SHORT TITLE:

STUDIES ON GALACTOSYLGALACTOSYLGLUCOSYLCERAMIDE

Ph.D. Biochemistry

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THE STRUCTURE AND METABOLISM OF GALACTOSYLGALACTOSYLGLUCOSYLCERAMIDE

ABSTRACT

Galactosylgalactosylglucosylceramide (CTH) was purified from normal human kidneys, and the kidney of a patient with Fabry's disease (characterized by a defect in the catabolism of CTH). NMR spectra of the intact molecules indicated the presence of two β-D-glycopyranosyl residues and one α-D-glycopyranosyl residue. CTH and lactosylceramide labelled in the terminal galactose residues were used to show that the terminal galactosidic linkage of CTH, but not lactosylceramide, was susceptible to hydrolysis by an α-galactosidase from coffee beans. Labelled CTH and p-nitrophenyl-α-D-galactopyranoside were used to demonstrate concomitant deficiencies of CTH galactohydrolase and nonspecific α-galactosidase in the kidney of a patient with Fabry's disease. Following renal transplantation the excretion of CTH in the patient's urine fell from 35 times to 4-8 times normal levels. The concentration of CTH in the plasma decreased transiently then returned to preoperative levels. These findings indicate that 1) CTH contains a terminal α-D-galactopyranosyl residue, 2) the nonspecific α-galactosidase deficiency observed in patients with Fabry's disease is a direct reflection of the deficiency of CTH galactohydrolase, and 3) renal transplantation has no place in the treatment of nonuremic patients with Fabry's disease at present.

THE STRUCTURE AND METABOLISM OF GALACTOSYLGALACTOSYLGLUCOSYLCERAMIDE

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ABBREVIATIONS

In general, the recommendations concerning abbreviations that appear in the Instructions to Authors of The Journal of Biological Chemistry were followed throughout this Thesis. Other abbreviations that occur are:

GLC - gas-liquid chromatography

LCB - long-chain base of the sphingosine class

nmr - nuclear magnetic resonance

tlc - thin-layer chromatography

TMSi - trimethylsilyl

CMH - monohexosylceramide

CMH(H_2) - CMH in which the double bonds have been reduced by catalytic hydrogenation

CDH - dihexosylceramide

CDH[O] - CDH in which the terminal galactose residue has been oxidized to the hexodialdose form

CTH - galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1')-ceramide

CTH[O] - CTH in which the terminal galactose residue has been oxidized to the hexodialdose form

CTH[^{3}H] - CTH labelled with a tritium atom at carbon-6 of the terminal galactose residue

CAH - 2-acetamido-2-deoxygalactosyl-(1→3)-galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1')-ceramide (also called "globoside")

NANA - N-acetylneuraminic acid

PLP - pyridoxal phosphate

THE STRUCTURE AND METABOLISM OF GALACTOSYLGALACTOSYLGLUCOSYLCERAMIDE

Fabry's disease, or angiokeratoma corporis diffusum, is an X-linked inborn error of glycosphingolipid metabolism caused by a defect in the catabolism of galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1')-ceramide (CTH). The resulting accumulation of the lipid in the walls of small arteries and in the kidneys usually causes death due to cerebrovascular disease or cardiac or renal failure before age fifty. The deficiency of a specific CTH galactohydrolase in Fabry's disease was first demonstrated by Brady and coworkers in 1967 using CTH extracted from the tissues of a patient with the disease and labelled with tritium. Although the terminal galactopyranosyl residue of CTH was believed to have the β anomeric configuration, no defect in the hydrolysis of other known β-galactosides (e.g. lactose, o-nitrophenyl-β-D-galactopyranoside) could be demonstrated. This was initially attributed to the unique specificity of CTH galactohydrolase. However, in 1970, using the artificial substrates, p-nitrophenyl-α-D-galactopyranoside and 4-methylumbelliferyl-α-D-galactopyranoside, Kint showed that the leukocytes from patients with Fabry's disease exhibited a marked deficiency of nonspecific α-galactosidase activity. This discovery stimulated speculation that the terminal galactopyranosyl residue of CTH may have the α-stereoconfiguration. In the same year, however, Sweeley, Snyder and Griffin reported the results of nmr studies indicating that all the glycopyranosides of CTH had the β-stereoconfiguration. They felt that the nonspecific α-galactosidase deficiency reported by Kint was a secondary phenomenon, and that it was not a direct reflection of the CTH galactohydrolase deficiency seen in Fabry's disease.

The aim of the work reported in this Thesis was to resolve this paradox by determining, if possible, the relationship between the accumulation of CTH, the deficiency of CTH galactohydrolase, and the deficiency of nonspecific α-galactosidase in Fabry's disease. To this end, CTH was extracted and purified from normal human kidney and from the kidney of a patient who had died of Fabry's disease. By a variety of physical, chemical and enzymic techniques which included fatty acid, long-chain base and carbohydrate analyses; infrared spectrophotometry; mass spectrometry; measurements of optical rotations; proton magnetic resonance spectrometry; acid hydrolysis; and attempts to hydrolyze the terminal galactosidic linkage with partially purified α- and β-galactosidases; normal and Fabry's CTH were both shown to contain terminal α-D-galactopyranosyl residues.

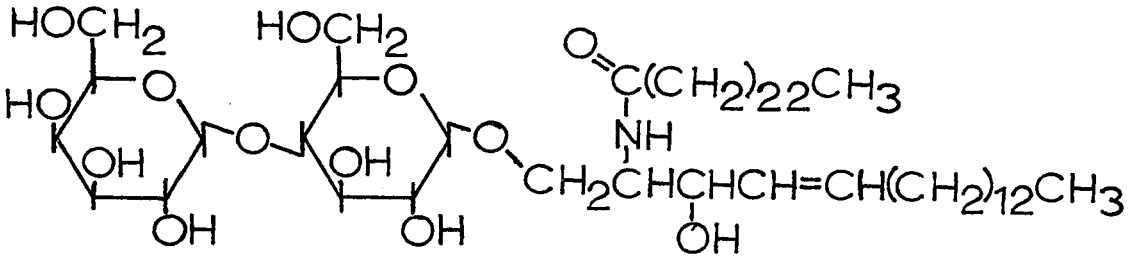
The discovery of two patients with clinical variations of Fabry's disease provided the opportunity to confirm Kint's observation of a nonspecific α-galactosidase deficiency in the peripheral leukocytes. One patient underwent bilateral nephrectomies and renal homotransplantation for treatment of progressive renal failure. Concomitant deficiencies of CTH galactohydrolase (measured using CTH labelled by oxidation of the lipid with D-galactose oxidase and reduction of the resulting hexodialdose with tritiated sodium borohydride) and nonspecific α-galactosidase (measured with p-nitrophenyl-α-D-galactopyranoside) were demonstrated in the patient's own kidney tissue removed at nephrectomy. Serial measurements of the concentrations of glycosphingolipids showed that the implantation of exogenous CTH galactohydrolase in the donor kidney had little or no effect

on the level of CTH in the plasma. There was a marked decrease in the excretion of CTH in the urinary sediment, but the level remained four to eight times normal. These data suggest that the kidney plays little role in the metabolism of circulating CTH. Renal transplantation, therefore, appears to have no place in the treatment of nonuremic patients with Fabry's disease at the present time.

I. INTRODUCTION

A. Neutral Glycosphingolipids

Neutral glycosphingolipids are a class of complex lipids composed of sphingosine (the trivial name for a heterogeneous group of long-chain amino alcohols), fatty acid and a variable number of monosaccharides. The sphingosine and fatty acid are coupled through an amide linkage to form ceramide, which forms the core of all the lipids of this class. The sugars are linked glycosidically to the primary carbinol group of the long-chain base. The structure of lactosylceramide (O-β-D-galactopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→1')-[N-lignoceryl]-sphing-4'<u>t</u>-enine) is shown to illustrate the salient structural features of this class of lipids.



The physical properties of the glycosphingolipids are profoundly affected by substitutions in the oligosaccharide moiety. Three subclasses can be defined in terms of the nature of the substitution.

a) Neutral glycosphingolipids are those containing only hexoses and N-acetylhexosamines, and in some cases fucose, in the oligosaccharide moiety.

b) Sulfatides is the trivial name given those glycosphingolipids containing a sulfate ester in the oligosaccharide moiety. The most important is O-β-D-[3-O-sulfate]-galactopyranosyl-(1→1')-ceramide, one of the principal constituents of myelin.

c) Gangliosides is the term applied to those glycosphingolipids containing sialic acid. They are particularly abundant in human cerebral grey matter where the sialic acid is N-acetylneuraminic acid.

The chemistry, biologic significance and metabolic relationships of these subclasses of lipids have been reviewed recently by Martensson (1), Stoffel (2), Wiegandt (3) and Kiss (4). Although much of what is said in this Thesis may apply to the sulfatides and gangliosides, for the most part, attention will be concentrated on matters relating to the neutral glycosphingolipids.

1. Chemistry

a) Long-chain bases

The study of the chemistry of glycosphingolipids began with the isolation of "cerebroside" from brain by Thudicum (5). He showed that it was composed of a long-chain base, fatty acid and a sugar. He went on to characterize the long-chain base which he named "Sphingosin" "....in commemoration of the many enigmas which it presented to the inquirer" (6). Since then, the sphingosines have been shown to be a highly heterogeneous group of compounds (7,8).

In 1912-14, oxidation studies by Levene and coworkers (9,10,11) established that Thudicum's 'sphingosine' was a dihydroxy-derivative of an unsaturated primary amine with the double bond between carbons 4 and 5. They proposed the structure:

$$CH_3(CH_2)_{11}CH=CHCHOHCHOHCH_2NH_2$$

In 1929, Klenk (12) showed that the compound contained 18 carbon atoms, rather than 17 as claimed by Levene. He later proposed the following structure for the long-chain base:

$$CH_3(CH_2)_{12}CH=CHCHNH_2CHOHCH_2OH \quad (13)$$

The relative positions of the functional groups was finally established by Carter and coworkers (14,15) on the basis of the failure of periodate oxidation of the N-benzoyl and N-acetyl derivatives of dihydrosphingosine, and the formation of a benzylidene derivative on treatment with benzaldehyde and zinc chloride. Further oxidative studies showed that sphingosine obtained by the hydrolysis of cerebroside had the structure:

$$CH_3(CH_2)_{12}CH=CHCHOHCHNH_2CH_2OH$$

In 1951, Carter and his group showed that carbon-2 had the D-configuration by observing the changes in optical rotation produced by the addition of water to anhydrous solutions of (-)-benzoyl-α-aminostearic acid derived from sphingosine (16). From a comparison of the properties of dihydrosphingosine (prepared from naturally-occurring sphingosine) with those of the synthetic threo and erythro derivatives of 1,3-dihydroxy-2-aminodecane, they showed that the naturally-occurring base had the erythro configuration (17). This was subsequently confirmed by Jenny and Grob (18) and, by an entirely different approach, by Kiss et al (19) in 1954. The trans configuration of the double bond in the sphingosine in cerebroside was assigned by Marinetti and Stotz (20) by infrared spectrophotometry. Spectra of the native compound contained a sharp peak at 10.3 μ (970 cm^{-1}) (21) which was eliminated by catalytic hydrogenation of

the lipid. The structure of sphingosine was thus finally established: trans-D-erythro-1,3-dihydroxy-2-amino-4-octadecene (sphing-4t-enine). The complete chemical synthesis of the base was achieved by Shapiro and Segal (22) in 1954. The evolution of the understanding of the structure of sphingosine was reviewed by Carter et al (23) in 1956. They drew attention to the fact that most of the studies of the structure of sphingosine had been done on the products of the hydrolysis of cerebrosides. They discussed at some length the problems of inversion that plagued the assignment of the erythro configuration to carbon-3. They concluded, nevertheless, that the sphingosine in intact sphingolipids had the same erythro configuration as that demonstrated in the isolated base. The enzymic studies reported by Sribney and Kennedy (24) and others (25,26) cast some doubt on this conclusion. Further investigations, however, tend to substantiate Carter's original hypothesis (27,28,29,30). The erythro configuration of the base in sphingolipids is now virtually universally accepted.

Although the heterogeneity of naturally-occurring LCB's of the sphingosine class had been suggested as early as 1941 (31), Carter's demonstration of dihydrosphingosine (sphinganine) in beef brain cerebroside (32) provided the main stimulus for the investigation of sphingosine homologues in glycosphingolipids. These studies were complicated by the variety of side reactions producing chemical modifications of the bases during the hydrolysis of the parent sphingolipid (reviewed in Ref. 7 and 8). The advent of gas-liquid chromatography and its application to the analysis of the long-chain aldehydes produced by the periodate oxidation

of the LCB's (33,34), or the fatty acids formed by various types of oxidations (35,36) provided a new method for the rapid separation and analysis of small quantities of sphingosine homologues. In 1965, Gaver and Sweeley (37) employed the GLC analysis of the trimethylsilyl derivatives of intact LCB's to determine the optimum conditions for the hydrolysis of sphingolipids. They developed conditions that practically eliminated the formation of the artifacts that had dogged previous efforts to study sphingosine homologues. This was a major contribution to the study of sphingolipids, and the method is still widely used for the analysis of LCB's. Gas-liquid chromatography coupled with mass spectrometry of TMSi derivatives has led to the identification of a wide variety of homologous bases (38,39). These new techniques are now being applied routinely to the analysis of the LCB compositions of various glycosphingolipids. Some representative analyses are shown in Table I.

The principal LCB's in the sphingolipids of plants are derivatives of phytosphingosine (4-hydroxysphinganine), named and characterized by Carter and his group (40,41). Several groups have since reported small quantities of the C_{20} derivative of phytosphingosine in the glycosphingolipids of mammalian kidney (46,47) and intestinal mucosa (42,43,44,45). Carter and Hirschberg (45) have also reported finding branched-chain LCB's in kidney glycosphingolipids. Branched-chain sphingosine derivatives had previously been thought characteristic of sphingomyelin-like lipids of the Phylum Protozoa (48).

Table I: Long-chain base composition of some glycosphingolipids. Adapted from Karlsson (8).

	CMH			CDH	
Long-chain base	Brain	Nerve	Plasma	Brain	Plasma
d16:0*	trace	1	1	trace	1
d17:0	trace	1	-	trace	-
d16:1	trace	5	2	trace	3
d18:0	2	20	1-2	3	2
d17:1	trace	4	2-3	1	1-2
i d18:1	-	1	trace	-	trace
d18:1	98	64	88	81	82
d20:0	-	-	-	1	-
d18:2	trace	3	2	-	2-3
ai d19:1	-	2	2	-	1-2
d19:1	-	-	-	trace	-
d20:1	-	-	-	trace	-
t18:0	-	-	2	-	4-5

* Abbreviations: d, dihydroxy; t, trihydroxy; i, iso; ai, anteiso.

b) Fatty acids

The investigation of the fatty acid composition of the glycosphingolipids was complicated by the difficulty obtaining cerebroside free of phospholipids, and by the problems of separating and identifying the fatty acids. Variations in the extraction procedure and fractional crystallization were the original methods used to purify sphingolipids. These techniques were refined by Thierfelder's group, and later employed with considerable success by Klenk and his coworkers. By these tedious methods, Klenk (49) showed that whereas C_{16} to C_{22} fatty acids predominate in the phosphatides, fatty acids with 24 carbon atoms are found exclusively in cerebrosides and sphingomyelin. For some time then, interest in the fatty acids of the glycosphingolipids centered around the metabolic relationship between the fatty acids in cerebroside, sphingomyelin and the phosphatides.

Studies of the fatty acid compositions of glycosphingolipids were transformed by the development of a microdistillation still capable of resolving complex mixtures of fatty acid methyl esters (50,51), and by the use of alumina to remove traces of phospholipids which invariably contaminated earlier preparations of glycolipids (52). Using these methods, Klenk and Leupold (52) undertook an analysis of cerebroside that stood for almost two decades. They demonstrated the predominance of hydroxy fatty acids and slight preponderance of saturated over unsaturated acids. Cerebronic (C24h:0) and oxynervonic (C24h:1) acids were the principal hydroxy fatty acids and nervonic (C24:1) and lignoceric (C24:0) acids the major nonhydroxy fatty acids.

The application of adsorption chromatography and gas-liquid chromatography to the purification and analysis of lipids revolutionized the understanding of the chemistry of glycosphingolipids. The analysis of the fatty acid composition of any complex lipid became so easy and quick that the literature is now replete with this form of data. Some of the more noteworthy analyses of neutral glycosphingolipids include those undertaken by Radin and his colleagues (53,54,55,56,57), O'Brien and Rouser (58), Martensson (59), Sweeley and his group (60). As the data accumulated a monotonous similarity between the fatty acid compositions of the various neutral glycosphingolipids became apparent. In almost all the lipids of this class the long-chain saturated and monoenoic acids predominate (notably C22:0, C24:0, C24:1). In the brain, over half the long-chain fatty acids in the CMH fraction are hydroxylated in the C-2 position (54,55,57,58). In the kidney, almost 50 percent of the CMH and CDH fractions also contain 2-hydroxy fatty acids (59). In the CTH and CAH fractions of the kidney, the nonhydroxy fatty acid compositions are identical to those of the CMH and CDH. However, they contain virtually no hydroxy fatty acids (59). In porcine erythrocytes, the fatty acid compositions of CTH and CAH were shown to be very similar to those of human kidney (60). On the other hand, Coles and Foote (61) reported a high percentage of hydroxy fatty acids and a substantial amount of C16:0 in porcine erythrocyte CTH. The fatty acid compositions of the CMH and CDH in erythrocytes and of all the neutral glycosphingolipids in plasma are unusual. These lipids contain a high proportion of shorter-chain fatty acids (e.g. C16:0, C18:1) (60). Brain ceramide (58) and the CDH from human peripheral leukocytes (62) also contain high porportions

of shorter-chain fatty acids (C18:0 in the case of brain ceramide; C16:0 in the case of leukocyte CDH).

Since the acylation of sphingosine is believed to be one of the first steps in the biosynthesis of the glycosphingolipids and the deacylation of ceramide one of the last steps in their degradation (63), the fatty acids provide a useful metabolic marker for lipids exhibiting precursor-product relationships. In the gangliosides, for example, the fatty acid composition is almost entirely stearic acid (C18:0) (64). Glycolipids derived from gangliosides can be identified as such by their high content of stearic acid even though they themselves may not be gangliosides (65).

The configuration of the double bonds in the fatty acids of the glycosphingolipids is cis (57). Nevertheless, Renkoken (66) has recently demonstrated about 4 percent trans-monoenoic fatty acids in the CMH from human plasma. The possibility of cis-trans isomerization does not appear to have been adequately ruled out.

The gas-liquid chromatography and coupled mass spectrometry of intact ceramides has recently been developed in Sweden (67). It promises to be a powerful tool in the analysis of the structure and metabolism of glycosphingolipids in the future (References 207 and 208).

c) Oligosaccharide

Monoglycosylceramide. The sugar in Thudicum's cerebroside was identified as galactose by Thierfelder (68) in 1890, and until 1940 little

more attention was paid to the carbohydrate moiety of the neutral glycosphingolipids. The classification of the various cerebrosides was, until then, based entirely on the fatty acid composition: phrenosin (also called cerebron), kerasin, nervon and oxynervon containing cerebronic (C24h:0), lignoceric (C24:0), nervonic (C24:1) and oxynervonic (C24h:1) acids respectively. In 1940, Halliday et al (69) reported that the cerebroside stored in the spleen of a patient with Gaucher's disease contained glucose rather than galactose as had been assumed from the studies of Lieb (70,71). Klenk confirmed these findings (72,73) and showed that glucocerebroside was present in small amounts in normal spleen (74). These discoveries stimulated renewed interest in the carbohydrate portion of cerebroside.

In 1950, Nakayama (75) showed by permethylation and lead tetra-acetate oxidation that the galactose in brain cerebroside was linked to the primary carbinol group of the long-chain base. This was confirmed by Carter and Greenwood (76) by a different approach involving the hydrogenolysis of peracetylated cerebroside. Nakayama (75) also showed that the galactose moiety was in the pyranose configuration.

The β-stereoconfiguration of the glycosidic linkage between the glucose and ceramide in glucocerebroside was established by its susceptibility to hydrolysis by a β-glucosidase (69) and by infrared spectrophotometry (77). The configuration of the galactose in galactocerebroside was shown to be β by infrared spectrophotometry (77,78,79) and by degradative studies (80).

Cerebroside esters. In 1963, Norton and Brotz (81) reported the isolation of small quantities of cerebroside fatty acid ester from brain. Klenk and Doss (82) made a similar discovery in 1966. Kishimoto et al (83) confirmed these findings, and reported the fatty acid composition of the esters. They also located the ester linkage at carbon-6 of the galactose moiety. Tamai et al (84) reported the isolation of a cerebroside ester in which, they claimed, the fatty acid was esterified to the secondary carbinol group of the sphingosine moiety. Makita et al (85) isolated glucocerebroside fatty acid ester from Gaucher spleen. They tentatively located the ester linkage at carbon-6 of the glucose moiety. Very little is known about this unusual group of lipids.

Diglycosylceramide. Klenk and Rennkamp (74), in 1942, were the first to demonstrate, among the lipids of bovine spleen, a neutral glycosphingolipid with more than one hexose moiety per sphingosine residue. Yamakawa and his group (86,87) showed that a similar lipid extracted from erythrocytes and from kidney tissue was O-β-D-galactopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→1')-ceramide (i.e. lactosylceramide). Adams and Gray (88) demonstrated the presence of the same lipid in pig lung. By immunochemical studies Rapport et al (89) showed that the antigenic dihexosylceramide, cytolipin H (90), was also lactosylceramide.

In 1963, Sweeley and Klionsky (91) demonstrated the accumulation of a dihexosylceramide containing only galactose in the kidney of a patient who had died of Fabry's disease. This lipid, which has the structure, O-D-galactopyranosyl-(1→4)-O-D-galactopyranosyl-(1→1')-ceramide (92,93),

is a normal constituent of pig lung (88) and human kidney (59,86). Gatt and Berman (95) reported the presence of digalactosylceramide in the brain of a patient with Tay-Sachs disease. Efforts to confirm this unexpected finding were unsuccessful (96). The anomeric configuration of the galactose residues is unknown.

Triglycosylceramide. The presence, in erythrocytes, of neutral glycosphingolipids containing three hexose residues but no amino sugar was revealed by Klenk and Lauenstein (97) in 1953. Svennerholm and Svennerholm (98,99) showed that trihexosylceramide from the serum, liver and spleen contained glucose and galactose in the proportion 1:2. In 1962, Makita and Yamakawa (100) had isolated a similar lipid from bovine spleen and claimed that it was dextrorotatory in contrast to the other glycolipids which were all levorotatory. Sweeley and Klionsky (91) demonstrated that the massive accumulation of this lipid was the principal biochemical abnormality in kidney tissue from a patient who had died of Fabry's disease. That it was one of the principal neutral glycosphingolipids of normal human kidney was shown by Makita (101) and Martensson (59). Makita and Yamakawa (86) demonstrated by permethylation, methanolysis and GLC analysis that the linkage of the oligosaccharide was gal-(1→4)-gal-(1→4)-glc-(1→1')-ceramide. In 1965, Gray (102) isolated, from BP8/C3H ascites-sarcoma cells, a trihexosylceramide which he tentatively identified as gal-(1→6)-gal-(1→4)-glc-(1→1')-ceramide on the basis of the chromatographic properties of the oligosaccharides obtained by partial acid hydrolysis. This has been neither confirmed, nor demonstrated

in any other tissue. Gal-(1→4)-gal-(1→4)-glc-(1→1')-ceramide is the principal neutral glycosphingolipid in pig lung (88,103) and in the kidneys of some strains of mice (105).

In 1967, Kawanami (106) extracted a similar trihexosylceramide from Nakahara-Fukuoka sarcoma tissue. He showed by the optical rotation, infrared spectrum and acid lability of the intact lipid, and by the nmr spectrum of the trihexosylsphingosine obtained from the alkaline hydrolysis of the lipid that it contained a terminal α-D-galactopyranosyl residue.

In 1969, Miyatake (93) showed that the trihexosylceramide accumulating in a number of tissues in Fabry's disease was also gal-(1→4)-gal-(1→4)-glc-(1→1')-ceramide. In addition, he reported optical rotations and infrared spectra which, in retrospect, are almost identical to those reported by Kawanami for the CTH from the murine sarcoma tissue. Nevertheless, he made no mention of the possibility of there being a terminal α-D-galactopyranosyl residue in Fabry's CTH. Kint's discovery of a nonspecific α-galactosidase deficiency in Fabry's disease (107) stimulated Sweeley and his group (as well as ourselves) to examine the stereoconfiguration of the oligosaccharide in the accumulated CTH. By permethylation, methanolysis and GLC analysis they confirmed Miyatake's assignment of the order and numbering of the interglycosidic linkages (92). They then prepared the intact oligosaccharide free of LCB and fatty acid by osmium tetroxide-catalyzed periodate oxidation and mild alkaline hydrolysis of the peracetylated lipid. The TMSi derivative of the oligosaccharide was formed

and nmr spectra recorded in deuterated chloroform. They also recorded spectra of the TMSi derivative of the intact glycolipid. On the basis of their results, they concluded that all the glycopyranosides in the CTH accumulating in Fabry's disease were of the β-stereoconfiguration. It was at this stage that the majority of the work described in this Thesis was undertaken.

Sweeley's conclusion concerning the anomeric configuration of the terminal galactopyranosyl residue of CTH was subsequently challenged by Bensaude et al (108) and Li and Li (109) who showed that the terminal galactosidic linkage of the lipid was completely resistant to hydrolysis by a β-galactosidase from jack beans, but that it was susceptible to hydrolysis by an α-galactosidase from figs. Clarke, Wolfe and Perlin (110) recently presented optical rotatory, nmr, chemical and enzymic evidence for a terminal α-D-galactopyranosyl residue in Fabry's CTH. (The article is based on the results of experiments reported in this Thesis). Within the last year, Hakomori et al (111) reported nmr, enzymic and immunochemical evidence for a terminal α-D-galactopyranosyl residue in the CTH from cultured guinea pig fibroblasts and from human erythrocytes.

Another trihexosylceramide, with the structure galNHAc-gal-glc-ceramide, has been isolated from the tissues of patients with Tay-Sachs disease (95) and Sandhoff's disease (94). Makita and Yamakawa (65) showed that it was the asialo derivative of G_{M2}-ganglioside (Svennerholm nomenclature (113)).

Tetraglycosylceramide. In their studies of the human erythrocyte stroma, Klenk and Lauenstein (114,115), in 1951, demonstrated the presence of a neutral aminoglycosphingolipid containing fatty acid (chiefly lignoceric acid), sphingosine, glucose, galactose and galactosamine but no neuraminic acid. This was confirmed by Yamakawa and Suzuki (116) who proposed the name globoside for the lipid. The structure of globoside was shown to be galNHAc-(β1→3)-gal-(1→4)-gal-(1→4)-glc-(1→1')-ceramide (117,118). It is the principal neutral glycosphingolipid of the human erythrocyte stroma (119) and the human kidney (59,101). In 1971, in an elegant investigation of the structure of human erythrocyte CTH and globoside, Hakomori et al (111) showed by nmr, enzymic hydrolysis and immunochemical studies that globoside contains a subterminal α-D-galactopyranosyl residue. The other anomeric protons are all of the β-configuration (this Thesis). Makita et al (120) showed that Forssman hapten extracted from equine spleen and kidney had a similar basic structure. On the basis of hemagglutination inhibition tests and differences in optical rotation, however, they assigned an α-stereoconfiguration to the terminal N-acetylgalactosaminyl residue. This was confirmed enzymatically by Abe, Handa and Yamakawa (112). The anomeric configuration of the subterminal galactosyl residue was unknown. Siddiqui and Hakomori (121) have just published findings which indicate that Forssman hapten has the structure: galNAc-(α1→3)-galNAc-(β1→3)-gal-(α1→4)-gal-(β1→4)-glc-(β1→1')-ceramide.

The haptenic neutral aminoglycosphingolipid isolated from human kidney and called cytolipin K by Rapport (122) is almost certainly identical to globoside. Philipson et al (123) showed that the lipid part of the

erythrocyte receptor for hemagglutinating enteroviruses was chromatographically identifiable with globoside.

Globoside accumulates in the tissues of patients with visceral Tay-Sachs (Sandhoff's) disease (94,124,125). Whereas patients with classical Tay-Sachs disease have a deficiency of hexosaminidase A (126,128) (nomenclature of Robinson and Stirling (127)), patients with visceral Tay-Sachs disease exhibit total deficiencies of both hexosaminidases A and B (94).

A tetraglycosylceramide with the structure gal-galNHAc-gal-glc-ceramide (asialo-G_{M1}-ganglioside) accumulates in the brains of patients with G_{M1}-gangliosidosis (129) who have a β-galactosidase deficiency (130).

Other neutral glycosphingolipids. In the last few years, progress has been made in the isolation and characterization of a variety of high molecular weight neutral glycosphingolipids. In 1964, Hakomori and Jeanloz (131,132) isolated a pentaglycosylceramide with the structure, gal-(fucosyl)-glcNAc-gal-glc-ceramide or fucosyl-gal-glcNAc-gal-glc-ceramide from human cancer tissue. The structure was recently reported to be O-β-galactosyl-(1→4)-[O-α-L-fucosyl-(1→3)]-O-β-(N-acetyl)glucosaminyl-(1→3)-O-β-galactosyl-(1→4)-glucosylceramide with a high content of 4-hydroxysphinganine and α-hydroxy fatty acids (133). The oligosaccharide portion of the compound is identical to the "lacto-N-fucopentaose III" isolated from human milk by Kobata and Ginsburg (134). In 1966, Vance et al (135) isolated a pentaglycosylceramide with the structure, galNAc-galNAc-gal-gal-glc-ceramide from normal dog intestine. Eto et al (136)

isolated a pentaglycosylceramide from rabbit erythrocytes and reticulocytes which they assigned the structure O-α-gal-(1→3)-O-β-gal-(1→3)-O-β-glcNAc-(1→3)-O-β-gal-(1→4)-O-β-glc-(1→1')-ceramide.

A great deal of interest has developed related to high molecular weight neutral glycosphingolipids with blood-group specificities. Eloquent reviews of this burgeoning field have recently been written by Rapport and Graf (206) and by Hakomori (137).

