75-126 ml)	18.6	1.12	1.00	0.01	6-8,000
Pool 2 (127-160 ml)	17.6	1.15	1.00	0.16	3-4,000

*Recovery by weight 66% and 84% for the Fractions P_2 and SN respectively.
**The appropriate fractions were pooled, and lyophilized. Blue-Dextran-2000 was excluded between 68 and 100 ml.

FIGURE 11

DESCENDING PAPER CHROMATOGRAPHY OF MONOSACCHARIDES DERIVED FROM LIVER GLYCOSAMINOGLYCANS

Paper chromatography of monosaccharides from hydrolysates of Bio-Gel P_{10} column eluates of the "residue fraction" (see Figure 10). The aqueous solution of hydrolyzed material was applied to Whatnam 4 MM paper for one descending run in the solvent system of ethyl acetate-pyridine-acetic acid-water (5:5:1:3, v/v/v/v). Spots were located by silver nitrate - sodium hydroxide sprays. Read chromatograms from left to right. Lane 1, 16 µg galactosamine, 20 µg galactose: standards; lane 2, hydrolysate containing 16 µg galactose from SN fraction, pool 1 (see Table 22) galactosamine (trace), glucosamine and galactose detected; lane 3, 20 µg galactosamine, 20 µg glucose: standards; lane 4, hydrolysate from SN fraction, pool 2; lane 5, 16 µg each of glucosamine, galactose and fucose.


1
2
3
4
5

of liver, consisted of four major molecular weight species each of which contained galactose and glucosamine as their major carbohydrate constituents. These glycosaminoglycans were not precipitable with CPC. Galactosamine and sialic acid were also present in these polysaccharides but in small quantities, and no fucose, mannose or uronic acid could be demonstrated. Each molecular weight species contained a small amount of protein (measured by Lowry method only), which was not lost on Bio-Gel chromatography.

Very small amounts of sulfate were found in the galactose-containing glycosaminoglycans. Only the CPC-precipitable glycosaminoglycans of the "residue fraction" (comprising only 2.9% by weight of the total glycosaminoglycans) contained significant quantities of uronic acid and sulfate.

In the following studies, the 3000-4000 M.W. G_{M1}-gangliosidosis liver glycosaminoglycan was used. This M.W. species was isolated in quantities sufficiently large enough to allow all the analyses to be performed on this liver fraction. The aim of the following experiments was to chemically characterize the structure of the glycosaminoglycan.

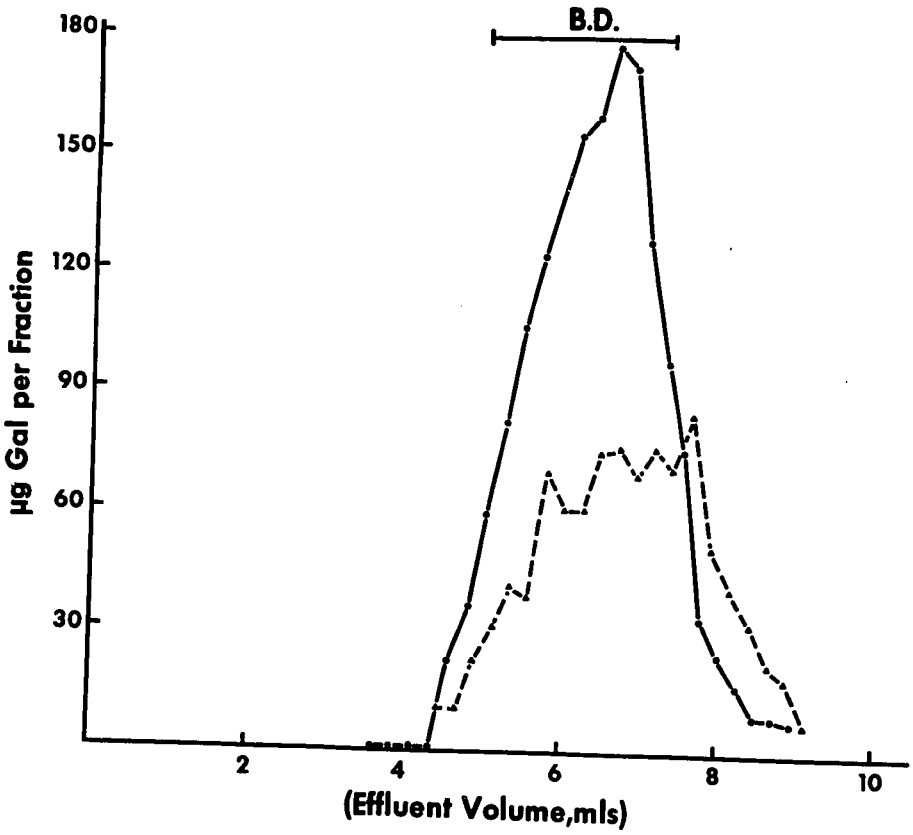
c Periodate Oxidation of the Low Molecular Weight Glycosaminoglycan

The 3000-4000 M.W. keratan sulfate fraction (fraction II SN, Tables 19 and 20) was re-chromatographed on a small Bio-Gel P_4 column and found to have a molecular weight near 3600. This material was subjected to periodate oxidation and re-

FIGURE 12

BIO-GEL P_4 SIEVE CHROMATOGRAPHY OF A PERIODATE TREATED LOW MOLECULAR WEIGHT KERATAN SULFATE

The 3600 molecular weight material fraction - II SN (Tables 19 and 20) was treated with periodate as described in the Methods section (p. 75). Both the untreated and the treated samples were individually chromatographed on a Bio-Gel P_4 column eluted with water. Fraction volumes were .22-.24 ml. Column dimensions 28x0.7 cm. Flow rate 1.5 ml/hr. Each fraction was analysed for hexose. Solid line is the control sample (periodate untreated) and the broken line is the sample treated for 24 hrs. with periodate (see Table 23).


B.D.
180
150
120
90
60
30
µg Gal per Fraction
2
4
6
8
10
(Effluent Volume,mls)

chromatographed on a P_4 column. The results are shown in Figure 12 and Table 23. It can be seen that the original molecule was degraded by periodate to a slightly lower molecular weight species with a loss of 33 per cent of galactose. However, after treatment of the pooled column eluates, from both control and periodate oxidized samples, with sodium borohydride, most of the oxidized galactose was apparently recovered (determined by anthrone analysis, Table 23). Such an effect of borohydride has been reported by Rothfus and Smith (265) in their studies on Gamma-globulin glycopeptides. Although the loss of galactose after periodate oxidation was apparently recovered after borohydride reduction, when hexosamine was measured a loss of 1-2 moles of hexosamine was found. This indicates that the recovery of galactose after borohydride reduction was an artefact. Determination of galactose, glucosamine and sialic acid in both control and periodate treated samples showed an overall loss from the native polymer of 4 moles of galactose, 2 moles of glucosamine and 0.01 moles of sialic acid per mole of starting material. Paper chromatography of the recovered materials showed two components with mobilities slightly behind that of galactose for both control and experimental samples. Threitol or erythritol was not found. The slight degree of retardation of the components on paper is likely due to incompletely-removed salt contamination.

The results indicate that there is very little degradation of the glycosaminoglycan to a lower M.W. species by periodate

TABLE 23

PERIODATE OXIDATION OF THE 3600 M.W. KERATAN SULFATE FROM G_{M1}-GANGLIOSIDOSIS LIVER

	Periodate Treatment		Total Change	
	untreated	24 hr. oxidation	observed (μmoles)	per mole* starting material (moles)
μmoles galactose				
before $NaBH_4$	8.35	5.55	2.80	4.15
after $NaBH_4$	8.43	8.59**		
μmoles hexosamine				
after $NaBH_4$	7.09	5.56	1.53	2.16
Molar ratios				
galactose/glucosamine	1.18/1.00	1.00/1.00		

*Calculations based on a molecular weight of 3600, containing 12 moles of galactose and 10 moles of hexosamine per mole starting material. Galactosamine content 1 mole or less.
**See text for explanation.

oxidation, suggesting that the internal galactose to glucosamine linkages are resistant to this treatment. This is consistent with glucosamine (1→3) galactose disaccharide repeating units. The oxidizable galactose is likely present at chain termini or branch points containing only galactose. The oxidizable hexosamine may also be terminal or on side chains but present to a lesser degree than galactose.

d Effect of Alkali Treatment on the 3600 M.W. Keratan Sulfate-like Glycosaminoglycan

The 3600 M.W. keratan sulfate fraction (Table 20) was treated with 0.5 N NaOH for 48 hours at room temperature at a concentration of 10 mg/ml of alkali. Half the sample was removed immediately, deionized with Dowex 50 X-8 H^+ (to a final pH of 2.7), concentrated and chromatographed on a Bio-Gel P_2 column (29x0.7 cm). The sample treated for 48 hours with alkali was treated in an identical manner. Both control and alkali-treated samples chromatographed in the void volume of this column and the total hexose measured in the alkali treated sample was within 3.3% of that for the control. The eluates were pooled, concentrated and tested for N-acetylhexosamine reactivity by the procedure of Reissig et al. (274). No chromogen was observed in the control sample while in the 48 hour alkali-treated sample, a small amount of chromogen (equivalent to 3.2% of the total hexosamine) was found. Thus, there was no appreciable degradation of the polysaccharide to small oligosaccharides. These results also show that the 3600 M.W.

FIGURE 13

THE EFFECT OF ALKALI ON LOW MOLECULAR WEIGHT KERATAN SULFATE

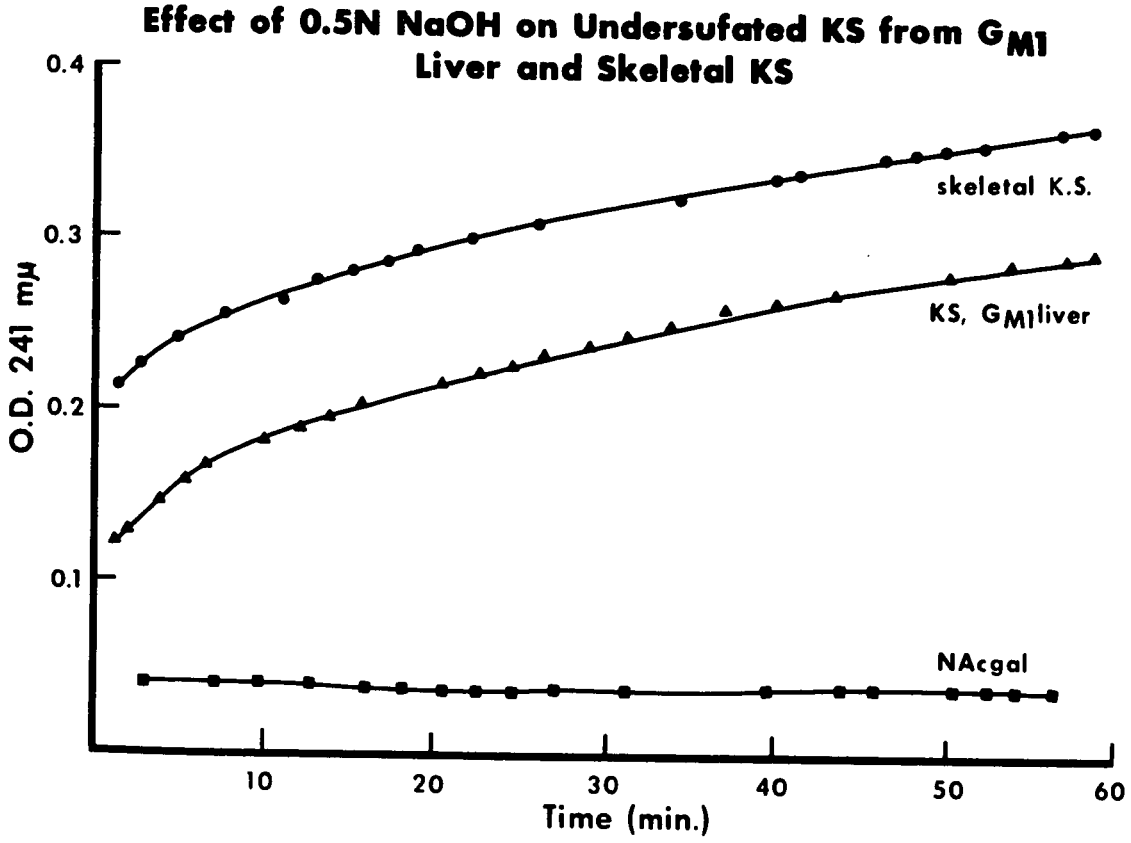
Fraction II SN (Tables 19 and 20) was treated with alkali in a quartz cuvette as described in the Methods section (p. 76). Old human rib cartilage keratan sulfate (KS_2) was measured simultaneously with N-acetylgalactosamine as control as described in the Methods.

•——• 500 µg standard of old human rib cartilage KS (KS_2).

▲——▲ 600 µg of liver "ganglioside fraction" II SN.

■——■ 70 µg of N-acetylgalactosamine.

The O.D. at 241 mµ was measured with time.


Effect of 0.5N NaOH on Undersufated KS from G_{M1} Liver and Skeletal KS
0.4
0.3
0.2
0.1
O.D. 241 mμ
skeletal K.S.
KS, G_{M1} liver
NAcgal
10
20
30
40
50
60
Time (min.)

protein-polysaccharide species does not consist of a series of short oligosaccharides (of about 1000-1200 M.W. attached to the same peptide through alkali-labile linkages.

Although the polysaccharide is stable to weak alkali, acid hydrolysis of the untreated material using the conditions of Bhavanandan and Meyer (86) (0.5N H_2SO_4, 100°C, 1 hr., 10 mg polysaccharide/ml) gave free monosaccharides on paper chromatography but no detectable reducing oligosaccharides.

In an attempt to characterize the linkage of the alkali-resistant polysaccharide to protein, the effect of 0.5N NaOH on the 3600 M.W. glycosaminoglycan was measured spectrophotometrically (see reference 91 and Figure 13). The time course of increase in optical density at 241 mμ was essentially identical to that obtained with standard skeletal keratan sulfate. This suggests that there is an alkali-labile protein-polysaccharide linkage region in the glycosaminoglycan accumulating in G_{M1}-gangliosidosis liver similar to that found in human skeletal keratan sulfate (90) where the small quantities of galactosamine in the molecule have been shown to reside in the protein-polysaccharide linkage region.