The chemical synthesis of a number of complex glycosphingolipids has been accomplished by Shapiro and his group at the Weizmann Institute in Israel. These achievements have been reviewed recently (138).

2. Occurrence

a) Species, sex and age

Although few systematic investigations of their distribution among the different species have been undertaken, it is clear that neutral glycosphingolipids are widespread in nature. They are found in organisms as disparate as virions (139,140), wheat seed (141), runner bean leaves (142), and fresh-water mussel spermatozoa (143), and in virtually all vertebrates (1).

Among the mammals neutral glycosphingolipids are ubiquitous. The principal neutral glycosphingolipid, because of its very high concentration in the nervous system, is galactocerebroside [O-β-D-galactopyranosyl-(1→1')-ceramide] . It is a major constituent of myelin, and its concentration varies little between different adult mammals (144,145). The

galactocerebroside content of the brain does, however, vary considerably with age; the increase in galactocerebroside during development parallels the advancement of myelination of the nervous system. Since the time table of myelination varies between species, the galactocerebroside content of the brain varies accordingly between immature animals of different species (144,145).

In the last several years, increasing attention has been directed to the neutral glycosphingolipids of extraneural tissues. Marked differences in the glycosphingolipid composition of erythrocytes from various mammals were discovered (23). Different animals were classified according to whether the principal erythrocyte glycolipid was hematoside (G_{M3} ganglioside), such as the horse and dog; globoside, such as man and the hog; neither, such as the chicken; or both, such as the ox. In a study of human, equine and bovine spleens, Makita and Yamakawa (100) found very little interspecies variation in content, type or fatty acid composition of the neutral glycosphingolipids. Nevertheless, in 1968, Kawanami (146) showed that whereas CDH and CTH, as well as CAH, are major constituents of the neutral glycosphingolipids of human kidney (59), CMH and CAH predominate in the rat kidncy, which also contains a high concentration of sulfatide [O-β-D-(3-O-sulfate)-galactopyranosyl-(1→1')-ceramide].

Even within species, marked subspecies variations of glycosphingolipid composition have been described. Gray and his group demonstrated differences in the glycolipid composition of the kidneys, not only between different strains of mice (105), but also between male and female animals of the same strain (104). Kwiterovitch, Sloan and Fredrickson (147)

showed that in the human liver the concentration of neutral glycosphingolipids was significantly higher in the female than in the male.

By a semiquantitative thin-layer chromatographic technique, Coles et al (104) demonstrated a much higher proportion of CMH in the kidneys of "old" mice than of young adults of the same strain and sex. This is similar to the results of studies done on the human kidney by Martensson (59), who also showed, however, that the total neutral glycosphingolipid concentration decreased with age. More recently Kuske and Rosenberg (148) have shown that the CMH, CDH and hematoside concentrations of rat spleen increase markedly between ages 1 and 96 days. The total lipid, cholesterol and CTH concentrations, on the other hand, only increase slightly during the same period.

In general, however, the differences between species and sexes have been largely due to minor variations in the distribution of glycolipids among the various subclasses of glycosphingolipid. The type, structure and even fatty acid compositions of the neutral glycosphingolipids are relatively constant among the mammals studied so far.

b) Tissue distribution

The distribution of the main glycosphingolipids in various human organs has been reviewed by Martensson (1) and is presented in Table II. Although neutral glycosphingolipids have been found in virtually every tissue in which they have been sought; except in brain, the concentrations are very low. As can be seen from Table II, the distribution of neutral glycosphingolipids is quite tissue specific. The principal or only

Table II: Distribution of glycosphingolipids in some human tissues (μmoles per gram dry weight)[a]

Neutral glycosphingolipids	Brain (149,154) Gray	Brain (149,154) White	Erythrocytes (119)	Leukocytes (289)	Plasma (99)	Spleen (79)	Kidney (59,155)	Liver (147)[b] Male	Liver (147)[b] Female	Placenta (156)
CMH	7.05	173	0.011	0.47	0.10 - 0.14	0.0 - 0.75	0.43	0.20	0.30	0.14
CDH	0.98	tr	0.026 - 0.070	11.09	0.060 - 0.075	2.1 - 2.15	0.65	0.25	0.50	0.30
CTH	1.10[c]	0	0.033 - 0.041	0	0.023 - 0.035	0.43 - 0.64	1.27	0.10	0.25	0.25
CAH	0.27	0	0.19 - 0.21	tr	0.020 - 0.033	0.37 - 0.56	1.93	0.10	0.20	0.43
Sulfatides										
Monohexose sulfatides	1.8	26.2	-	-	-	0.05	0.55	-	-	-
Dihexose sulfatides	-	-	-	-	-		0.20	-	-	-
Gangliosides	10.6	0.82	0.82	-	-	0.73	0.24	18.0 - 59.3 μg NANA/g wet wt		0.97

[a] adapted in part from Martensson (1)

[b] assuming dry weight = 0.20 x wet weight

[c] The "CTH" in brain is entirely asialo-G_{M2} ganglioside, i.e. galNAc-gal-glc-ceramide.

monohexosylceramide in extraneural tissues (with the exception of kidney) is glucocerebroside. In kidney almost half the cerebroside is galactocerebroside (59); in the nervous system it constitutes almost 100 percent of the cerebroside (149). Digalactosylceramide, which accumulates in the kidneys and urinary sediment in Fabry's disease (91,150), has not been found in any other normal human tissue (93). The demonstration of digalactosylceramide in the brain of a patient with Tay-Sachs disease (95) is puzzling in view of the absence of the lipid in normal brain. Its relationship to the aberration in ganglioside metabolism in Tay-Sachs disease is an even greater enigma.

c) Subcellular distribution

Galactocerebroside is one of the principal constituents of adult myelin (151,152). In a study of myelinogenesis, Davison et al (151) showed that the cerebroside content of "early myelin" in the rat was low, similar to the cerebroside content of glia. They could demonstrate no cerebroside at all in the neuronal fraction of their preparation. "It therefore seems possible", they wrote, "that early myelin consists essentially of oligodendroglial plasma membrane (italics mine) and that the later insertion of cerebroside into the myelin lamella produces the stable mature membrane structure". Cumings et al (152) demonstrated low concentrations of cerebroside in adult human cerebral cortical microsomal preparations which, however, probably included fragments of glial plasma membrane (151). They also demonstrated traces of CTH and small amounts of CDH in myelin and cerebral cortical microsomal preparations.

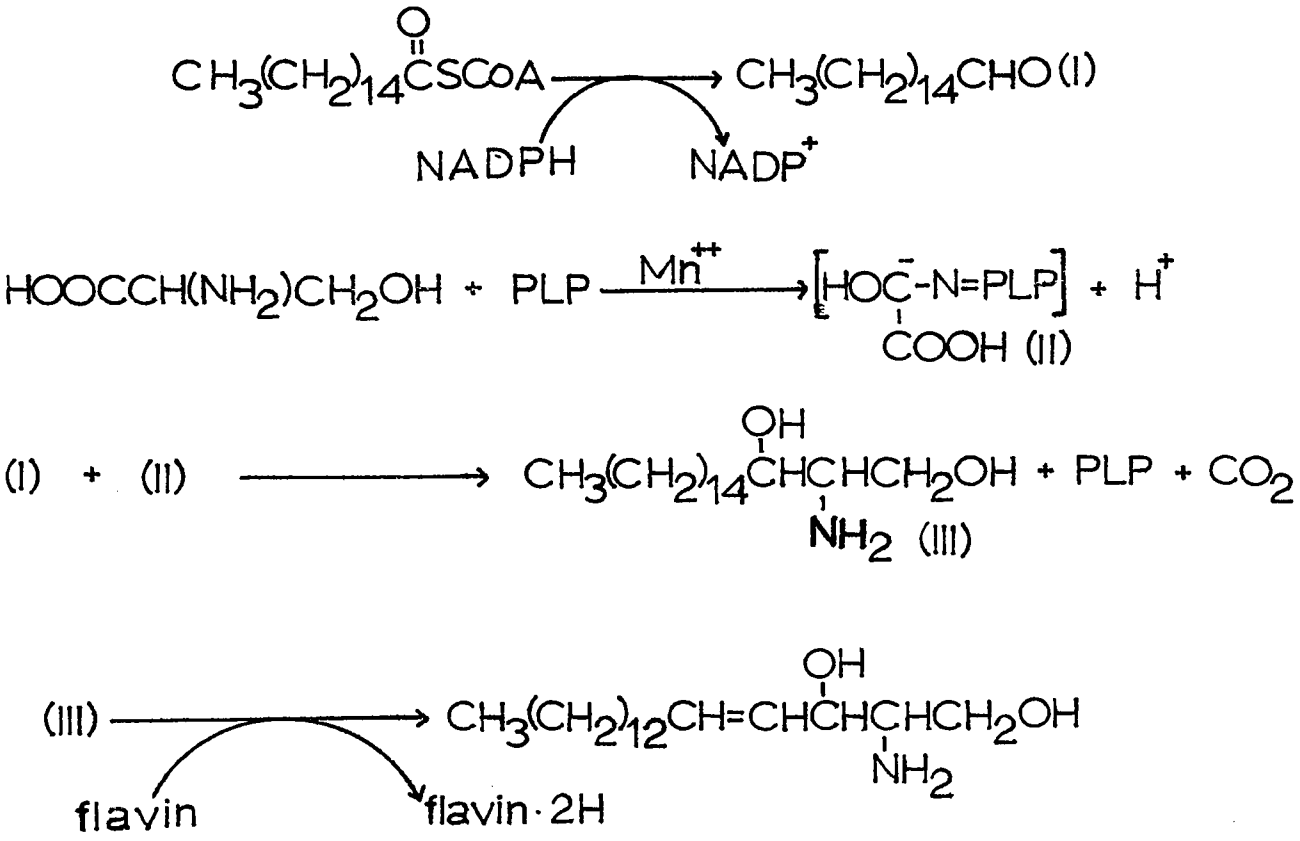
The elucidation of the subcellular distribution of the neutral glycosphingolipids is seriously hampered by the difficulty of obtaining purified membrane fractions and by the vanishingly low concentrations of this class of lipids in them. As a result, very little is known about it. The studies on myelinogenesis (151) and the high concentrations of neutral glycosphingolipids in the erythrocyte stroma (116), however, suggest that they are likely constituents of the cell membrane. The only definitive attempt to localize the neutral glycosphingolipids in cells containing a full complement of subcellular organelles, reported by Dod and Gray (153), supports this hypothesis. They showed that in rat liver cells the neutral glycosphingolipids occurred almost exclusively in the plasma membrane. The small quantity appearing in the endoplasmic reticulum fraction was attributed to contamination by fragments of plasma membrane (shown by enzyme markers).

In spite of the paucity of direct evidence, the localization of the neutral glycosphingolipids in the plasma membranes of all cells in which they occur is almost universally accepted. Although neutral glycosphingolipids occur in low concentrations in plasma (119), they are probably not in true solution, but rather, are part of lipoprotein complexes. These types of "soluble complexes" undoubtedly also occur in the cytoplasm of cells which synthesize glycosphingolipids, but by far the major share of this class of lipids is firmly bound in insoluble membranes.

3. Metabolism

a) Biosynthesis

In 1953-54, Zabin and Mead (157,158) and Sprinson and Coulon (159) showed from studies of the incorporation of low molecular weight precursors into rat brain sphingolipids in vivo that carbons 1 and 2 of the sphingosine moiety were derived from serine and carbons 3 to 18 were derived from a 16-carbon fatty acid-like intermediate. In 1958, Brady and coworkers (160,161) reported detailed studies of the mechanism of the condensation of serine with the 16-carbon intermediate by a microsomal preparation from rat brain, and suggested, on the basis of their findings, the following sequence of reactions:

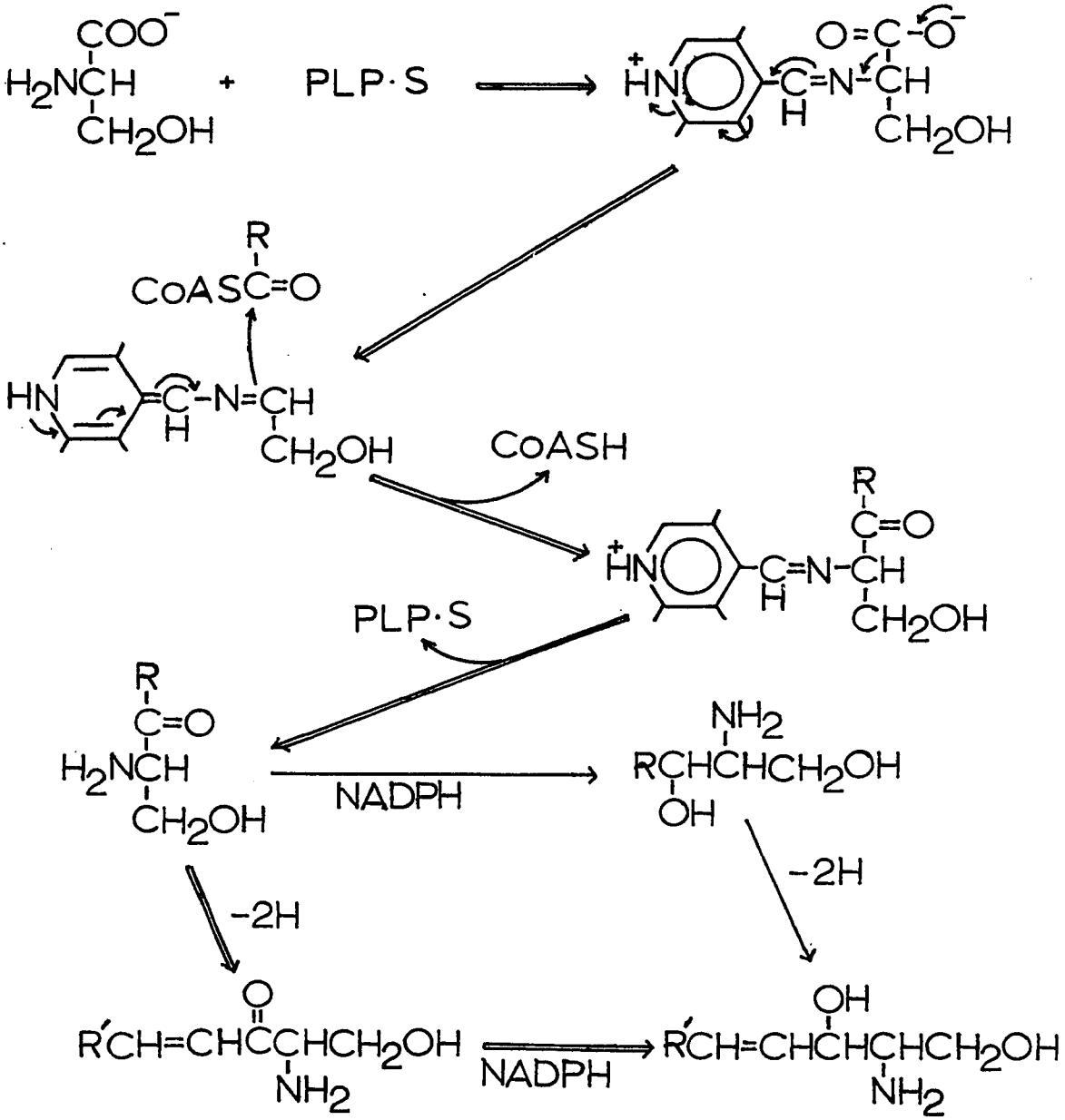


This mechanism was widely accepted for almost a decade. Although Weiss (162) indicated several alternative pathways in 1963, it was not until 1967 that Braun and Snell (163) and Stoffel and coworkers (164) independently reported evidence for the direct condensation of palmityl-CoA with serine and the intermediate formation of 3-ketodihydrosphingosine (2-amino-1-hydroxyoctadecane-3-one) in the biosynthesis of sphingosine. Parallel studies in the yeast, Hansenula ciferri (28,30,165), rat liver, and mouse brain (29,30,166) confirmed these findings. The condensing enzyme and reductase both exhibit chain-length specificities for CoA esters (2). The mechanism of the introduction of the 4-trans double bond in sphing-4-enine is still unknown (2). Some have proposed the double bond is produced by the desaturation of the precursor, palmitylCoA (167). On the other hand, Fujino and Nakano (168) have presented evidence that 3-keto-sphinganine is the substrate for the desaturation.

Similar studies have been undertaken on the biosynthesis of phyto-sphingosine (4-hydroxysphinganine) (169-173), the sphingosine derivative isolated from plant lipids. The origin of the C-4 oxygen atom is obscure. It does not appear to be derived from molecular oxygen or water (169), nor does it appear to be derived from the condensation of 2-hydroxypalmityl-CoA with serine (170). Recently, Polito and Sweeley (174) and Stoffel and Binczek (197) published evidence that 4-hydroxysphinganine is synthesized by a direct stereospecific hydroxylation of sphinganine. The formation of the double bond in sphing-4-enine appears to occur by a stereospecific anti-periplanar elimination process involving either palmitylCoA or 3-ketosphinganine.

The biosynthesis of sphingosine and dihydrosphingosine by cell-free systems from Hansenula ciferri was reviewed by Snell, DiMari and Brady (167)

in 1970. The currently accepted pathway for sphingosine biosynthesis is:



PLP·S = PLP-Synthetase complex

R = $CH_3(CH_2)_{13-19}$ R′ = R minus $-CH_2-CH_2-$

The biosynthesis of the fatty acids incorporated into neutral glycosphingolipids has been reviewed recently (1), and will not be discussed here. It is worth noting, however, that in the biosynthesis of glycosphingolipids containing α-hydroxy fatty acids, the hydroxylation occurs before the incorporation of the fatty acid into the glycolipid (175). Once incorporated into the glycolipid, the fatty acid remains unchanged. This is important in metabolic studies in which the fatty acid composition of a specific glycosphingolipid is used to follow its metabolism (see Reference 60).

In 1957-58, Radin et al (176), Moser and Karnovsky (177), and Burton and his coworkers (178,179,180) showed that when ^{14}C-glucose or ^{14}C-galactose was administered parenterally to mice or rats, ^{14}C-labelled glycosphingolipids could be demonstrated in the brains of the animals within a few hours. Moreover, the rate of incorporation of the label into brain cerebroside was shown to be a function of the age of the animals (180,181). In an in vivo study of the metabolism of the neutral glycosphingolipids in porcine blood following the injection of ^{14}C-glucose, Dawson and Sweeley (60) showed that whereas the glycolipids in the red cells were, with the exception of CMH, derived entirely from de novo synthesis during erythropoiesis in the bone marrow, the glycolipids in the plasma were derived from at least two sources. The smaller pool (they estimated 20 percent of the total plasma glycolipid pool) was derived from the rapid synthesis of the lipid, probably in the liver. The other 80 percent was derived from the loss of senescent red cell CAH directly into the plasma, and its subsequent catabolism somewhere to yield the simpler glycosphingolipids. Within the circulation, the plasma

and red cell pools of glycosphingolipids were completely separate except for CMH, which exchanged rapidly between plasma and erythrocytes. The authors emphasized that the problem is very complex, and that, in fact, the plasma glycolipids were probably derived from many sources.

Burton et al (178,179,180) developed a system for studying the biosynthesis of cerebroside in brain in vitro. With it they showed that the reaction involved the transfer of galactose from uridine diphosphogalactose to some unidentified endogenous lipid acceptor. In 1960, Cleland and Kennedy (182) reported that the lipid acceptor for the galactose from UDP-galactose was sphingosine and the reaction product psychosine. Later, Brady (183) showed that the biosynthesis of cerebroside in particulate preparations from rat brain was completed by the N-acylation of psychosine. He could only demonstrate minimal formation of ceramide by the N-acylation of sphingosine. These studies and the in vivo studies by Kopaczyk and Radin (184) suggested rather strongly that psychosine was an intermediate in the biosynthesis of cerebroside.

On the other hand, using very similar preparations, Gatt (185,186) and Sribney (26) demonstrated the presence of enzymes catalyzing the condensation of fatty acids or fatty acyl-CoA thiolesters with sphingosine to form ceramide in rat brain and other tissues. Basu and collaborators (187,188,189,190) showed, using embryonic chick brain, that whereas ceramide served as an excellent substrate for the biosynthesis of cerebrosides, no reaction could be demonstrated between UDP-gal or UDP-glc and sphingosine. Similar studies on mice by Morell and Radin (191) produced

identical results. It is interesting that in both studies only ceramides containing 2-hydroxy fatty acids were effective as substrates in the biosynthesis of galactocerebrosides *in vitro*. Burton *et al* (180) had noticed a similar preference for the incorporation of 2-hydroxy fatty acids into cerebroside (193,194). The demonstration of ceramides in a number of tissues (57,58) as well as a small pool of ceramides containing 2-hydroxy fatty acids in brain (192) supports the hypothesis that cerebrosides are synthesized by the glycosidation of ceramide.

Morell and Radin (195) undertook a systematic study of the specificity in ceramide biosynthesis from various nonhydroxy fatty acyl-CoA thiolesters in brain microsomes. They concluded that 1) the source of the N-acyl fatty acid is fatty acyl-CoA thiolesters, not free fatty acids, as was suggested by Yavin and Gatt (186); and 2) the distribution of N-acyl fatty acids is determined, at least in part, by the specificity of the acyl transferase.

These data are strong evidence for the biosynthesis of cerebrosides via the intermediate formation of ceramide. Nevertheless, in a re-examination of the problem, Kanfer (196) in 1970, reported that whereas added sphingosine stimulated the incorporation of labelled UDP-glc and UDP-gal into glycosphingolipids by a particulate preparation from rat brain, added ceramide had no effect. This suggests that cerebroside is formed by the N-acylation of psychosine as originally proposed by Cleland and Kennedy (182) and Brady (183). These apparently contradictory conclusions have not yet been resolved (2). It is possible that both pathways operate *in vivo*, and that the bias shown one or the other in

experiments done *in vitro* is related to the difficulties and differences in the preparation of the highly insoluble lipid substrates. In fact, Hammarström (207,208) has recently published mass spectrometric evidence for the participation of both pathways in the biosynthesis of cerebrosides containing nonhydroxy fatty acids by rat and mouse brain microsomes.

Although Kanfer (198) was unable to demonstrate the biosynthesis of more highly glycosylated glycosphingolipids from labelled glucosylceramide in experiments done *in vivo* in immature rats, Hauser (199,200) and Basu (187,190) succeeded in demonstrating the presence of UDP-galactose: glucosylceramide galactosyltransferase and UDP-galactose:lactosylceramide galactosyltransferase activities in a number of rat tissues. Kampine *et al* (201) demonstrated the incorporation of labelled glucose and galactose into CMH and CDH by human leukocytes *in vitro*. In Kanfer's experiments, the failure of the labelled glucosylceramide to be incorporated into more highly glycosylated glycolipids is probably attributable to the failure of the substrate to penetrate to the site of the appropriate glycosyltransferases.

The synthesis of the higher molecular weight neutral glycosphingolipids undoubtedly proceeds in a manner similar to that demonstrated for the biosynthesis of glycoproteins, mucins and gangliosides (2,202). Monosaccharides are transferred from their nucleotide derivatives to the appropriate acceptors in reactions catalyzed by glycosyltransferases that are highly specific for the nucleotide sugar and the acceptor molecule. Roseman (203) advanced the following thesis to describe the biosynthesis of the

oligosaccharides in glycoproteins and gangliosides (and presumably, glycosphingolipids):

> (1) The oligosaccharide chains grow at their non-reducing ends or at branch points.
>
> (2) Sugars are added in a stepwise sequence as monosaccharide units...
>
> (3) The enzymes catalyzing these reactions, glycosyltransferases, comprise homologous families, where each family transfers only one type of monosaccharide unit.
>
> (4) The glycose donors are the corresponding sugar-nucleotides. To date, all members of a single family of transferases require the same sugar-nucleotide.
>
> (5) The members of one family of glycosyl-transferases can be differentiated by two properties: either (a) they require different acceptor molecules; or (b) when the acceptor molecule requirement is the same, they accept the glycose unit at different positions, giving different products.

He postulated that the synthesis of the completed complex carbohydrate is governed by "a specific complex of glycosyltransferases, called a multiglycosyltransferase (MGT) system" (italics author's) with "cooperative sequential specificity" (italics author's).

Although the bulk of the glycosyltransferase activity involved with the synthesis of glycoproteins is located in the synaptosome fraction in embryonic chick brain (202) and the Golgi apparatus in rat liver (204), the precise subcellular localization of the enzymes involved with neutral glycosphingolipid synthesis is unknown. From the results of the various in vitro studies done on glycosphingolipid synthesis, however, the enzymes involved appear to be located in the "microsomal fraction" (see Reference 200).

The mechanism of the control of the synthesis of neutral glycosphingolipids is poorly understood. Roseman (202) has suggested, in reference to the glycoproteins, that the "genetic information for the synthesis of the oligosaccharide chains is translated into the synthesis of different multi-glycosyltransferase complexes". The results of the studies of the biosynthesis of ceramide by Morell and Radin (195) indicate that this hypothesis may be extended to the control of the synthesis of the aglycone as well. Recently, Gray (205) presented evidence that testosterone controls the biosynthesis of digalactosylceramide in the kidneys of C57/BL mice by modulating the amount of the galactosyltransferase involved in the synthesis of galactosylceramide from ceramide and UDP-galactose.

b) Biodegradation

The discovery of various hereditary diseases caused by inborn defects of the catabolism of glycosphingolipids (209) has stimulated a great deal of activity in the investigation of the normal biodegradation of these compounds. The breakdown of this group of lipids is initiated by the stepwise removal of the sugar residues by specific glycosylceramide

hydrolases (2). The resulting ceramide is hydrolyzed to yield a long-chain base and a fatty acid (63). These may re-enter the precursor pools for the biosynthesis of new glycosphingolipids, or they may be further degraded. Stoffel and his group showed that the catabolism of the long-chain bases proceeds by the phosphorylation of the primary carbinol group and cleavage of a two-carbon fragment to yield phosphoryl-ethanolamine and a long-chain aldehyde (reviewed in Reference 2). The long-chain aldehydes are oxidized to the corresponding fatty acids (e.g. hexadec-2t-enal → palmitic acid), which then enter the general metabolic fatty acid pool.

Progress in our understanding of the properties of the various specific glycosylceramide hydrolases involved in glycosphingolipid catabolism has evolved along three different but convergent lines: 1) studies on the catabolism of glycolipids *in vivo*, 2) studies on the hydrolysis of the natural substrates *in vitro*, and 3) studies on nonspecific glycosidases using artificial substrates such as p-nitrophenyl-β-D-galactopyranoside.

The *in vivo* studies have been the least numerous and the least satisfying. In 1963, Shapiro and Statter (210) demonstrated the conversion of CAH to CTH *in vivo* in the rat. In a much more detailed study of the metabolism of neutral glycosphingolipids in porcine blood, Dawson and Sweeley (60) presented data which they felt was consistent with "the loss of senescent red cell globoside directly into the plasma and subsequent metabolism somewhere to yield the simpler glycosphingolipids". The

evidence for the interconversion of the various glycosphingolipids is largely indirect, however, and is not supported by the results of the analyses of their fatty acid compositions. In two of the most lucid demonstrations of glycosylceramide hydrolase activity *in vivo*, Kopaczyk and Radin (184) and Kanfer (198) reported the conversion of galactocerebroside and glucocerebroside to ceramide in rat tissues.

The understanding of the glycosylceramide hydrolases was greatly improved by the development of *in vitro* techniques to study glycosphingolipid catabolism. The earliest attempts to demonstrate cerebrosidase activity in animal tissues *in vitro* were only partially successful (211, 212). In 1963, however, Gatt (185) in his studies on ceramidase, demonstrated the importance of detergents, such as sodium cholate, for the activation of enzymes involved in sphingolipid catabolism. This discovery, together with the development of techniques to synthesize glycosphingolipids labelled specifically with radioisotopes, led to the demonstration of glucocerebrosidase in human spleen (213) and rat intestine (214). Following on these studies, Brady *et al* (215) showed that patients with Gaucher's disease, who store glucocerebroside, exhibit profound deficiencies of glucocerebrosidase activity in their tissues. The demonstration of a number of glycosylceramide hydrolases in a wide variety of tissues ensued (Table III). All the glycosylceramide hydrolases so far described have had pH optima in the acid range (pH 3-6). In addition, studies of their subcellular distribution indicate that they are associated with the lysosomes (*vide infra*) (216).