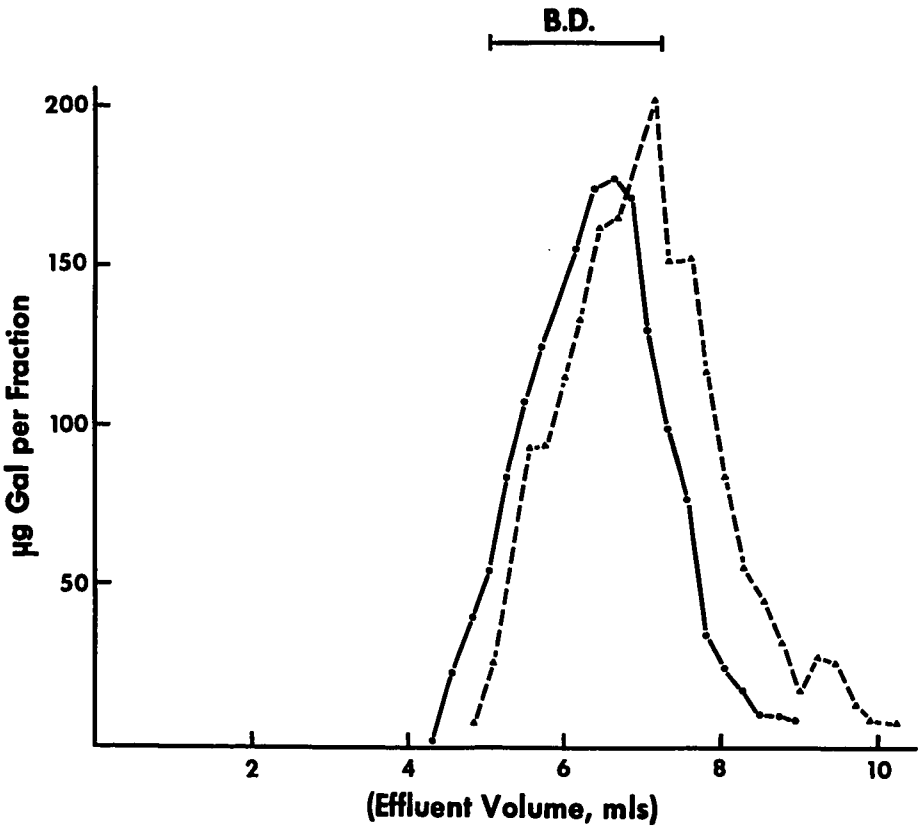
e <u>The Action of β-Galactosidase from Beef Liver on the 3600 M.W. Glycosaminoglycan Fraction</u>

In preliminary experiments, using the Galactostat method, we were unable to measure free galactose released from the 3600 M.W. keratan sulfate (Table 19) following incubation with a beef liver β-galactosidase preparation because the liver

FIGURE 14

THE ACTION OF BEEF LIVER β-GALACTOSIDASE ON THE 3600 M.W. LIVER KERATAN SULFATE

Fraction II SN (Tables 19 and 20) was treated with beef liver β-galactosidase at 37°C for 23 hours. Column dimensions 28x0.7 cm. Column was eluted with water. Flow rate 1.5 ml/hr. 0.23-0.24 ml fractions were collected. B.D. indicated the Blue Dextran 2000 peak. Each fraction was analysed for hexose. Broken line, 23 hours incubation with enzyme. The solid line shows the elution profile of the untreated polymer.


B.D.
200
150
100
50
µg Gal per Fraction
2
4
6
8
10
(Effluent Volume, mls)

polysaccharide interfered with the Galactostat assay. Measurement of free galactose by Galactostat in solutions containing 18-54 µg galactose (per 2 ml) was inhibited up to 90% by the presence of 500 µg of liver polysaccharide. Therefore, to determine if β-galactosidase from beef liver degraded G_{M1}-gangliosidosis liver keratan sulfate, approximately 5 mg of the 3600 M.W. fraction (about 2 mg galactose) was incubated for 24 hours at 37°C with the β-galactosidase preparation followed by separation of the residual polysaccharide on small Bio-Gel P_4 columns. The recovery of galactose from the incubated sample was compared with that of an equivalent amount of the untreated polysaccharide (Figure 14). Only very little degradation to smaller molecular weight species was found despite the presence of 66.4 per cent of the initial β-galactosidase activity at the end of the incubation period. Other experiments showed that the enzyme released 51 per cent of the terminal galactose from G_{M1}-ganglioside in 3 hours with a specific activity of 154.3 mµmoles galactose released/hr/mg enzyme protein at pH 5.0. Lactose was hydrolysed very slowly at approximately 7 per cent of the rate observed with G_{M1}-ganglioside as the substrate.

To summarize, the keratan sulfate-like glycosaminoglycans isolated from either the "ganglioside fraction" or the "residue fraction" of G_{M1}-gangliosidosis liver were identical in all respects (Table 19 and 22). Galactose and glucosamine were the major carbohydrate constituents with small amounts of

sulfate, sialic acid and galactosamine. They were not precipitated by cetylpyridinium chloride. Significant amounts of the total keratan sulfate fractions were not precipitated by ethanol, were dialysable, of molecular weights from 1600 to 3600 and also contained little protein (Table 19 and 20). The larger M.W. species, the 6000-8000 and 10,000 M.W. compounds were precipitated by ethanol. These compounds likely represent various sizes of polymers of the galactose-glucosamine repeating unit. The purified liver glycosaminoglycan fraction were thus polydisperse and had galactose to glucosamine molar ratios greater than unity (Figures 9 and 10). Uronic acid-containing glycosaminoglycans were found only in the fractions precipitated by cetylpyridinium chloride (Table 21). Differences in the extractability of the glycosaminoglycans in the two preparations reported in this study were likely due to variability in the water content of the extracting solvents. In future work on pathological liver of this type, we suggest special care in the control of the water content of solvents during lipid extraction since the bulk of the glycosaminoglycans can be extracted into chloroform-methanol and subsequently can partition in the Folch procedure along with the gangliosides.

The preliminary studies on the chemical structure of the liver polysaccharides suggests the presence of N-acetyllactosamine repeating units assembled in a linear sequence. The loss of galactose during the periodate oxidation but no significant decrease in the molecular weight of the polysaccharide suggests

the presence of side chains containing mostly galactose. Exposure of the 3600 M.W. glycosaminoglycan to 0.5 N NaOH did not result in the formation of small oligosaccharides. However, this 3600 M.W. species appears to contain an alkali-labile linkage of the carbohydrate moiety to one or more hydroxyamino acids of the peptide fragment (91).

Our studies thus indicate that the glycosaminoglycans stored in the liver of G_{M1}-gangliosidosis patients have a structure similar, if not identical to, the skeletal form of keratan sulfate. The most striking difference between G_{M1}-gangliosidosis liver keratan sulfate and normal human skeletal keratan sulfate was the extremely low content of sulfate in the liver polysaccharides. A low sulfate content in the glycosaminoglycans had previously been suggested (31), although no precise measurements were reported. Further studies on the detailed chemical structure of these polysaccharides employing methylation, mild acid hydrolysis and mass spectrometry, are in progress.

C TYPE II, G_{M1}-GANGLIOSIDOSIS: OBSERVATIONS ON A FIBROBLAST CELL STRAIN

In the study of the urinary glycosaminoglycans from case 1 we found an excessive excretion of undersulfated polysaccharides of the keratan sulfate-type. A fibroblast cell strain was developed from the skin of this patient to study the expression of this disease in cell culture especially with regard to gangliosides and glycosaminoglycans. Preliminary results of this work have appeared (245,286).

In preliminary experiments, the cultured fibroblasts (case 1) were grown on cover slips and fixed with absolute acetone. Histochemical observations carried out by the late Dr. J.S. Fawcett showed the presence of cytoplasmic vacuoles and granular metachromatic material in these cells (245).

In the following experiments, five different cell strains were used as control cell lines.

1 Fibroblast β-Galactosidase

Beta-galactosidase activity was measured in several cultures of skin fibroblasts of case 1 with two types of synthetic substrates (Table 24). Considerable variability was found in β-galactosidase activity in normal cell lines with both substrates, but all cultures from case 1 had markedly decreased β-galactosidase activity and in some cases no enzyme activity was found.

In mixed cultures of normal and case 1 fibroblasts, the

TABLE 24

β-GALACTOSIDASE ACTIVITIES OF CULTURED FIBROBLASTS

Cell line	mμmoles substrate cleaved/hr/mg fibroblast protein	
	ONPG*	MeUBF**
Normal culture 1	229.0	229.8
2	435.1	261.0
3	-	173.4
4	126.2	-
Case 1, culture 1	5.6	-
2	2.7	-
3	1.5	0
4	-	0.6

Averages of triplicate measurements.

*o-nitrophenyl-β-D-galactopyranoside
**4-methylumbelliferyl-β-D-galactopyranoside

expected intermediate level of β-galactosidase activity was obtained (Table 28) indicating that the activity of this enzyme in case 1 cells is not influenced by the presence of normal cells.

As we found in the liver the activities of acid phosphatase, β-glucuronidase, α-L-fucosidase and β-N-acetylglucosaminidase were increased above normal values in the cultured cells (Pinsky, L.: Pers. Comm.). The decreased β-galactosidase activity in cultured fibroblasts is consistent with the findings in all other tissues of patients with G_{M1}-gangliosidosis examined to date (157,243,245,291).

2 Fibroblast Gangliosides and Glycolipids

In preliminary experiments, the total glycolipid hexose (calculated in terms of galactose) was measured in total chloroform-methanol extracts of the fibroblasts. The mutant cell total lipid extracts had higher quantities than did the normal cells (39.7 ± 19.3 and 17.6 ± 7.8 μg glycolipid galactose per mg cell protein for case 1 and normal cells, respectively- average of 16 determinations in both cases ± SD).

Gangliosides and neutral glycolipids were isolated from fibroblasts cultured either in the presence or absence of ^{14}C-galactose. The ganglioside-NANA content of mutant cells in one experiment was not considered significantly different from normal (1.00 and 0.76 μg ganglioside-NANA per $1x10^6$ cells for mutant and normal cells, respectively). However, the ganglioside

types found in mutant cells were different from normal. Three gangliosides, migrating like the G_{M3}-, G_{D3a}- and G_{D1a}-gangliosides of brain were identified in both the control and mutant cell lines (Figure 15, a, c and d, lanes 2 and 3). In the mutant cell strain, two additional gangliosides were observed migrating like the G_{M2}- and G_{M1}-gangliosides of brain (see Table 1 and Figure 15, zones b and c, lane 3). These latter gangliosides were not found in normal cells. It is noteworthy that in the ganglioside fraction from case 1 cells, separated by TLC, there was more resorcinol-positive material remaining at the origin (zone f, lane 3) than in the corresponding zone of the normal cell ganglioside fraction (zone f, lane 2). Equal amounts of sialic acid from the control and mutant cell extracts were applied to the thin layer. Resorcinol-positive material remaining at the origin of thin layer chromatograms has also been observed in ganglioside fractions isolated from the liver and spleen of G_{M1}-gangliosidosis patients (see Figure 7, this thesis and reference 240).

Gangliosides were also isolated from fibroblasts previously incubated with ^{14}C-galactose and separated on TLC (Table 25). G_{M3}-ganglioside was the most extensively labeled ganglioside in both normal and case 1 cells, with much smaller quantities of radioactivity in the other ganglioside zones. In the mutant cell ganglioside fraction the extent of labeling of the gangliosides was about one-half that in the normal, when equivalent amounts of radioactivity were applied to the thin layer. A large fraction (61%) of the radioactivity detected in the case 1

FIGURE 15

ASCENDING CHROMATOGRAPHY OF FIBROBLAST GANGLIOSIDES ON SILICA GEL G

Solvent system: chloroform-methanol-2.5 N NH_4OH (60:35:8, v/v/v). Gangliosides were visualized with resorcinol. Lane 1: 120 µg of normal human brain gangliosides. Lane 2: gangliosides containing 6.5 µg sialic acid from normal fibroblasts. Lane 3: gangliosides containing 6.5 µg sialic acid from G_{M1}-gangliosidosis Type II (case 1) fibroblasts. Lane 4: 120 µg of G_{M1}-gangliosidosis, Type II (case 1) brain gangliosides. Zones (a), (b) and (c) are G_{M3}-, G_{M2}- and G_{M1}-ganglioside respectively. G_{D3a}-ganglioside migrates between (b) and (c). Zone (d) is G_{D1a}-ganglioside; zone (e) contains two gangliosides G_{D1b}- and G_{T1}. Zone (f) is the material remaining at the origin.

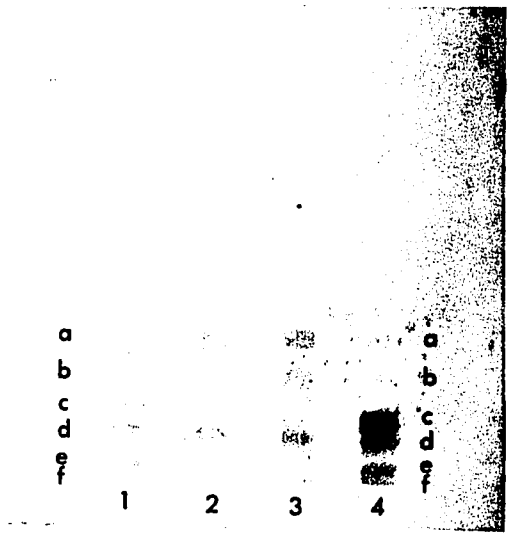

a
b
c
d
e
f
1
2
3
4
a
b
c
d
e
f

TABLE 25

RADIOACTIVITY IN GANGLIOSIDES FROM NORMAL AND CASE 1 FIBROBLASTS AFTER INCUBATION FOR 24 HOURS WITH 1-^{14}C-D-GALACTOSE

Cells were incubated and gangliosides were isolated as described (p. 55 and 62) and separated on Silica Gel G plates. Marker lanes were visualized with iodine and the corresponding labeled fibroblast zones were scraped into scintillation vials and counted (see p. 79).

Ganglioside Type	Per cent Total Activity*	
	Normal	Case 1
G_{M3}	44.1	18.7
G_{M2}	9.6	7.5
G_{M1}	10.6	5.0
G_{D1a}	4.7	3.5
G_{D1b}	2.4	1.9
G_{T1}	2.3	2.5
Origin	26.9	61.0

*Total counts measured: normal, 4916; case 1, 4739. Recovery: normal, 76.8% and case 1, 83.5%.

ganglioside extract did not migrate like ganglioside but remained at the origin. A smaller fraction of material (26.9% of the total) behaved in this manner in the normal cell fraction. This observation is similar to that found for the fibroblast gangliosides stained with resorcinol as indicated above (Figure 15) and for the liver ganglioside fraction (Figure 7).

Neutral glycolipids (asialogangliosides) were recovered from the chloroform-methanol phase of the Folch extraction procedure after removal of the phospholipids by saponification see Methods, p. 55). The partially purified neutral glycosphingolipids were then separated into five fractions by Silica Gel HR column chromatography and chromatographed on TLC (Figure 16). Essentially identical results were obtained for both normal and case 1 fibroblasts. As standard, pure G_{D1a}-ganglioside from Ox brain was hydrolysed in dilute acid and the neutral ceramide hexosides were recovered after Folch partition (249). G_{M3}-ganglioside, generated by this acid treatment, does not partition completely into the aqueous phase and is thus present as a contaminant of the neutral glycolipids.