Table III: Some glycosylceramide glycohydrolases

Enzyme	Tissue	Reference
glucocerebrosidase	human spleen	Brady, R.O. et al (213)
	rat intestine	Brady, R.O. et al (214)
	ox brain	Gatt, S. (225)
	human leukocytes	Kampine, J.P. et al (226)
	rat brain	Radin, N.S. et al (229)
galactocerebrosidase	rat intestine	Brary, R.O. et al (214)
	rat brain	Hajra, A.K. et al (227)
		Bowen, D.M. and Radin, N.S. (230)
	rat spleen, kidney, lung	Hajra, A.K. et al (227)
	pig brain	Hajra, A.K. et al (227)
	human spleen	Hajra, A.K. et al (227)
	human leukocytes	Kampine, J.P. et al (226)
	human brain	Suzuki, K. and Suzuki, Y. (231)
lactosylceramide galactosidase	pig brain	Hajra, A.K. et al (227)
	rat brain	Gatt, S. and Rapport, M.M. (228)
		Radin, N.S. et al (229)
	human liver	Dawson, G. and Stein, A.O. (232)
galactosylgalactosyl-glucosylceramide galactosidase	rat intestine, liver, kidney, spleen, brain	Brady, R.O. et al (233)
	human intestinal mucosa	Brady, R.O. et al (234)
	human kidney	Wolfe, L.S. and Clarke, J.T.R. (235)
globoside N-acetyl-galactosaminidase	calf brain	Frohwein, Y.Z. and Gatt, S. (236)

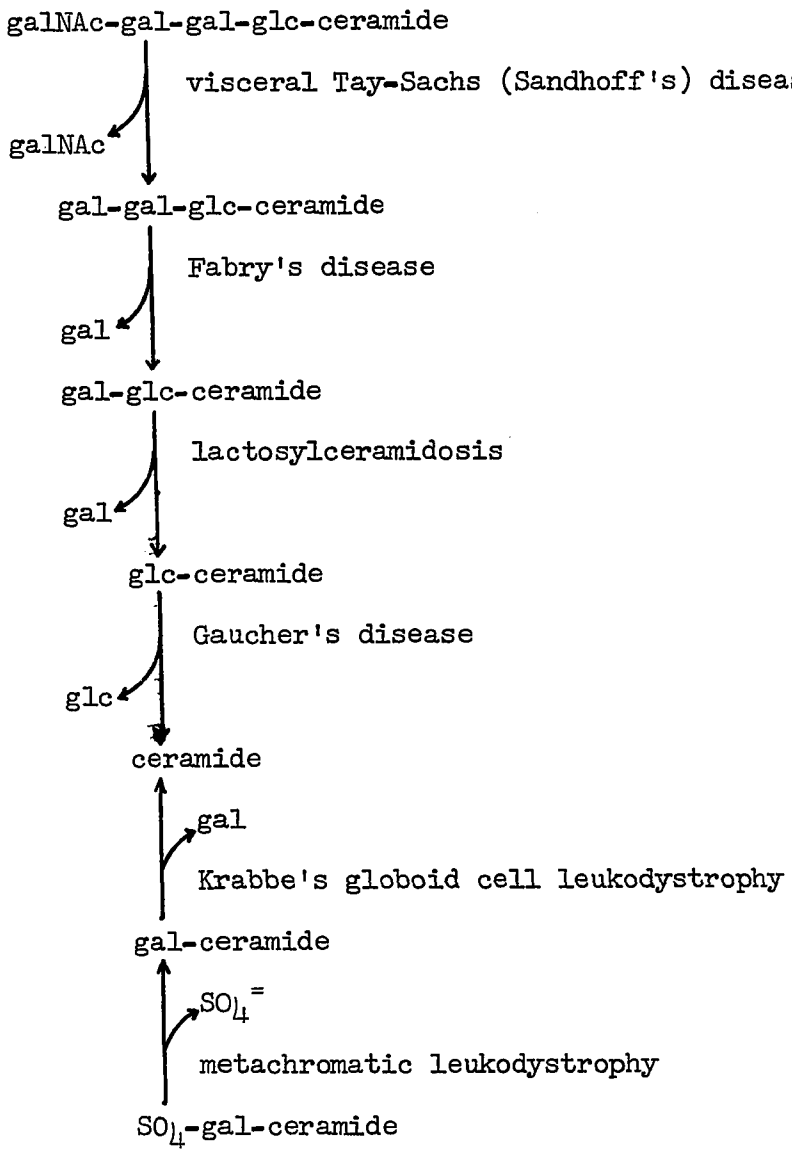
The third major contribution to the understanding of the glycosylceramide hydrolases developed out of the use of artificial substrates to study the enzymes in vitro. Enzymes that hydrolyze synthetic, non-biological glycosides such as p-nitrophenyl-β-D-galactopyranoside, are widespread in nature. In mammalian tissues they invariably exhibit acid pH optima. This observation and the results of studies of their subcellular distribution led, in 1955, to the development of the concept of the "lysosome", ". . . a single population of enzymically homogeneous granules" (217). Lysosomes form an "intracellular digestive system" (218) which is involved in the continuous vacuolization and hydrolysis of material of exogenous or endogenous origin. These fascinating organelles are the subject of a monumental monograph edited by Dingle and Fell (219). In most cells in the body, particularly neurones, virtually all the material entrained and hydrolyzed by lysosomes is of endogenous origin (autophagy). These organelles, with their complement of acid hydrolases, play an important role in the breakdown and renewal of cell constituents. Conchie et al (220), using artificial substrates, demonstrated a vast number and the wide distribution of lysosomal glycosidases in mammalian tissues. The natural substrates for these enzymes were, for the most part, however, unknown. In 1963, Hers (221) showed that the accumulation of glycogen in the tissues of patients with Pompe's disease was the result of a deficiency of the lysosomal enzyme, "acid α-maltase". At about the same time, Austin et al (222) discovered that patients with metachromatic leukodystrophy lacked the lysosomal enzyme, arylsulfatase A (measured with the artificial substrate, 2-hydroxy-5-nitrophenyl sulfate). The relationship

between the enzyme deficiency and the accumulation of cerebroside sulfate in the disease was not, however, clearly understood. Nevertheless, the concept of "inborn lysosomal disease" (223) developed. These diseases are characterized by the progressive, widespread accumulation of substance(s) throughout the body, associated with a deficiency of one of the lysosomal acid hydrolases. In 1965, Patrick (224), using the artificial substrate p-nitrophenyl-β-D-glucoside, demonstrated a deficiency of β-glucosidase activity in the tissues of patients with Gaucher's disease (later challenged by Brady et al (215)). Two facts became obvious: 1) artificial substrates might be useful in the diagnosis and investigation of a number of "storage diseases", and 2) these substrates might prove useful in the purification of the enzymes involved in the catabolism of a variety of compounds, including the glycosphingolipids. According to the first hypothesis, once the structure of the material(s) stored in patients with "inborn lysosomal diseases" is known, one need only test for the enzymic hydrolysis of the appropriate synthetic structural analogue in order to establish the identity of the enzyme defect responsible for the disease. This was accomplished with great success in the diagnosis of G_{M1}-gangliosidosis (130), visceral Tay-Sachs (Sandhoff's) disease (94), and Fabry's disease (107), but failed in studies of other very similar diseases, such as Krabbe's globoid cell leukodystrophy (231), lactosyl ceramidosis (232), Gaucher's disease (215), and initially, Fabry's disease (237). In the case of Tay-Sachs disease, measurements of total nonspecific hexosaminidases revealed increased levels of activity in the tissues of affected patients. In 1969, Sandhoff (128) and Okada and O'Brien (126)

showed that Tay-Sachs disease was associated with a generalized deficiency of hexosaminidase A. When the total hexosaminidase activity was measured, the deficiency of hexosaminidase A was completely obscured by a marked increase in the activity of hexosaminidase B. An outline of the catabolism of the neutral glycosphingolipids and the location of the enzyme deficiencies responsible for various storage diseases is shown in Figure 1. Gatt (63) has pleaded an eloquent case for ceramidase occupying a central role in glycosphingolipid metabolism. On the basis of its pH optimum (pH 3.5-9, which is much wider than the pH optima of the catabolic enzymes) and easy reversibility, he postulated that it is "a common meeting-point" between the biosynthesis and catabolism of glycosphingolipids.

The value of artificial substrates for use in the purification of glycosylceramide hydrolases has been amply proved by the work of Gatt and his coworkers in Israel. In each instance in which they obtained a partially purified enzyme preparation active on the natural substrate (see Table III), the extraction, solubilization and steps in the purification were worked out initially using artificial substrates. There seems little doubt that studies on lysosomal hydrolases using artificial substrates often directly reflect properties of some of the glycosylceramide hydrolases. Nevertheless, some of the findings are paradoxical and, in some cases, unsettling. For example, the β-galactosidase Gatt and Rapport (238) purified using the artificial substrate was ineffective in the hydrolysis of galactocerebroside (228). Similarly, no deficiency of β-galactosidase, measured using p-nitrophenyl-β-D-galactopyranoside as the substrate, could be demonstrated in patients with Krabbe's disease, who exhibit profound deficiencies of

Figure 1. Outline of the biodegradation of neutral glycosphingolipids and related inborn errors of metabolism.



galactocerebrosidase (231). The problem of enzyme specificity is compounded by the demonstration of more than one enzyme in a tissue catalyzing the hydrolysis of a single artificial substrate (e.g. β-galactosidases (241), N-acetylhexosaminidases (239)) and conversely, by the demonstration of the accumulation of more than one compound in the tissues of a patient with a single enzyme defect (91,95,240). "One may ask how many enzymes there are for each substrate and conversely, how many substrates there are for each enzyme" - Gatt (63).

Not unexpectedly the properties of the various acid glycosidases are different when studied using the natural or artificial substrates. The pH optima, although always between 3.0 and 6.0 (characteristic of lysosomal hydrolases), are often different and dependent upon the buffer used (228,236). The enzymic hydrolysis of the natural substrates requires the presence of detergents. On the other hand, the hydrolysis of the artificial substrates may be inhibited by them (236). The requirements for a detergent in <u>in vitro</u> studies of glycosphingolipid catabolism are redolent of the auxiliary lipid requirements in studies of the immunochemistry of these compounds (206). The complexity of the matter is underscored by the recent demonstration of the stimulation of glycosylceramide hydrolase activity by adding the substrate to the enzyme, coated on a filter paper disk impregnated with lecithin (242)! Although most of the lysosomal hydrolases are very stable whether measured with the natural or artificial substrates, there is one striking exception. Whereas CTHase has been shown to be very labile (233,243), nonspecific α-galactosidase is very stable (this Thesis). This together with the differences in pH

optima (this Thesis) and the demonstration of a terminal β-D-galacto-pyranosyl residue in CTH (92), initially made it difficult to accept the identification of the α-galactosidase deficiency with the CTHase deficiency observed in patients with Fabry's disease. These findings are constant reminders that the results of studies done using artificial substrates cannot be extrapolated with alacrity to the metabolism of glycosphingo-lipids (244). Nor, indeed, can in vitro studies done using the natural substrates be assumed to reflect accurately the situation in vivo, particularly as regards the kinetics of the reactions.

4. Biological significance

Neutral glycosphingolipids appear to be important constituents of many membrane systems, particularly myelin. Apart from their contribution to the structures of these membranes, however, little is known about their biological significance. Nevertheless, during the past 20-25 years a growing body of evidence is accumulating to support the thesis that these lipids play an important role in cell-cell interaction.

In 1947, Tyler (245) and Weiss (246) postulated that specific chemical groups on cell surfaces contributed to the maintenance of cell adhesion in tissues, and influenced differentiation. The belief that intercellular adhesion might be mediated by antigen-antibody-type reactions stimulated a great deal of research into the nature and distribution of the blood-group antigens A and B. It soon developed that two different types of haptens existed, an "alcohol-soluble" type (i.e. lipid) and a "water-soluble" type (i.e. glycoprotein) (137,247). Moreover, these haptens, especially the

"alcohol-soluble" variety, were found to be present in a number of tissues besides erythrocytes (255). The antigenic specificity of both types of haptens was found to reside in complex oligosaccharides containing glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and fucose (137,247).

In 1961, Kay and Wallace (248) demonstrated a deletion of A- and B-haptens in human cancer tissue, in which cell-cell interactions are abnormal. In 1964, Hakomori and his group (131,249) demonstrated an accumulation of a particular Le^a-active glycosphingolipid in human cancer tissue. These discoveries suggested that malignant change was accompanied by defects in the synthesis of compounds important in intercellular interactions resulting in uncontrolled, chaotic cell proliferation, i.e. cancer. In a more definitive exploration of this hypothesis, Hakomori and Murakami (250) showed that in malignant-transformed cultured hamster fibroblasts there was an accumulation of lactosylceramide and decrease in the quantity of G_{M3} ganglioside, normally the main glycolipid of hamster fibroblasts. Moreover, there was a fairly close correlation between the G_{M3} ganglioside content and contact inhibitory properties of various lines of these cells. Loss of contact inhibition had long been thought the cytokinetic equivalent of malignant change. Hence the glycolipid composition of cells appeared to play an important role in ordered, multicellular growth. Hakomori's findings are supported by similar observations made in rat hepatocyte and hepatoma cell lines (251), and in mouse cell lines transformed by SV-40 or polyoma virus (252). In further studies of contact inhibition and glycosphingolipid metabolism, Hakomori (253) showed that in normal hamster kidney fibroblasts susceptible to contact inhibition, the concentrations

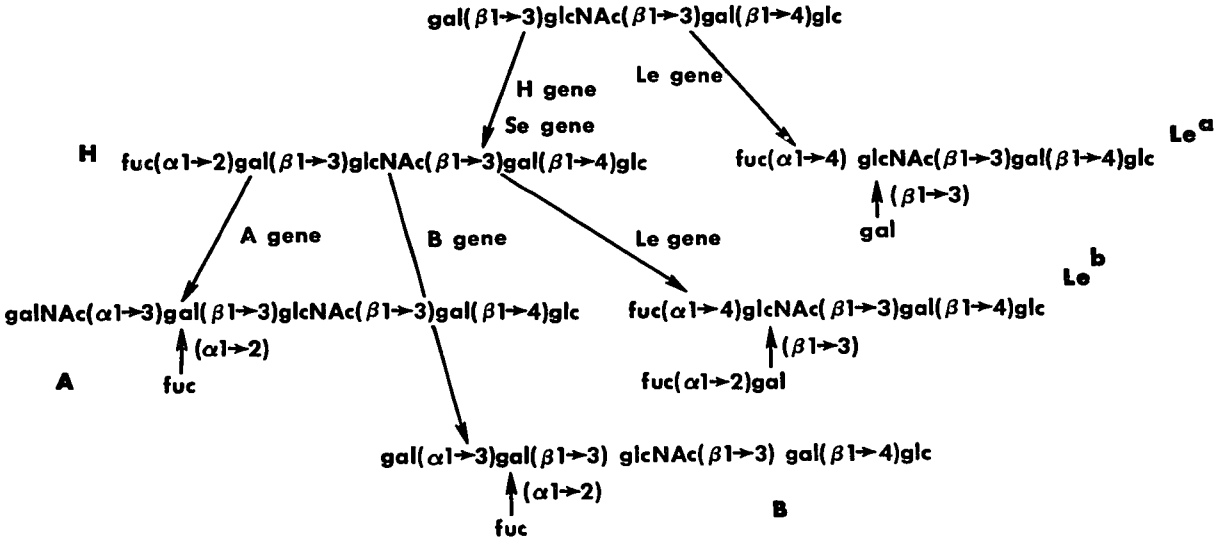
of CTH, G_{M3} ganglioside and G_{D3} ganglioside increased on cell-to-cell contact. In contrast, the concentrations of these lipids fell when susceptibility to contact inhibition is lost through repeated passages *in vitro* or transformation by SV-40 or polyoma virus. The concentration of these glycosphingolipids became independent of cell density in culture. He also demonstrated similar changes in human cultured fibroblasts in which G_{M3} and G_{D3} ganglioside concentrations are affected. Using radioisotopically labelled precursors, he demonstrated, in normal hamster fibroblasts, higher specific activities of the glycosphingolipids in confluent cultures than in growing cultures. He suggested that the biochemical concomitant of the loss of contact inhibition (e.g. by virus transformation) is a decrease in the synthesis or incomplete synthesis of the higher glycosylceramides.

These studies provide strong evidence that the glycosphingolipids in cell membranes play a part in the control of cell density. The mechanism by which this control is mediated, however, is still unknown.

The role of neutral glycosphingolipids with blood-group activity in the interaction of erythrocytes is a special case of cell-cell interaction in which the mechanism is fairly well understood. The reactions in this case are clearly of an immunologic nature. The subject of the immunochemistry of glycosphingolipids and their blood-group activities has been reviewed recently by Rapport and Graff (206) and Hakomori (137). The structures of the various blood-group antigens and their metabolic relationship is shown in Figure 2 derived in part from Kobata *et al* (254).

Figure 2. Outline of the biosynthesis of the oligosaccharides with blood-group activity in human milk (from Kobata *et al* (254)).

Biosynthesis of Oligosaccharides with Blood Group Activity



The role of the neutral glycosphingolipids in disease is completely unknown except for those illnesses caused by defects in glycosphingolipid metabolism. It is noteworthy that all the sphingolipidoses for which the mechanism is known are caused by defects in catabolism. This suggests that defects in the biosynthesis of the sphingolipids are incompatible with life, and is implicit testimony to the importance of these compounds in the general metabolism of the human organism - whatever their role must be. It seems at least theoretically possible, however, that partial defects in glycosphingolipid biosynthesis might occur and produce disease. Since the present methods for measuring the activities of the enzymes involved are highly artificial, these partial defects, which might be very significant in vivo, would likely go undetected. Diseases attributable to aberrations in glycosphingolipid biosynthesis will probably be discovered by studies of glycolipid turnover in vivo and through improvements in the measurement of the enzyme activities in vitro.

B. Fabry's Disease

1. Clinical features

In 1898, the British and German dermatologists, Anderson (255) and Fabry (256) independently described patients with a bizarre skin disorder characterized by punctate, blue-purple, angiokeratoma symmetrically distributed on the trunk and genital area. The disease has since become known as angiokeratoma corporis diffusum or Fabry's disease. The clinical features of the disease have been reviewed by Fessas, Wintrobe and Cartwright (257), Wise, Wallace and Jellinek (258) and Kahlke (259).

The disease is almost exclusively limited to males although a modified illness may occur in some women. The pathognomonic skin rash appears in early childhood and is probably the first clinical manifestation of the disease. It is inconspicuous, however, and is often overlooked. In middle childhood, the patient often develops severe, paroxysmal pains in the extremities and formication in the fingers and toes. The pain is excruciating and is frequently misdiagnosed as acute rheumatic fever or rheumatoid arthritis. Intolerance to heat and dyshidrosis may be described. Proteinuria usually develops by early adulthood. As the patient advances in age, the crises of pain become less frequent and less severe, and progressive renal and vascular disease dominate the clinical picture. Asymmetrical varicose veins and edema may develop on the legs. Males with the disease exhibit decreased fertility. Death due to renal failure or cerebrovascular or cardiac disease usually overcomes the patient in the fourth or fifth decade of life.

Although this is the usual clinical course of Fabry's disease, Hamburger et al (260) described two patients with renal lesions characteristic of the disease but lacking any of the other clinical manifestations. Clarke et al (261) added two more cases, but demonstrated, in addition, that they had biochemical abnormalities characteristic of Fabry's disease (107,150). These authors suggested that the disease might be occurring more frequently in the form of isolated renal disease than in the classical form with skin lesions. Since the characteristic skin lesions have been the basis of the recognition of almost all reported cases of Fabry's disease, their absence would almost surely obscure the true diagnosis unless special histochemical and biochemical studies were undertaken.

Although attention was originally concentrated on the cutaneous manifestations of the disease, even Anderson's original description contained clues to its systemic nature (255). His patient had proteinuria, varicose veins and edema of the legs. Fabry's original patient also developed proteinuria and puffy eyelids (262). Nevertheless, its generalized nature was not fully appreciated until 1947 when Ruiter's group published the results of the first systematic autopsies of patients who had died of Fabry's disease (263). They described bizarre lesions in many tissues throughout the body. The swelling and vacuolization of the glomerular epithelial cells in the kidney, cardiac muscle cells and smooth muscle cells in the walls of medium-sized and small arteries were particularly striking. A variety of histochemical studies offered little in terms of defining the material in the vacuoles beyond the suggestion that it was probably of a lipid nature. Since Ruiter's report, many studies of the pathologic changes in Fabry's disease have been reported. These have been reviewed by Kahlke (259). In general, they confirmed the generalized nature of the disease but added little more than Ruiter to the understanding of the basic defect responsible for the disease.

2. Genetics

Fabry's disease is transmitted as an X-linked disorder with variable penetrance in female carriers. In 1965, Opitz et al (264) reported on 17 kindreds (including 8 previously reported by Wise, Wallace and Jellinek (258)) and attempted to locate the mutant gene on the X-chromosome from linkage data. Their data and those reported by Johnston et al (265) showed that the mutant locus was about 27 centimorgans from the Xg locus

and very close to the glucose-6-phosphate dehydrogenase and deutan color-vision loci.

Although the pattern of the disease in affected males is relatively constant, the clinical manifestations of heterozygosity in carrier females are highly variable. Carrier women rarely exhibit the pathognomonic skin lesions of the disease. Nonetheless, they almost always have the characteristic corneal lesions (266) and excrete increased amounts of neutral glycosphingolipids in their urine (150,267). Unlike affected males, who excrete large excesses of CTH and smaller excesses of digalactosylceramide in their urine, the excess glycolipid in the urine of carrier women is principally a dihexosylceramide (267). The severity of renal impairment in female carriers is variable. Apart from the excretion of increased levels of glycolipids, many of them have no clinically significant kidney disease. Most exhibit a mild to moderate proteinuria. However, the renal lesions rarely become severe enough to shorten the patient's life. The most reliable test for heterozygosity is the measurement of enzyme levels in the tissues (e.g. duodenal mucosa or leukocytes) of suspected carriers (107,234).

3. Biochemical abnormality

In the reports on pathologic studies done by Scriba (268) and Ruiter (269), Kühnau is quoted as claiming that the lipid-like material distending the vacuolated cells found in sections from throughout the body in patients dying of Fabry's disease was, in fact, an aminophosphatide. Although definitive analyses of postmortem tissues were not published, Fabry's disease came to be considered a phospholipid storage disease.

In 1963, Sweeley and Klionsky (91) reported the first substantive evidence that Fabry's disease was an inborn error of neutral glycosphingolipid metabolism. They demonstrated large excesses of trihexosylceramide (CTH) and digalactosylceramide in the kidney of a young man who had died from the disease. In 1967, Brady et al (234) prepared tritium-labelled Fabry's CTH by the Wilzbach technique (270). With this substrate they were able to demonstrate a deficiency of CTH galactohydrolase in biopsies of the duodenal mucosa from living patients with Fabry's disease. In addition, a known heterozygous woman exhibited CTH galactohydrolase activities intermediate between those of affected patients and normal controls.

A number of papers appeared confirming and adding to the concept of Fabry's disease as an hereditary disease resulting from a defect in the catabolism of CTH and digalactosylceramide. Lou (271), Miyatake (93) and Schibanoff, Kamoshita and O'Brien (272) showed that the accumulation of CTH occurred in a number of tissues. The accumulation of digalactosylceramide appeared to be confined to the kidney (93) and possibly brain (272). Philippart, Sarlieve and Manacorda (267) and Desnick, Sweeley and Krivit (150) showed that the diagnosis of Fabry's disease or the carrier state could be made by analysis of the urinary sediment. Vance, Krivit and Sweeley (273) reported that the concentration of CTH in the plasma of patients with Fabry's disease was elevated. The neutral glycosphingolipid composition of the erythrocytes was normal. Matalon et al (274) demonstrated an accumulation of CTH in cultured skin fibroblasts from patients with Fabry's disease. They also described an increase in the concentration of

glycosaminoglycans. Since the increase in glycosaminoglycans was not identifiable with any specific class of these compounds, the authors felt it was a secondary phenomenon not directly related to the basic enzyme defect.

The next major advance in our understanding of Fabry's disease and the metabolism of CTH in general was Kint's discovery of a deficiency of nonspecific leukocytic α-galactosidase in patients with the disease (107). This was confirmed by Clarke et al (261). Romeo and Migeon (275) demonstrated the defect in cultured fibroblasts. They also showed that when the cells from a known carrier were cloned, two populations of cells were obtained: one with normal α-galactosidase activity, the other with no detectible enzyme activity. This is in keeping with the Lyon hypothesis concerning X-chromosome inactivation in the female (276), and supports the X-linked genetic character of the disease. Brady et al (277) made the prenatal diagnosis of Fabry's disease in a fetus at risk by demonstrating the α-galactosidase deficiency in cultured amniocytes. The pregnancy was terminated and the enzyme defect demonstrated in a number of fetal tissues.

The demonstration of an α-galactosidase deficiency in Fabry's disease posed a very perplexing problem. For years everyone studying the chemistry of glycosphingolipids had assumed that all the galactosidic linkages were of the β-stereoconfiguration. However, Brady et al (233) who purified an active CTH galactohydrolase from the intestinal mucosa of the rat, could demonstrate no activity of the enzyme towards known

β-galactosides (e.g. lactose). Known α-galactosides were not tested as substrates. The failure of the purified enzyme to hydrolyze low molecular weight β-galactosides was attributed to the unique specificity of the enzyme.

As part of a continuing study of glycosphingolipids in cancer, Kawanami, in 1967, extracted a CTH from the Nakahara-Fukuoka sarcoma, and he showed that it had a terminal α-D-galactopyranosyl residue (106). This was the clue that had stimulated Kint to undertake the studies which ultimately led to the discovery of the α-galactosidase deficiency in Fabry's disease. The discoveries of Kawanami and Kint were considered strong presumptive evidence that the CTH that accumulates in the tissues of patients with Fabry's disease also contains a terminal α-D-galacto-pyranosyl residue. The nmr studies by Sweeley, Snyder and Griffin (92), which appeared to show that all the glycosides of CTH extracted from a patient who had died of Fabry's disease were of the β-stereoconfiguration, were therefore greeted with surprise and puzzlement. Sweeley and his group claimed, on the basis of their findings, that CTH galactohydrolase was really a β-galactosidase, and that the α-galactosidase deficiency reported in patients with Fabry's disease was merely a secondary phenomenon. The relationship between the accumulation of CTH, the deficiency of CTH galactohydrolase and the deficiency of nonspecific α-galactosidase in Fabry's disease, posed something of a riddle. The solution of this conundrum was one of the aims of the work reported in this Thesis.

C. Nuclear Magnetic Resonance Spectrometry

During the last fifteen years, the application of nmr spectrometry to the elucidation of the structures of a wide variety of organic compounds has become routine in most laboratories conducting research in organic chemistry. Biologists have been slower to realize the potential of this technique. Nevertheless, in the last few years the introduction of high resolution nmr spectrometers has produced a surge of investigative activity in the study of high molecular weight compounds of biological interest (278). Since nmr spectrometry was one of the tools used in the investigations described in this Thesis, and since it appears to hold considerable promise for the investigation of other biological problems in the future, a brief review of the theory and how it is applied to the determination of the anomeric configuration of carbohydrates seems appropriate at this point.

Atomic nuclei with odd-numbered masses (e.g. ^{1}H, ^{13}C, ^{15}N, ^{17}O, ^{19}F, ^{31}P), and nuclei of even mass but odd atomic number (e.g. ^{2}H, ^{10}B, ^{14}N) possess magnetic moments (μ). This discussion will be confined to the nmr spectra of hydrogen (^{1}H), sometimes called proton magnetic resonance (pmr) spectrometry.

When placed in a magnetic field, H_o, the proton, which acts like a tiny spinning magnet, tends to become oriented either in the same direction as the field, or against it. Because it is spinning, the nuclear magnet precesses about the direction of H_o with an angular velocity of ωo which is a function of H_o and the gyromagnetic coefficient, γ, of the proton.

(The gyromagnetic coefficient is a constant defined by the spin number I, magnetic moment, μ, and nuclear magneton, β_N, of any paramagnetic nucleus). Transitions between the state in which the nuclear magnet is oriented in the same direction as H_o and the state in which it is oriented against the field (defined in terms of the spin number as I=+1/2 and I=-1/2 respectively), can be induced by the application of a second magnetic field perpendicular to H_o and oscillating with a frequency, ν, equal to ωo. In practice, the second magnetic field is applied by the introduction of a coil in which an oscillating magnetic field at right angles to H_o is induced with a radiotransmitter (r.f. oscillator). The frequency of the r.f. oscillator, ν, is held constant at a value characteristic of the spectrometer. H_o is then varied until ωo is almost equal to ν - a situation of resonance. Energy is absorbed by the proton as it "flips" from I=+1/2 to I=-1/2. The absorption of energy is detected by the increase in the flow of current through the coil, and is measured with an ammeter.

In most organic compounds, the protons are surrounded by electrons. The circulation of electrons exerts a "shielding" effect on the nuclear magnet, the spinning proton. That is, because of the protective "electron cloud" about the proton, the effective magnetic field, H , is reduced. Hence, a well-shielded proton resonates at a higher magnetic field strength, H_o, than an unshielded proton. The degree of shielding of any given proton in a compound is determined by 1) the electronegativity of adjacent nuclei, and 2) the spatial relationship of the proton to certain functional groups, such as olefinic, acetylenic or aromatic groups. For example, strongly electronegative nuclei, such as oxygen, exert a deshielding effect on

protons causing a downfield shift in their resonance frequencies. These types of shifts are called "chemical shifts". They are always measured by reference to a standard, most commonly the absorption of tetramethylsilane, and they are a function of the frequency of the r.f. oscillator of the particular spectrometer. The amplitude of the signal is directly related to the number of protons with the same chemical shift. The degree of shift is measured in parts per million (ppm), which is the ratio of the field strength at which the proton absorbs (because $\omega o = \gamma H$, it can be expressed in Hertz) to the frequency of the r.f. oscillator (ν). The chemical shift of tetramethylsilane is arbitrarily defined as $\delta = 0$ ppm or $\tau = 10$ ppm, depending on the convention used.

In pmr spectra single protons are often represented by more than one spectral line. This "splitting" is caused by changes in the total magnetic field, H , produced at a given proton by the magnetic moments of neighboring protons and transmitted to it by the bonding electrons. The spacing of these peaks, J, measured in Hertz, is independent of ν because the magnetic moments of the adjacent protons are independent of it. The number of peaks representing any given proton is a function of the number of neighboring protons influencing it. In situations where first-order rules apply, the number of peaks is equal to one more than the number of neighboring protons. Hence, the spectrum of each proton in a compound can be defined 1) in terms of its chemical shift (δ or τ), and 2) in terms of the degree and magnitude of line splitting (J) caused by "spin-spin coupling".

Several excellent monographs have been written which describe the physical basis and application of nmr spectrometry in detail (279,280,281).

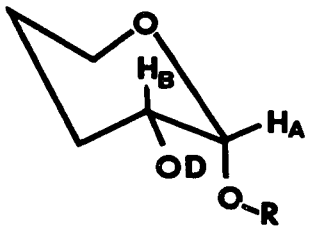
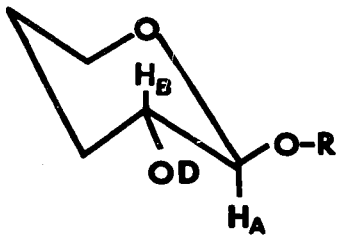
For the purpose of interpretation of nmr spectra, particularly with reference to their anomeric configuration, glycopyranosides can be thought of as rigid, substituted cyclohexanes. On the basis of free energy calculations (282) and studies of the formation of complexes (283), the C 1 (D) conformation of D-glucopyranose (as shown in Figure 3) is more stable than the corresponding alternative one, 1 C (D). Although α-hexopyranosides are less stable in the C 1 (D) conformation than the corresponding β-hexopyranosides, Onodera et al (284) have shown that the α-glycosides of D-mannose, L-rhamnose and D-glucose (and presumably also D-galactose) exist mainly in the C 1 (D) conformation in D_2O and in dimethylsulfoxide-d_6.