The neutral glycolipids eluted in the 19:1 chloroform-methanol (C/M) (lane 1, Figure 16) fraction migrated near the solvent front and were similar in their chromatographic behaviour to ceramide and 1-O-glucosylceramide (a and b, Figure 16). Similarly, two components were eluted in the 9:1 C/M column fraction (lane 2) and migrated like cerebrosides (using 1-O-

FIGURE 16

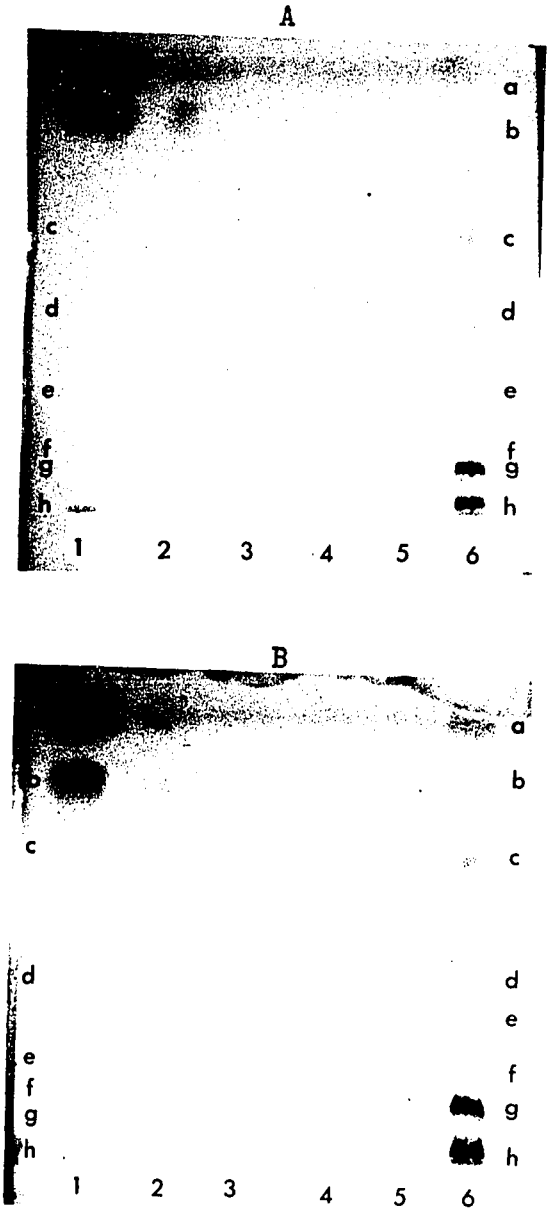
ASCENDING THIN LAYER CHROMATOGRAPHY OF FIBROBLAST NEUTRAL GLYCOLIPIDS

Solvent system: chloroform-methanol-H_2O (70:30:4, v/v/v). Glycolipid zones were visualized by charring with 10 N sulfuric acid.

A. Neutral glycolipids from control cells separated by silica gel HR column chromatography.

B. Neutral glycolipids from G_{M1}- Type II cells separated by silica gel HR column chromatography.

For both A and B: Lane 1: 19:1 C/M eluate. Lane 2: 9:1 C/M eluate. Lane 3: 4:1 C/M eluate. Lane 4: 1:4 C/M eluate. Lane 5: Methanol eluate. Lane 6: 300 µg of acid hydrolysed ganglioside. See the text for all methods (p. 65).


A
a
b
c
c
d
d
e
e
f
f
g
g
h
h
1
2
3
4
5
6
B
a
b
c
c
d
d
e
e
f
f
g
g
h
h
1
2
3
4
5
6

glucosylceramide as a marker; b, Figure 16). Dihexosylceramide (zone c) was observed in small amounts in the 4:1 C/M eluate (lane 3). Several components were identified in the 1:4 C/M eluate (lane 4). The fastest moving components (one major and one minor) in this fraction migrated intermediate between lactosylceramide and trihexosylceramide of brain (N-acetyl-galactosaminyllactosylceramide) for both cell lines (zone d, lane 4). This fast moving major component from case 1 appears to be increased in quantity over that of normal cells (based on visual inspection only). Two additional components, in both normal and case 1, migrated between brain trihexosylceramide (zone e) and brain tetrahexosylceramide (zone f) while two more unidentified components migrated more polar than G_{M3}-ganglioside (zone g). Two components were found in the methanol eluate, the fastest migrating like the tetrahexosylceramide of brain and the other slightly more polar (lane 5).

On the basis of chromatographic behaviour alone the major trihexosylceramides of fibroblasts do not correspond to that derived from brain ganglioside, while the other hexosylceramides do migrate in this manner. Matalon et al. (287) have found relatively high amounts of a trihexosylceramide in normal fibroblasts and an accumulation of this glycolipid, having a mole ratio of galactose to glucose of 2:1, in fibroblasts of Fabry's disease.

Neutral glycolipids from both normal and case 1 cell lines labeled with ^{14}C-galactose were also subjected to column and

thin layer chromatography (Table 26 and Figure 17). Radioactive material was present in all the eluates (Table 26). The labeled glycolipids from both normal and case 1 cells migrated on TLC like the unlabeled glycolipids obtained from the corresponding column fractions (Figure 16), as described above (TLC not shown). The largest quantities of radioactivity were eluted in the 1:4 C/M eluate for both normal and case 1 cell lines (Table 26). It may be seen that in the case 1 glycolipids the radioactivity eluted in the less polar solvents (19:1, 9:1 and 4:1 C/M) was about 50 per cent of normal (on the basis of per cent of total radioactivity in each fraction) while that in the 1:4 C/M eluate was higher in the mutant than normal cell glycolipids. In both cell lines, however, the tetrahexosylceramides (methanol eluate) contained the same relative proportion of radioactivity.

The 1:4 C/M eluates were then subjected to TLC (Figure 17). In the control 26.4% of the total radioactivity was found at the origin (zone 1) while only 8.6% of the total was found at the origin in case 1 (zone 16). Two other regions of the thin layer contained significant amounts of radioactivity in the control (zone 6 and zones 8 and 9) and in the mutant cell strain (zone 21 and zones 23 and 24). The components migrating between the tri- and tetrahexosylceramides of brain (zone 6, control and zone 21, case 1) were comparable in their extent of labeling for both cell strains. The components migrating between lactosylceramide and trihexosylceramide of brain (zones 8

TABLE 26

FRACTIONATION OF ^{14}C-GALACTOSE-CONTAINING GLYCOLIPIDS ON SILICA GEL COLUMN CHROMATOGRAPHY

The crude glycolipids were separated on 15x0.7 cm columns. Glycolipids from each cell line were chromatographed simultaneously on separate columns.

Eluant	CPM per Eluate	
(Chloroform-Methanol)	Normal	Case 1
19:1	2,300	2,300
9:1	11,900	8,500
4:1	8,900	4,700
1:4	59,500	99,800
Methanol	2,600	3,600

Recovery: normal, 82%; Case 1, 95%

FIGURE 17

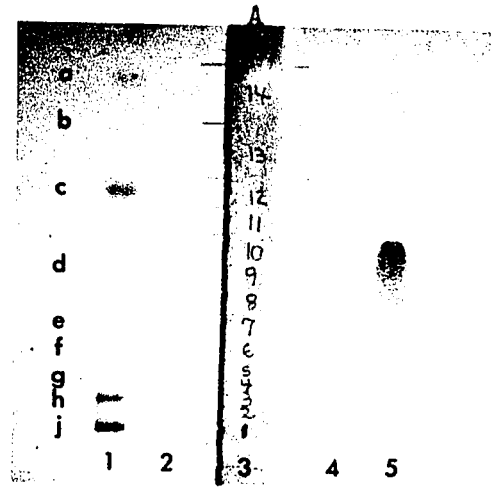
ASCENDING THIN LAYER CHROMATOGRAPHY OF FIBROBLAST NEUTRAL GLYCOLIPIDS LABELED WITH ^{14}C-GALACTOSE

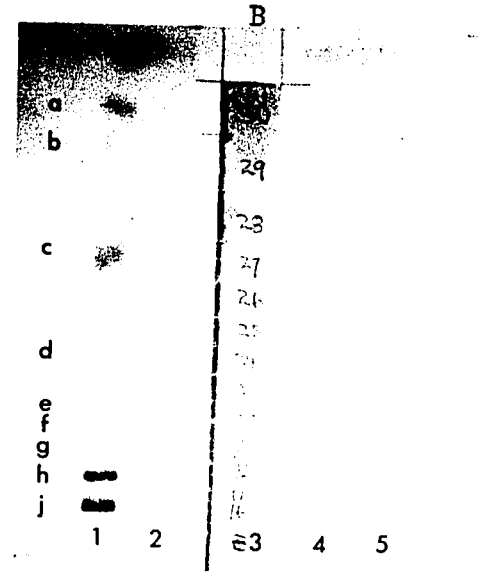
Solvent system and other details - see legend to Figure 16.

A. Control: Lane 1: 200 µg acid hydrolysed ganglioside. Lane 2: 1:4 C/M eluate, unlabeled (see Figure 16). Lane 3: 1:4 C/M eluate of ^{14}C-galactose labeled glycolipids (see Table 26). Lane 4: MeOH eluate of control (see Figure 16). Lane 5: 200 µg beef brain sulfatide.

B. G_{M1}-Type II: Lane 1: as in A. Lane 2: 1:4 C/M eluate from G_{M1}-Type II - unlabeled (see Figure 16). Lane 3: 1:4 C/M eluate of ^{14}C-galactose labeled glycolipids (see Table 26 - G_{M1}-Type II). Lane 4: MeOH eluate of G_{M1}-Type II (see Figure 16). Lane 5: as in A.

Recovery of radioactivity: control, 91%; G_{M1}-Type II, 83%.


A
a
b
c
d
e
f
g
h
j
1
2
3
4
5


B
a
b
c
d
e
f
g
h
j
29
1
2
3
4
5

and 9, control; zones 23 and 24, case 1) were clearly different in their extent of labeling. The radioactivity of this region in the mutant cell fraction was twice that of normal.

All of these results taken together suggest that in the mutant cells there is a metabolic block at the level of the glycolipid tentatively identified as the trihexosylceramide described by Matalon et al. (287). Since in the mutant cells the G_{M2}-like ganglioside (Figure 15, zone b) was found at a higher level than the G_{M1}-like ganglioside (neither ganglioside was found in normal cells), it is likely that this G_{M2}-like ganglioside is similar to (N-acetylneuraminyl) galactosyl-galactosylglucosylceramide. The linkage position of the sialic acid in this compound has not been determined. Such a ganglioside has been found in dog intestine (288), in the lens of the human eye (14) and, in small quantities, in normal brain (see Reference 13).

3 Incorporation of Labeled Precursors by Fibroblasts

The incorporation of several labeled precursors during 24 hours of incubation was compared for normal and case 1 cell strains. The values obtained have been expressed in terms of total cell protein and of amino acid incorporation. Results of a representative experiment are given in Table 27. When expressed in terms of total cell protein, the incorporation of ^{14}C-galactose and ^{35}S-sulfate in the mutant cells was increased over normal while the incorporation of ^{14}C-glucosamine

TABLE 27

INCORPORATION OF LABELED PRECURSORS INTO FIBROBLASTS DURING A 24 HOUR PERIOD

Cells ($1x10^6$) were incubated for 24 hours in the presence of 10 ml of medium containing one of the labeled precursors. The cells were then harvested, dried and digested with 0.1N NaOH. Aliquots of the digest were removed for protein and radioactivity measurements. For details see Methods (pp. 62,79).

Precursor	Specific Activity (DPM/mg cell Protein)		Specific Activity (DPM/mg cell Protein/10^3 DPM of RPH)	
	Normal	Case 1	Normal	Case 1
1-^{14}C-D-galactose	149,422	220,698	823	1,583
1-^{14}C-Sodium acetate	71,329	67,435	338	484
^{35}S-H_2SO_4	688	1,363	4	10
1-^{14}C-D-glucosamine	39,100	25,196	216	199
^{14}C-RPH*	181,374	139,400	-	-

Averages of four determinations.

*RPH - reconstituted protein hydrolysate (Schwartz Bioresearch)

was reduced to 65% of the normal value. No change was observed with ^{14}C-acetate while the incorporation of ^{14}C-reconstituted protein hydrolysate (^{14}C-R.P.H.) was slightly reduced in the mutant cells. In five separate experiments the mean values of ^{14}C-glucosamine incorporation in the mutant cells, was 50% of the normal (range: 34.5-66.5%, established with four normal cell lines). The incorporation of these labeled precursors was also expressed in terms of the amino acid incorporation (^{14}C-R.P.H.). When expressed in this way galactose and sulfate uptake was twice that of normal, acetate incorporation was slightly increased while the glucosamine incorporation showed a slight decrease from normal. In these and other experiments, not reported here, we found considerable variability in the degree of increase in galactose incorporation but the results obtained with the other precursors were very reproducible.

The decreased glucosamine uptake in the mutant cells was particularly interesting and somewhat surprising in light of our previous findings; namely, the accumulation in the viscera and the elevated excretion of glucosamine- and galactose-containing polysaccharides in G_{M1}-gangliosidosis patients. For this reason, glucosamine incorporation in mutant cells was studied in more detail.

As mentioned earlier, mutant fibroblasts grown in the presence of an approximately equal number of normal cells gave the expected intermediate level of β-galactosidase activity (Table 28). However, the capacity of the mixed culture to incorporate

TABLE 28

COMPARISON OF THE β-GALACTOSIDASE ACTIVITIES AND THE INCORPORATION OF 1-^{14}C-D-GLUCOSAMINE IN FIBROBLAST CULTURES

Equal numbers of cells ($1x10^6$) were incubated for 24 hours in the presence of labeled glucosamine, harvested and finally suspended in saline. Aliquots were withdrawn for protein, radioactivity and enzyme activity measurements as described (p. 62).

Cell Line	β-Galactosidase		Incorporation of ^{14}C-Glucosamine	
	Spec. Activity*	% of Normal	DPM/mg cell Protein	% of Normal
Normal				
1	178.8	100	22,613	100
2	168.0		23,335	
Case 1				
1	0.6	0.3	11,848	51.2
2	0.6	0.3	11,690	
Mixed (Normal and Case 1)				
1	97.9	56.5	13,996	59.8
2	98.3	56.7	13,463	

Averages of three determinations.
*Specific Activity is the mμmoles of substrate cleaved per mg protein per hour at 37°C with 4-methylumbelliferyl-β-D-galactopyranoside as substrate.

^{14}C-glucosamine was 59.8% of normal instead of the expected 76%. This suggests that the incorporation by normal cells may be inhibited by contact with or by materials released from the mutant cells.

The pattern of incorporation of ^{14}C-glucosamine into G_{M1}-gangliosidosis (case 1) fibroblasts differs markedly from that obtained with normal cells (Figure 18). In normal cells, the labeled precursor was rapidly incorporated during the first 36 hours and then decreased. In contrast, in the mutant cells, the rate of incorporation was very much slower and the steady state level was not reached by the end of the experiment (72 hours).

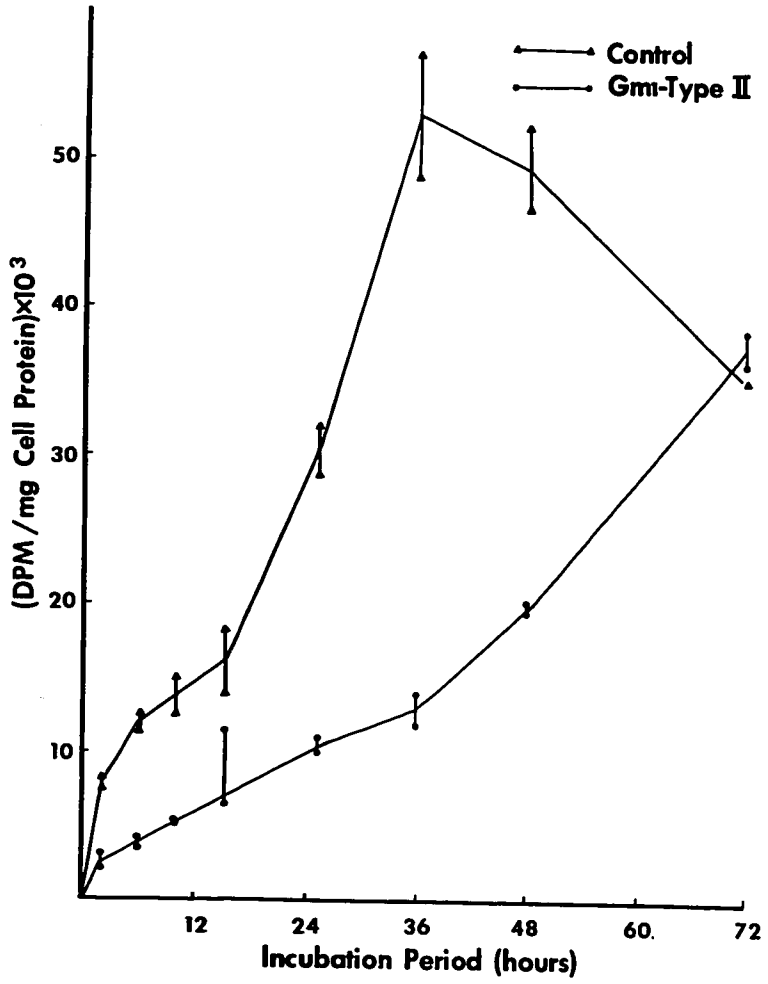
Lipids were extracted from both cell strains but contained little label. Seventy-five to 80% of the total label incorporated was found in the lipid-free cell residue. The time course of incorporation into this fraction, after papain digestion and dialysis, was identical to that found for the whole cells for both cell lines, whether expressed in terms of dry weight (after papain digestion, dialysis and lyophilization) or of hexosamine. Differential colorimetric analysis and Dowex-50 column chromatography showed that about 95% of the label was incorporated as glucosamine in both normal and case 1 cells.

The histochemical demonstration of metachromatic granules in these cells derived from the G_{M1}-gangliosidosis patient (case 1) suggested that their content of polysaccharides was increased. This finding together with the demonstration of the

FIGURE 18

TIME COURSE OF INCORPORATION OF $1\text{-}^{14}C$-GLUCOSAMINE

The fibroblasts ($1x10^6$ cells) were incubated in medium (10 ml) containing 0.4 μC of $1\text{-}^{14}C$-D-glucosamine, after an initial incubation of 12 hours in unlabeled medium. Cells were harvested at appropriate time. Each point represents replicate cultures pooled prior to analysis.


Control
Gmi-Type II
(DPM/mg Cell Protein)×10³
50
40
30
20
10
12
24
36
48
60.
72
Incubation Period (hours)

deficiency in β-galactosidase activity and the decreased incorporation of ^{14}C-glucosamine into cellular components suggested that the catabolism of intracellular polysaccharides was decreased. This hypothesis was tested by studying the rate of loss of label from cells pre-incubated for 24 hours in the presence of ^{14}C- glucosamine (Figure 19). The mutant cells showed a relative decrease in the rate of loss of radioactivity compared to the control cell strain examined. This finding was confirmed by measuring the rate of appearance of radioactivity in the unlabeled incubation medium (Table 29). The rate of appearance of radioactivity in the medium obtained from incubation of normal cells was greater than that from case 1 cells.

The radioactivity was then measured in the combined Trypsin-EDTA harvest media and the combined saline washes. Total recovery of radioactivity was comparable at all time points for both cell lines (95-103%). In addition, the radioactivity in the Trypsin-EDTA and saline wash solutions was compared to the total intracellular label at each time point (Table 30). The results of these measurements show that more radioactivity was lost from normal cells than from case 1 cells. This suggests that the mutant cells either were more stable to the harvest and washing conditions or that the intracellular label was more rapidly organized into non-diffusable substances in the mutant cell strain.

The media recovered from the decay experiment (Figure 19) were digested with pronase, dialysed and concentrated. The

FIGURE 19

THE TIME COURSE OF LOSS OF RADIOACTIVITY FROM FIBROBLASTS AFTER AN INITIAL 24 HOUR LABELING PERIOD

The cells ($1x10^6$ per dish) were incubated overnight in unlabeled medium (10 ml). The medium was then replaced with medium containing 0.4 μC of 1-^{14}C-D-glucosamine and incubated for 24 hours. At this time, labeled medium was replaced with unlabeled medium. Cells were harvested at appropriate times. Each point represents the mean of replicate cultures pooled prior to analysis.

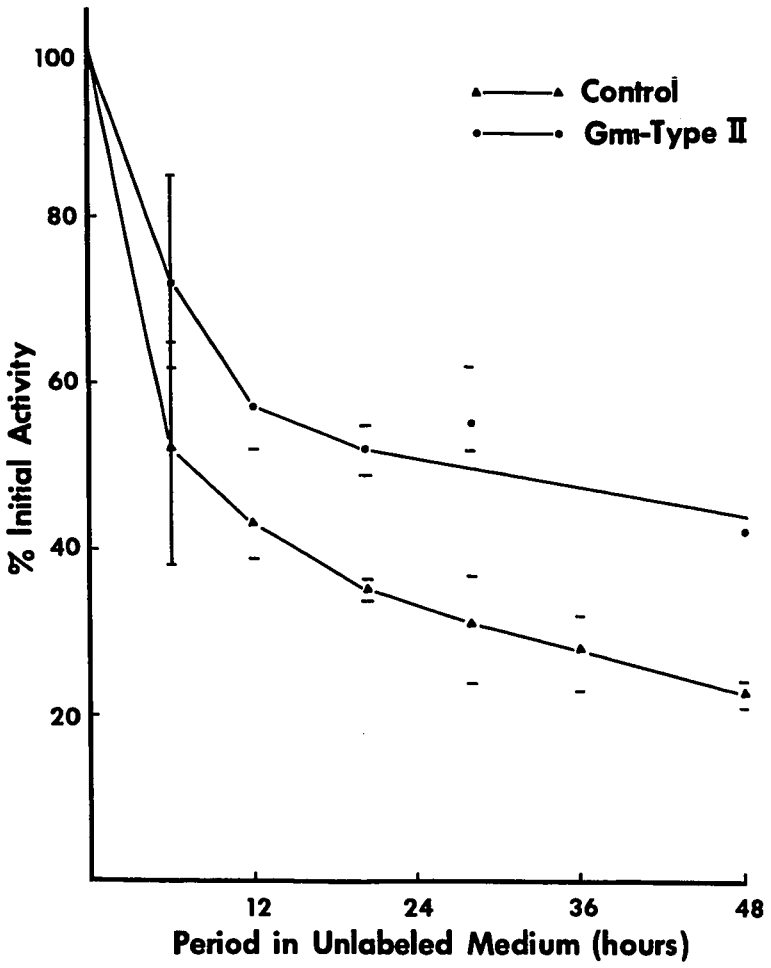

Control
Gm-Type II
100
80
60
40
20
% Initial Activity
12
24
36
48
Period in Unlabeled Medium (hours)

TABLE 29

LOSS OF RADIOACTIVITY FROM THE CELLS AND ITS APPEARANCE IN THE MEDIUM

Aliquots of the initially unlabeled medium were digested with Hyamine and counted as described. The cells taken at time zero were washed with Earle's A balanced salt solution prior to harvest and no measurement of the radioactivity in this solution was made. All cells were harvested, digested and the radioactivity measured as described (pp. 62,79).

Time in Unlabeled Medium (Hours)	Total Radioactivity (CPM)			
	Cells		Medium	
	Normal	Case 1	Normal	Case 1
0	24,274	10,261	-	-
6	13,641	7,711	20,340	15,080
12	12,062	7,367	22,620	15,740
28	9,535	6,260	26,540	15,600
47	9,161	5,788	29,700	16,280

Averages of replicate cultures, each measured in duplicate.

TABLE 30

THE APPEARANCE OF RADIOACTIVITY IN THE HARVEST AND WASHING SOLUTIONS

The cells, incubated with unlabeled medium after an initial 24 hour incubation with ^{14}C-glucosamine-containing medium, were harvested with EDTA and Trypsin in successive incubations and the collected cells were washed with saline (see p.62).

Time in Unlabeled Medium (Hours)	CPM in Trypsin-EDTA* / CPM in Cells		CPM in Saline* / CPM in Cells	
	Normal	Case 1	Normal	Case 1
0	0.85	1.01	0.28	0.16
6	0.97	0.35	0.26	0.13
12	0.92	0.27	0.18	0.07
28	0.93	0.46	0.22	0.11
47	0.97	0.57	0.15	0.09

*The total EDTA and Trypsin harvest solutions were pooled, digested with Hyamine (p. 79) and counted. Aliquots of the pooled saline wash solutions (15 ml total volume) were counted directly.

radioactivity of the dialysable material was 63 and 50% of the total for the control and mutant cell strains, respectively. The non-dialysable labeled material was then subjected to chromatography on a Bio-Gel P_{10} column eluted with water. The recovery was poor in both cases (20-25% of the total added to the column). The labeled component recovered from the medium obtained from incubation of mutant cells was slightly retarded with a calculated molecular weight of 6,000-8,000. The labeled component from the control sample, however, was greatly retarded on the column and had a calculated M.W. of 3,000-4,000.

To summarize, the studies on the incorporation of ^{14}C-glucosamine showed that the rate of its incorporation and the rate of loss of labeled cellular material was decreased in the mutant cells. In addition, co-culture of normal and mutant cells had no effect on β-galactosidase activity but a decrease in ^{14}C-glucosamine incorporation was found in the mixed culture.

4 Isolation and Characterization of Fibroblast Glycosaminoglycans Labeled with ^{14}C-Glucosamine and ^{14}C-Galactose

Glycosaminoglycans were isolated from the cell residues obtained after removal of the lipids by C/M extraction. The cell residues were then digested with papain, dialysed and the sac contents lyophilized (see p. 63).

The cell residues obtained from the previous experiment on the rate of loss of ^{14}C-glucosamine were digested with papain, dialysed, pooled and then subjected to Bio-Gel P_{10} sieve chromatography (Figure 20). Samples from the control and mutant

FIGURE 20

BIO-GEL P_{10} SIEVE CHROMATOGRAPHY OF ^{14}C-GLUCOSAMINE LABELED COMPOUNDS FROM LIPID-FREE CELL RESIDUES AFTER PAPAIN DIGESTION

Column dimensions: 46x1.2 cm. Flow rate 20 ml/hr. Volume per fraction 2.1-2.2 ml. Eluant - H_2O. B.D. refers to Blue Dextran. Quantitative recovery of label was obtained in both cases. The eluates containing labeled material were pooled and concentrated.

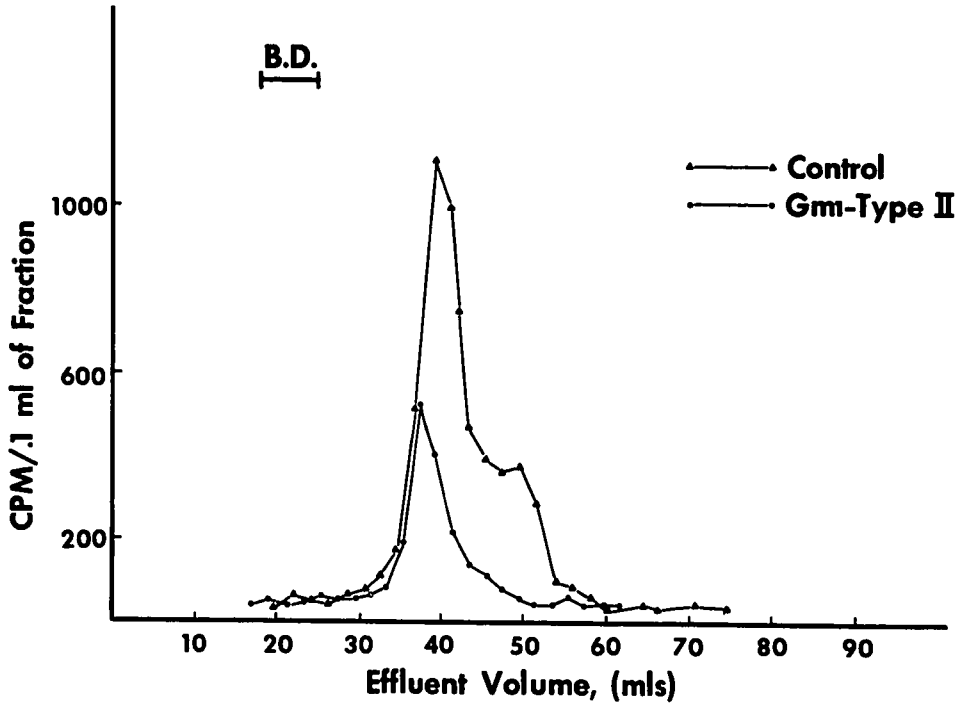
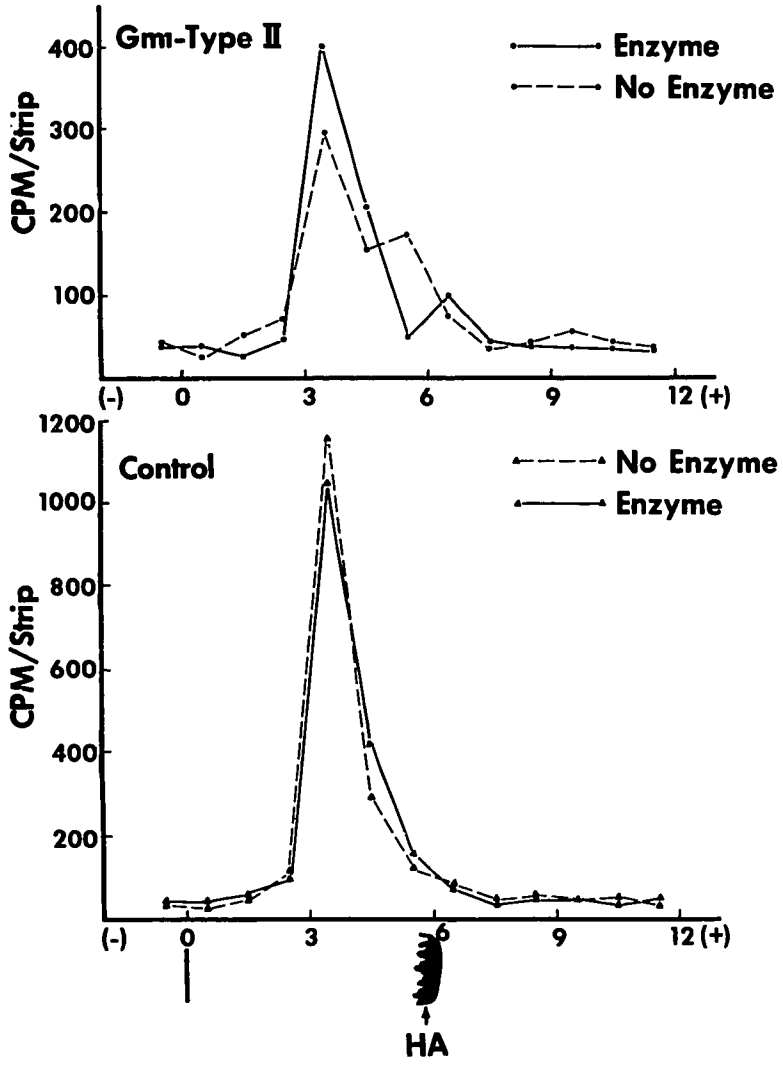
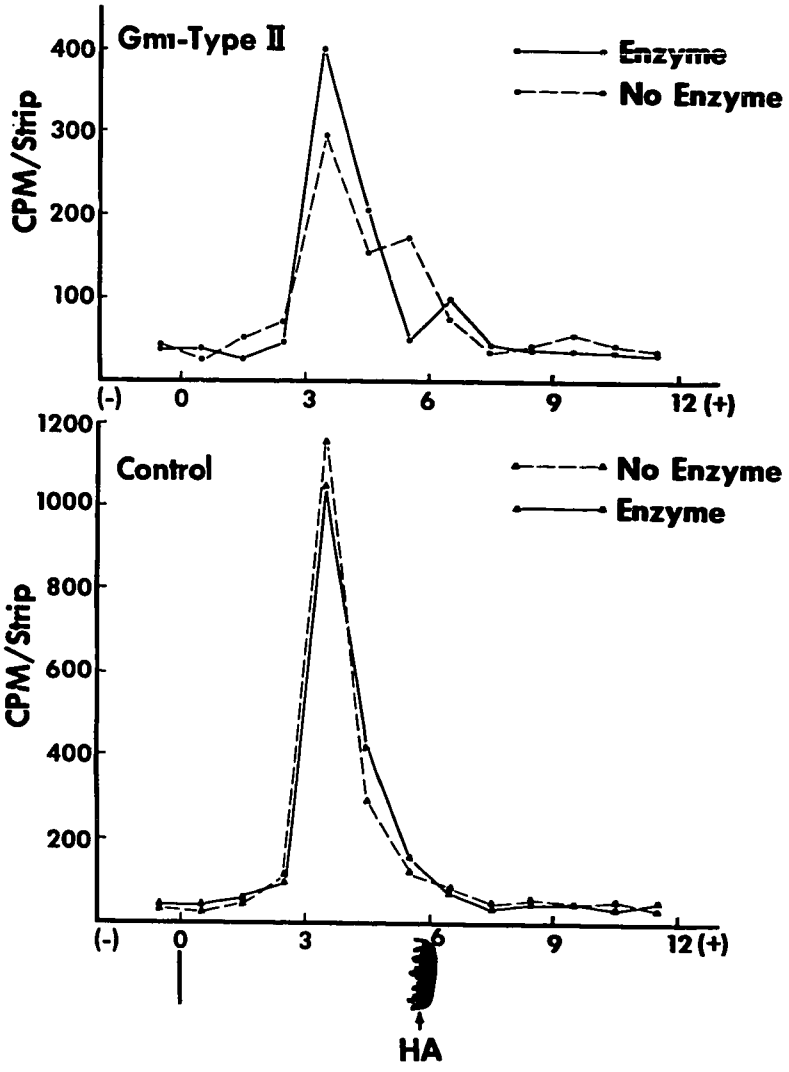