In rigid, unsubstituted cyclohexanes, equatorial protons absorb downfield of axial protons due to the spatial effect of the carbon-carbon bonds (286). The absorption of the anomeric protons (H-1) of hexopyranosides is shifted further downfield by the deshielding effects of the two oxygen atoms linked to C-1 (285,288). The result is 1) the anomeric protons absorb in a region of the nmr spectrum relatively free of interference by the absorption of the other ring protons, and 2) the anomeric proton of an α-glycoside, because it is an equatorial proton, absorbs downfield of the anomeric proton of a β-glycoside, in which it is axial.

Due to the difference in the angular relationship between the bonds to the protons and the carbon-carbon bond, the coupling constant between H-1 and H-2 is smaller for axial-equatorial protons than for diaxial

Figure 3. The principles of the determination of the anomeric configuration of glycopyranosides by nuclear magnetic resonance spectrometry. Abbreviations: $\Delta\nu_A$= chemical shift of H_A measured with reference to tetramethylsilane; J_{AB}= the coupling constant of H_A with H_B.

Nuclear Magnetic Resonance Spectrometry of Glycopyranosides in D_2O

	Linkage	$\Delta\nu_A$ (ppm)	J_{AB} (Hz)
	α	δ = 5.12±0.33	3.2±0.6
	β	δ = 4.44±0.06	7.2±0.2

van der Veen 1963

protons (286,287). Hence, the spacing of the splitting of the spectral line of H-1 in α-glycosides is smaller than the spacing in β-glycosides*. In 1963, van der Veen (285) surveyed the nmr spectra of a number of hexopyranosides with particular attention payed to the spectral characteristics of the anomeric proton. His findings are summarized in Figure 3.

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*This is only true if H-2 is axial as shown in Figure 3. In D-glucopyranose and D-galactopyranose H-2 is axial, whereas in D-mannose it is equatorial, for example, and the coupling constants of H-1 of both anomers, α-mannosides and β-mannosides, are small (286,287).

II. MATERIALS AND METHODS

A. Clinical Material

1. Case presentations

Cases 1 and 2 were referred to me by Dr. J. C. Crawhall, Department of Clinical Biochemistry, Royal Victoria Hospital, Montreal. The results of preliminary studies on these patients have been reported (261). Invaluable assistance in tracing the relatives of A.H. (Case 2), and in obtaining urine specimens from two of them for analysis of the glycolipids was provided by Dr. M. W. Spence, Department of Pediatrics, Dalhousie University, Halifax. Dr. Gilles Paré of Sherbrooke, Quebec, kindly made it possible for me to study N.T. (Case 3).

Case 1. R.C., a 39-year old Italian immigrant, was admitted to the Royal Victoria Hospital in 1969 with a 7-year history of proteinuria and mild fatigability. He denied any family history of kidney disease or persistent skin rashes.

Physical examination revealed moderate dilatation and tortuosity of the conjunctival vessels. The retinal vessels and corneas were normal. He had moderate, bilateral, asymmetrical varicose veins of the legs. There were no skin lesions or angiokeratomas, or telangiectasia of the buccal mucosa.

Routine laboratory procedures revealed that he was moderately polycythemic and had moderately severe hyperlipidemia shown by electrophoresis to be due to a marked increase in the pre-beta lipoproteins. Urinalysis showed moderate proteinuria (excretion of 2.25 to 5.06 g per day), and oval fat

bodies and many hyaline and granular casts in the urinary sediment. The blood urea nitrogen was 7.5 mg and serum creatinine 1.3 mg per 100 ml. The endogenous creatinine clearance was 97-98 ml per minute. Other tests of renal function were normal.

An open renal biopsy was performed. The histologic, histochemical and electron microscopical findings were characteristic of the renal lesion of Fabry's disease (290).

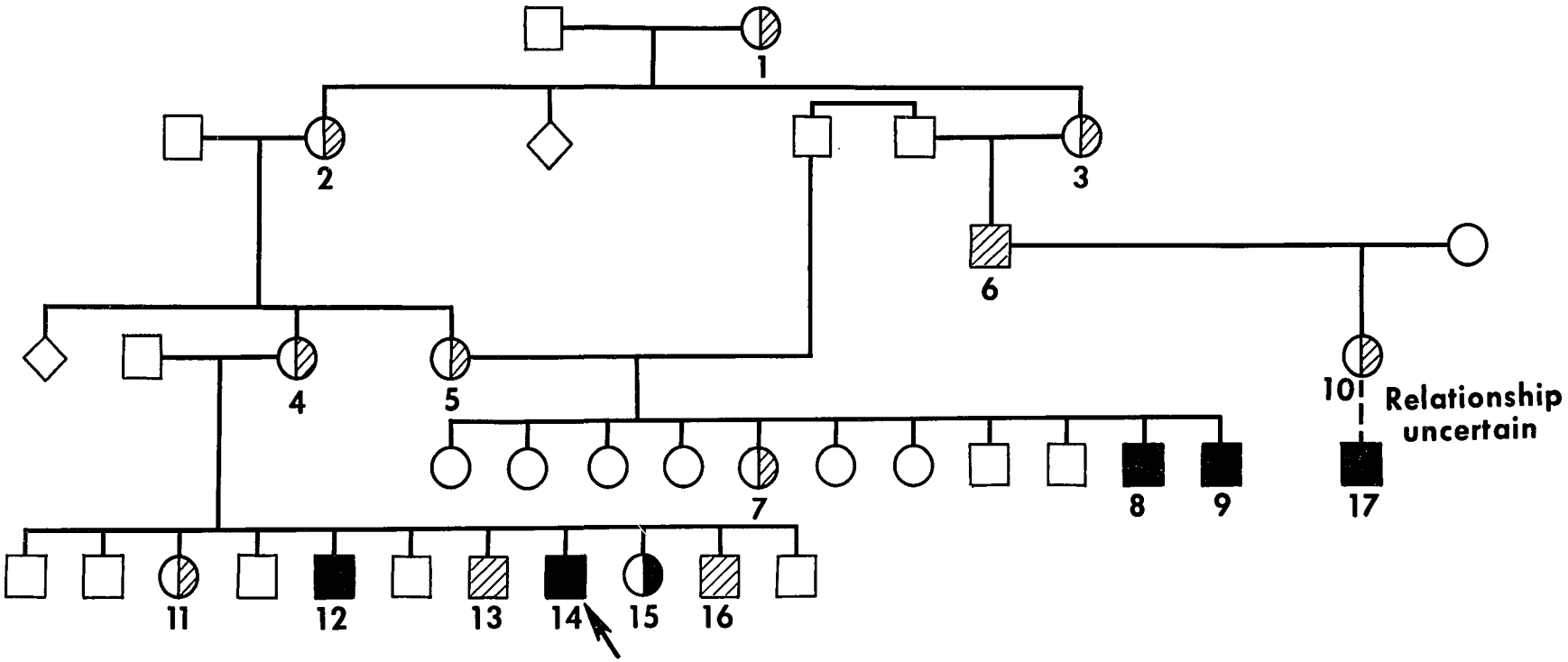
Case 2. A.H., a 48-year old man, presented with a history of vomiting, fatigability and weight loss dating from an episode of smoke inhalation suffered in a fire 2-1/2 months prior to admission.

He came from a large family in which 3 of his 8 brothers had died in middle age of unknown causes (Figure 4). One (No. 12) died at age 58 years with the typical clinical and pathological features of Fabry's disease. (This was determined retrospectively by an examination of the hospital records and autopsy findings undertaken by Dr. Spence). Two cousins (Nos. 8 and 9) were described by Bethune et al (323) in 1961.

Physical examination revealed a well built man with a generalized papular, nonpigmented, cutaneous eruption on the face but no other skin lesions. He was moderately hypertensive and had mild hypertensive retinopathy. The conjunctivas, corneas and lens, however, were normal. He had mild pitting edema of the ankles and feet.

Urinalysis showed a specific gravity of 1.006, a pH of 7.5, moderately severe proteinuria (excretion of 4.76 to 8.36 g per day) and mild polyuria

Figure 4. Partial pedigree of A.H. (Case 2).


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Relationship uncertain
Sex unknown
Affected male
Probably affected
Probably carrier female
Known carrier female
Healthy male

(volume of 2000 to 3500 ml per day). The blood urea nitrogen was 53 to 126 mg, and the serum creatinine 7.0 to 9.4 mg per 100 ml. The endogenous creatinine clearance was 12 ml per minute. The patient had mild hyperglycemia which was easily controlled by the administration of chlorpropamide.

An open surgical biopsy of the kidney disclosed histologic and histochemical changes similar to those seen in Case 1 except that they were much more severe (Figure 5). Unlike Case 1, this patient had advanced vascular lesions in the medium-sized arteries in the kidneys. The findings (Figure 5) were typical of the lesions found in patients with Fabry's disease (290).

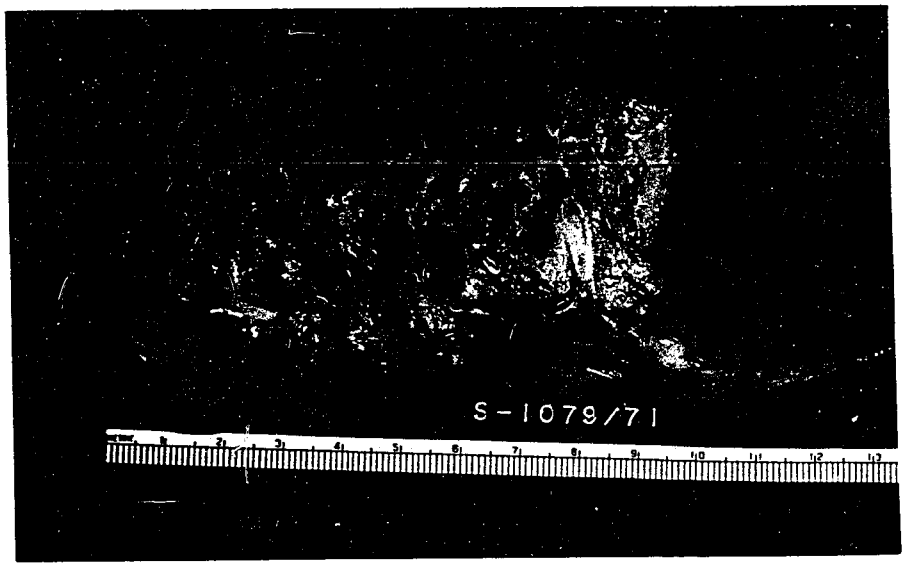
The patient initially improved clinically on hydrochlorothiazide, alph-methyl dopa and chlorpropamide. After 6 months, however, he began to deteriorate rapidly. He required repeated peritoneal dialysis and finally regular hemodialysis because of his renal failure.

On February 26, 1971 he underwent bilateral nephrectomies. The kidneys were contracted and a uniform pale yellow in color (Figure 6). The histologic findings confirmed those found in the biopsy taken less than a year before. We received one kidney less than 15 minutes after its excision and undertook the enzymic studies described on page 89 and following. Most of the tissue was frozen and stored at -20° for the chemical studies presented in Table XV.

Figure 5. Upper. Histologic appearance of a renal biopsy from a patient with Fabry's disease (Case 2). Two glomeruli are seen. Both are moderately hypercellular. Virtually all the glomerular epithelial cells have been converted into foamy, vacuolated storage cells. Many of the tubular epithelial cells are similarly affected. Foam cells are discernible in the lumens of the renal tubules. The glomerulus on the right has undergone secondary hyalinization. The biopsy also revealed advanced vascular lesions (not shown) in the medium-sized arteries. The media was irregularly thickened by vacuolated smooth-muscle cells and large hyalinized nodules. The intima was thickened by vacuolated endothelial cells. Periodic acid-Schiff stain.

Figure 6. Lower. Appearance of the cut surface of a kidney obtained at operation from a patient with Fabry's disease (Case 2). Gross: Each kidney weighed about 100 g and was about 10 x 5 x 3.5 cm in size. The outer surface was finely nodular and a uniform pale yellow color. The parenchyma was very soft and "flabby". The cut surface was a uniform pale yellow color with a total loss of corticomedullary distinction. The pelvis was mildly dilated and the pelvic and ureteral walls edematous. Microscopic (not shown): Compared with the findings in the biopsy done about one year prior to nephrectomy (Figure 5), the alterations in the microscopic appearance of the kidney had approached an "end-stage". Nearly all glomeruli were converted into amorphous, unevenly staining eosinophilic material. Most of the tubules were very small or disintegrated. The arteries were severely narrowed by intimal proliferation and fibrosis. There was a severe inflammatory reaction in the ureteral and pelvic mucosa, and numerous ill-defined patches of mononuclear infiltration were present in the kidney parenchyma.

All the pathologic studies described in this Thesis were done by Dr. J. Knaack, Dept. of Pathology, McGill University, Montreal, and his staff. His assistance and cooperation were an invaluable prerequisite to the satisfactory completion of this project.


S-1079/71

On April 8, 1971 the patient received a cadaveric renal homograft from an unrelated donor*. He was treated with azathioprine and prednisone. He suffered an early bout of pyelonephritis that responded to antibiotic treatment. About two months after the transplantation the patient experienced a minor rejection crisis which was successfully aborted by increasing the dosage of immunosuppressive drugs. Periodically before and after renal transplantation blood samples and urine were collected for the analysis of plasma and urinary glycosphingolipids.

The patient had been doing well when suddenly 6 months after renal transplantation he became febrile and disoriented. He expired less than two weeks later from a gram-negative septicemia.

Case 3. N.T., a 14-year old boy, adopted in infancy by his present parents, exhibited the characteristic clinical features of Fabry's disease from early childhood. He had the pathognomonic skin and ocular lesions, and suffered severe paroxysmal pain in his limbs which had for some years been variously attributed to rheumatic fever or "rheumatism".

Case 4. J.L., a 46-year old Alsatian with typical clinical and biochemical features of Fabry's disease, was extensively investigated in France and

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*The renal transplantation and subsequent care of the patient were supervised by Dr. R. D. Guttmann, Director, Transplantation Service, Royal Victoria Hospital, Montreal. The excellent cooperation provided by him and his staff in the subsequent biochemical studies described in this Thesis is gratefully acknowledged.

reported by Wolfe et al (291). He died of renal failure. Kidney tissue taken at autopsy was frozen and stored at -20° until extraction and analysis. The tissue from this patient provided the Fabry's CTH used to compare with CTH extracted from normal kidney.

2. Clinical biochemical studies

a) Analysis of glycosphingolipids in plasma and urinary sediment

The plasma and urinary glycosphingolipid levels were determined by the methods described by Vance and Sweeley (119) and Desnick, Sweeley and Krivit (150) respectively. The general procedure for both was the same. The plasma or urinary sediment was collected by centrifugation and in the case of the urinary sediment was washed once with 0.9 percent NaCl. The lipids were extracted with chloroform-methanol and partitioned against 0.1 M KCl. The lower phase lipids were then chromatographed on 4 gram columns of silicic acid (Bio-Sil HA, -325 mesh) eluted with 100 ml chloroform, 150 ml acetone-methanol (9:1, v/v) and 100 ml methanol. The acetone-methanol fractions, which contained all the neutral glycosphingolipids, were subjected to mild alkaline hydrolysis and finally separated into classes based on the number of sugar residues in the oligosaccharide moieties by preparative thin-layer chromatography using System A. The lipids, visualized by exposure to I_2 (Figure 7), were scraped from the plates and eluted from the silica gel with chloroform-methanol-water (10:10:1, v/v/v). The lipids were then subjected to anhydrous methanolysis (p.75). The fatty acid methyl esters were removed by extraction with n-hexane and the methyl glycosides analyzed as the TMSi derivatives by

Figure 7. Preparative thin-layer chromatograms of urinary neutral glycosphingolipids. The lipids were extracted and purified as described in the text. The tlc was carried out on 0.5 mm thick layers of silica gel H activated for 18-20 hrs at 125°. The chromatograms were developed with $CHCl_3$-CH_3OH-H_2O (100:42:6, v/v/v) and the lipids visualized by exposure to I_2 vapor. The various glycolipids were identified by reference to appropriate standards: GL-1=CMH, GL-2=CDH, GL-3=CTH, GL-4=CAH. J.W. and A.S. are normal controls; R.C. and A.H. are patients with Fabry's disease (Cases 1 and 2 respectively).

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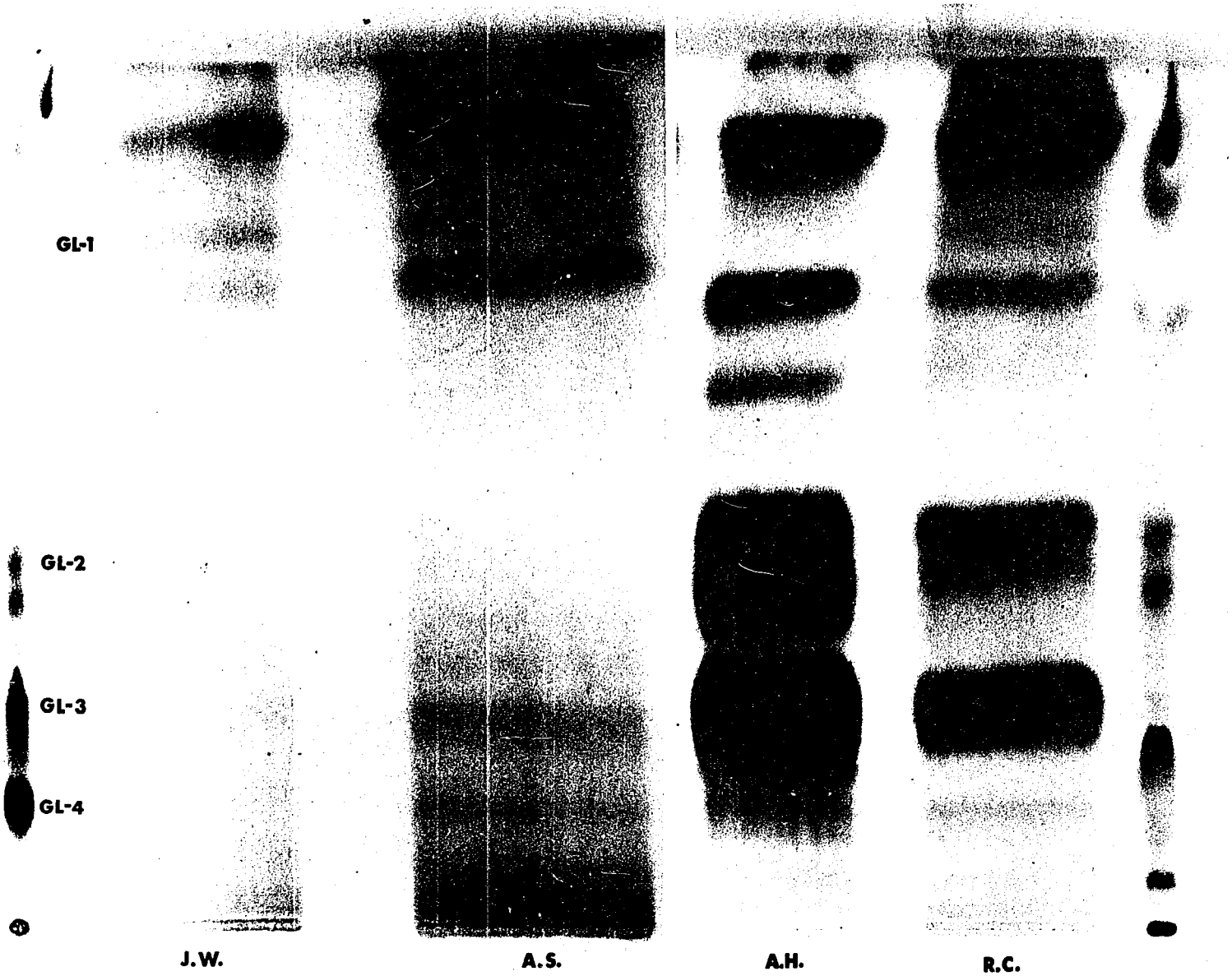
GL-1
GL-2
GL-3
GL-4
J.W.
A.S.
A.H.
R.C.

gas-liquid chromatography using D-mannitol as an internal standard (p. 76).

b) Measurement of leukocytic α-D-galactosidase

Peripheral leukocytes were prepared from 10 ml of heparinized blood by sedimentation in heparinized dextran-saline (292). The cells were washed twice with 1 ml 0.9 percent NaCl, then suspended in enough of the same saline solution to make the leukocyte concentration 2.0-6.0x10^4 cells per microliter (i.e., 1-2 ml 0.9 percent NaCl). The leukocytes were then either counted with the aid of a hemocytometer, or the total protein measured by the Lowry technique (293).

Initially, the α-galactosidase was measured by a method similar to that described by Kint (107) using p-nitrophenyl-α-D-galactopyranoside as the substrate (colorimetric). Later in the studies, the 4-methylumbelliferyl derivative was substituted (fluorimetric). The incubation mixtures for the various assays included (i) for α-galactosidase: colorimetrically, 6 μmoles of p-nitrophenyl-α-D-galactopyranoside, 20 μmoles of acetate buffer (pH 4.5), and 20 μl of leukocyte preparation in a final volume of 0.5 ml; and fluorimetrically (modification of method described by Callahan (294)), 5 μmoles of 4-methylumbelliferyl-α-D-galactopyranoside, 65 μmoles of acetate buffer (pH 5.0), 50 μg of sodium taurocholate, and 20 μl of leukocyte preparation in a final volume of 1.0 ml; (ii) for β-galactosidase: (according to Callahan (294)), 1 μmole of 4-methylumbelliferyl-β-D-galactopyranoside, 65 μmoles of acetate buffer (pH 5.0), 50 μg of sodium taurocholate, and 20 μl of leukocyte preparation in a

final volume of 1.0 ml. All incubations were carried out at 37°. At the end of the incubation (1 hr for β-galactosidase, 2 hr for α-galactosidase), the assays in which the colorimetric substrate was used were terminated by the addition of 2.5 ml of 0.1 M glycine-NaOH buffer, pH 10.5. The samples were centrifuged and the optical density of the supernatant measured at 410 nm. The optical densities of enzyme and substrate blanks were subtracted and the p-nitrophenol present calculated by reference to appropriate standards. In the assays in which the fluorimetric substrates were used, the incubations were terminated by cooling and adding 1.5 ml of ice-cold 2.8 percent trichloroacetic acid. The samples were centrifuged and the supernatants added to 0.9 ml aliquots of 0.5 M NaOH. The fluorescence was measured with a Turner Model 110 fluorometer equipped with a 7-60 primary filter, 2A and 47B secondary filters, and a 10 percent neutral density filter. The fluorescence of the enzyme and substrate blanks was subtracted and the 4-methylumbelliferone present calculated by reference to appropriate standards.

B. Extraction and Purification of CTH from Human Kidney

1. General analytical methods

Glycolipid hexose was determined by the anthrone method described by Roe (295) or the orcinol method of Svennerholm (113). In the case of pure glycolipids, standards containing the theoretical molar ratio of glucose to galactose were used; otherwise measurements were based on standards of galactose.

Ganglioside sialic acid was estimated by the Miettinen and Takki-Luukkainen modification (296) of the resorcinol method described by Svennerholm (307). Crystalline N-acetylneuraminic acid (Sigma) was used as the standard.

Protein was measured by the method described by Lowry et al (293) using crystalline bovine serum albumin (Sigma) as the standard.

Lipid phosphorus was determined by digesting an aliquot of the lower phase lipids with 70 percent perchloric acid and measuring the resulting inorganic orthophosphate spectrophotometrically as the reduced phosphomolybdic acid complex (297).

2. Thin-layer and paper chromatography

a) System A

Most of the thin-layer chromatography, analytical and preparative, was carried out using the solvent system chloroform-methanol-water (100:42:6, v/v/v) described by Sweeley and Klionsky (91). Plates of silica gel G or H were washed with chloroform-methanol (2:1), air-dried, then heated at 125° for at least 18 hours. Saturation tanks, lined with filter paper, were prepared 2 hours prior to use. Following development, the lipids were visualized either by exposure to I_2 vapor, or by spraying with orcinol spray*. Orcinol spray stains glycolipids purple, cholesterol red,

*Orcinol spray = mixture of equal volumes of 0.2 percent orcinol in ethanol and 20 percent aqueous H_2SO_4.

and any other lipids yellow-brown after heating at 110° for 10 min.

b) System B

A two dimensional tlc technique was also used to check the purity of glycosphingolipids. The plates were prepared as described for System A. The sample (100-200 μg of lipid) was spotted in one corner 3 cm from each edge of the plate. The plate was developed first with chloroform-methanol-water (100:42:6, v/v/v), air-dried for 20 minutes, rotated 90° and developed in the second direction with tetrahydrofuran-methylal-methanol-4M aqueous NH_3 (50:25:25:5, v/v/v/v) (298). The lipids were visualized with orcinol spray.

c) System C

The purity of the commercially obtained ox brain galactocerebroside was checked by separating the gluco- and galactocerebrosides by tlc on borate-impregnated plates according to Young and Kanfer (299) using the solvent system chloroform-methanol-water (65:25:4, v/v/v).

d) System D

Descending paper chromatography of the glycosphingolipids was carried out using a technique developed by Michalic and Kolman (300). An aliquot (100-200 μg) of a lipid sample was spotted on a 10x46 cm strip of Schleicher and Schüll No. 289 silica gel-impregnated paper and developed for 90 min with chloroform-methanol-water (48:6.4:0.4, v/v/v) in a tank previously equilibrated for one hour with the solvent mixture. The

chromatographs were dried with a hair dryer, rolled and soaked in 0.001 percent Rhodamine 6G in 0.25 M K_2HPO_4 for 1 hour. They were then rinsed thoroughly with tap water, dried again with a hair dryer and viewed under ultraviolet light. The lipids fluoresce yellow.

3. Extraction and purification of CTH from human kidney

CTH was extracted from pooled normal human kidney tissue obtained at autopsy, and from the kidney of a patient who had died of Fabry's disease (Case 4). The tissues were stored at -20° until extraction. The procedure used was essentially the same as that described by Martensson (59) and outlined in Table IV.

The tissue was dehydrated by homogenization and extraction with 4 volumes of acetone at 2° for 2 days, filtration and re-extraction of the residue for another 3 days at 2°. The final residue was dried *in vacuo*, then suspended in 20 volumes of chloroform-methanol (2:1, v/v) and stirred at room temperature for 48 hours. Following filtration the residue was refluxed in another 20 volumes of chloroform-methanol (2:1) for 8 hours. The extract was filtered and the filtrate combined with the first chloroform-methanol extract.

The extracts were concentrated *in vacuo* and 1 N KOH (1 ml/4 g fresh tissue) added. Enough chloroform was introduced to make a two-phase system, and the whole was incubated at 37° for 45 hours. It was then neutralized with concentrated HCl added dropwise while shaking the mixture vigorously. The lipids were then extracted by partitioning with

Table IV: Outline of the procedure for the extraction of human renal neutral glycosphingolipids (modified from Martensson (59)).

1. Dehydration of tissue with acetone
2. Exhaustive extraction with chloroform-methanol (2:1)
3. Mild alkaline hydrolysis
4. Partition between water and chloroform-methanol (2:1)
5. Precipitation of glycosphingolipids with acetone
6. Silicic acid chromatography
7. DEAE-cellulose chromatography
8. Preparative thin-layer chromatography

4 volumes of chloroform-methanol (2:1). The lower phase was evaporated to dryness and the residue dissolved in 50 ml chloroform-methanol (98:2). Several volumes of ice-cold redistilled acetone were added and the resulting precipitate collected by passing the mixture through a fine sintered glass filter. The residue was washed with cold acetone, then dissolved in chloroform-methanol (2:1).

An initial purification of the glycosphingolipids was accomplished by silicic acid column chromatography. The neutral lipid contaminants were eluted with chloroform, the glycosphingolipids with acetone-methanol (9:1, v/v) and any remaining phosphatides with methanol (91). By the employment of a second silicic acid column eluted with a stepwise gradient of methanol in chloroform (4, 10, 20 and 33 percent respectively), partial separation of the glycosphingolipids on the basis of the number of sugar residues in the oligosaccharide moieties was achieved.

Contaminating sulfatides (O-β-D-(3-O-sulfate)-galactosylceramide and O-β-D-(3-O-sulfate)-galactosyl-(1→4)-O-β-D-glucosylceramide) were removed by DEAE-cellulose chromatography (301). The glycosphingolipid-containing fractions dissolved in chloroform-methanol (2:1) were passed through 5 g columns of DEAE-cellulose (Cellex D, Bio-Rad) made up in chloroform-methanol (2:1). The neutral glycosphingolipids were eluted with 100-150 ml of the same solvent. The columns were then washed with 100 ml chloroform-methanol (2:1) containing 5 percent glacial acetic acid, and the sulfatides were finally eluted with 100 ml chloroform-methanol (2:1) containing 5 ml aqueous 0.5 N LiCl.

The final purification of the neutral glycosphingolipids was achieved by preparative thin-layer chromatography. This was first done on 1 mm layers of silica gel G spotted with about 25 mg of lipid per plate and developed with chloroform-methanol-water (100:42:6, v/v/v). The crude CTH was freed of all contaminating lipids by final preparative thin-layer chromatography on 0.5 mm layers of silica gel H using the same solvent system.

Traces of silica gel were removed by washing the CTH dissolved in chloroform-methanol (2:1) with 0.2 volume of distilled water. At this stage the lipid was easily crystallized from a concentrated solution in chloroform-methanol (2:1) by cooling from 55° to 23° and letting stand 2-3 hours.

4. Compositional analysis

a) Methanolysis

The carbohydrate and fatty acid portions of the glycosphingolipids (notably CTH) were analyzed by the method described by Vance and Sweeley (119). Aliquots of the lipid (1-3 mg) were evaporated to dryness in 13x100 mm culture tubes with screw-top Teflon-lined caps. Three milliliters of 0.6-0.8N fresh anhydrous methanolic HCl, prepared by bubbling anhydrous HCl through redistilled methanol, were added and the tubes sealed. The samples were heated at 80° for 18-20 hr, cooled, opened and 0.2 ml H_2O and 0.1 μmole D-mannitol in 0.2 ml 99 percent aqueous methanol added to each. The fatty acids methyl esters were extracted three times

with 3 ml portions of n-hexane. These were evaporated to dryness with a stream of N_2 and analyzed by GLC. The aqueous lower phases were neutralized with 0.5 g Ag_2CO_3 (308) and centrifuged to remove the AgCl and unreacted Ag_2CO_3. The supernatants were evaporated to dryness with a stream of N_2 and the residues dissolved in 100 µl TriSil Z (Pierce). After heating at 70-80° for 15-20 min the samples were cooled and the TMSi ethers of the methyl glycosides analyzed by GLC.