B.D.
Control
Gm-Type II
CPM/.1 ml of Fraction
1000
600
200
10
20
30
40
50
60
70
80
90
Effluent Volume, (mls)

FIGURE 21

SEPRAPHORE III ELECTROPHORESIS OF ^{14}C-GLUCOSAMINE LABELED COMPOUNDS AFTER BIO-GEL P_{10} CHROMATOGRAPHY

Samples were subjected to electrophoresis on Sepraphore III with 0.1 M Sodium Phosphate buffer, pH 7.3, as electrolyte, for 1 hour at 10 Volts/cm. Marker strips were blotted and stained with alcian blue. Test strips were cut into 1 cm slices and counted. Samples were run before and after treatment with testicular hyaluronidase. Recovery of radioactivity: control, 81%; G_{M1}-Type II, 75%.


Gm-Type II
Enzyme
No Enzyme
CPM/Strip
400
300
200
100
(-)
0
3
6
9
12 (+)
Control
No Enzyme
Enzyme
CPM/Strip
1200
1000
800
600
400
200
(-)
0
3
6
9
12 (+)
HA


Gm-Type II
Enzyme
No Enzyme
CPM/Strip
400
300
200
100
(-)
0
3
6
9
12 (+)
Control
No Enzyme
Enzyme
CPM/Strip
1200
1000
800
600
400
200
(-)
0
3
6
9
12 (+)
HA

cell lines were chromatographed separately on the same P_{10} column. The labeled components from both cell samples were eluted at the same point and were slightly retarded. The control sample also contained a smaller peak incompletely resolved from the major peak (Figure 20). The calculated molecular weight for the major peak from both control and mutant cells was 7,000-8,000. The eluates containing the labeled material were pooled, concentrated and examined on electrophoresis with and without hyaluronidase digestion.

There was no significant difference between the Hyase treated and untreated samples from the control cells when examined by electrophoresis (Figure 21). However, in the mutant cell sample after Hyase digestion there was a small decrease in the amount of labeled material migrating to the same region as standard Hyaluronic acid (Figure 21). For both cell samples, the major radioactive component migrated electrophoretically about 60% of the distance travelled by Hyaluronic acid. Thus, the major glucosamine-labeled components in both cells lines were weakly anionic, of high molecular weight and not digested with hyaluronidase.

In another experiment, fibroblasts were incubated for 24 hours in medium containing ^{14}C-galactose. The lipids were extracted and the cell residues obtained were digested with papain, dialysed and subfractionated by CPC and ethanol. The results of this fractionation are presented in Table 31. As shown earlier (Table 27), more label from ^{14}C-galactose was

TABLE 31

FRACTIONATION OF FIBROBLAST GLYCOSAMINOGLYCANS LABELED WITH ^{14}C-GALACTOSE BY PRECIPITATION WITH CETYLPYRIDINIUM CHLORIDE AND ETHANOL

The fibroblasts ($1x10^6$ cells/10 ml medium) were incubated for 24 hours in medium containing 1-^{14}C-D-galactose. The cells from 20 dishes were harvested, pooled, washed with saline and lyophilized. The dried cells were extracted with C/M to remove the lipids and the lipid-free cell residues were digested with papain and fractionated as below.

Fraction From Papain Digest	Normal Case 1 (CPM/mg dry weight cells)		Normal Case 1 (% Total)	
Papain Digest				
1. undialysed	30,800	54,201	-	-
2. dialysed	14,614	49,286	(100%)	(100%)
CPC-precipitable				
Glycosaminoglycans	2,428	10,504	(16.6%)	(21.3%)
Non-CPC-precipitable				
Ethanol precipitable Glycosaminoglycans	3,870	29,253	(26.5%)	(59.3%)
Supernatant fluid	6,602	5,325	(49.0%)	(10.8%)

Recovery: Normal, 91%; Case 1, 92%.

The mean protein content of dry cells: 51.9 ± 7.4% for normal and 48.9 ± 8.7% for Case 1.

incorporated in the mutant cell extract than in the control. Further, only 9% of the total radioactivity was lost from the mutant cell extract on dialysis of the papain digest while 52.5% of the total was dialysed from the normal cell extract. The dialysed extracts were then subdivided into two fractions by precipitation with CPC. In both normal and case 1 cells about 20% of the non-dialysable label was precipitated with CPC. However in absolute terms, (on the basis of total dry weight of cells), the CPC-precipitable glycosaminoglycan fraction from case 1 contained about 4 times more label than the same fraction from the control (the CPC-precipitable glycosaminoglycan fraction from mutant cells contains a large amount of non-CPC-precipitable material, as shown later in Figure 23).

The clear supernatant fluid obtained after removal of the CPC-precipitable glycosaminoglycans was then treated with 3 volumes of ethanol (100%). An immediate formation of white precipitate was observed on addition of ethanol to the mutant cell supernatant fluid. Both samples were left overnight at $4^{o}C$ (in the presence of sodium acetate) at which time a small amount of precipitate was also obtained from the control. These non-CPC-precipitable glycosaminoglycan fractions differed markedly in their radioactivity. The mutant cell non-CPC-precipitable material contained 59% of the total label while the corresponding control fraction contained 27% of the total. Further, on the basis of dry weight of cells, this fraction from the mutant cells contained 7 to 8 times more radioactivity

than the control fraction.

Most of the radioactivity (49%) from the control was ethanol soluble while only 11% of the total label was found in this fraction in the mutant cells (Table 31).

The non-CPC-precipitable glycosaminoglycans from the mutant cells labeled with ^{14}C-galactose were chromatographed on a Bio-Gel P_{10} column (Figure 22) eluted with water. The chromatography was carried out with G_{M1}-gangliosidosis liver "residue fraction" polysaccharide (subfraction P_2, Table 21 and Figure 10) as carrier. One major radioactive peak was obtained which corresponded closely in its chromatographic behaviour to the polysaccharide isolated from the liver.

The CPC-precipitable glycosaminoglycans from the mutant cells were then chromatographed on the same Bio-Gel P_{10} column in the presence of another G_{M1}-gangliosidosis liver "residue fraction" polysaccharide (fraction SN, Table 21 and Figure 10). The CPC-precipitable fibroblast glycosaminoglycans were resolved into two radioactive components, one eluted at the void and one retarded (Figure 23). The component at the void corresponded to the non-CPC-precipitable glycosaminoglycan fraction and had an apparent M.W. of about 10,000 (Figure 22). The retarded peak had an apparent molecular weight of 3,000-4,000. The undersulfated polysaccharides purified from the liver appeared to have no effect on the chromatographic behaviour of the ^{14}C-galactose-labeled components from the fibroblasts.

The CPC-precipitable and non-CPC-precipitable

FIGURE 22

BIO-GEL P_{10} CHROMATOGRAPHY OF NON-CPC-PRECIPITABLE GLYCOSAMINOGLYCANS LABELED WITH ^{14}C-GALACTOSE ISOLATED FROM CASE 1 FIBROBLASTS

The column (28x0.7 cm) was eluted with water at a flow rate of 5 ml/hour. Column fractions (0.27-0.30 ml) were counted for radioactivity and analyzed for hexose. The fibroblast fraction was co-chromatographed in the presence of 6.2 mg of a glycosaminoglycan fraction (subfraction P_2, Table 21) isolated from the liver of a Type I, G_{M1}-gangliosidosis patient. B.D. is the Blue Dextran 2000 peak. Recovery of radioactivity 87%.

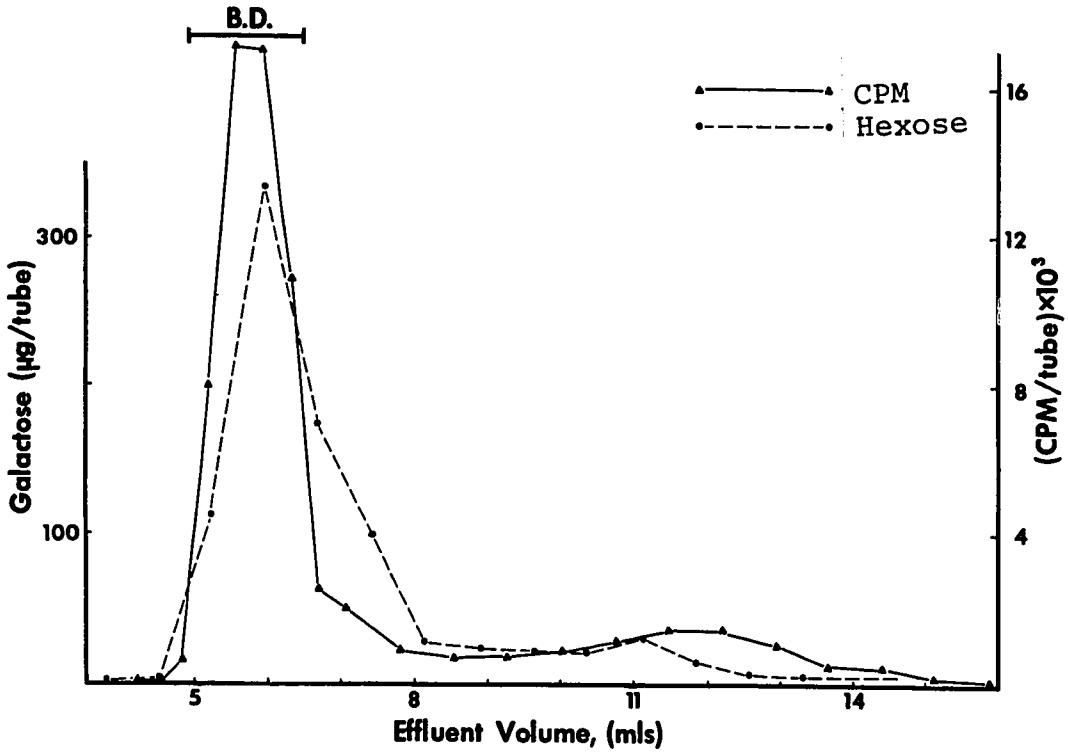

B.D.
CPM
Hexose
Galactose (µg/tube)
300
100
(CPM/tube)×10³
16
12
8
4
5
8
11
14
Effluent Volume, (mls)

FIGURE 23

BIO-GEL P_{10} CHROMATOGRAPHY OF G_{M1}-TYPE II CPC-PRECIPITABLE GLYCOSAMINOGLYCANS LABELED WITH ^{14}C-GALACTOSE

Conditions used were the same as outlined in the legend to Figure 22. The fibroblast glycosaminoglycans were co-chromatographed with a glycosaminoglycan fraction (SN, Table 21) isolated from the liver of a G_{M1}-gangliosidosis patient. Column eluates were counted for radioactivity and analyzed for hexose. B.D. is the Blue Dextran 2000 peak. Recovery of radioactivity 60%.

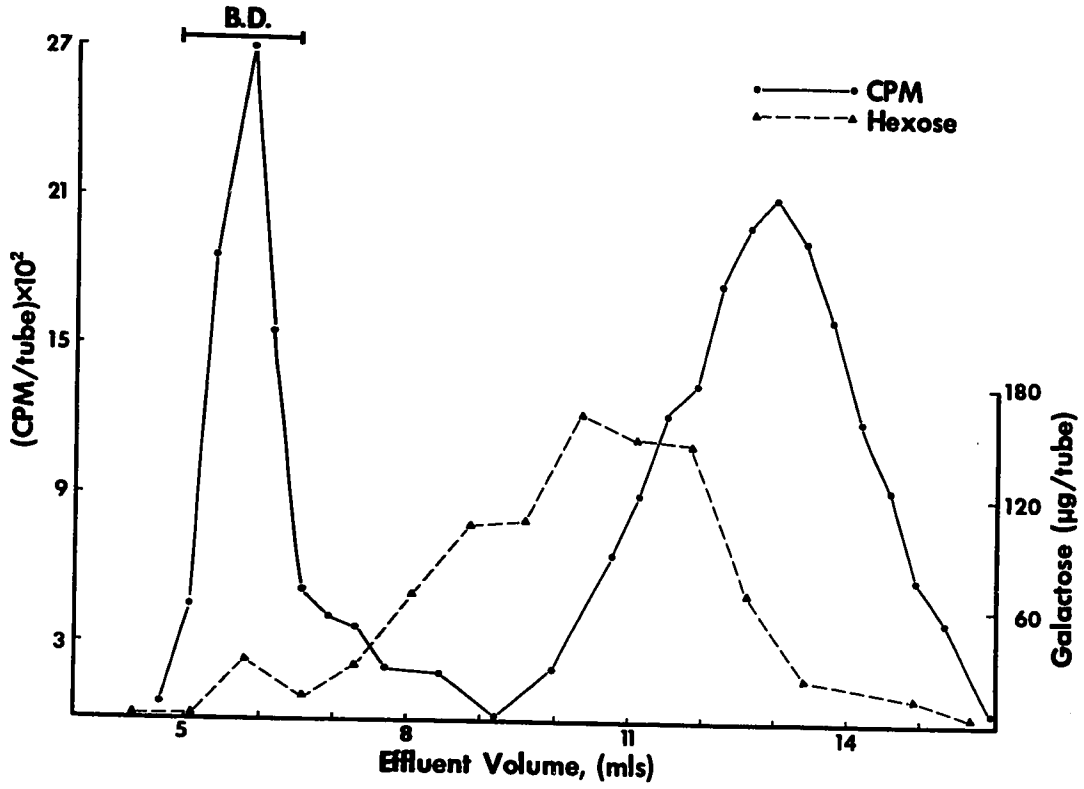
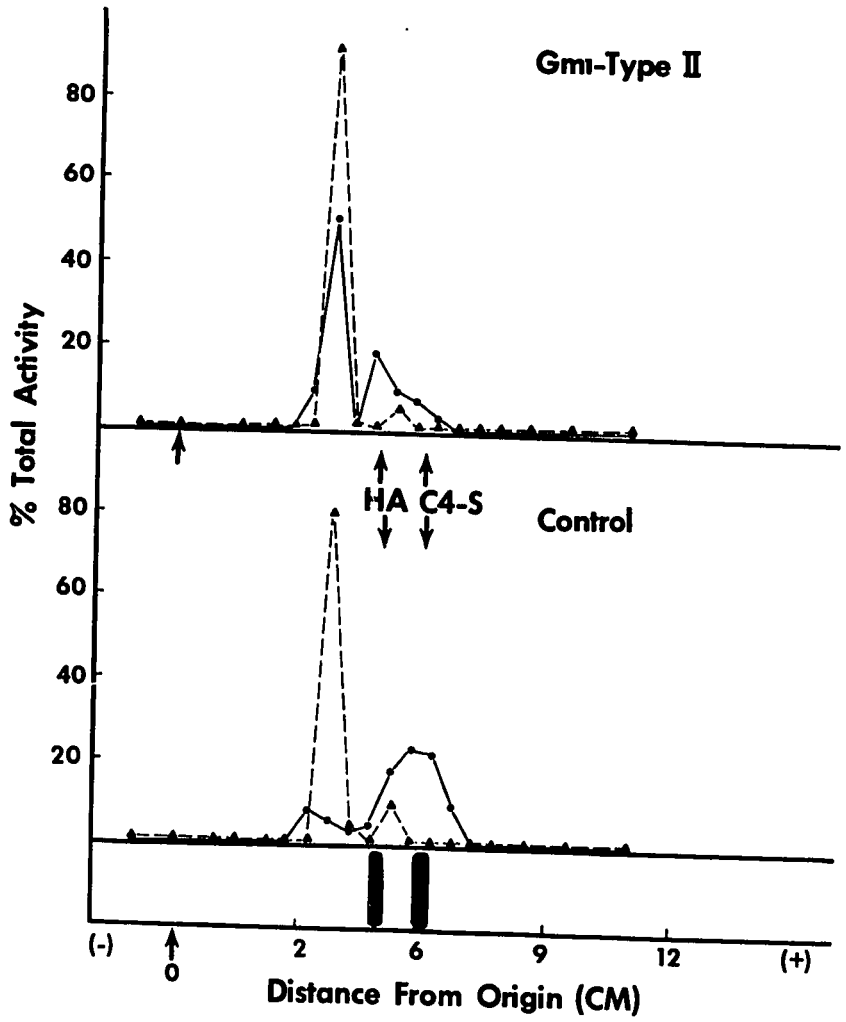

B.D.
CPM
Hexose
(CPM/tube)x10²
27
21
15
9
3
180
120
60
Galactose (µg/tube)
5
8
11
14
Effluent Volume, (mls)

FIGURE 24

SEPRAPHORE III ELECTROPHORESIS OF CPC-PRECIPITABLE AND NON-CPC-PRECIPITABLE GLYCOSAMINOGLYCANS FROM CONTROL AND G_{M1}-TYPE II CELLS LABELED WITH ^{14}C-GALACTOSE

Each fraction was subjected to electrophoresis as outlined in the legend to Figure 21. The strips were cut into 1.0 and 0.5 cm strips, placed in scintillation vials and counted after addition of Naphthalene scintillant. For both the control and G_{M1}-Type II samples, the solid line represents the CPC-precipitable and the brokenline the non-CPC-precipitable glycosaminoglycans.


Gmi-Type II
80
60
40
20
% Total Activity
HA
C4-S
Control
80
60
40
20
(-)
0
2
6
9
12
(+)
Distance From Origin (CM)

glycosaminoglycans were also subjected to electrophoresis on Sepraphore III (Figure 24). The CPC-precipitable fraction from the control cells contained two components on electrophoresis, one of which migrated to the same zone as standard chondroitin 4-sulfate while the second smaller peak was less anionic than Hyaluronic acid. The same fraction from mutant cells also gave two components on electrophoresis, but in this case the major one was less anionic than Hyaluronic acid. The major component in the non-CPC-precipitable glycosaminoglycans from both control and case 1 cells migrated to the same position less polar than Hyaluronic acid. The electrophoretic behaviour of the labeled components in control and case 1 cells was not altered by Hyase digestion.

In summary, the major glucosamine- and galactose-labeled components from both control and case 1 cell lines were electrophoretically indistinguishable, were not digested with Hyase, not CPC-precipitable and had an apparent molecular weight greater than 7,000. The mutant cell non-CPC-precipitable glycosaminoglycans labeled with ^{14}C-galactose were found in amounts several times greater than corresponding components from normal cells.

These glycosaminoglycans which had accumulated in the mutant cells have not been further characterized chemically as yet. Nevertheless, the studies completed to date suggest that these materials are similar to the keratan sulfate-like glycosaminoglycans found in other tissues in patients with both types of G_{M1}-gangliosidosis.

D DISCUSSION

1 G_{M1}-gangliosidosis

The accumulation of G_{M1}-ganglioside has been demonstrated in a biopsy specimen of the brain of a patient (case 1) with Type II, G_{M1}-gangliosidosis. This patient is still alive at the age of five years and we therefore do not have information regarding the visceral organs. In agreement with the findings of others (29,52,239), G_{M1}-ganglioside was found at a level 10-20 times higher than that of normal brain (Tables 3 and 4). In the later stages of this work we found an accumulation of G_{M1}-ganglioside in the brain and the liver of a patient with the Type I form of this disease (case 2). G_{M1}-ganglioside was also found in the spleen and the appendix of this patient (case 2) but these results have not been reported. In addition, G_{M2}-ganglioside was elevated in three specimens of appendix obtained from patients with Tay-Sachs disease (results not reported).

These results show that in both types of G_{M1}-gangliosidosis, there is a specific accumulation of one ganglioside type (G_{M1}) in the brain and, to a lesser extent, in the viscera.

Two gangliosides were found in the fibroblasts of case 1 which were not detected in normal cells (Figure 15). These gangliosides migrated on TLC like the G_{M2}- and G_{M1}-gangliosides of brain. The G_{M2}-ganglioside-like species was more prominent than the G_{M1}-like component. The presence of the G_{M2}-like

ganglioside was unexpected but is in accord with the following findings on the neutral glycolipids. The trihexosylceramide of case 1 fibroblasts was the only neutral glycolipid which did not correspond in chromatographic behaviour to the species derived by acid hydrolysis from brain G_{D1a}-ganglioside (Figures 16 and 17). The major trihexosylceramide of brain ganglioside has an N-acetylgalactosamine as its terminal sugar while the major trihexosylceramide of normal fibroblasts has a galactose terminal sugar (287). The latter glycolipid accumulates in fibroblasts derived from patients with Fabry's disease (287). It is proposed that the G_{M2}-ganglioside of case 1 fibroblasts corresponds structurally to trihexosylceramide (gal-gal-glc-ceramide) and contains an N-acetylneuraminic acid linked to the terminal galactose of the glycolipid. According to this hypothesis, the lack of β-galactosidase activity in the fibroblasts would lead to a relative increase in the amount of the trihexosylceramide and indirectly to the accumulation of G_{M2}-ganglioside. The increase in trihexosylceramide concentration in mutant cells is based on visual inspection of the TLC (Figure 16) and is reflected in the two-fold increase in its radioactivity. A G_{M2}-like ganglioside has also been observed in fibroblasts of Hurler's disease (217) while the G_{M3}- and G_{D1a}-gangliosides demonstrated in this work in normal cells have been also observed by others (289,290).

The results presented clearly show that the accumulation of G_{M1}-ganglioside is not restricted to the brain in either Type I or the Type II, G_{M1}-gangliosidosis. This finding should

help to clarify the considerable confusion in the literature with regard to the nomenclature applied to G_{M1}-gangliosidosis. The Type II form has been called Systemic Late Infantile Lipidosis (214,239) while the most common name applied to the Type I form is Generalized Gangliosidosis (29,238,242). This nomenclature is both misleading and non-specific. In Type I, G_{M1}-gangliosidosis, G_{M1}-ganglioside accumulates in the brain and to a lesser extent in the liver, spleen and appendix while in G_{M2}-gangliosidosis (Tay-Sachs disease), G_{M2}-ganglioside accumulates in the brain and also in the liver and spleen (240) and appendix (this thesis, data not shown). Thus, the results of Suzuki (31,240), Eeg-Olofsson et al. (173) and O'Brien (29,238) as well as the results reported in this thesis show that both types of G_{M1}-gangliosidosis and the classical Tay-Sachs disease can be considered as "Generalized Gangliosidoses." In addition, the major storage substances in the visceral organs of both types of patients with G_{M1}-gangliosidosis are galactose- and glucosamine-containing glycosaminoglycans. Thus, it seems inappropriate to call this disease "Generalized Gangliosidosis." It is for these reasons that we prefer to identify G_{M1}-gangliosidosis according to Type I or II, as originally proposed by Derry, Wolfe et al. (239).

2 Visceral Storage Substances

Glycosaminoglycans were isolated from three different sources in this study on G_{M1}-gangliosidosis - the urine of case 1 (Type II), the liver from case 4 (Type I) and cultured

fibroblasts of case 1 (Type II).

Galactose- and glucosamine-containing polysaccharides were isolated from the urine of case 1. One fraction was found mixed with the uronic acid-containing, CPC-precipitable glycosaminoglycans and the rest was not precipitable with CPC and not associated with uronic acid-containing glycosaminoglycans.

The polysaccharides isolated from the liver were similar to those isolated from the urine. They were not CPC-precipitable and were polydisperse on Bio-Gel sieve chromatography. These compounds were partially chemically characterized. The results of the chemical studies performed to date strongly indicated that the carbohydrate moiety of these compounds was similar, if not identical, to that of keratan sulfate. However, the liver polysaccharides lacked sulfate.

The fibroblasts of case 1 incorporated both ^{14}C-galactose and ^{14}C-glucosamine into weakly anionic compounds of molecular weight greater than 7000, which were not CPC-precipitable and not digested with hyaluronidase. With ^{14}C-galactose as precursor, the radioactivity incorporated into these non-CPC-precipitable glycosaminoglycans of case 1 was several fold higher than that incorporated into a corresponding fraction from normal cells.

The detailed chemical analyses of the urinary glycosaminoglycans of case 1 disclosed a number of interesting features. In the cetylpyridinium chloride-precipitable glycosaminoglycans after ECTEOLA-cellulose or DEAE-Sephadex A-50 column fractionations (Tables 11 and 14), we obtained a keratan sulfate fraction

low in sulfate which was associated with a uronic acid-containing glycosaminoglycan that was not dermatan sulfate. Exhaustive testicular hyaluronidase digestion did not completely remove the uronic acid or the galactosamine. These keratan sulfate-chondroitin sulfate mixtures were not resolved into two distinct bands by electrophoresis on Sepraphore III but after Hyase digestion, the major alcian-blue stained material did have the mobility of a keratan sulfate. This glycosaminoglycan contained large amounts of galactose and glucosamine but had a very low content of sulfate (Table 12).

The difficulties experienced in this work in isolating individual glycosaminoglycan species have been encountered by others (283,293,294). The urinary glycosaminoglycans containing uronic acid are heterogeneous in their sulfate content and their molecular size since they are byproducts of the partial degradation of connective tissue protein-polysaccharide complexes (198, 199,283). Thus, the presence of mixtures of sulfated polysaccharides in our column fractions may be a reflection of the high degree of sulfate heterogeneity normally found in urinary glycosaminoglycans.

A unique finding in the study of the urinary glycosaminoglycans was the greatly increased excretion of undersulfated keratan sulfates NOT precipitated by CPC which after DEAE-Sephadex A-50 column chromatography contained very little uronic acid (Tables 15 and 16). The material was isolated as a protein-polysaccharide complex which contained significant amounts of

sialic acid (Table 16). The chemical composition of this material was similar in some respects to the polysaccharides isolated from the G_{M1}-gangliosidosis liver (case 4). The presence of non-CPC-precipitable glycosaminoglycans in the urine of case 1 emphasizes the necessity for analysis of all urine fractions obtained during a fractionation procedure. This has recently been stressed by Linker et al. (295) in their studies on the urinary glycosaminoglycans of patients with diseases of the Hurler syndrome.

Keratan sulfate is a normal constituent of adult human urine (283,296). Varadi et al. (283) found that keratan sulfate comprised about 1% of the total urinary glycosaminoglycans, while Berenson and Dalferes (296) reported values of 10-11%. This keratan sulfate was highly sulfated and was isolated free of uronic acid-containing glycosaminoglycans(283). The total keratan sulfate glycosaminoglycans isolated from the urine of case 1 comprised about 30-35% of the total glycosaminoglycans isolated with about equal amounts in the CPC- and non-CPC-precipitable fractions. In the urine of patients with the Morquio syndrome, Pedrini et al (297) also found keratan sulfate to be present (38-46% of the total glycosaminoglycans) but this material had a sulfate to hexosamine mole ratio of 1:1. The low sulfate content of the keratan sulfate-like material isolated in this study is also particularly striking since the glycosaminoglycans present in the urine of infants generally have a higher sulfate content than those of adults (294).

The keratan sulfate-like materials from the urine of case 1 (Type II) and those isolated from the liver of case 4 (Type I) differed in their sulfate content. While the urinary materials had a sulfate to hexosamine mole ratio of about 0.4:1.0, the polysaccharides from the liver had a lower mole ratio of approximately 0.1:1.0. However, both the urinary and liver glycosaminoglycans of the keratan sulfate type had a galactose to glucosamine mole ratio of slightly greater than 1.0:1.0.

The polysaccharides isolated from the liver in preparations 1 and 2 were not markedly different in carbohydrate composition but did differ slightly in their protein content. The increased protein content of the polysaccharides isolated from the lipid-free liver residue could be reduced by Bio-Gel chromatography (Table 21). The ease with which the polysaccharides were extracted from the liver suggested that they were not intimately linked to a protein matrix. Furthermore, since the uronic acid-containing mucopolysaccharides could be freed from the undersulfated keratan sulfate-like glycosaminoglycans by precipitation with CPC (Table 21), no structural complexes can exist between these uronic acid-containing and galactose-containing glycosaminoglycans.

In Hurler's disease, heparan sulfates have been shown to be accumulated in the liver in quantities much higher than that found in normal liver (298). It has been suggested that the glycosaminoglycans in the liver and spleen of patients with Hurler's disease were not combined with protein as they were in normal connective tissue. In the latter, protein-polysaccharide complexes containing chondroitin sulfates and variable amounts of keratan sulfates have been demonstrated (101,104,108). The

keratan sulfates in G_{M1}-gangliosidosis liver like the heparan sulfates from the liver of patients with Hurler's disease do not appear to be associated with protein to the same extent as occurs normally in cartilage. In the case of the accumulations of acid mucopolysaccharides in the liver of patients with Hurler's disease, Meyer has suggested that the liver parenchymal cells acquire their deposits by ingestion and attributed the hepatomegaly in this disease to the accumulation of the "wrong" compounds (299). Such a process may also be responsible for the accumulation of the polysaccharides in the liver and spleen of patients with G_{M1}-gangliosidosis.

The liver glycosaminoglycans characterized in this study were isolated from a Type I patient while Suzuki and co-workers (31,237) recently identified similar galactose- and glucosamine-containing polysaccharides from the liver and spleen of a Type II patient. The electron microscopic appearance of these materials <u>in situ</u> is that of fine tubular structures 200 Å in diameter, quite unlike the characteristic membranous cytoplasmic bodies seen in the nervous system (31,237).