Gaver and Sweeley (37) discovered that the artifactual formation of 3-O-methyl sphingosines and other products which occurs during the anhydrous methanolysis of sphingolipids was substantially reduced by making the reaction mixture 10 M with respect to water. The same modification was introduced in our experiments to analyze the LCB composition of CTH. Following the hydrolysis of aliquots of the lipid (1-3 mg), the reaction mixtures were extracted with n-hexane, neutralized with Ag_2CO_3 and the LCB's analyzed by GLC as the TMSi derivatives.

b) Gas-liquid chromatography

The GLC analysis of the TMSi derivatives of methyl glycosides, long-chain bases, and fatty acid methyl esters was carried out on an F and M Model 402 gas-liquid chromatograph equipped with a 6-foot x 1/8 inch (i.d.) glass column of 3 percent SE-30 on Gas Chrom Q, 80-100 mesh, and a flame ionization detector. The carrier gas was helium at 20 psi and 50 ml/min.

For the analysis of methyl glycosides, the column was operated isothermally at 160°. The analysis of fatty acid methyl esters was conducted at a column temperature of 195°. The analysis of long-chain bases was done at 200°. In all cases, the inlet temperature was maintained at 245° and the detector at 290°.

The area of each peak on the chromatogram was calculated by triangulation or by polar compensation planimetry. The amounts of methyl glycosides in the samples were determined by reference to an internal standard of D-mannitol. Like Vance and Sweeley (119), we found that the detector response to TMSi mannitol was 1.25 times that of the TMSi methyl glycosides. The appropriate correction was made in the calculations of quantities of methyl glycosides, e.g.

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

5. Infrared spectrophotometry

Infrared spectra of CTH were recorded in the Department of Endocrinology, Royal Victoria Hospital on a Perkin-Elmer Model 221 spectrophotometer with the technical assistance of Mr. H. Kallou. Approximately 400 µg of CTH were dispersed in 30 mg of KBr and pressed into translucent pellets to record the spectra.

6. Mass spectrometry

Mass spectra of the CTH were recorded as the trimethylsilyl derivatives on an LKB Model 9000 mass spectrometer as described by Sweeley and

Dawson (302)*.

The samples were prepared by heating 0.2-0.5 mg aliquots of the dry lipid with 50 µl of TriSil Z for 30 min at 80°. After cooling, 3-5 µl aliquots of the reaction mixture were transferred to the mass spectrometer probe and blown to dryness with a fine stream of dry nitrogen. The probe was introduced into the mass spectrometer operated at 3500 volts and 70 eV electron energy with a 60 µamp electron current and ion source temperature of 290°. Several spectra were recorded at various probe temperatures between 80° and 160°.

7. Optical rotations

The specific optical rotations of the glycosphingolipids were measured in pyridine at room temperature using a Zeiss automatic polarimeter with a 1 dm light path**. Several measurements were made at 578 and 546 nm and the $[\alpha]_D$ calculated (Appendix).

The molecular rotations (M_D) of the glycosphingolipids were calculated from the relationship:

$$M_D = [\alpha]_D \times \text{molecular weight}$$

The molecular weights were estimated from the percentage hexose content as

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*The expert technical assistance of Dr. O. A. Mamer, Department of Clinical Biochemistry, Royal Victoria Hospital in recording the spectra is gratefully acknowledged.

** The assistance of Dr. B. H. Korsch, Department of Chemistry, McGill University, Montreal, is acknowledged.

determined by the anthrone method (295) using standards containing the same theoretical ratio of glucose to galactose as the lipid.

8. Nuclear magnetic resonance spectrometry

The nmr spectra recorded at 100 MHz were obtained in the Department of Chemistry, McGill University, Montreal; those recorded at 220 MHz were obtained through the facilities of the Canadian 220 MHz NMR Center, Sheridan Park, Ontario*.

In all cases the samples were prepared by the same technique. The labile hydroxyl protons of the glycosphingolipids were exchanged with deuterium by suspending the sample in D_2O (Merck, Sharp and Dohme), containing a trace of redistilled pyridine, and evaporating it to dryness several times in vacuo. The sample was then dissolved in 0.5-1 ml pyridine-d_5 (Merck, Sharp and Dohme) and transferred to the nmr sample tube. In some cases a trace of D_2O was added to "sharpen" the DOH peak which often obscured part of the spectrum. A few drops of tetramethylsilane were added as the internal standard. The tube was sealed and nmr spectra recorded with a Varian HA-100 nmr spectrometer. Samples to be studied at 220 MHz were sealed in the nmr tube and sent air mail to Toronto where the spectra were recorded with a Varian HR-220 nmr spectrometer.

The field homogeneity, gain, sweep frequency and other variables were optimized and spectra recorded which best demonstrated the region of the spectra where the anomeric protons absorb. In cases in which the DOH peak

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*These studies were done under the supervision and guidance of Dr. A. S. Perlin, Department of Chemistry, McGill University, Montreal.

obscured part of the spectrum, spectra were recorded at various temperatures. The effect was to displace the DOH peak downfield at low temperatures, and upfield at higher temperatures (Figure 15). By this manipulation we succeeded in demonstrating the entire spectra of the various glycosphingolipids studied.

C. Catalytic Reduction of Galactosylceramide

Twenty milligrams of platinum oxide (Adam's catalyst) were suspended in 5 ml redistilled ethanol and stirred vigorously during exposure to hydrogen gas. When the uptake of H_2 had ceased, the activated catalyst was transferred to a 25 ml flask containing 30 mg galactosylceramide suspended in 1 ml ethanol. The mixture was then stirred vigorously in an atmosphere of H_2 at room temperature for 45 min in a microhydrogenator by which time H_2 uptake had ceased. The hydrogenated lipid was diluted with chloroform-methanol (2:1) and filtered to remove the catalyst.

D. Preparation of Lactosylceramide

Lactosylceramide was prepared from 1.3 g of purified ox brain gangliosides (gift from Dr. J. A. Lowden, Hospital for Sick Children, Toronto) according to the technique described by Radin et al (229). The lipid was heated at 90° for 2 hours in 150 ml 1 N H_2SO_4. The hydrolysate was cooled to 0° and neutralized with gaseous NH_3 and by the addition of solid Na_2CO_3. It was then partitioned against 5 volumes of chloroform-methanol (2:1). The lower phase was washed twice with 0.2 volumes of "ideal upper phase" (303). The lower phase was then evaporated to dryness under reduced pressure and the residue dissolved in chloroform-methanol

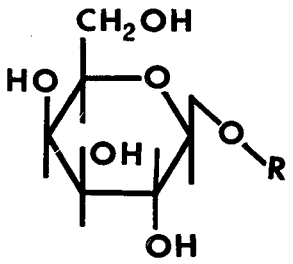
(98:2, v/v) for column chromatography on silicic acid. A stepwise elution was carried out with 4 percent, 10 percent and 33 percent methanol in chloroform. The 10 percent methanol fraction contained virtually all the lactosylceramide, the major glycolipid obtained by this procedure. Final purification of the lactosylceramide was achieved by preparative tlc as described for the purification of CTH.

E. Preparation of Labelled CTH and Lactosylceramide

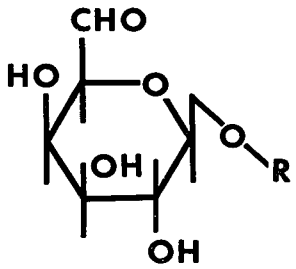
CTH and lactosylceramide labelled with tritium on C-6 of the terminal D-galactose moieties were prepared by a modification of the procedure described by Radin et al (229) and summarized in Figure 8. Twenty milligrams of lipid were dissolved in 5 ml redistilled tetrahydrofuran. Five milliliters of 0.01 M potassium phosphate buffer, pH 7, were added followed by 125 units of D-galactose oxidase (Worthington Biochemical Corp.) in 1 ml phosphate buffer. The mixture was incubated under an atmosphere of O_2 for 22 hours at room temperature with gentle stirring. The reaction was stopped by the addition of 15 ml chloroform-methanol (4:1, v/v) and shaking. The resulting upper phase was washed twice with 8 ml chloroform-methanol (4:1) then discarded. The pooled lower phases were washed twice with 10 ml methanol-water (1:1, v/v) and evaporated to dryness with a stream of N_2. Thin-layer chromatography at this stage, using System A, indicated that about 40 percent of the CTH and 80 percent of the lactosylceramide had been oxidized and migrated slightly faster than the unreacted lipid (Figure 9).

Figure 8. Outline of the preparation of ^{3}H-labelled galactolipids. The details of the reaction conditions and purification of the product are described in the text.

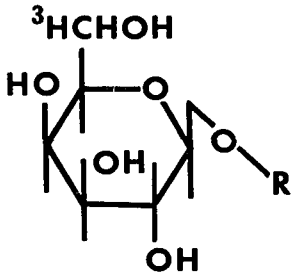
Preparation of [^{3}H] Galactosyl Glycolipids



D-Galactose Oxidase
24 hours at 20°

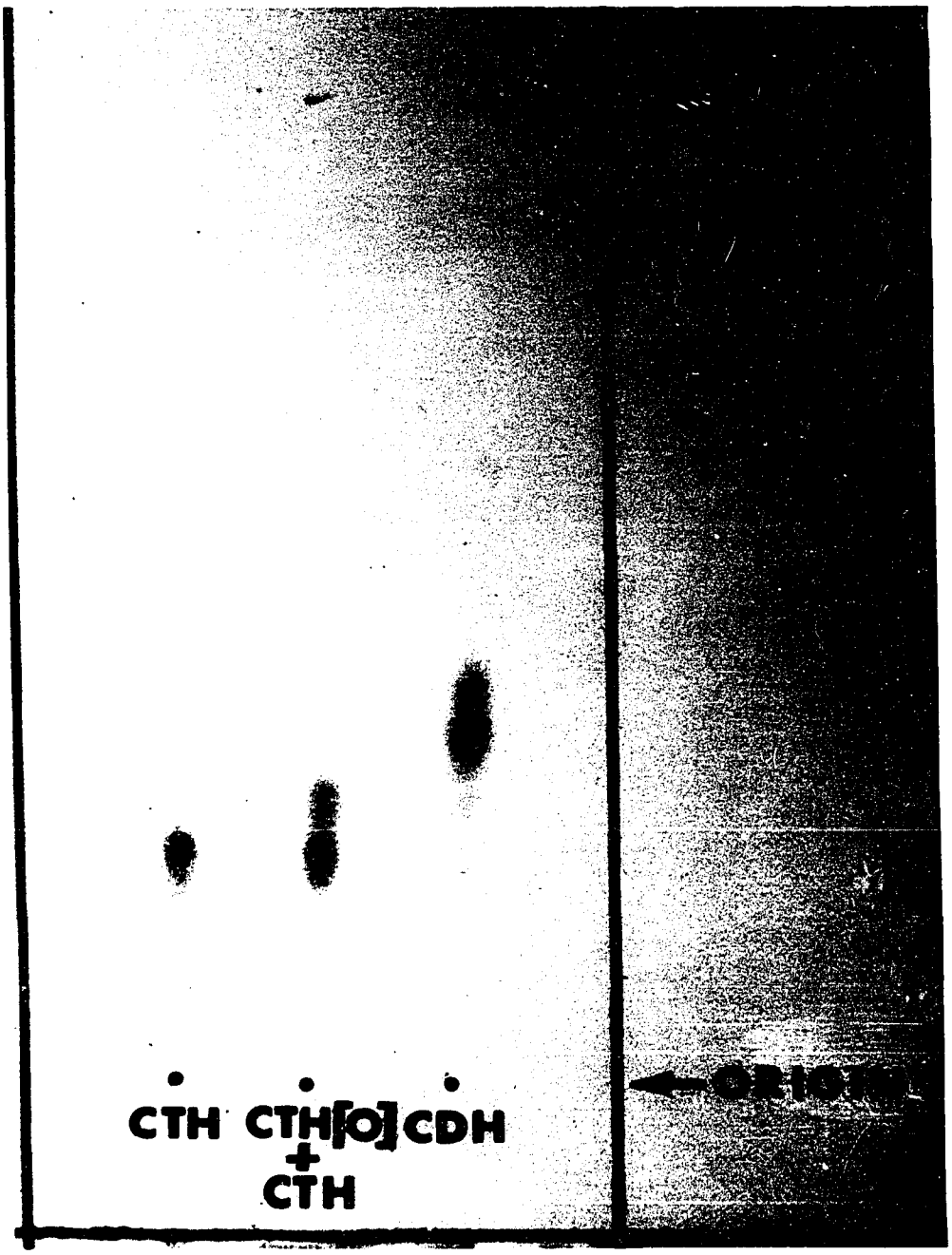


Sodium Borohydride[^{3}H]
24 hours at 20°



R = Ceramide
Galactosyl- or Glucosylceramide
or Lactosylceramide

Figure 9. Thin-layer chromatogram of the products of the reaction of CTH with D-galactose oxidase. Adsorbent: silica gel H; Solvent system: $CHCl_3$-CH_3OH-H_2O (100:42:6, v/v/v); Conditions: System A.


CTH
CTH[O]CDH
+
CTH

The oxidized lipid was dissolved in 5 ml redistilled tetrahydrofuran and 1 mg of ^{3}H-$NaBH_4$, about 6 mCi/mg (New England Nuclear), added in 0.35 ml 1 mM NaOH. The mixture was allowed to stand 18-20 hours at room temperature. Ten milligrams of nonradioactive $NaBH_4$ were then added and the mixture allowed to stand another hour. The excess $NaBH_4$ was then degraded and removed by the addition of 7 ml 1 N acetic acid and 25 ml chloroform-methanol (2:1). The upper phase was discarded and the lower phase washed 8-10 times with 12 ml aliquots of methanol-water (1:1, v/v) containing 1 mg NaCl/ml. The washed lower phase was then evaporated to dryness and the residue dissolved in chloroform-methanol (2:1) for purification by preparative tlc (System A). The final labelled products were diluted with unreacted, purified glycolipid to a specific activity of about 4.5 mCi/mmole.

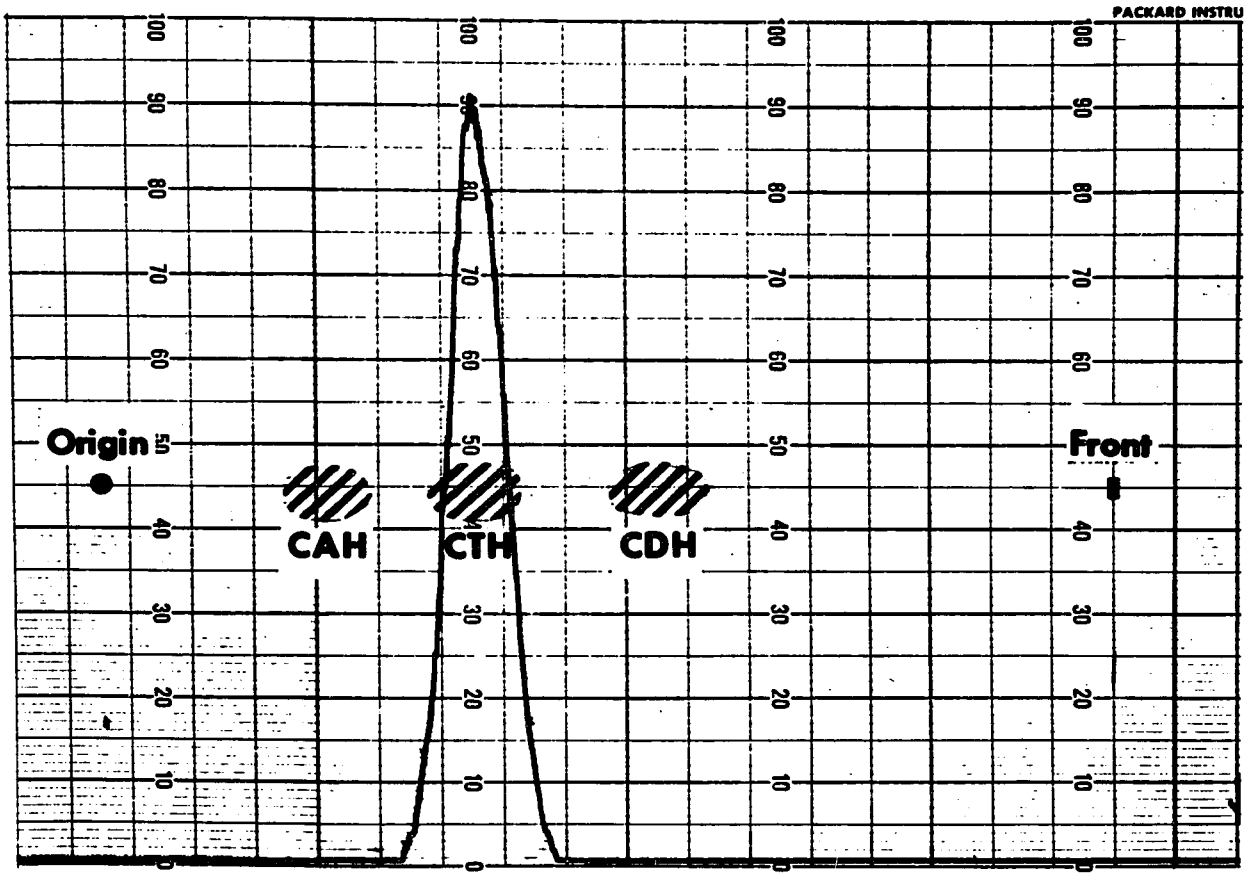
The radiochemical purity of the labelled products was checked by tlc (System A) and paper chromatography (System D) and scanning the developed chromatograms with a Packard Model 7201 Radiochromatogram Scanner (Figure 10).

F. Measurement of Radioactivity

1. Organic system

Samples of tritiated lipids were prepared for counting by evaporating aliquots to dryness in standard glass counting vials, dissolving the residue in 0.1 ml methanol and adding 15 ml "toluene scintillant" (5 g 2,5-diphenyloxazole, PPO, and 0.3 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene, dimethyl-POPOP, per liter of toluene). Counting was done with a

Figure 10. Thin-layer radiochromatogram of CTH[^{3}H]. The ^{3}H-labelled lipid was prepared as described in the text. Adsorbent: silica gel H, 0.25 mm thick. Solvent: $CHCl_3$-CH_3OH-H_2O (100:42:6, v/v/v). Conditions: System A. The chromatogram was scanned with a Packard Model 7201 radiochromatogram scanner. Full scale deflection = 10,000 cpm.


PACKARD INSTRU
Origin
CAH
CTH
CDH
Front

Packard Tri-Carb Model 3003 Liquid Scintillation Spectrometer and the efficiency of counting (30-40 percent) determined by the channels ratio method.

2. Aqueous system

Samples of tritiated galactose were prepared by evaporating aliquots to dryness with a stream of nitrogen while heating the vials in a water bath at 80-90°. The residue was dissolved in 1 ml water and 10 ml "naphthalene scintillant" (80 g naphthalene, 5 g PPO, and 50 mg dimethyl-POPOP in 1 liter of a mixture of equal volumes of toluene, dioxane and ethanol). Counting was carried out as described for the organic system. The efficiency of counting in this system was 10-20 percent.

G. Acid Hydrolysis of CTH and Lactosylceramide

The acid labilities of the terminal galactosidic linkages of CTH and lactosylceramide were determined by incubating aliquots of the lipids (0.7 mg CTH and 0.9 mg lactosylceramide) in 2 ml 0.3 N HCl in chloroform-methanol (2:1) at 60° in glass-stoppered test tubes. Periodically 0.2 ml aliquots of the incubation mixtures were removed and added to 0.6 ml ice-cold 0.1 N NaOH and 0.4 ml H_2O. The neutralized solutions were partitioned with 4.8 ml chloroform-methanol (2:1) and centrifuged. Aliquots (0.1 ml) of the upper phases were evaporated to dryness in counting vials. The residues were dissolved in 1 ml H_2O and 10 ml "naphthalene scintillant" added for the determination of ^3H-galactose released by hydrolysis.

H. Extraction and Partial Purification of α-D-Galactosidase from Coffee Beans

The α-galactosidase extracted from green Colombian coffee beans as described by Courtois and Petek (304).

1. Enzyme assays

The α- and β-galactosidase activities of the coffee bean extracts were measured using the respective p-nitrophenyl-D-galactopyranosides (Koch-Light). The enzyme in 1.0 ml distilled water was incubated with 2 mg of substrate, 0.2 ml water saturated with toluene, 0.5 ml citrate-phosphate buffer (ionic strength, 0.5 M) and water to a final volume of 1.8 ml. Incubations were carried out at 37°C. The reactions were terminated by adding 20 µl 1 N NaOH and 1.2 ml 0.1 M glycine-NaOH buffer, pH 10.5. The optical density was measured at 410 nm against substrate and enzyme blanks, and compared with standards of p-nitrophenol.

2. Partial purification of α-galactosidase

Fifty grams of beans were finely ground, washed exhaustively with redistilled benzene and air-dried. The powder was extracted with 250 ml of water containing 1 ml of toluene at 2° for 24 hours. The extract was filtered through several layers of cheesecloth and the filtrate dialyzed against several changes of distilled water for 48 hours. The sac contents were centrifuged at 2500xg for 60 minutes and the supernatant added to 5 volumes of redistilled acetone at -20°. After 10-15 minutes the precipitate was collected by filtration. The protein was rinsed off the filter papers with 50 ml of distilled water and dialyzed against distilled

water for 48 hours at 2°. The dialyzed crude enzyme preparation was applied to a 300 g column of alumina prepared exactly as described by Courtois and Petek (304) and eluted with a citrate-phosphate buffer, ionic strength 0.5 M and pH 4.4 (305). The column effluent was monitored by its absorbance at 280 nm.

I. Hydrolysis of Various Galactosides by α-Galactosidase

The effect of pH on the hydrolysis of p-nitrophenyl-α-D-galactopyranoside, p-nitrophenyl-β-D-galactopyranoside, melibiose and CTH[^{3}H] by the partially purified α-galactosidase from coffee beans was determined. The reaction of the enzyme with the p-nitrophenylgalactosides was conducted as previously described. In the reaction with CTH[^{3}H], the enzyme (0.015 mg in 1.0 ml H_2O) was mixed with 0.5 ml citrate-phosphate buffer (ionic strength 0.5 M), 0.2 ml water saturated with toluene, and 2 mg sodium cholate in a final volume of 1.9 ml. The reaction was started by adding 10-15 nmoles CTH[^{3}H] in 10 µl water containing 10 mg sodium cholate per ml. Blanks were prepared in which the enzyme solution was heated to 100° for 3 minutes prior to the addition of the substrate. Incubations were carried out at 37° for 24 hours. The reaction was terminated by cooling and the addition of 40 µg of carrier galactose and 7.5 ml chloroform-methanol (2:1, v/v). The tubes were stoppered and shaken vigorously, then centrifuged to separate the two phases. The upper phases were washed with 2 ml "theoretical lower phase" (303). One milliliter aliquots of the washed upper phases were transferred to counting vials and evaporated to dryness with a stream of nitrogen while heating at 80-90°. The residue was dissolved in 1 ml water and 10 ml "naphthalene scintillant"

for counting. The recovery of ^{3}H-galactose was 95-100 percent.

The hydrolysis of melibiose by the partially purified α-galactosidase was determined using the same incubation procedure as was used with the p-nitrophenylgalactosides substituting 2 mg of melibiose for the colorimetric substrates. The galactose released was measured by transferring 0.5 ml aliquots of the incubation mixtures to 1.0 ml 0.04 M Tris-HCl buffer, pH 8.6, 4 mM reduced glutathione, 0.4 mM NAD^{+} and adding 25 μg galactose dehydrogenase (EC 1.1.1.48) (Boehringer Mannheim). The mixture was incubated at 25° for 60 min and the absorbance at 340 nm measured and compared with standards containing known amounts of galactose (306).

J. Studies on Human Renal Galactohydrolases

The object of the studies described in this segment of the Thesis was originally to show that human renal CTH galactohydrolase was identifiable with p-nitrophenyl-α-D-galactopyranoside galactohydrolase. Unfortunately, CTH galactohydrolase is very labile - storage of the 1200xg supernatant at 0-2° resulted in a 50 percent loss of activity in 6 days. Freezing or lyophilization did not retard this loss of activity. On the other hand, the nonspecific α-galactosidase activity is stable for several weeks frozen and stored at -20°. At the time of writing, therefore, only the results of preliminary studies on some of the properties of nonspecific α-galactosidase are available. Future studies will be directed towards stabilizing the activity of the enzyme towards the natural substrate in order that a reasonable partial purification might be achieved.

1. Enzyme assays

a) p-nitrophenyl-α-D-galactopyranoside galactohydrolase and p-nitrophenyl-β-D-galactopyranoside galactohydrolase

Aliquots of crude enzyme preparations (0.1-2 mg protein) were incubated with 2 mg of p-nitrophenyl-α-D-galactopyranoside or p-nitrophenyl-β-D-galactopyranoside, 0.5 ml citrate-phosphate buffer (ionic strength = 0.5 M) (305), and water (final volume = 1 ml) at 37° for 1 hr. The incubations were terminated by adding 1 ml ice-cold 2.5 percent trichloroacetic acid. The samples were centrifuged and 1.0 ml aliquots of the supernatant transferred to 20 μl 1 N NaOH and 2.0 ml 0.1 M glycine-NaOH buffer (pH 10.5) added. The optical densities were measured at 410 nm. The p-nitrophenol present was estimated by comparison with appropriate standards after the subtraction of the optical densities of the substrate and enzyme blanks.

In assays of the nonspecific α-galactosidase in soluble enzyme preparations, the precipitation of protein with trichloroacetic acid was omitted. In assays of dialyzed enzyme preparations, the incubation media were prepared containing 20 mM Mg Cl_2. Otherwise all assays were conducted as described above.

b) CTH galactohydrolase

Aliquots of the enzyme preparation (0.5 ml containing 5-7 mg of protein) were incubated with 30 nmoles CTH[^{3}H] (4.1×10^3 dpm/nmole) dissolved in 30 μl sodium cholate solution (10 mg/ml), 2 mg sodium cholate,

0.5 ml citrate-phosphate buffer (ionic strength = 0.5 M) (305) and water (final volume = 1.23 ml) at 37° for 90 min. The reactions were started by the addition of the substrate and terminated by cooling and the addition of 7.5 ml chloroform-methanol (2:1), 75 μg galactose and 0.2 ml water. The samples were shaken vigorously and centrifuged to separate the mixture into two distinct phases. The upper phases were carefully removed and washed with 2 ml aliquots of "ideal lower phase" (303). One milliliter aliquots of the washed upper phases were transferred to standard glass counting vials and evaporated to dryness with a stream of N_2 while heating at 80-90°. The residues were dissolved in 1 ml H_2O and 10 ml "naphthalene scintillant" and the ^3H-galactose measured by liquid scintillation spectrometry.

2. Human renal galactohydrolases

In the studies on human renal galactohydrolases, two sources of tissue were used. Most the studies were done on specimens obtained at autopsies performed 8-12 hrs after death. All the experiments in which the enzymic hydrolysis of CTH[^3H] was examined, however, were performed on normal renal tissue obtained at surgery or at autopsies performed less than 8 hrs after death.

a) p-nitrophenyl-α-D-galactopyranoside galactohydrolase

pH optimum - Kidney tissue (mixed cortex and medulla) was chopped, rinsed with 0.9 percent NaCl and homogenized at 0° in 4 volumes of 0.01 M potassium phosphate buffer (pH 6.0) with an Omnimixer (90 sec at top speed). The homogenate was centrifuged at 1230xg for 10 min at 2°.

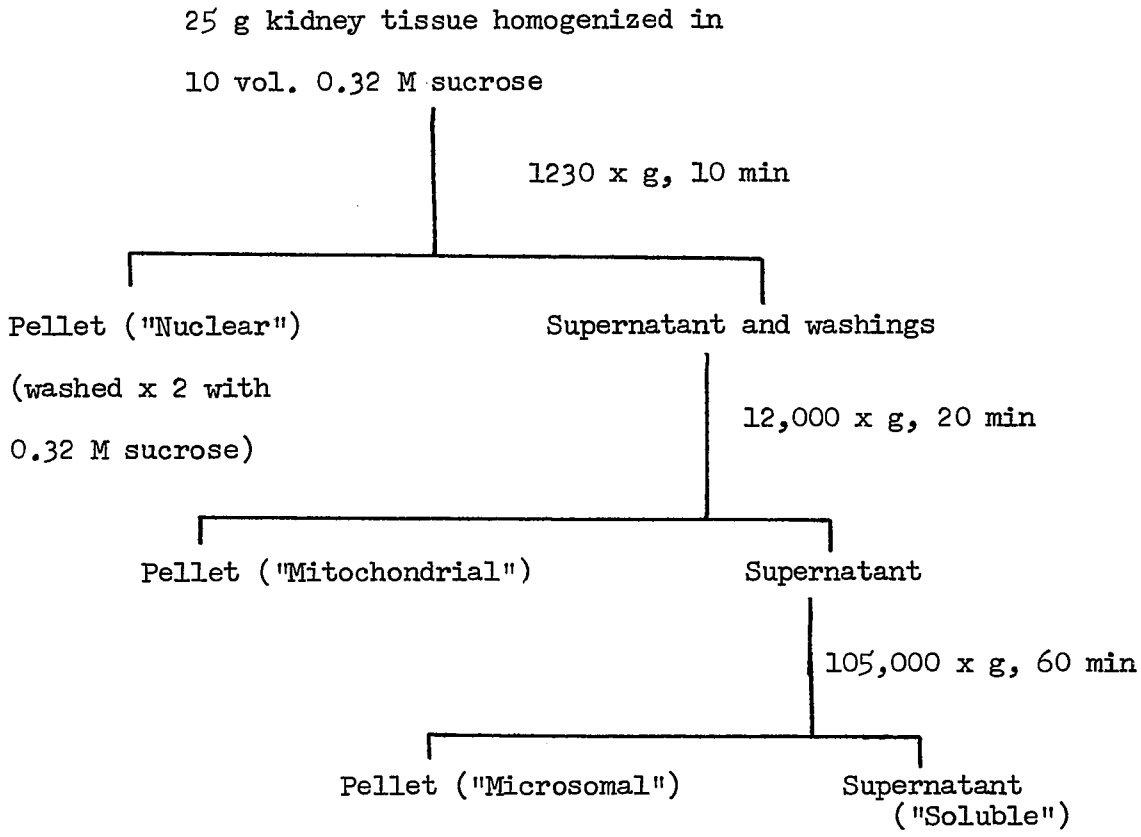
Aliquots (0.1 ml) of the supernatant were assayed for nonspecific α- and β-galactosidase activity between pH 2.6 and 8.0.