The site of synthesis of these polysaccharides is unknown. Although no reports have appeared on the amount and type of keratan sulfates in the cartilage and connective tissue of patients with G_{M1}-gangliosidosis, it may well be that the osseous abnormalities, prominent in the Type I form of this disease, and the general visceral involvement in both types are associated with excessive accumulation of undersulfated keratan

sulfates in these tissues. In G_{M1}-gangliosidosis, the presence of bony abnormalities and visceromegaly in Type I and their absence in Type II despite a marked deficiency of β-galactosidase in all tissues from both Types may be due to differences in the degree of accumulation and the higher content of sulfate, but still lower than the normal, of the keratan sulfates in Type II. An increase in the cartilage content of keratan sulfate in patients with Marfan's disease has been observed (129).

Kaplan and Meyer (125) have shown that normal infant cartilage contains little glucosamine and therefore only small amounts of keratan sulfates. In this study, we have found very high quantities of galactose- and glucosamine-containing polysaccharides in the liver and urine of children with G_{M1}-gangliosidosis.

3 Beta-galactosidase

The demonstration of a β-galactosidase enzyme deficiency in the liver of Type I, G_{M1}-gangliosidosis was reported independently by Seringe et al. (241), Sacrez et al. (242) and Okada and O'Brien (243). The latter authors found a marked deficiency in β-galactosidase activity with both synthetic substrates and with G_{M1}-ganglioside terminally labeled with ^{14}C-galactose in frozen post-mortem brain, liver, spleen and kidney. Our studies have shown that there is a severe deficiency of β-galactosidase activity in brain, leucocytes and cultured

fibroblasts during life in a G_{M1}-gangliosidosis patient without chondrodystrophy or visceromegaly (case 1). We have used the leucocyte β-galactosidase assay to diagnose a case of Type I, G_{M1}-gangliosidosis (case 2). The diagnosis of this disease in case 2 was subsequently confirmed by the demonstration of G_{M1}-ganglioside accumulation and a very low β-galactosidase activity in the liver (Table 18 and Figure 7).

The level of β-galactosidase activity in the leucocytes appears to be an extremely good indicator not only of the homozygous (diseased) condition but also of the heterozygous (carrier) state (Table 5). The β-galactosidase activity of the leucocytes from the parents of both Types of G_{M1}-gangliosidosis (cases 1 and 2) was approximately 50% of the mean of normal values and outside the normal range. This finding is consistent with an autosomal recessive mode of inheritance in both Types of this disease.

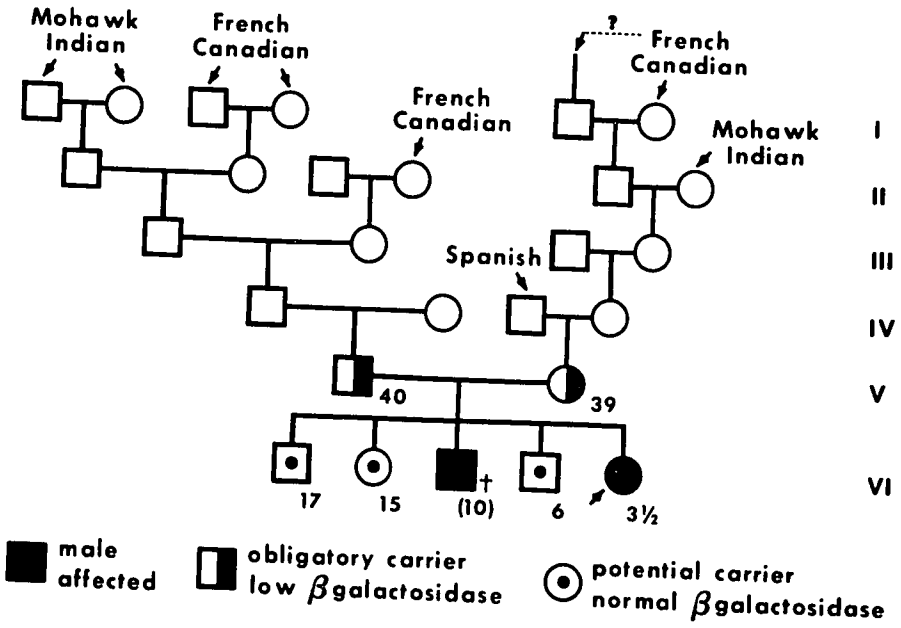
The birth records of the ancestors of case 1 were obtained from the parish archives by the mother and an abbreviated pedigree has been constructed on the basis of this information (Figure 25). It reveals North American Mohawk Indian and Caucasian French-Canadian ancestry on both sides of the family. An older sibling of case 1 died at the age of 10 with a clinical history of progressive psychomotor degeneration similar to that of case 1 but there are no cousins known to have a similar clinical phenotype. It has not been determined whether the gene originated in the Mohawk Indian or the French-Canadian

FIGURE 25

ABBREVIATED PEDIGREE OF THE PATIENT WITH TYPE II, G_{M1}-GANGLIOSIDOSIS (CASE 1)

The ethnic origins of case 1 include Mohawk Indian and French-Canadian ancestry on both sides of the family. An older sibling of case 1 died at the age of ten with a clinical history reminiscent of the Type II form of this disease but three other siblings are apparently normal. In generation VI, the arrow indicates the patient. The numbers give the ages at the time at which the study was started.

The J. Family


Mohawk Indian
French Canadian
French Canadian
?
French Canadian
Mohawk Indian
Spanish
I
II
III
IV
V
VI
40
39
17
15
(10)
6
3½
male affected
obligatory carrier low βgalactosidase
potential carrier normal βgalactosidase

population but it should be noted that a previous case of Type II, G_{M1}-gangliosidosis (case 3, Table 3), reported from this laboratory (239) also had a French-Canadian parentage. It would be of considerable interest to obtain more complete pedigree information from both the above families and the family of case 2.

The results with leucocyte β-galactosidase activities have been confirmed recently by Kint et al. (291) for a case of Type II, G_{M1}-gangliosidosis and the parents of this patient. Okada and O'Brien (Personal Communication) have also used the measurement of β-galactosidase activity in leucocytes to detect the heterozygous condition in the parents and siblings of the Type I form of this disease.

The pH optimum for the residual β-galactosidase activity in G_{M1}-gangliosidosis cerebral cortex was lower and broader than in normal brain (Figure 5). Whether this activity represents a different enzyme or a structurally altered enzyme cannot be determined until much more is known of the β-galactosidases of normal brain. Beta-galactosidase activity was also found to be very low in the liver while the elevated activities of several other acid hydrolases in the liver and the decreased neuraminidase activity in the brain do not appear to be specific to G_{M1}-gangliosidosis but rather reflect alteration in lysosomal activity generally (Tables 7 and 18 and reference 157).

A decrease of β-galactosidase activity does not appear to be unique to G_{M1}-gangliosidosis. The recent studies of

Ockerman (221-224), Van Hoof and Hers (157) and Ho and O'Brien (225) have shown using synthetic substrates a statistically significant decrease in a β-galactosidase activity in liver and skin from patients with Hurler's disease. However, in the plasma there was a 2-3 fold increase in β-galactosidase activity using 4-methylumbelliferyl-β-D-galactopyranoside as substrate and an increased activity of several other lysosomal hydrolases (221,222). These authors showed that in their material there was a loss of a specific β-galactosidase activity having an optimal activity at pH 4-5 (223-225), but the degree of deficency of this enzyme appears to be variable according to the tissue examined. Such is not the case for G_{M1}-gangliosidosis in which the enzyme deficiency is much more pronounced. For example, in the fibroblasts of case 1, the activity was only 1-2% of normal (Table 24).

As discussed earlier, there is considerable heterogeneity in β-galactosidase activities both in pH optima (224,280,281) and in substrate specificities (266). All the β-galactosidases having optimal activities in the range of pH 3-5.5 are very low in G_{M1}-gangliosidosis, but Van Hoof and Hers (157) have observed much higher enzyme activities in the liver of one patient at pH 6-7 and at pH 9. It should be noted, however, that these hydrolases with optimal activities at neutral or alkaline pH are primarily lactases and not glycolipid hydrolases (266,280, 281).

It is likely that the presence of galactose- and

glucosamine-containing polysaccharides in the urine of case 1, their accumulation in the liver of case 4 and the increased content of weakly anionic, high molecular weight and non-CPC-precipitable glycosaminoglycans in the fibroblasts of case 1 can all be attributed to the loss of β-galactosidase activity. Little information is available on the degradation of keratan sulfates by β-galactosidase. Yeast β-galactosidase did not cleave galactose from a trisaccharide (galactose-terminal) derived from corneal keratan sulfate despite its ability to cleave lactose (85). We found that a beef liver β-galactosidase preparation did not greatly affect the molecular weight of the 3600 M.W. undersulfated keratan sulfate characterized in this work, nor did its action significantly decrease the content of galactose bound to the polysaccharide (Figure 14). MacBrinn et al. (292) have reported that almost 40% of the galactose present could be released from a keratan sulfate preparation isolated from G_{M1}-gangliosidosis liver when a partially purified human liver β-galactosidase enzyme preparation was used. It should be noted, however, that no data on the chemical composition of this keratan sulfate were provided. Later, the same preparation was reported to have a galactose content of 4% by weight (O'Brien,J.S.: Personal Communication) far lower than the 30-50% content of galactose found in the various purified fractions in the present work (Tables 19, 21, 22). The differences between our results and those of MacBrinn et al. (292) may be explained by several observations. Galactose was readily hydrolysed from

G_{M1}-ganglioside by the beef liver enzyme and this enzyme is similar in this respect to that from the human liver (243). Another lysosomal hydrolase of brain, neuraminidase, (see Table 7) was active towards G_{D1a}-ganglioside but did not release sialic acid from the sialyloligosaccharide derived from this ganglioside or from sialyllactose. It may be that the lipid moiety of the ganglioside makes the enzyme reactive site more accessible or that it serves to bind the substrate in a conformation favourable for catalysis by the above enzymes. Similarly, if the protein portion of the glycosaminoglycans is required to perform the same function as the lipid moiety of gangliosides, the inability of the beef liver β-galactosidase to cleave galactose from the G_{M1}-gangliosidosis liver polysaccharide may be due to the very small amount of protein attached to the polysaccharide in this preparation. For the same reason, the G_{M1}-gangliosidosis liver polysaccharides may be inactive as substrates for other liver hydrolases normally active on similar glycose moieties.

There appears to be a direct relationship between the β-galactosidase deficiency and the accumulation of substances in the brain and the visceral organs in G_{M1}-gangliosidosis since there are certain similarities in the chemical structure of gangliosides and the keratan sulfates (Table 1 and 2). In G_{M1}-ganglioside, the terminal galactose is in a β, 1→3 linkage to N-acetylgalactosamine whereas in the keratan sulfate repeating unit the galactose is in β, 1→4 linkage to N-acetylglucosamine

6-sulfate. In the liver polysaccharides characterized in this study the sulfate is absent. It should be noted however, that the β, 1→4 linkages involving galactose in both ganglioside and keratan sulfates (KS_1 or KS_2) are internal. In addition, KS_2 from old human rib cartilage contains side chains composed of galactose (as the branching sugar) but of undetermined length and composition. The liver polysaccharides of the keratan sulfate-type studied in this work also contained some side chains containing galactose but they were of unknown length. The galactose at branch points of KS_2 is attached to the C_6 hydroxyl of the galactose in the main polysaccharide chain. Current evidence suggests that another galactose is attached to this branch point galactose in a 1→6 linkage (86). The β-galactosidase which cleaves the terminal β, 1→3 galactose of ganglioside and the β, 1→4 internal galactose of other glycolipids (see reactions 6 and 9, p. 16) may be the same enzyme that cleaves the β, 1→4 galactose linkages and those in the side chains of keratan sulfates. However, this requires that the enzyme be relatively non-specific with respect to its substrate requirements. Since the β-galactosidase of rat brain has been shown to hydrolyze galactose-terminal glycolipid substrates at different rates (see 74-77) but not lactose or other small carbohydrates (75), it is likely that the β-galactosidase of visceral organs is different from that of brain in its substrate specificity even though the linkage hydrolyzed is similar.

Since there is a marked deficiency of the lysosomal type of β-galactosidase in both brain and visceral organs in the Type I

and Type II forms of this disease, both G_{M1}-ganglioside and polysaccharides of the keratan sulfate-type can be expected to accumulate in the affected patients.

Thus, the deficiency of β-galactosidase in G_{M1}-gangliosidosis appears to be a primary defect since the substances accumulated in various tissues contain terminal galactose linkages requiring the action of this enzyme for normal catabolism. On the other hand, the decrease of a specific β-galactosidase in Hurler's disease appears to be secondary since the characteristic accumulation of heparan and dermatan sulfates cannot be explained solely on the basis of the lack of this enzyme (as discussed earlier, pp.42-44). The moderate elevation of G_{M3}-, G_{M2}- and G_{M1}-gangliosides in the brain of Hurler's patients may be related to the lack of this specific β-galactosidase activity (see p. 42).

4 The Incorporation of Labeled Precursors in Cultured Skin Fibroblasts

Cultured skin fibroblasts derived from case 1 and from normal subjects were incubated in the presence of several labeled precursors including ^{14}C-galactose, glucosamine, acetate and reconstituted protein hydrolysate and ^{35}S-sulfate.

The incorporation of ^{14}C-galactose into case 1 cells was increased over normal as was that of sulfate but the total level of ^{35}S-sulfate incorporated was very low. The most striking finding was the decrease of the incorporation of glucosamine in case 1 cells to a level about 50% of normal during a 24 hour incubation period. This decrease was confirmed by measuring

the time course of incorporation of ^{14}C-glucosamine into case 1 cells in another experiment and with a different cell strain as the control. The rate of loss of radioactivity from the fibroblasts of case 1, after an initial 24 hour labeling period, was also decreased as compared to control. However, with galactose as labeled precursor, the glycosaminoglycans, isolated from a papain digest of case 1 cells by CPC and ethanol subfractionation (Table 31), contained very high levels of radioactivity, several fold higher than a corresponding fraction from normal fibroblasts.

The major glycosaminoglycans, from both normal and case 1 cells incubated with either ^{14}C-glucosamine or galactose, were weakly anionic, not CPC-precipitable, not digested with Hyase and of molecular weight greater than 7000.

Our results show that compounds which accumulated in incubated case 1 cells are similar to those found in normal human fibroblasts, but that they are found in much higher amounts in the G_{M1}-gangliosidosis cells.

The differences between the incorporation of glucosamine and galactose were quite unexpected and seemingly contradictory to our previous findings on the glycosaminoglycans from the urine of case 1 and from the liver of case 4. However, it should be noted that fibroblasts of case 1 did show metachromatic granules after fixation in absolute acetone (245). This suggests that there is a high intracellular content of glycosaminoglycans.

If we assume that the glycosaminoglycans in case 1 cells which stain metachromatically, are similar to the galactose- and glucosamine-containing compounds isolated from the liver of case 4, then the disparity between the incorporation of ^{14}C-galactose and ^{14}C-glucosamine into case 1 cells can be explained.

It is proposed that ^{14}C-galactose is incorporated into pre-existing glycosaminoglycans in a process of chain elongation or that it is added at branch points containing galactose. Since the glycosaminoglycans isolated from the liver contained more periodate-oxidizable galactose than glucosamine, it is unlikely that significant quantities of ^{14}C-glucosamine would be incorporated into these branch chains. Rather, it is likely that the glucosamine would be incorporated into the linear portion of the growing polysaccharide chains and would thus reflect de novo synthesis which can be expected to be somewhat depressed by the high intracellular content of pre-formed glycosaminoglycans. It is further suggested that the decreased rate of loss of incorporated glucosamine and the high intracellular content of metachromatic material in case 1 reflects an abnormally low rate of catabolism of these compounds, a consequence of the deficiency of β-galactosidase activity.

The major radioactive glycosaminoglycans of the fibroblasts from case 1 were of high molecular weight (7000-10,000 or greater) while the compounds isolated from the liver of case 4 were heterogeneous with respect to M.W. (1600-10,000 M.W.). The results of the chemical studies on the 3600 M.W. liver glycosaminoglycan

and the radioactivity incorporation studies with case 1 fibroblasts suggest that the polysaccharides accumulated in the tissues of G_{M1}-gangliosidosis patients possess some branch chains, containing primarily galactose, attached to the main carbohydrate polymer. One possible explanation for the M.W. heterogeneity of the liver material is that the glycosaminoglycans are partially degraded in the tissues by exo- and endoglycosidases up to the vicinity of the branch points but due to the absence of β-galactosidase they are degraded no further. The small amounts of protein found associated with the G_{M1}-gangliosidosis liver glycosaminoglycans also suggest that the smaller molecules may then be released by proteolysis from a parent structure with a common core protein backbone. The glycosaminoglycans could then be eliminated from the tissues into the circulation as essentially neutral glycopeptides with a carbohydrate composition of the keratan sulfate-type.

5 Type I and Type II, G_{M1}-gangliosidosis: One or Two Diseases?

The data presented in this thesis as well as the recent findings of others (29,31,214,239-242) allow clear definition of the characteristic biochemical abnormalities in both Type I and Type II G_{M1}-gangliosidosis:

1. The excessive accumulation in the nervous system of the normal major monosialoganglioside, G_{M1}, and to a lesser extent, an asialoganglioside.

2. A profound deficiency of β-galactosidase which in normal brain, has optimal activity at pH 4.8 to 5.0. The deficiency of this acid hydrolase is likely

general, since it has been demonstrated in leucocytes, cultured skin fibroblasts, liver, spleen and kidney.

3. Accumulation in varying degree in liver, cultured fibroblasts, spleen and kidney but not in brain, and elevated excretion of undersulfated, water-soluble glycosaminoglycans of the keratan sulfate-type.

The presence of these three biochemical abnormalities enables the unambiquous and specific diagnosis of G_{M1}-gangliosidosis as defined by Suzuki and Chen (175).

The apparent biochemical identity of the two Types of G_{M1}-gangliosidosis is further manifested by the identity of the carrier state. In the parents of both case 1 (Type II) and case 2 (Type I) the leucocyte β-galactosidase activity was about 50% of normal consistent with an autosomal recessive inheritance of this disease.

A problem arises when the clinical manifestations of G_{M1}-gangliosidosis are considered. Type I, G_{M1}-gangliosidosis is a neonatal and infantile disease with pronounced bony abnormalities, visceromegaly and early death, while Type II, G_{M1}-gangliosidosis exhibits a late infantile onset and a longer survival without or with only minor bony deformities and usually without visceromegaly (see reference 237). O'Brien (238) regards the neonatal and infantile form (Type I, G_{M1}-gangliosidosis) and the late infantile form (Type II, G_{M1}-gangliosidosis) as two separate diseases. On the other hand, Suzuki and co-workers (31) emphasized that the similarity of biochemical characteristics of Type I and Type II justified the designation of this condition as a single disease entity. We previously suggested that the

differences in phenotypic expression of the Type I and Type II form of this disease were related to the time during development of the expression of the action of a single mutant gene (245). This appears unlikely, in retrospect, since visceral histiocytosis and the degree of accumulation of G_{M1}-ganglioside in the brain (this thesis and reference 31) is comparable in both Types of this disease.

The problem of whether we are dealing with one or two diseases has recently been somewhat clarified. Certain aspects of the disease should be noted further. If both Types of G_{M1}-gangliosidosis involved a single gene mutation then it would not be unreasonable to expect both Types of this disease or a phenotypic expression intermediate between the two extremes to appear in the same family. In our studies in this laboratory (this thesis, case 1 and family and those of Derry et al. (239)) two families were investigated. Each family had two affected siblings each of which exhibited only the Type II phenotype. A constant intrafamilial phenotypic expression has also been seen in the Type I form of G_{M1}-gangliosidosis (see 226 and 238). Thus on genetic grounds, the constancy of intrafamilial expression suggests that the two Types of G_{M1}-gangliosidosis are different genetic diseases. Recent evidence (300) on the β-galactosidases of the fibroblasts derived from the skin of case 1 and case 2 reported in this thesis show that there is less total enzyme activity in the fibroblasts of case 2 (Type I form) than case 1 (Type II form) and that differences exist in the heat

stabilities of the residual enzyme activities in these cells. These results support the view that the two Types of G_{M1}-gangliosidosis are different diseases. O'Brien (301) has recently stated that in Type I the activities of three "isoenzymes" of β-galactosidase (A,B and C) are deficient in the liver while in Type II, two "isoenzymes" (B and C) are deficient while the "fast-moving component," A, is preserved. This provides presumptive evidence for the identification of different genetic defects in G_{M1}-gangliosidosis although it is not clear whether the β-galactosidases are the products of abnormal structural genes. More information on the structure of the human β-galactosidases is required for definitive identification of the basic genetic defects in both Types of G_{M1}-gangliosidosis.

SUMMARY

A three year old girl with Mohawk Indian and French-Canadian ancestry (case 1) showed a progressive neurological deterioration from the age of ten months but did not manifest any bony abnormalities or visceromegaly. She is still alive at the age of five. This patient was diagnosed as Type II, G_{M1}-gangliosidosis on the basis of chemical analysis of brain biopsy tissue reported here.

There was a large increase of G_{M1}-ganglioside in the cerebral cortex. Beta-galactosidase activity was greatly decreased in brain and in leucocytes. The normal brain enzyme had a pH optimum of 4.8-5.0 while the residual enzyme activity in the brain of the Type II patient had a broad pH profile with no distinct pH optimum.

The β-galactosidase activity was markedly decreased in the leucocytes of a patient with the Type I form. This patient also had a greatly elevated content of G_{M1}-ganglioside in the brain, liver, spleen and appendix.

The parents of patients with both Types of this disease showed significantly decreased leucocyte β-galactosidase activity. The siblings of the patient in one family showed normal enzyme activity while the one sibling in the other family showed an enzyme activity intermediate between the normal and the diseased state.

Neuraminidase activity of brain was decreased in both G_{M1}-

and G_{M2}-gangliosidosis but was normal in the brain of a patient with Niemann-Pick disease. The level of sialomucopolysaccharide-NANA was also decreased in the brain of patients with gangliosidoses but these changes were not specific.

The urine of a patient with the Type II form of G_{M1}-gangliosidosis yielded positive toluidine blue and alcian blue reactions even though the uronic acid excretion (expressed as mg uronic acid excreted /g creatinine/24 hours) was normal. A marked increase in undersulfated keratan sulfate-like glycosaminoglycan fractions was found. One fraction was precipitated by cetylpyridinium chloride along with chondroitin sulfates. The keratan sulfate species isolated in the CPC-precipitable fraction was purified by both ECTEOLA-cellulose and DEAE-Sephadex A-50 column chromatography. It contained galactose and glucosamine and migrated on Sepraphore III electrophoresis like standard cartilage keratan sulfate, but could not be freed completely of uronic acid even after Hyaluronidase digestion and ethanol precipitation.

The other fraction was not precipitated by cetylpyridinium chloride. It was also purified by column chromatography as above. This non-CPC-precipitable glycosaminoglycan was isolated essentially free of uronic acid after DEAE-Sephadex chromatography. It contained galactose and glucosamine as its major carbohydrate constituents, with smaller amounts of sialic acid. This glycosaminoglycan also had a sulfate to glucosamine mole ratio of 0.49:1.0 and it is thus similar in its chemical composition to an undersulfated keratan sulfate.

In an attempt to further characterize the glycosaminoglycans accumulating in the viscera and excreted in the urine of patients with G_{M1}-gangliosidosis, we examined the glycosaminoglycans isolated from the liver of a patient with the Type I form of this disease. Major monosialoganglioside (G_{M1}) was present in this liver specimen and that obtained from another patient with this form of the disease. In addition, the liver specimens from these patients had decreased pH 5.0 β-galactosidase activity whereas the activities of three other acid hydrolases were greatly increased over the normal values.

The glycosaminoglycans were isolated from the affected liver in two separate preparations. In the first preparation, the glycosaminoglycans were extracted into Chloroform:Methanol and partitioned into the upper phase of the Folch partition. The total material recovered comprised 2.7% of the wet weight of the liver. In the second preparation, the glycosaminoglycans remained in the residue obtained after C/M extraction but were rendered soluble by papain and α-amylase digestion of the lipid-free liver residue. The total material recovered comprised 1.7% of the wet weight of the liver. In both preparations, these compounds contained galactose and glucosamine as their major carbohydrate constituents with much smaller amounts of galactosamine and sialic acid and a molar ratio of galactose to glucosamine greater than unity. These compounds were highly soluble in water, had a very low sulfate content, were polydisperse on molecular sieve chromatography and contained an alkali-labile

protein-polysaccharide linkage region. Preliminary studies on periodate oxidation of a purified fraction indicated that the structure of the polysaccharide consisted of periodate-resistant galactose and glucosamine residues in the main chain with some periodate-susceptible galactose, presumably on branch chains. Except for a low sulfate content, these galactose-glucosamine-containing polysaccharides are similar if not identical to the skeletal form of keratan sulfate.

A fibroblast cell strain was developed from the skin of the patient with Type II form of G_{M1}-gangliosidosis. These cultured skin fibroblasts will be referred to as mutant cells. Beta-galactosidase activity of these mutant cells was markedly decreased from that of normal cells but mixtures of normal and mutant cells gave the expected intermediate level of activity.

Two gangliosides (G_{M2}- and G_{M1}-) were found in Folch extracts of mutant cells in addition to the three gangliosides (G_{M3}-, G_{D3a}- and G_{D1a}-) found also in normal cells. G_{M3}-ganglioside was the one most extensively labeled ganglioside when the cells were incubated in medium containing ^{14}C-galactose. Neutral glycolipids were also purified and examined. The major glycolipid of both normal and mutant cells was tentatively identified as trihexosylceramide (see reference 287). This compound showed a relative increase in concentration in the mutant cells compared to normal and contained twice as much radioactivity as the corresponding fraction in normal cells. The results obtained on the gangliosides and the neutral glycolipids

suggest that the G_{M2}-ganglioside of the mutant fibroblasts (but absent in normal cells) has a structure similar to gal-gal-glc-ceramide and contains sialic acid.

Studies on the incorporation of ^{14}C-glucosamine showed that its rate of incorporation and the rate of loss of labeled cellular material was decreased in the mutant cells. The incorporation of other labeled precursors including ^{14}C-galactose, acetate and reconstituted protein hydrolysate and ^{35}S-sulfate was also studied. Glucosamine incorporation was decreased from the expected intermediate value when normal cells were incubated in the presence of mutant cells.

Glycosaminoglycans, labeled with either ^{14}C-galactose or glucosamine, were isolated from the mutant and normal cells after papain digestion. The major glucosamine- and galactose-labeled components from both control and mutant cells were electrophoretically indistinguishable, were not digested with Hyaluronidase, were not CPC-precipitable and had an apparent molecular weight greater than 7000. The mutant cell non-CPC-precipitable glycosaminoglycans labeled with ^{14}C-galactose were found in amounts several times higher than the corresponding fraction from normal cells. It is concluded that the mutant fibroblast glycosaminoglycans are similar to those shown to accumulate in the liver and other visceral organs of patients with both Types of G_{M1}-gangliosidosis.

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CLAIMS TO ORIGINAL RESEARCH

1. A patient with Type II, G_{M1}-gangliosidosis and her family has been studied. This study is to our knowledge the first investigation of a living patient with this disease. A marked deficiency of β-galactosidase activity in the brain, leucocytes and cultured skin fibroblasts of this patient was demonstrated. Leucocyte β-galactosidase analyses have also been used to diagnose biochemically the Type I form of this disease and to recognize the heterozygous (carrier) condition in both Types I and II. This assay can be readily adapted as a routine laboratory test for the screening of newborn babies and for genetic counselling of families.

2. The residual β-galactosidase activity in the brain of this patient exhibited no distinct pH optimum between 3.0 and 5.95 while that of normal brain showed an optimal activity at 4.8-5.0.

3. A non-specific decrease both in brain neuraminidase activity and the level of sialomucopolysaccharide-NANA in cortical specimens from patients with G_{M1}- and G_{M2}-gangliosidosis was demonstrated. The neuraminidase of normal brain was similar in its substrate requirement to that of calf brain described by others.

4. Little was known about the visceral storage substances or related compounds present in the urine in either Type of G_{M1}-gangliosidosis at the time this study was initiated. The urinary glycosaminoglycans of the patient with Type II, G_{M1}-gangliosidosis were studied. These glycosaminoglycans were

separated into two main fractions on the basis of their precipitability with cetylpyridinium chloride. The uronic acid- and neutral hexose-containing glycosaminoglycans present in the CPC-precipitable fraction were fractionated by column chromatography and their composition determined. Similarly, the composition of the non-CPC-precipitable fraction has been determined. The major finding was the highly elevated amount of galactose- and glucosamine-containing glycosaminoglycans present in the urine of this patient. These materials had a composition like that of keratan sulfate but contained very low amounts of sulfate. They were found in both major fractions but were isolated free of uronic acid only after column chromatography of the non-CPC-precipitable fraction. The isolation of a non-CPC- but ethanol-precipitable glycosaminoglycan fraction emphasizes the necessity for the examination of all subfractions obtained during any fractionation procedure.

5. A method has been developed for the subfraction of the glycosaminoglycans accumulated in the liver of patients with G_{M1}-gangliosidosis. This method employs precipitation with both CPC and ethanol. The galactose-glucosamine-containing materials were not precipitated with CPC but could be freed from acidic glycosaminoglycans by the use of this precipitant. The polysaccharides were separated into four molecular weight species by ethanol fractionation and by column chromatography.

6. The liver glycosaminoglycan fractions were further purified and characterized. They had a M.W. range of 1600-10,000, an

alkali-labile protein-polysaccharide linkage and a structure similar to that of skeletal keratan sulfate. They differed from KS_2 only in their very low content of sulfate. These materials were shown to be similar to those isolated from the urine of the Type II patient.

7. The studies reported in this thesis on the cultured skin fibroblasts are to our knowledge the first attempt to characterize the expression of the genetic defect in Type II, G_{M1}-gangliosidosis in cell culture. The most noteworthy features of these cells are: a pronounced deficiency of β-galactosidase activity; the presence of two gangliosides not found in normal cells and an elevated amount of a specific neutral glycosphingolipid; a decreased rate of incorporation and loss of ^{14}C-glucosamine in cultured cells of the patient; an increased level of glycosaminoglycans labeled with ^{14}C-galactose, which are not CPC-precipitable, not digested with Hyase, weakly anionic and of molecular weight greater than 7000. These glycosaminoglycans, labeled with either glucosamine or galactose, are similar to those accumulated in other tissues in this disease.

8. Ox brain ganglioside mixtures were degraded by ozonolysis and base hydrolysis to yield the sialyloligosaccharides which were then fractionated on Dowex-1-acetate into separate pure species. Excellent yields could be achieved by the conditions used. This method has an advantage over those previously used since mixtures of gangliosides can be used as starting material. This avoids the necessity of starting with purified individual ganglioside species.

9. The sialyloligosaccharide derived from the G_{M1}-ganglioside accumulated in the brain of Type II, G_{M1}-gangliosidosis was shown to be identical to that of known standard monosialyloligosaccharide (see claim 8).