Subcellular distribution - Kidney tissue (mixed cortex and medulla) from a patient who had died from a brain tumor 16 hrs 20 min previously was homogenized at 0° in 10 volumes of 0.32 M sucrose with an Omnimixer (3 min). The homogenate was centrifuged at 1230xg for 10 min. The supernatant was decanted and the pellet washed twice with half the original volume of 0.32 M sucrose. The washed pellet ("Nuclear") was suspended in 0.9 percent NaCl (approx. 1/3 the original volume of sucrose). The combined supernatants were centrifuged at 12000xg for 20 min at 4° in a Spinco Model L preparative ultracentrifuge. The pellet ("Mitochondrial") was suspended in 0.9 percent NaCl. The supernatant was centrifuged at 105,000xg for 60 min. The pellet ("Microsomal") was suspended in a small volume of 0.9 percent NaCl. The supernatant contained the "Soluble" enzyme activity. Each fraction was assayed for nonspecific α- and β-galactosidase activity. The fractionation procedure is summarized in Figure 11.

Cofactor requirements and inhibitors - The "Soluble" enzyme preparation was dialyzed against several changes of distilled water for 18 to 48 hrs. Periodically aliquots of the sac contents were assayed for nonspecific α-galactosidase activity. The effects of a variety of divalent cations and potential inhibitors were studied. In each case the compound under investigation was added to the assay mixture in 0.1 ml aqueous solution.

Ammonium sulfate fractionation - An ammonium sulfate fractionation of nonspecific α-galactosidase activity in the "Soluble" fraction (Figure 11) was undertaken on the undialyzed enzyme preparation. All

Figure 11. Outline of the subcellular fractionation of normal human renal tissue.



operations were conducted at 0-2°. Solid ammonium sulfate was added very slowly (approx. 45 min) to the enzyme preparation to 25 percent saturation with constant, gentle stirring. After 2 hrs the suspension was centrifuged at 80,000xg for 40 min. The precipitate (0-25 Fraction) was redissolved in distilled water. Solid ammonium sulfate was then added slowly to the supernatant to 50 percent saturation. After 2 hrs gentle Stirring the suspension was again centrifuged at 80,000xg for 40 min. The precipitate (25-50 Fraction) was dissolved in distilled water. Each fraction, including the final supernatant (>50 Fraction) was assayed for nonspecific α- and β-galactosidase activities before and after dialysis against 0.05 M citrate-phosphate buffer (pH 5, ionic strength 0.5 M) for 20 hrs at 0-2°.

b) CTH galactohydrolase

pH optimum - Fresh (surgical or autopsy material obtained less than 8 hrs after death) renal tissue (mixed cortex and medulla) was chopped, rinsed with ice-cold 0.9 percent NaCl and homogenized in 4 volumes of 0.01 M potassium phosphate buffer (pH 6.0) for 90 sec at 0° with an Omnimixer. The homogenate was centrifuged at 1230xg for 10 min at 0-2°. Aliquots of the supernatant were assayed for CTH galactohydrolase activity between pH 2.6 and 8.0.

III. RESULTS

A. Properties of Purified Galactosylgalactosylglucosylceramide

The yield of CTH from 900 g of pooled normal kidney was 218 mg (1.47 mg/g dry weight). This compares favorably with the CTH concentrations reported by Martensson (59) (0.98-1.42 mg/g dry weight). The yield of CTH from 19.3 g of Fabry's kidney (Case 4) was 40.7 mg (10.6 mg/g dry weight).

1. Thin-layer and paper chromatography

The CTH purified from normal human kidney and from Fabry's kidney both migrated as single spots on paper chromatography, System D, and two-dimensional tlc, System B (Figure 12). The Rf's for the lipids from both sources were identical in all systems.

The lactosylceramide prepared from ox brain ganglioside, and the globoside prepared from normal human kidney were also chromatographically pure by the same criteria.

2. Chemical composition

The hexose content and composition of normal and Fabry's CTH, lactosylceramide and globoside are presented in Table V. Thin-layer chromatographic analysis of the fatty acid methyl esters obtained by the anhydrous methanolysis of CTH indicated the absence of any hydroxy fatty acids. The results of the GLC analysis of the fatty acid methyl esters is shown in Table VI. The fatty acid compositions of the normal and Fabry's CTH

Figure 12. Two-dimensional thin-layer chromatogram of galactosylgalactosylglucosylceramide (CTH). About 200 μg of CTH extracted and purified from normal human kidney was spotted in one corner 3 cm from each edge. Solvent 1, $CHCl_3$-CH_3OH-H_2O (100:42:6, v/v/v); Solvent 2, tetrahydrofuran-$CH_2(OCH_3)_2$-CH_3OH-4M NH_4OH (50:25:25:5, v/v/v/v). Detection: Orcinol-H_2SO_4 spray and heating at 110° for 15 min.

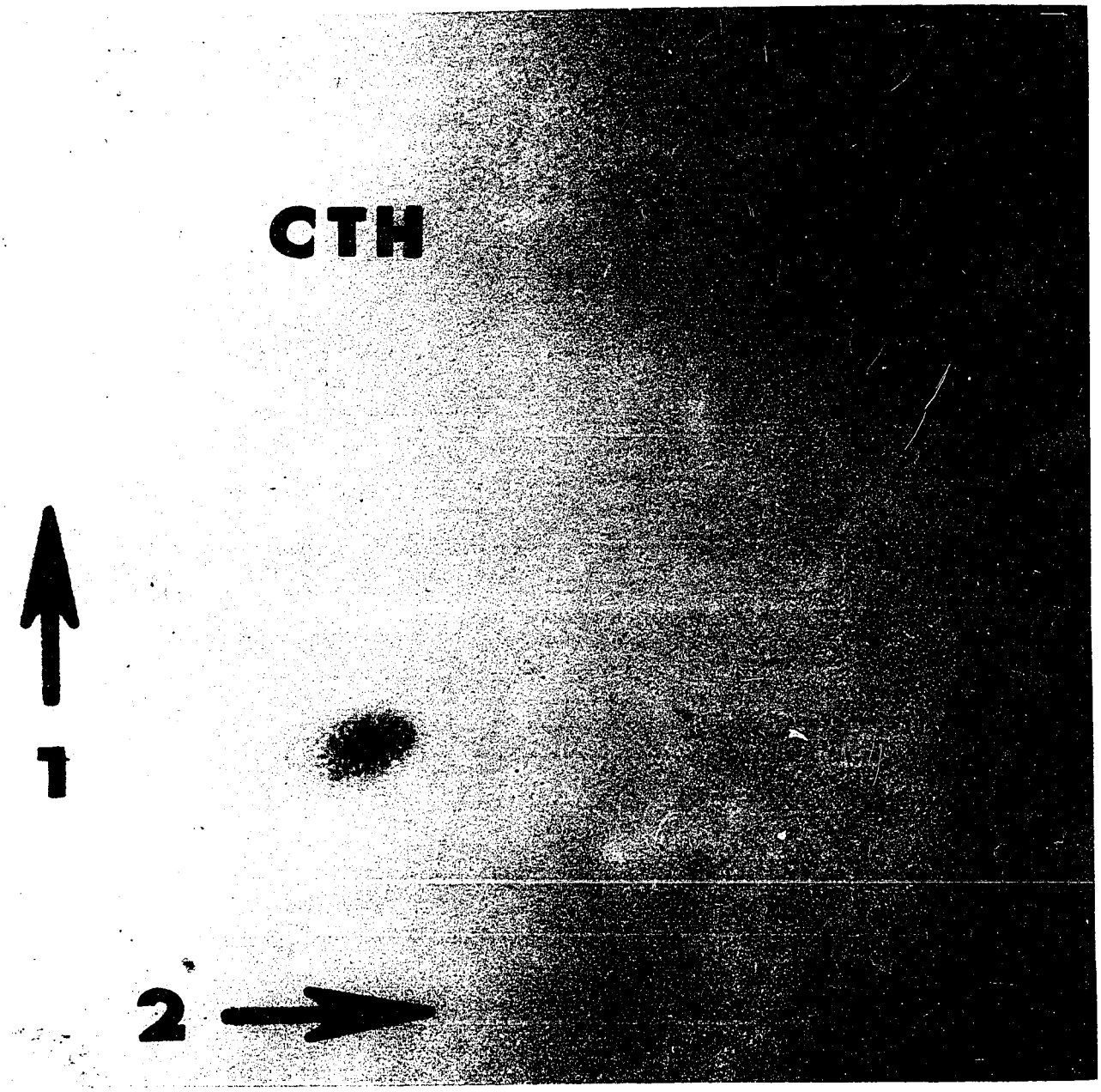

CTH
1
2

Table V: Some properties of purified glycosphingolipids

Glycosphingolipid	Source	% Hexose[a]	Galactose:glucose ratio[b]		$[\alpha]_D^{23}$
			Theoretical	Observed	in pyridine
Galactosylgalactosyl-glucosylceramide (CTH)	Normal human kidney	49.2	2.0	2.11	+23 (c=7.6)
Galactosylgalactosyl-glucosylceramide (CTH)	Fabry's kidney	46.9	2.0	2.05	+21 (c=2.9)
Galactosylglucosylceramide (Lactosylceramide)	Brain ganglioside	36.4	1.0	1.03	-12 (c=2.1)
N-acetylgalactosaminyl-galactosylgalactosylglucosyl-ceramide (globoside)	Normal human kidney	44.5	2.0	2.22	+10.2 (c=4.7)

[a] Estimated by anthrone method (295) using standards containing the same galactose:glucose as the glycosphingolipid.

[b] Measured by GLC analysis of the TMSi derivatives of the methylglycosides obtained by the anhydrous methanolysis of the lipid.

Table VI: Distribution of fatty acids of CTH.

The fatty acid methyl esters were obtained by the anhydrous methanolysis of the glycosphingolipids as described in the text. The analysis of the fatty acid methyl esters was carried out by gas-liquid chromatography on a 6 foot column of 3% SE-30 on Gas Chrom Q, 80/100 mesh, maintained at 195° with helium, the carrier, at 50 ml/min. The fatty acids were identified by reference to authentic standards and quantitated by triangulation.

Table VI.

			Previous work (59)
Fatty acid	Normal CTH[b]	Fabry's CTH[b]	Normal CTH[b]
16:0[a]	7.9	2.2	6.3
16:1	trace	trace	0.1
17:0	0	0	0.2
17:1	0	0	trace
18:0	3.2	2.8	3.3
18:1	trace	1.2	0.2
19:0	0	0.2	0.2
19:1	0	0.5	0
20:0	6.8	5.1	7.3
20:1	0.2	trace	0.3
21:0	0	0.5	0.2
21:1	0	0.1	0
22.0	21.3	17.5	21.8
22:1	2.6	2.6	2.0
23:0	2.4	3.6	2.7
23:1	0	0.3	0.4
24:0	24.1	17.8	23.8
24:1	31.7	44.3	30.1
25:0	0	0	0.4
25:1	0	0	0.1
26:0	0	0	0.2
26:1	0	0	0.4

[a] Ratio of the number of carbon atoms to the number of methylene-interrupted double bonds.

[b] Expressed as percentages of the total nonhydroxy fatty acid composition.

Table VII. Long-chain base composition of CTH.

The long-chain bases were obtained by the hydrolysis of the glycosphingolipids as described by Gaver and Sweeley (37). Gas-liquid chromatographic analysis of the TMSi derivatives was carried out on a 6 foot column of 3% SE-30 on Gas Chrom Q, 80/100 mesh, maintained at 200°. The carrier gas was helium at 50 ml/min.

Long-chain base	C-value[b]	Normal	Fabry's
S15:0[a]	19.00	1.8[c]	4.0[c]
S15:1	18.47	2.7	1.4
S16:0	20.00	3.0	3.0
S16:1	19.37	1.3	5.3
S17:0	21.00	trace	trace
S17:1	20.37	1.5	2.7
S18:0	22.04	6.2	11.7
S18:1	21.50	83.6	71.9

[a] Ratios of the number of carbon atoms to the number of double bonds in long-chain bases of sphingosine class.

[b] The C-value of a long-chain base is the theoretical number of carbon atoms in a fatty acid methyl ester with the same retention time.

[c] Percentages of the total long-chain base composition.

are very similar to each other and are almost identical to that reported by Martensson (59) for CTH isolated from normal human kidney. The distribution of the LCB's in normal and Fabry's CTH is shown in Table VII.

3. Infrared spectrophotometry

Several IR spectra were recorded of various concentrations of CTH in KBr in an attempt to obtain the optimum spectrum. The final concentration, 400 μg of CTH in 30 mg KBr, represents the preparation producing the best sensitivity and resolution although the pellet was not completely transparent and the baseline absorbance was high. Due to the inferior quality of the pellet the fine features of the spectra are obscured. Nevertheless, some important characteristics stand out. First, the spectra of normal and Fabry's CTH are indistinguishable (Figure 13), and are virtually identical to that recorded on CTH purified from human serum by Svennerholm and Svennerholm (98). Secondly, both spectra contain a small peak at 890 cm^{-1} attributable to the axial protons of β-glycopyranosyl residues. Unlike the spectra reported by Kawanami (106) and Hakomori et al (111), neither contains the peak at 850 cm^{-1} that is often observed in spectra of compounds containing α-glycopyranosyl residues.

4. Mass spectrometry

Figure 14 shows the mass spectra of the TMSi derivatives of normal and Fabry's CTH. The spectra are almost identical to each other and very similar to the spectrum of CTH from Fabry's kidney published by Sweeley and Dawson (302). They showed, in 1969, that the number of neutral aldohexoses in glycosphingolipids could be determined from the ratio of the

Figure 13. Infrared spectra of normal and Fabry's CTH. The samples (about 400 μg of lipid) were dispersed in 30 mg of KBr and spectra recorded on a Perkin-Elmer Model 221 Spectrophotometer.

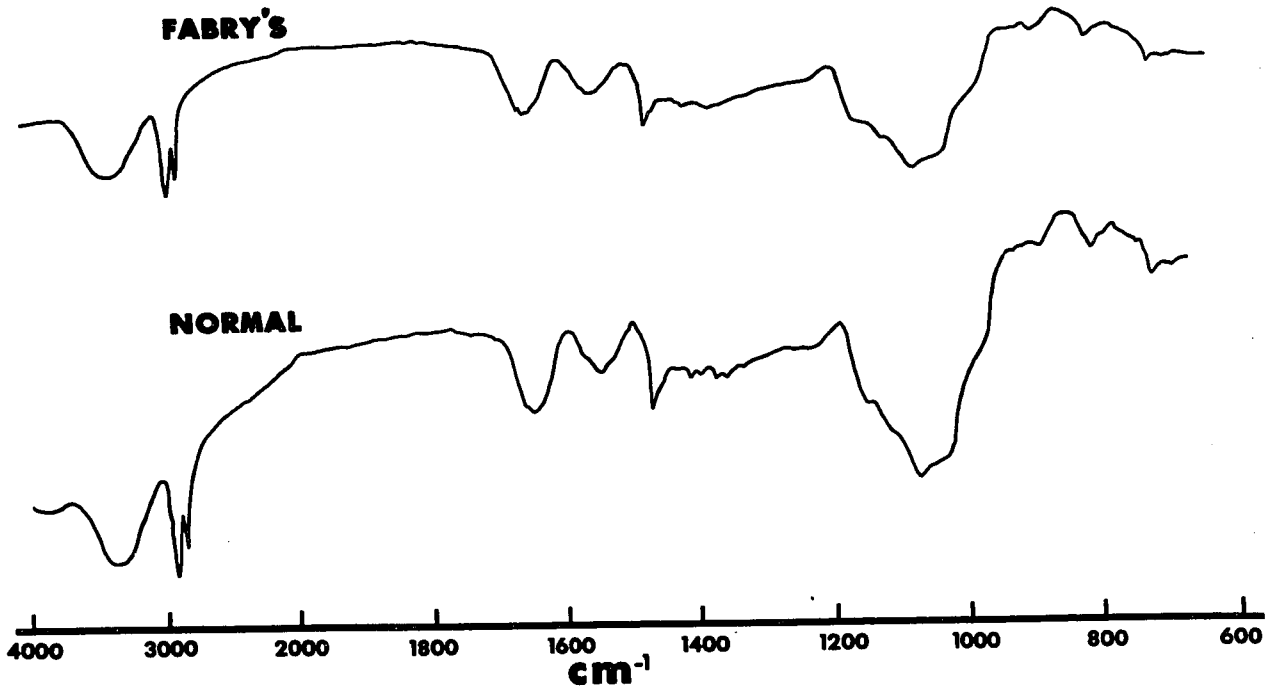
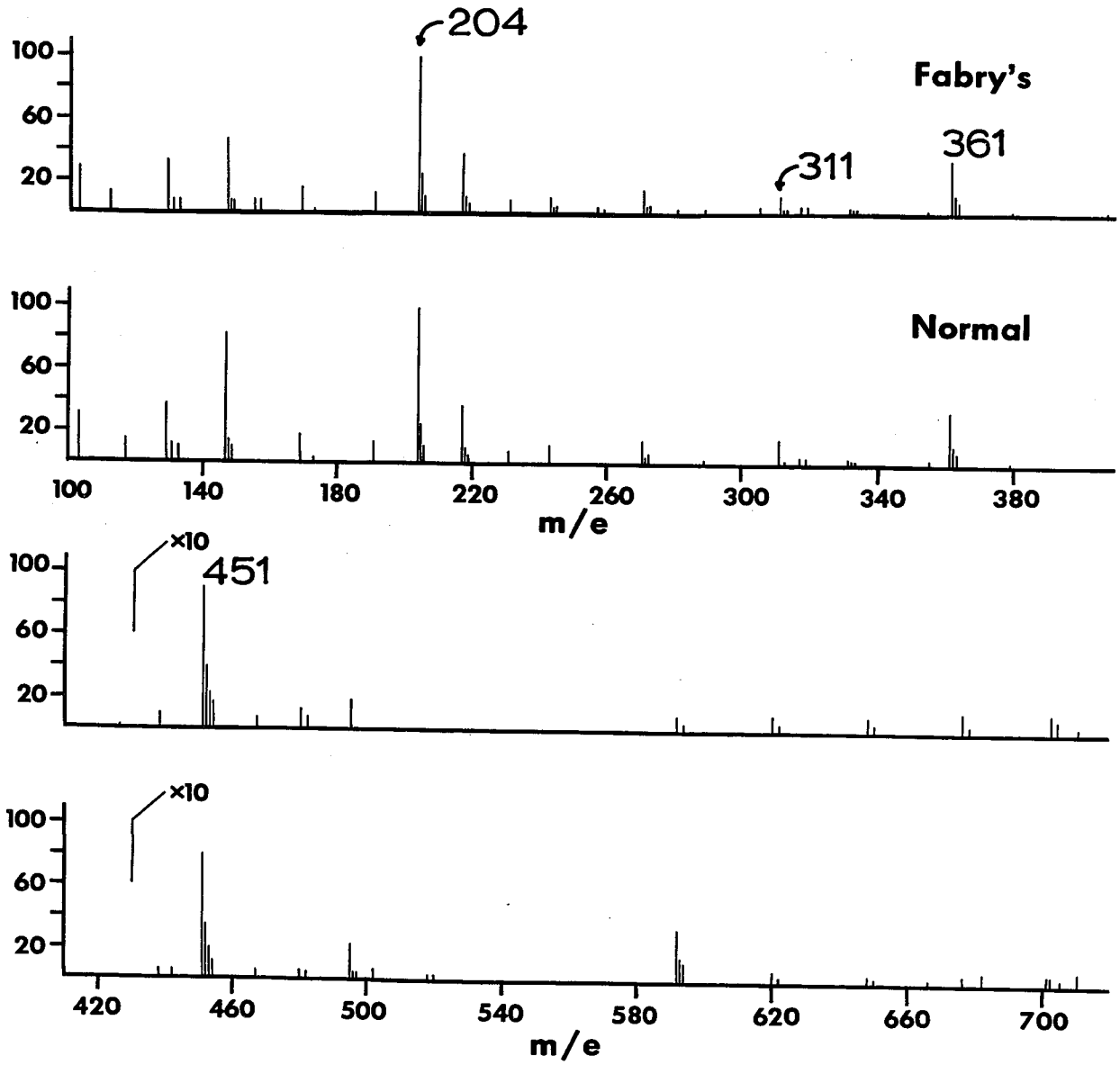

FABRY'S
NORMAL
4000
3000
2000
1800
1600
1400
1200
1000
800
600
cm-1

Figure 14. Mass spectra of the TMSi derivatives of normal and Fabry's CTH. The samples were prepared as described in "Materials and Methods". The spectra were recorded with an LKB Model 9000 mass spectrometer by the direct inlet method. The spectrometer was operated at 3500 volts with an electron energy of 70 eV and ion source temperature of 290°. The spectra in this Figure were recorded when the probe temperature was 140-145°.


204
Fabry's
311
361
100
60
20
Normal
100
140
180
220
260
300
340
380
m/e
×10
451
×10
420
460
500
540
580
620
660
700
m/e

intensity of ions derived from the hexoses, notably m/e 204, [TMSiOCH-CHOTMSi]$^+$, to the intensity of ions characteristic of the sphingosine bases, e.g. sphing-4-enine, m/e 311, [$CH_3(CH_2)_{12}CH{=}CH{-}CHOTMSi$]$^+$. Thus the ratio 204/311 was 1.8 for glucosylceramide, 4.0 for lactosylceramide and 6.6 for CTH. The 204/311 ratio, however, varies somewhat with the probe temperature as seen in Table VIII. In the experiment from which these data were obtained, the peak total ion current occurred at 150-160°, and the spectra recorded at 140-145° probe temperature were plotted. The 204/311 ratios were 6.5 for normal CTH and 8.9 for Fabry's CTH. The fragment m/e 311 is derived entirely from sphing-4-enine. In CTH from normal and Fabry's kidney, however, sphing-4-enine accounts for only 83.6 percent and 71.9 percent, respectively, of the long-chain bases in these lipids (Table VII). When the appropriate correction is made, the 204/311 ratio becomes 5.4 for normal and 6.4 for Fabry's CTH. Although this method appears useful as a first approximation in the determination of glycosphingolipid structure, it is plagued by variables that prejudice its precision and accuracy. From their analysis of CTH from Fabry's kidney, for example, Dawson and Sweeley (309) concluded that it contained over 95 percent sphing-4-enine. The results of the GLC analysis of the long-chain bases from comparable material (Table VII) indicates that this is clearly not the case.

5. Optical rotations

In Table IX, the calculated and observed molecular rotations ($M_D/100$) of various galactosides are shown. The calculated molecular

Table VIII: Effect of probe temperature on m/e 204/311 ratio in mass spectra of CTH.

Probe temp.	204/311
80°	28.0
100°	9.7
110°	6.0
120°	6.3
128°	7.4
132°	6.8
140°	6.5
150°	6.7

From the mass spectra of normal CTH recorded as the trimethylsilyl derivatives by direct inlet. Apart from the probe temperature, the conditions were the same as described for Figure 14.

Table IX: Optical rotations of glycosphingolipids

Compound	Molecular Weight (calculated)	$[\alpha]_D^{23}$ (in pyridine)	$M_D/100$ Calculated	$M_D/100$ Found
Methyl-β-galactopyranoside	194	+0.6	-	+1.2
Methyl-α-galactopyranoside	194	+196	-	+380
Lactosylceramide (Brain ganglioside)	953	-12 (c=2.1)	-	-114
CTH(α)	1050	-	+266	-
CTH(β)	1050	-	-113	-
CTH (normal kidney)	1044	+23 (c=7.5)	-	+240
CTH (Fabry's kidney)	1097	+21 (c=2.8)	-	+230

CTH(α) = gal-(α1→4)-gal-(β1→4)-glc-(β1→1')-ceramide.

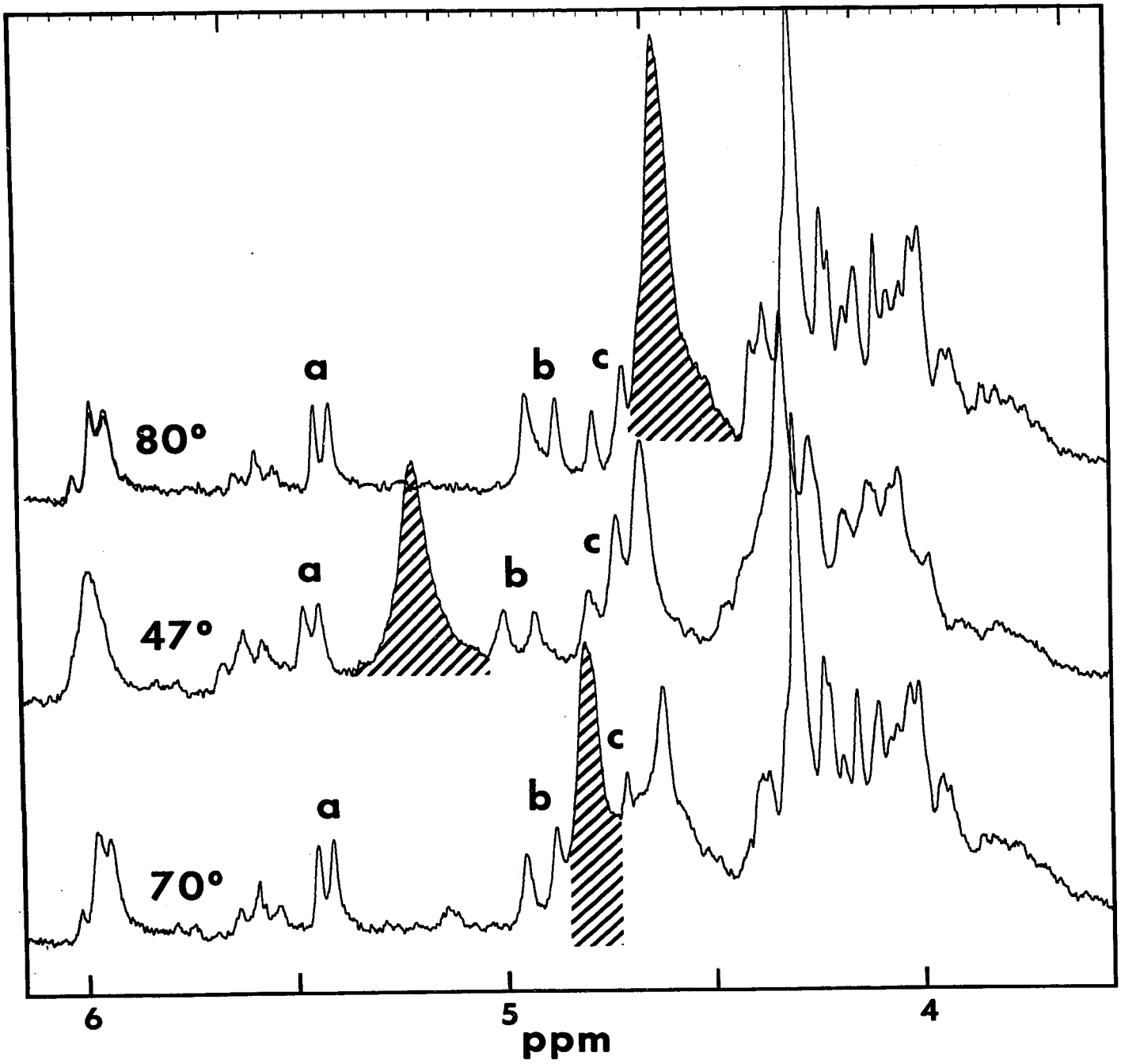
CTH(β) = gal-(β1→4)-gal-(β1→4)-glc-(β1→1')-ceramide.

rotations for O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→1')-ceramide [CTH(β)] and O-α-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→1')-ceramide [CTH(α)] were determined by the application of Hudson's Rules of Isorotation (310). Thus the $M_D/100$ of CTH would be expected to be approximately equal to the sum of the $M_D/100$ of lactosylceramide (-114) and that of a methylgalactopyranoside (α= +380, β= +1.2). The observed rotations of the normal and Fabry's CTH, +240 and +230 respectively, are very close to that calculated for CTH(α).

6. Nuclear magnetic resonance spectrometry

Several nmr spectra were recorded of intact glycosphingolipids and derivatives in pyridine-$\underline{d}_5$ at 100 MHz and 220 MHz. In every case the spectra of normal CTH and Fabry's CTH were indistinguishable. Figure 15 shows partial spectra of normal CTH recorded at 47°, 70° and 80°. The large shaded peak is due to the absorption of DOH caused by the presence of traces of moisture in the sample. Varying the temperature caused shifts in the DOH peak which made it possible to see everything in the region of the anomeric protons clearly. Three doublets, marked a, b and c, are easily discernible. Two, b and c, have the spectral characteristics of β-anomeric protons. The third, a, has a chemical shift and coupling constant characteristic of an α-anomeric proton. The downfield shift in the entire spectrum as compared to those recorded in D_2O (285) is caused by the solvent, pyridine (288). Apart from this generalized downfield shift, the spectral characteristics of the anomeric protons of

Figure 15. Partial nmr spectra of normal CTH at 47°, 70° and 80°. The spectra were recorded at 100 MHz on 75 mg of CTH from normal kidney in 0.5 to 1 ml of pyridine-$\underline{d}_5$ containing a trace of D_2O with tetramethylsilane as an internal standard and a sweep width of 500 Hz. The shaded peak shows DOH absorption. The doublets attributable to the anomeric protons (H-1) are indicated by the letters a, b, and c. a, δ=5.45 ppm (J=4.1 Hz), H-1 of the terminal α-D-galactopyranosyl residue; b, δ=4.94 ppm (J=7.5 Hz), H-1 of internal β-D-galactopyranosyl residue; c, δ=4.74 ppm (J=7.6 Hz), H-1 of the β-D-glucopyranosyl residue linked to ceramide.


a
b
c
80°
a
b
c
47°
a
b
c
70°
6
5
4
ppm

various hexopyranosides were the same when recorded in pyridine-$\underline{d}_5$ as when they are recorded in D_2O (311).

Figure 16 shows partial spectra of CTH and O-β-D-galactopyranosyl-(1→1')-ceramide (CMH) recorded at 220 MHz. The spectrum of CMH contains a single doublet in the region of the anomeric protons. A comparison of the spectra of CMH and CTH makes it possible to assign the doublet d (in Figure 15) to the β-anomeric proton of the D-glucopyranosyl residue linked to the ceramide in CTH.

The partial nmr spectra of other neutral glycosphingolipids and derivatives in Figure 17 permit the identification of all the peaks between 4.74 and 6.0 ppm in the spectrum of CTH. The doublet marked a occurs in all the spectra and corresponds to that marked c in Figure 15. It was attributed to the β-anomeric proton of the hexopyranoside linked to ceramide in the various glycosphingolipids. The multiplets marked b and c in the spectrum of CMH are not observed in the spectrum of the hydrogenated lipid, $CMH(H_2)$, indicating that they represent the absorption of olefinic protons. The spectrum of lactosylceramide (CDH) contains only the multiplet c. Since the CDH was prepared from brain ganglioside, which contains no unsaturated fatty acids (64), the only double bond in the compound is that in the long-chain base. The double bond in sphingosine bases has the trans configuration (20). The multiplet marked c in Figure 17 was therefore attributed to the olefinic protons of the trans double bonds of the long-chain bases, and the multiplet marked b in the spectra of CMH and CTH was assigned to the olefinic protons of the cis double bonds of the unsaturated fatty acids.

Figure 16. Partial nmr spectra of CTH and O-β-D-galactopyranosyl-ceramide (CMH). These spectra were recorded at 220 MHz (through the facilities of the Canadian 220 MHz Nuclear Magnetic Resonance Center, Sheridan Park, Ontario) on 75 mg of CTH from normal kidney and 50 mg of bovine brain galactosylceramide (CMH) in 0.5 to 1 ml of pyridine-$\underline{d}_5$ containing a trace of D_2O, at 30°, with a sweep width of 500 Hz. The chemical shifts (δ) were calculated with reference to tetramethylsilane.

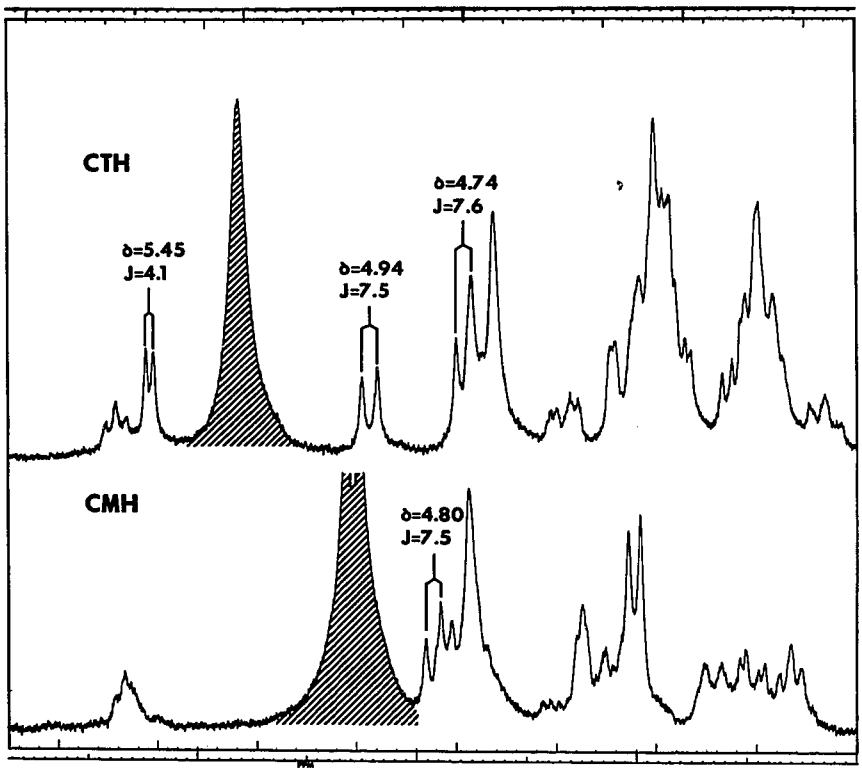
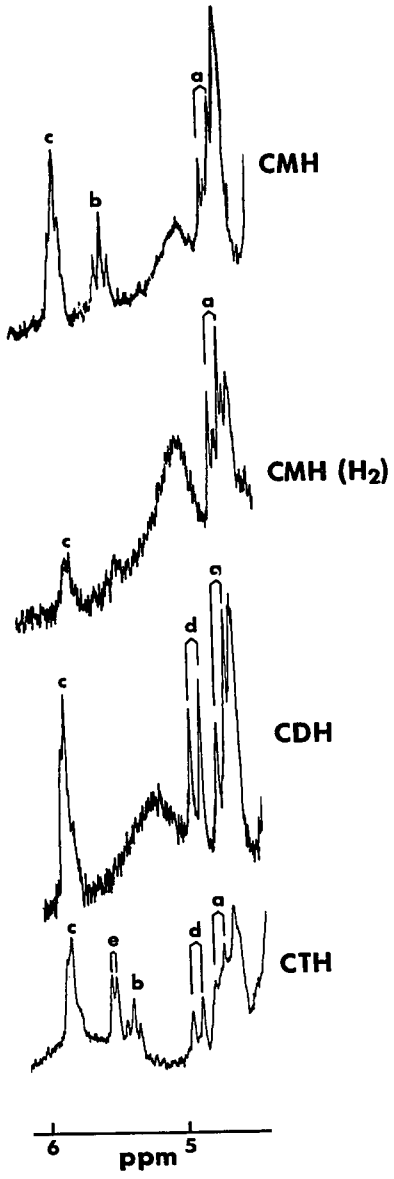

CTH
δ=5.45
J=4.1
δ=4.94
J=7.5
δ=4.74
J=7.6
CMH
δ=4.80
J=7.5

Figure 17. Partial nuclear magnetic resonance spectra of various glycosphingolipids and derivatives. Spectra of galactosylceramide (CMH), hydrogenated galactosylceramide [CMH(H_2)], lactosylceramide (CDH) and CTH were recorded at 100 MHz in 0.5-1 ml pyridine-d_5, at 30°, with a sweep width of 1000 Hz. Identification of peaks: a: anomeric proton of β-D-glycopyranosyl residue linked to ceramide; b: multiplet attributable to the olefinic protons of cis double bonds; c: multiplet attributable to the olefinic protons of trans double bonds; d: anomeric proton of a β-D-galactopyranosyl residue; e: anomeric proton of an α-D-galactopyranosyl residue. The broad humps in the baselines of the spectra are due to the presence of some moisture in the samples. The small peak at 5.9 ppm in the spectrum of CMH(H_2) indicates that the catalytic reduction of the trans double bonds of the long-chain bases was only 80-90 percent complete.


a
c
b
CMH
a
CMH (H2)
c
a
d
c
CDH
a
c
e
d
b
CTH
6
ppm
5

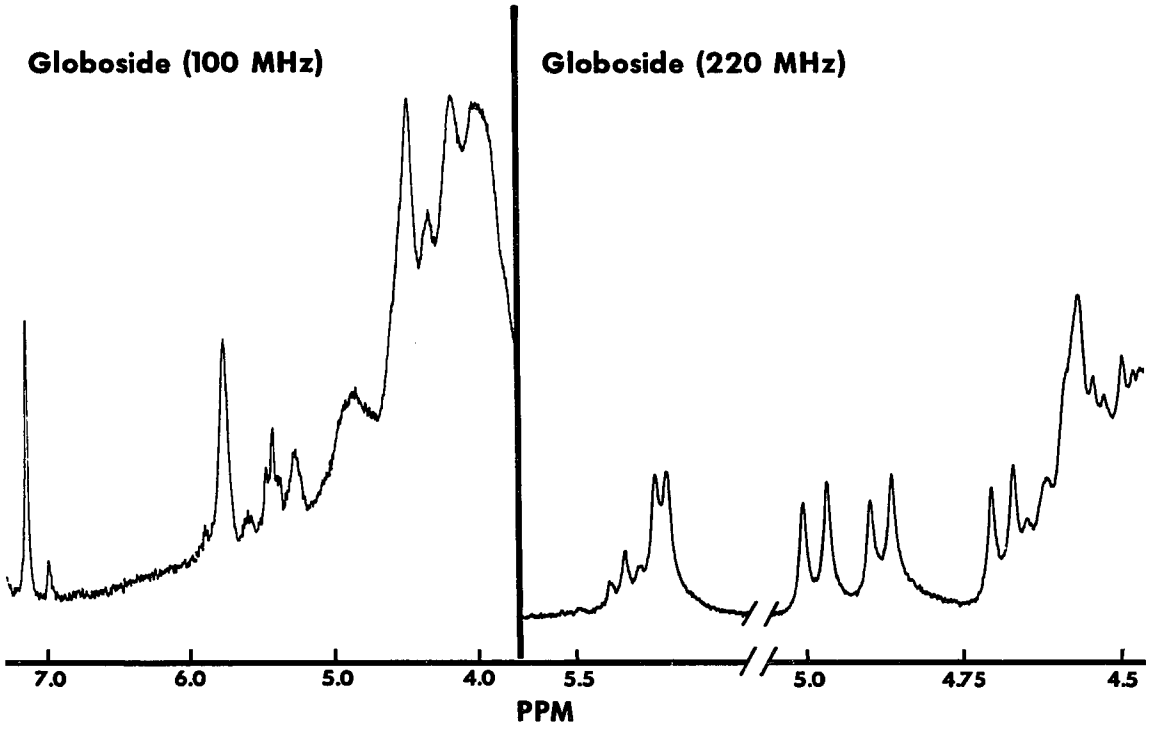
The doublet marked d in Figure 17 occurs in the spectra of CDH and CTH but not in that of CMH. It was attributed to the anomeric proton of a β-D-galactopyranosyl residue. The doublet marked e corresponds to that marked a in Figure 15. It occurs only in the spectrum of CTH and has the spectral properties of the anomeric proton of an α-D-galactopyranosyl residue.

The ratio of the areas under the doublets attributable to the anomeric protons in the spectrum of CTH shown in Figure 16 is, from downfield towards the tetramethylsilane absorption (i.e. left-to-right), 1.00:0.92:1.24 as determined by planimetry.

No attempt was made to interpret the upfield portions of the spectra where the absorptions due to the non-anomeric ring protons of the oligosaccharides and the protons of the other portions of the molecules occur.

Figure 18 shows spectra of globoside purified from normal human kidney. The resolution of the spectrum recorded at 100 MHz is poor. On the other hand, at 220 MHz, four doublets occurring between δ=4.70 and 5.50 ppm are clearly defined. The doublet furthest downfield (δ=5.36 ppm, J=3.9 Hz) has the spectral characteristics of an α-anomeric proton. The rest (δ=4.99 ppm, J=8.1 Hz; δ=4.88 ppm, J=7.5 Hz; δ=4.69 ppm, J=8.0 Hz) all have the characteristics of β-anomeric protons. The ratio of the areas under the four doublets is approximately unity. The evidence for the presence of a single α-galactosidic linkage in globoside is supported by the optical rotatory data (Table V), and is in keeping with the structure of erythrocyte CAH proposed by Hakomori et al (111).

Figure 18. Partial nuclear magnetic resonance spectra of human kidney globoside recorded at 100 and 220 MHz. The spectra were recorded on 109 mg globoside dissolved in 0.5-1 ml pyridine-$\underline{d}_5$ at 30° with sweep widths of 500 Hz.


Globoside (100 MHz)
Globoside (220 MHz)
7.0
6.0
5.0
4.0
5.5
5.0
4.75
4.5
PPM

7. Susceptibility to acid hydrolysis

The progress of the acid hydrolysis of CTH[^{3}H] and CDH[^{3}H] is shown in Figure 19. The release of ^{3}H-galactose from CTH[^{3}H] is clearly significantly faster than the release from CDH[^{3}H]. By 2 hours incubation, 70 percent of the CTH[^{3}H] had been hydrolyzed while only 46 percent of the CDH[^{3}H] was hydrolyzed. Alpha glycosidic linkages are, as a rule, more susceptible to hydrolysis than beta linkages (324). Since the product of the hydrolysis of CTH[^{3}H], as shown by tlc, was almost entirely lactosylceramide, the increased susceptibility of the lipid to acid hydrolysis was attributed to the lability of the terminal galactosidic linkage. This is presumptive, but by no means conclusive, evidence for a terminal α-D-galactopyranosyl residue in CTH.

8. Susceptibility to enzymic hydrolysis

Studies of the susceptibility of CTH[^{3}H] to hydrolysis by coffee bean α-galactosidase were done on two enzyme preparations, before alumina column chromatography and after. Figure 20 shows the effect of pH on the rates of hydrolysis of CTH[^{3}H], p-nitrophenyl-α- and -β-D-galactopyranoside by the enzyme preparation before alumina column chromatography. The rates of hydrolysis of p-nitrophenyl-β-D-galactopyranoside and CTH[^{3}H] were very slow and exhibit pH optima at pH 3.75-4.25. The nonspecific α-galactosidase activity was, on the other hand, very great and exhibited a complex pH profile with a major optimum at pH 6.0 and a minor optimum at pH 3.5-4.0. This is almost identical to the results reported by Courtois and Petek (304)

Figure 19. Acid hydrolysis of CTH and lactosylceramide (CDH). CTH and CDH (0.7 mmole, 4.5 mCi/mmole) were heated at 60° in 2 ml 0.3 N HCl in $CHCl_3$-CH_3OH (2:1, v/v) in test tubes with Teflon-lined stoppers for 4 hours. Aliquots (0.2 ml) were removed at 30 min intervals and neutralized with 0.6 ml 0.1 N NaOH and 0.4 ml H_2O added. The solutions were partitioned against 4.8 ml $CHCl_3$-CH_3OH (2:1) and centrifuged. Aliquots (0.1 ml) of the upper phases were evaporated to dryness in counting vials and dissolved in 1 ml H_2O and 10 ml naphthalene scintillant for counting.

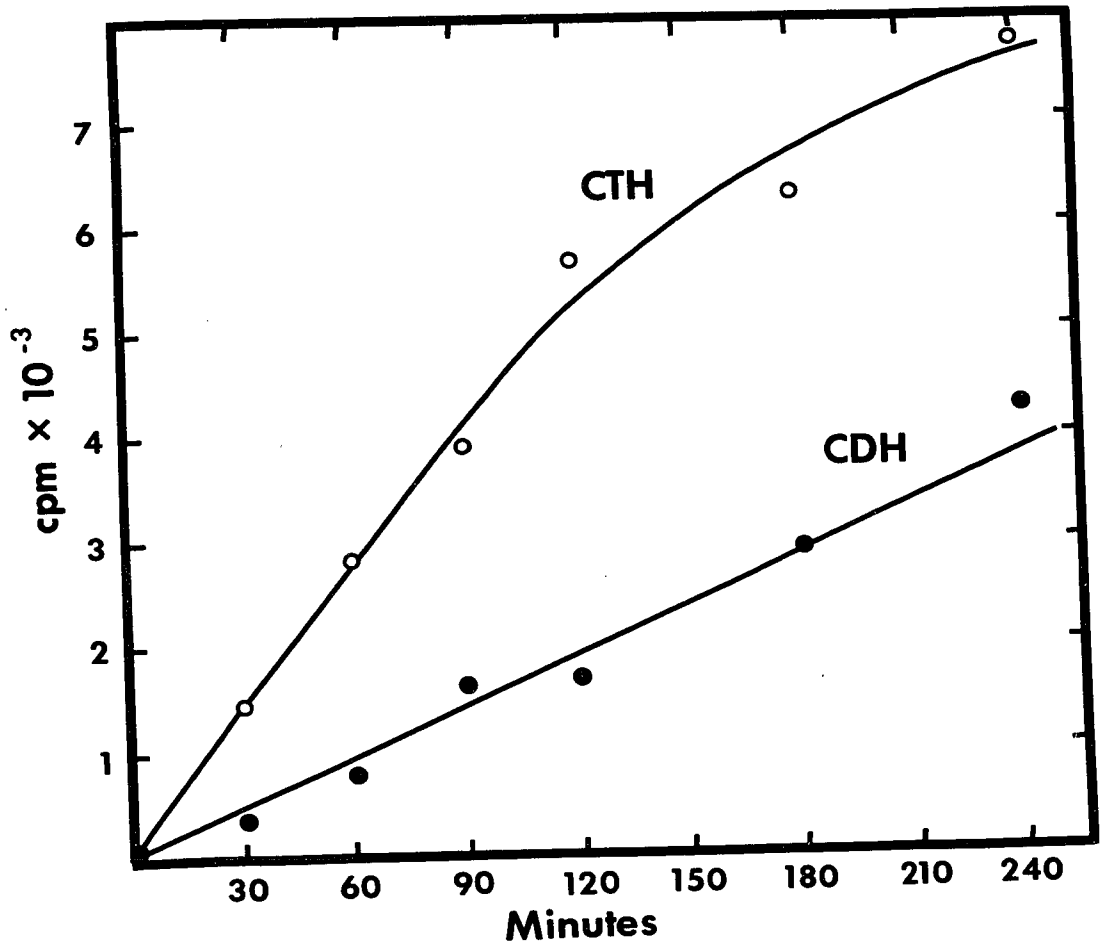
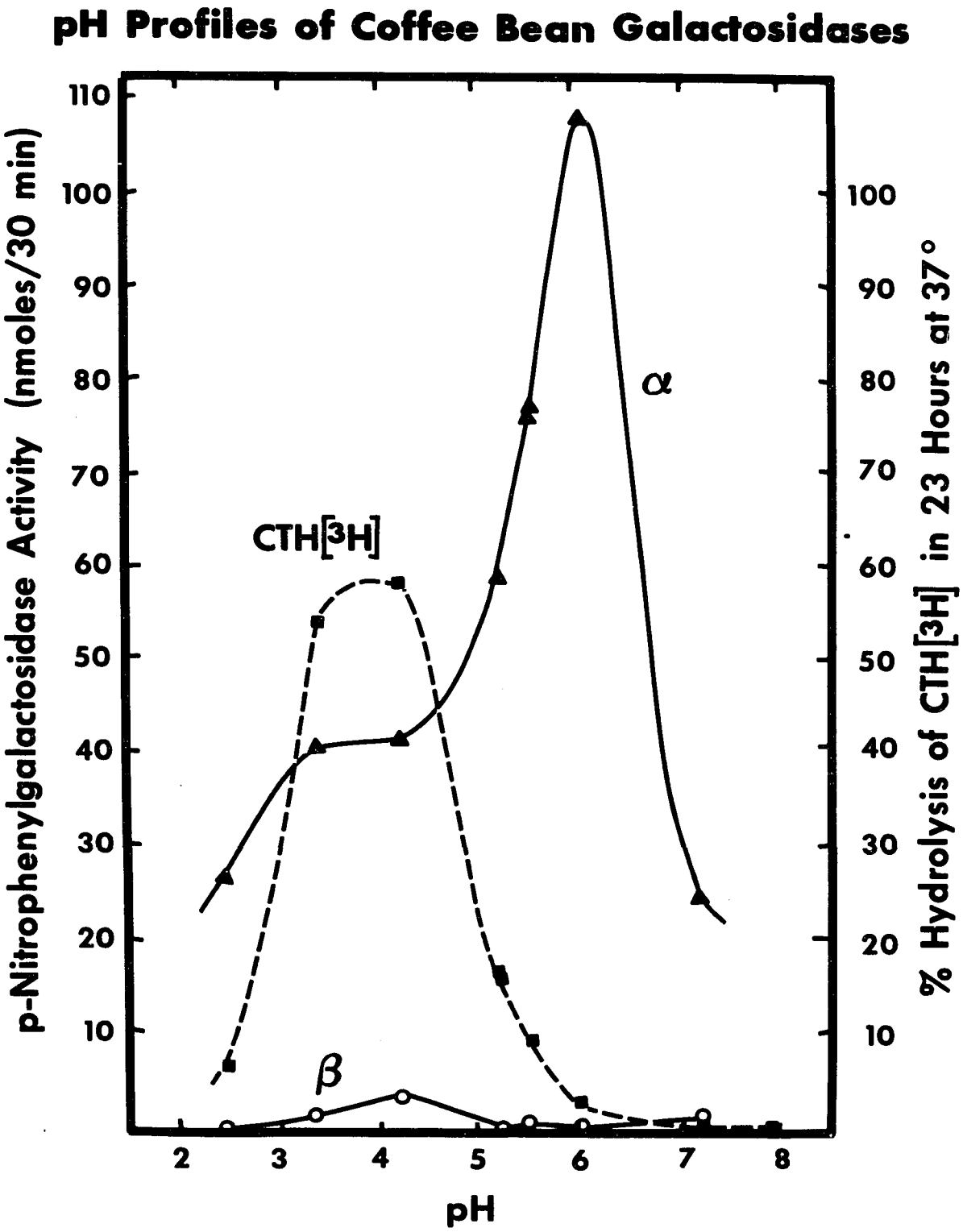

cpm × 10⁻³
7
6
5
4
3
2
1
CTH
CDH
30
60
90
120
150
180
210
240
Minutes

Figure 20. Effect of pH on the hydrolysis of CTH and p-nitrophenyl-α- and -β-D-galactopyranosides by crude α-galactosidase from coffee beans. The incubation mixtures contained a) for the hydrolysis of the p-nitrophenyl-galactosides, 2 mg of substrate, 0.15 ml of citrate-phosphate buffer, 0.05 ml of water saturated with toluene, enzyme preparation (approx. 0.03 mg of protein) and water to a final volume of 0.5 ml; and b) for the hydrolysis of CTH[^{3}H], 10.4 nmoles of CTH[^{3}H] (41.2×10^3 dpm/nmole) in sodium cholate solution (10 mg/ml), 0.5 ml of citrate-phosphate buffer, 0.15 ml of water saturated with toluene, 1 mg of sodium cholate, enzyme preparation (approx. 1.5 mg of protein) and water to a final volume of 1.5 ml. The incubations were carried out at 37° and the amounts of substrate hydrolyzed determined as described in the text. Abbreviations: α, p-nitrophenyl-α-D-galactopyranoside; β, p-nitrophenyl-β-D-galactopyranoside.


pH Profiles of Coffee Bean Galactosidases
p-Nitrophenylgalactosidase Activity (nmoles/30 min)
% Hydrolysis of CTH[3H] in 23 Hours at 37°
pH
CTH[3H]
α
β
110
100
90
80
70
60
50
40
30
20
10
2
3
4
5
6
7
8

for their crude enzyme preparation. The decision to pursue the purification further was based on the ambiguity caused by the presence of non-specific β-galactosidase activity in the crude enzyme preparation.

By alumina column chromatography a successful separation of non-specific α- and β-galactosidase activities was achieved, but the α-galactosidase activity could not be resolved into two enzymes as described by Courtois and Petek (304). The elution profile of the column is shown in Figure 21. Peak I of protein contained mainly nonspecific β-galactosidase activity. Peak II contained only nonspecific α-galactosidase activity and was used in all subsequent studies. Peaks III and IV contained no α- or β-galactosidase activity.

Figure 22 shows the effect of pH on the hydrolysis of p-nitrophenyl-α- and β-D-galactopyranosides, melibiose, and CTH[^{3}H] by the purified coffee bean α-galactosidase. Absolutely no nonspecific β-galactosidase activity could be demonstrated in the enzyme preparation in spite of 18 hrs incubation. The pH profile for the hydrolysis of CTH[^{3}H] is very similar to that for the hydrolysis of melibiose, another "natural" α-galactoside. This is in keeping with Courtois and Petek's observations on the difference in pH profile of the coffee bean α-galactosidase when tested with synthetic, aromatic substrates and "natural" substrates (oligosaccharides) (304).

The products of the reaction of CTH[^{3}H] with a crude enzyme preparation were shown, by tlc, to be only unreacted CTH[^{3}H] and

Figure 21. Elution profile of alumina column chromatographic separation of proteins in coffee bean extract. The details of the preparation and elution of the column are described in the text.

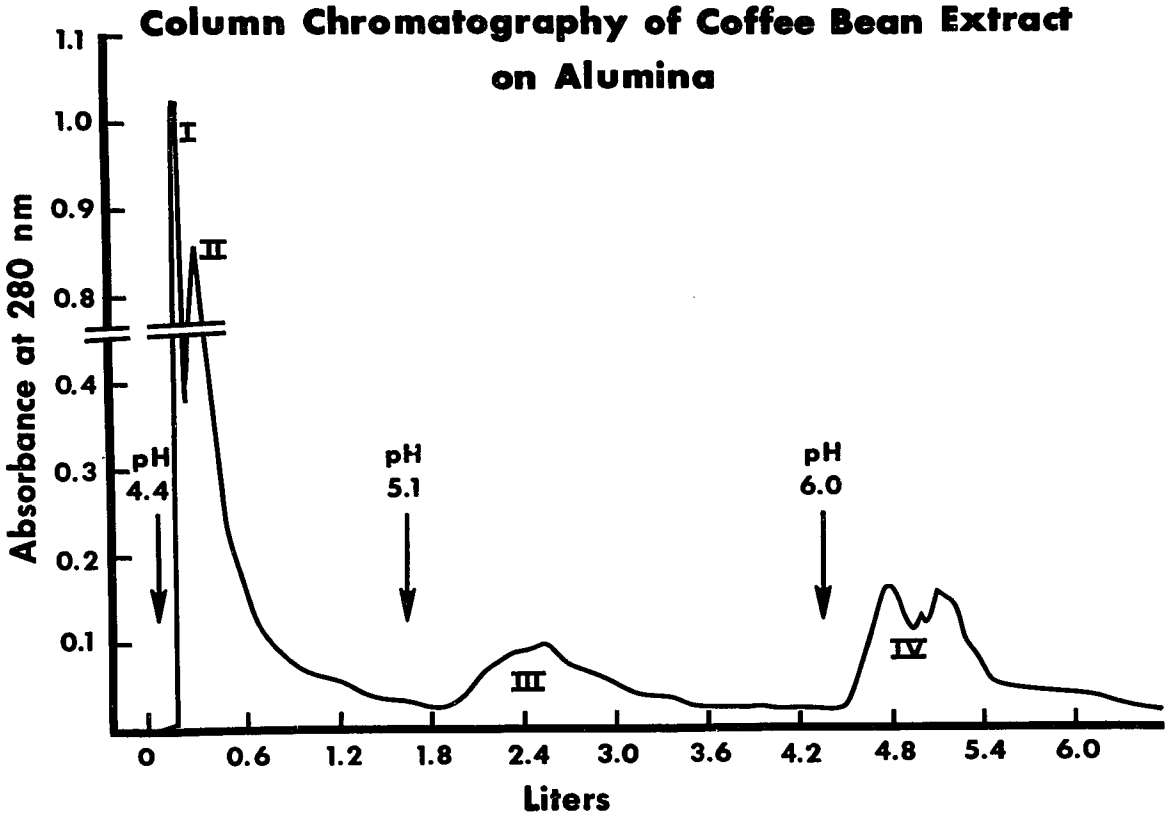
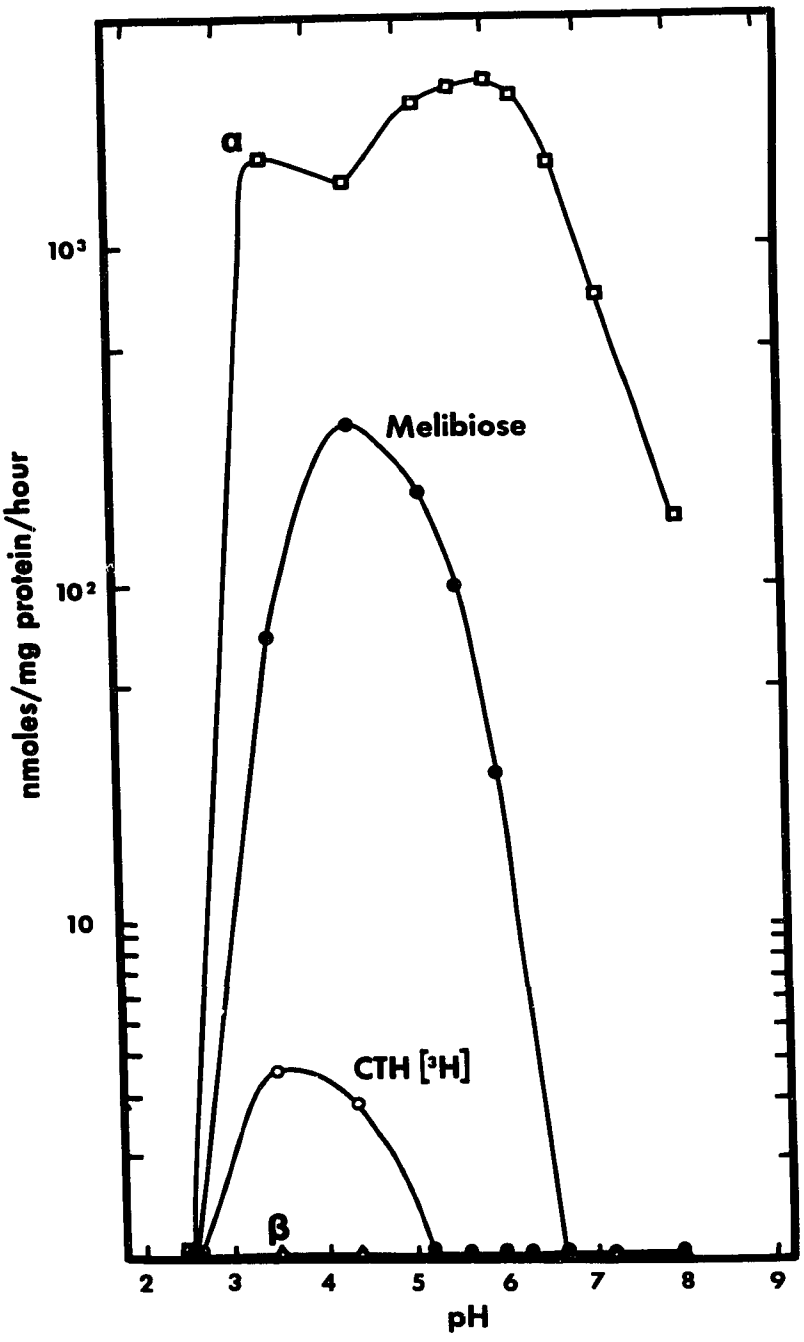

Column Chromatography of Coffee Bean Extract on Alumina
Absorbance at 280 nm
1.1
1.0
0.9
0.8
0.4
0.3
0.2
0.1
I
II
III
IV
pH 4.4
pH 5.1
pH 6.0
0
0.6
1.2
1.8
2.4
3.0
3.6
4.2
4.8
5.4
6.0
Liters

Figure 22. Effect of pH on the hydrolysis of various D-galactosides by coffee bean α-galactosidase after alumina column chromatography. The source of the enzyme was the protein in Peak II of the alumina column effluent (Figure 21). The experimental conditions are described in the text. Substrates: α, p-nitrophenyl-α-D-galactopyranoside; β, p-nitrophenyl-β-D-galactopyranoside; CTH[^{3}H] and melibiose.


α
Melibiose
CTH [³H]
β
10³
10²
10
nmoles/mg protein/hour
2
3
4
5
6
7
8
9
pH

lactosylceramide (Figure 23). This confirmed the location of the α-D-galactopyranosyl residue at the nonreducing terminal of the oligosaccharide in CTH.

Table X shows the results of a comparison of the hydrolysis of CTH[^{3}H] and CDH[^{3}H] by purified coffee bean α-galactosidase and commercial *E. coli* β-galactosidase (Worthington). Whereas there was considerable hydrolysis of CTH[^{3}H] by α-galactosidase, the hydrolysis of CDH[^{3}H] was negligible. Conversely, CTH[^{3}H] was completely resistant to hydrolysis by β-galactosidase under conditions in which there was a small but significant hydrolysis of CDH[^{3}H].

B. Properties of Renal Galactohydrolases

The relationship between the protein concentration and amount of product formed was linear under all conditions the nonspecific α- and β-galactosidase and CTH galactohydrolase activities in renal tissue were measured. The relationship between the duration of incubation and amount of product formed was also linear up to 90 min incubation.

1. pH optimum

The pH profile of human renal nonspecific α-galactosidase exhibited a sharp optimum at pH 5.2 (Figure 24). CTH galactohydrolase, on the other hand, exhibited a sharp, symmetrical optimum at pH 3.6.

2. Subcellular distribution

Figure 25 shows the subcellular distributions of nonspecific α- and β-galactosidase activity. Since these enzymes are postulated to be located

Figure 23. Thin-layer chromatogram of the products of the hydrolysis of CTH by coffee bean α-galactosidase. Approximately 50 μg of CTH[^{3}H] were mixed with 20 mg sodium cholate, 0.2 ml water saturated with toluene, 0.5 ml citrate-phosphate buffer (pH 4.0, ionic strength 0.5 M), and approximately 4 mg of crude coffee bean α-galactosidase in a final volume of 2.0 ml. The mixture was incubated at 37° for 20 hrs. The reaction was terminated by the addition of 0.2 ml 1 N NaOH (to make the mixture alkaline) and 7.5 ml chloroform-methanol (2:1). Following mixing, centrifugation and removal of the upper phase, the lower phase was washed twice with 2 ml alkaline "ideal upper phase" (303) to ensure the removal of sodium cholate. The lower phase lipids were chromatographed with appropriate standards on a 0.25 mm thick layer of silica gel H using System A. The lipids were visualized by spraying with orcinol-H_2SO_4 and heating. The reaction products are in the left lane; the standards are on the right.

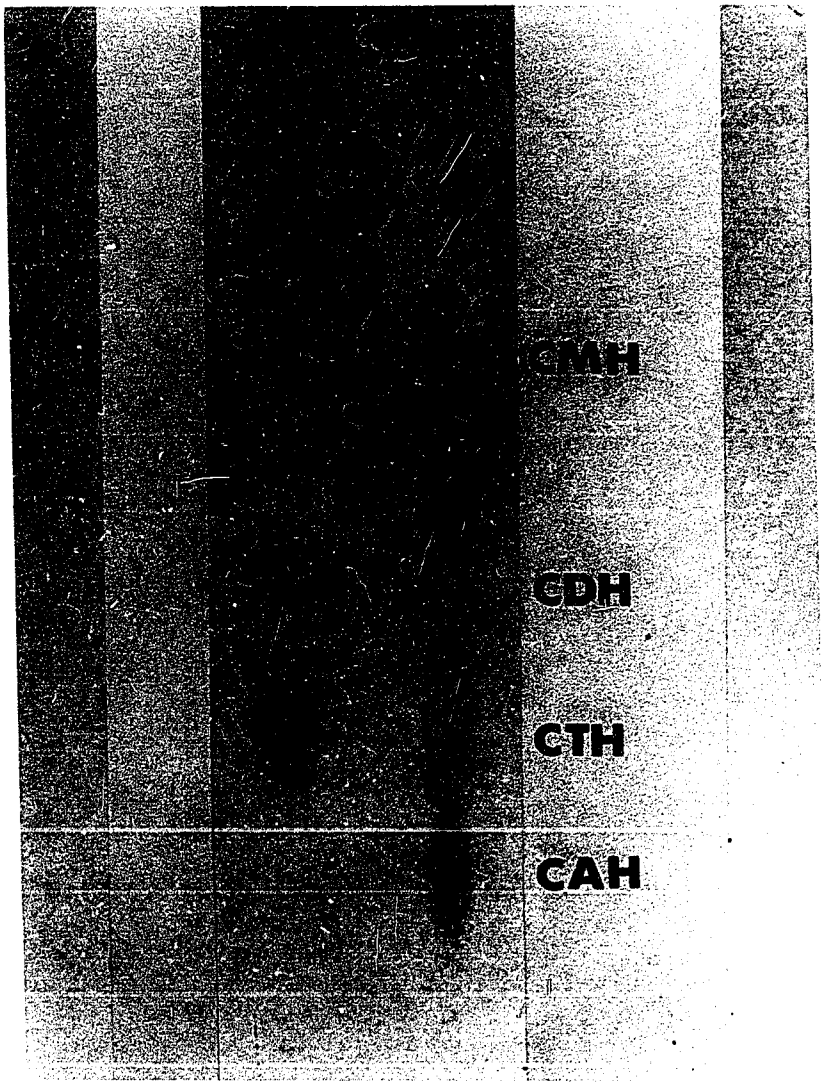

CMH
CDH
CTH
CAH

Table X: Enzymatic hydrolysis of CTH and lactosylceramide

Substrate	Specific Activity	α-Galactosidase[a]	β-Galactosidase[b]
	dpm/nmole	dpm	dpm
Galactosylgalactosyl-glucosylceramide (CTH)	1.0×10^4	10,000	0
Galactosylglucosyl-ceramide (Lactosylceramide)	1.1×10^4	750	5600

[a] The reaction mixture contained 12 nmoles of substrate, 2 mg sodium cholate, 0.015 mg partially purified coffee bean α-galactosidase, 0.2 ml water saturated with toluene, 0.5 ml citrate-phosphate buffer, pH 3.9 in a final volume of 1.5 ml.

[b] This reaction mixture contained 12 nmoles of substrate, 1 mg sodium cholate, 3.2 mg *E. coli* β-galactosidase, 1 ml citrate-phosphate buffer, pH 6.0, in a final volume of 1.5 ml.

The incubations were carried out at 37° for 24 hours. The reactions were stopped and the radioactivity in free ^{3}H-galactose determined as described in Materials and Methods. The values have been corrected for background radioactivity and the radioactivity of the blank, in which enzyme heated for 3 min at 100° was substituted for the native enzyme.

Figure 24. Effect of pH on the hydrolysis of CTH and p-nitrophenyl-α-D-galactopyranoside by human kidney.

- ● Enzymic hydrolysis of CTH[^{3}H] by normal human kidney.
- o Enzymic hydrolysis of p-nitrophenyl-α-D-galactopyranoside by normal human kidney.
- ▲ Enzymic hydrolysis of p-nitrophenyl-α-D-galactopyranoside by kidney tissue from a patient with Fabry's disease (Case 2).

The experimental conditions are described in the text.

Effect of pH on Hydrolysis of CTH and p-Nitrophenyl- α -galactoside by Human Kidney

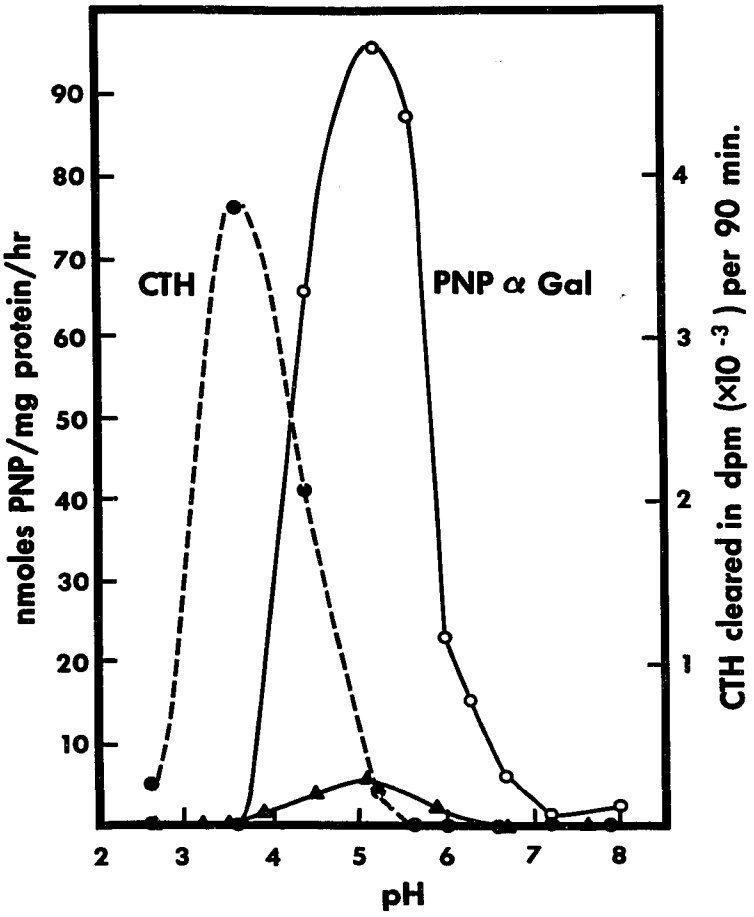
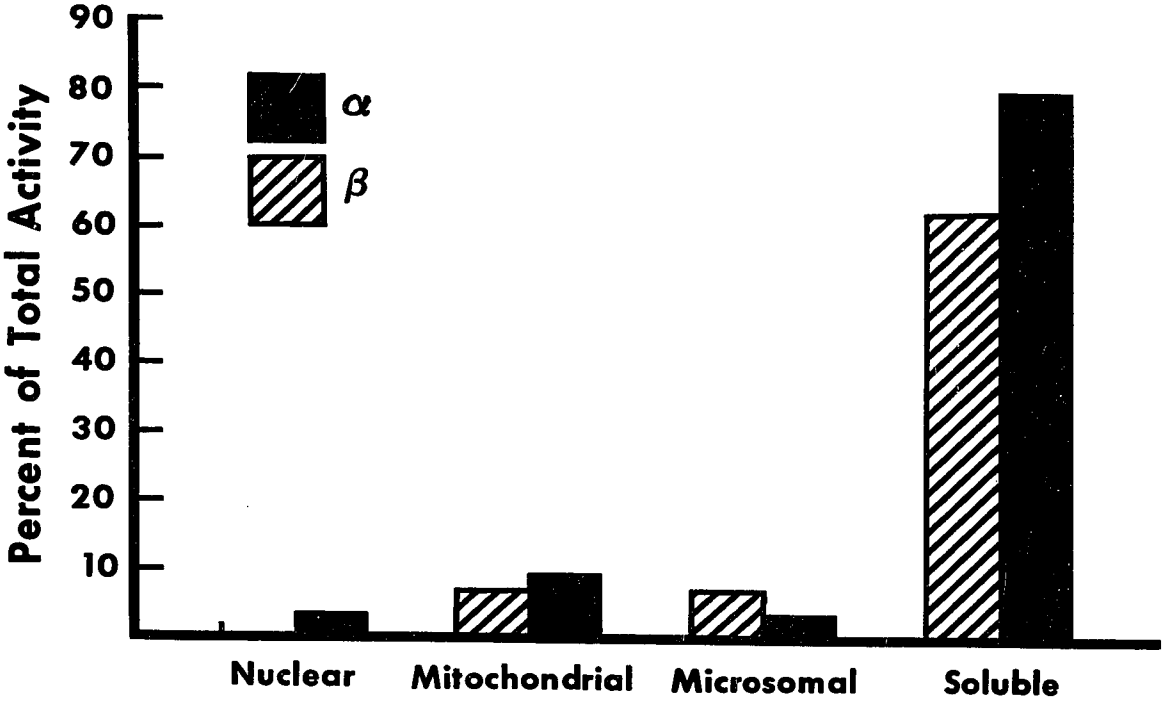


Figure 25. Subcellular distribution of nonspecific galactosidases in human kidney: α, nonspecific α-galactosidase; β, nonspecific β-galactosidase. The source of tissue, enzyme assays and details of the preparation of the subcellular fractions are described in the text and in Figure 11.

Subcellular Distribution of Human Renal Nonspecific Galactosidases



in lysosomes (219), the demonstration of 80 percent of the α-galactosidase and 61 percent of the β-galactosidase in "Soluble" fraction was surprising. This anomalous distribution was probably caused by the release of the enzymes into the cell sap by postmortem autolysis. All the results described in succeeding sections were obtained from studies of the "Soluble" α-galactosidase.

3. Effect of divalent cations, EDTA and other additions on α-galactosidase activity

Dialysis of the enzyme preparation against deionized water for 18 hrs caused a 30 percent loss of activity which was completely restored by making the assay mixture 20 mM with respect to Mg^{+2}. Further dialysis did not increase the loss of activity, but the addition of tetrasodium ethylenediamine tetraacetate (final concentration 10 mM) to the assay caused a further 42 percent loss of activity. Concentrations of Mg^{+2} over 25 mM caused inhibition of the enzyme activity: apparent K_I=241.7 mM (Figure 26). The effects of various divalent cations on the α-galactosidase activity in the 25-50 Fraction are summarized in Table XI. Several divalent cations (Ca^{+2}, Co^{+2}, Zn^{+2}) appear as effective as Mg^{+2} in stimulating the α-galactosidase activity of dialyzed enzyme preparations. Some, notably Cu^{+2} and especially Hg^{+2}, were frankly inhibitory.

The Km of α-galactosidase was 25 mM with respect to p-nitrophenyl-α-D-galactopyranoside in the absence of added Mg^{+2} (Figure 27). The addition of Mg^{+2} (20 mM) caused a decrease in the Km (5.5 mM), but it also caused a

moderate decrease in the Vmax. (from 0.143 μmoles/hr to 0.118 μmoles/hr). Melibiose was a competitive inhibitor (Figure 27), but myoinositol, which has been shown to inhibit α-galactosidases from other sources (312), had little or no effect (Table XI).

4. Ammonium sulfate fractionation

The presence of ammonium sulfate had no effect on nonspecific α-galactosidase activity. Nevertheless, fractional precipitation of the enzyme with salt caused a 35 percent loss of total enzyme activity. Of the activity recovered, 66.5 percent precipitated between 25 and 50 percent saturation and the rest remained in solution at 50 percent saturation. No activity was detected in the 0-25 Fraction. The ammonium sulfate fractionation resulted in a 25 fold increase in the specific activity of nonspecific α-galactosidase.

The ammonium sulfate fractionation caused an even greater loss of nonspecific β-galactosidase activity (approx. 85 percent), but the distribution of the recovered activity was the same as that of the nonspecific α-galactosidase. Thus no true separation of nonspecific galactosidases was achieved at this stage of fractionation.

5. Renal CTH galactohydrolase

Detailed studies of the properties of CTH galactohydrolase were hampered by the shortage of satisfactory autopsy or surgical specimens of normal human kidney and by the marked lability of the enzyme. Nevertheless it was possible to establish the pH optimum under the assay conditions

Figure 26. Effect of Mg^{++} on human renal nonspecific α-galactosidase (Dixon plot).

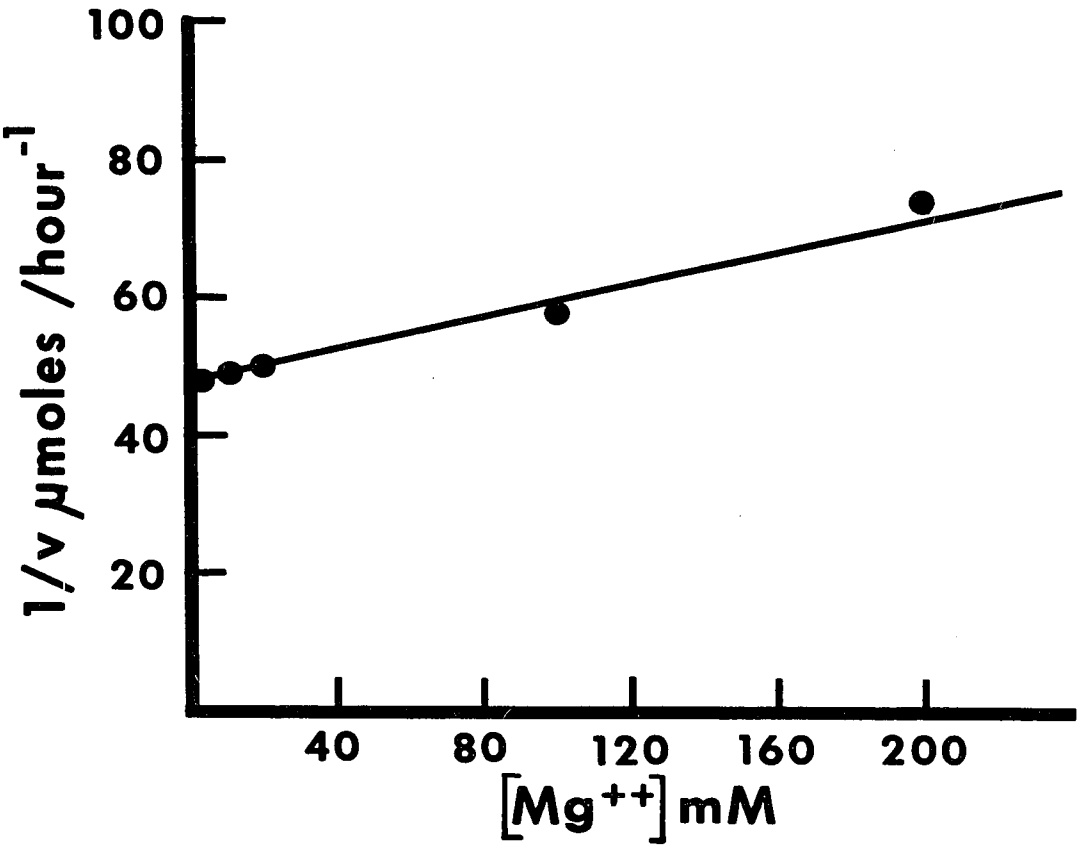

100
80
60
40
20
1/v μmoles /hour⁻¹
40
80
120
160
200
[Mg⁺⁺] mM

Figure 27. Effect of melibiose on human renal nonspecific α-galactosidase (Lineweaver-Burk plot).

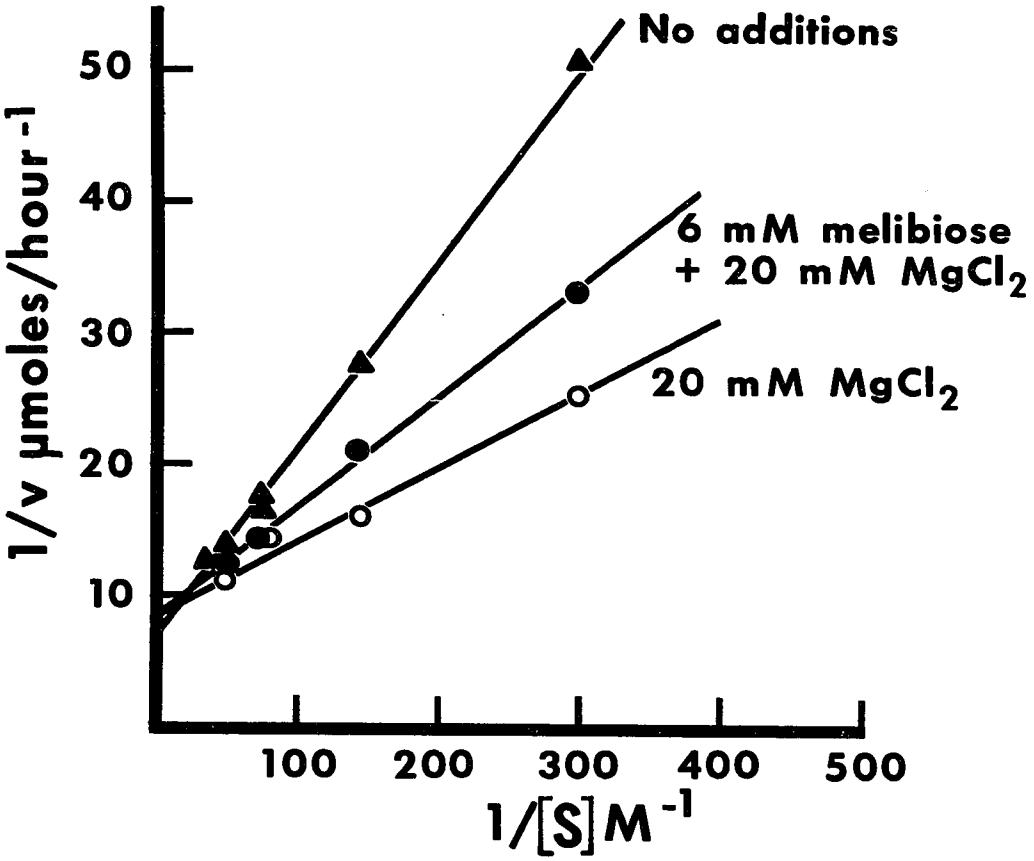

No additions
6 mM melibiose
+ 20 mM MgCl2
20 mM MgCl2
1/v µmoles/hour-1
50
40
30
20
10
100
200
300
400
500
1/[S]M-1

Table XI: Effect of various reagents on human renal nonspecific α-galactosidase activity

Additions	Concentration (mM)	% Activity
None	-	100
Co^{++}	20	164
Ca^{++}	20	158
Mg^{++}	20	153
Zn^{++}	20	143
Cu^{++}	20	75
Hg^{++}	10	21
EDTA	10	28
Myoinositol	20	93
Myoinositol	60	92

employed (Figure 24). The rate of hydrolysis of CTH[^{3}H] by normal human kidney at pH 3.6 was 0.06 nmoles/hr/mg protein in one patient and 0.11 nmoles/hr/mg in another. In crude homogenates of kidney tissue obtained at surgery from a patient with Fabry's disease (Case 2), no CTH galactohydrolase activity could be demonstrated at all between pH 2.5 and 8.0. The nonspecific α-galactosidase activity (Figure 24) was greatly attenuated, but it exhibited a normal pH optimum. The nonspecific β-galactosidase activity of the homogenate was normal.

C. Clinical Studies

1. Urinary neutral glycosphingolipids

Table XII shows the results of the quantitative analysis of the neutral glycosphingolipids in the urinary sediment of a number of patients in various states of health. The nephrotic syndrome in R.H. was due to chronic glomerulonephritis. Although the levels of glycolipids in his urinary sediment were elevated, the pattern was very much like the pattern of neutral glycosphingolipids in normal renal parenchyma (59). There was no preferential increase in the urinary CTH level. R.C. and A.H. are Cases 1 and 2 described in detail in Materials and Methods. O.H. is the sister of A.H., and on the basis of the increased excretion of digalactosylceramide and CTH, she is a carrier of Fabry's disease. The marked increase in excretion of glucosylceramide by O.H. is perplexing. The source of the lipid is not clear. It may be derived from epithelial cells of the external genitalia rather than from the urinary tract. S.C. is a

Table XII: Urinary neutral glycosphingolipids

Subject	Sex	Age	Diagnosis	gal-cer	glc-cer	digal-cer	lact-cer	trihex-cer	globoside
						μmoles/24 hr			
J.C.	M	30	Normal	tr.	0.027	tr.	0.013	0.015	0.014
M.K.	M	34	Normal	tr.	0.019	tr.	0.010	(0.007)	0.012
J.W.	M	26	Normal	tr.	0.013	tr.	0.024	0.033	0.016
A.S.	M	68	Normal	tr.	0.014	tr.	0.012	0.014	0.013
R.H.	M	41	Nephrotic syndrome	0.010	0.053	tr.	0.018	0.096	0.097
R.C.	M	40	Fabry's variant	0.017	0.024	0.036	0.018	0.295	0.012
				tr.	0.051	0.039	0.029	0.310	0.035
A.H.	M	48	Fabry's variant	0.011	0.060	0.361	0.089	0.749	0.058
S.C.	M	c.30	Fabry's disease	0.012	0.039	0.233	0.065	1.650	0.040
O.H.	F	c.60	Fabry's carrier	tr.	0.400	0.188	0.062	0.284	0.062
N.T.	M	15	Fabry's disease	0.025	0.070	0.339	0.024	1.975	0.039

distant cousin of A.H. (No. 17 in the pedigree in Figure 4). He has many of the classical clinical stigmata of Fabry's disease including the pathognomonic skin rash. N.T. is an unrelated patient who has Fabry's disease (Case 3).

These data indicate the diagnostic value of the quantitative analysis of urinary neutral glycosphingolipids as described by Philippart et al (267) and Desnick et al (150,313).

2. Plasma neutral glycosphingolipids

In the plasma all the CMH was glucosylceramide, and all the CDH was lactosylceramide. In spite of the marked accumulation of digalactosylceramide in the kidney in Fabry's disease (91), none appeared in the plasma.

Table XIII shows that the plasma levels of CTH were elevated in patients with Fabry's disease. Moreover, the CTH levels were not significantly affected by renal homotransplantation. In one case (A.H. before renal transplantation) the plasma levels of CDH and globoside were significantly lower than in the controls. The plasma levels of CMH and CDH were very high in R.C. (Case 1). This patient had hyperlipoproteinemia with a Frederickson type IV pattern (hyper-pre-β-lipoproteinemia) (314). Until the glycosphingolipid composition of the various plasma lipoproteins is known, the significance of the elevated plasma CTH concentration will be in doubt.

Table XIII: Plasma neutral glycosphingolipids

	CMH	CDH	CTH	CAH
		μmoles/100 ml		
Controls (6)	0.99 ± 0.14	0.63 ± 0.04	0.31 ± 0.03	0.36 ± 0.04
R.C. (Case 1)	2.73	1.15	0.87	0.44
N.T. (Case 3)	0.80	0.58	0.95	0.45
A.H. (Case 2)				
Peritoneal dialysis				
Before	0.51	0.34	0.65	0.17
After	0.48	0.36	0.61	0.26
Hemodialysis				
1. Before	0.98	0.36	1.09	0.34
After	0.95	0.31	0.57	0.18
2. Before	0.62	0.18	0.50	0.18
After	0.68	0.36	0.58	0.26
Renal transplantation				
Before (10)	0.70 ± 0.06	0.38 ± 0.03	0.65 ± 0.06	0.21 ± 0.02
After (6)	0.63 ± 0.08	0.56 ± 0.07	0.74 ± 0.09	0.33 ± 0.09

Values represent means ± SEM where applicable.

3. Leukocyte α-galactosidase

Table XIV shows the marked deficiency of nonspecific α-galactosidase activity in the leukocytes of patients with Fabry's disease. Unlike Kint (107) however, we were able to demonstrate some enzyme activity, albeit only 6-8 percent of normal, in patients with Fabry's disease (N.T.) and in Fabry's disease variants (R.C. and A.H.).

4. Kidney lipids

The lipid compositions of Fabry's kidney tissue, Case 2, obtained at nephrectomy, was compared with that of renal tissue obtained at autopsy from patients who had died of diseases unrelated to the kidneys (Table XV). The water content of the Fabry's tissue was much lower than in normal human kidney. This was interpreted as a direct reflection of the replacement of normal renal parenchyma by cells packed with hydrophobic lipid material. The glycolipid hexose in the tissue was 15 times the normal content. Since it is normally a minor constituent of the kidney lipids, however, the total lipid content is only doubled. The failure to demonstrate an increase in the lipid phosphorus was also surprising in light of Kahnau's claim that the material accumulating in Fabry's disease was a phospholipid (268).

The analysis of the lipids in the renal allograft of the patient (Case 2) who died six months following renal transplantation indicated that no accumulation of glycosphingolipid had occurred in the graft during that period (Table XV). The glycolipid hexose concentration is slightly higher than in the control kidneys, but the graft was from a young woman whereas

Table XIV: Leukocyte nonspecific α- and β-galactosidase activities

	Enzyme activity in nmoles substrate* cleaved/hr/mg protein	
	α	β
Diagnosis		
Normal	35.6	71.1
Normal	40.7	94.0
Normal	60.8	110.0
Cerebral degeneration	77.2	195.0
Renal failure	57.1	130.0
Krabbe's globoid cell leukodystrophy	45.3	117.3
Hurler's disease	41.3	77.6
G_{M1}-gangliosidosis (variant)	57.8	10.0**
Chronic glomerulonephritis	38.5	81.3
Mean ± S.D.	50.5 ± 12.9 (9)	109.5 ± 37.6 (8)
Fabry's disease:		
R.C. (Case 1)	3.8	124.6
A.H. (Case 2)	4.2	65.0
N.T. (Case 3)	3.7	71.4

* Substrates were α, 4-methylumbelliferyl-α-D-galactopyranoside

β, 4-methylumbelliferyl-β-D-galactopyranoside

Conditions of assays are described on p. 68.

** Omitted from calculations

Table XV. Lipid composition of human kidney. Portions of kidney tissue (10-12 g) were homogenized in 7-8 vol. $CHCl_3$-CH_3OH (1:2, v/v) and stirred at room temperature for 20 hrs. After filtration the residue was further extracted by refluxing with 10 vol. $CHCl_3$-CH_3OH (2:1, v/v) for 6 hrs, then stirring at room temperature for 18 hrs. The extract was filtered. The combined filtrates were made 2:1 chloroform to methanol by the addition of chloroform and partitioned against 0.2 vol. 0.1 M KCl. The analyses of glycolipid hexose, lipid phosphorus and total lipid were done on the lower phase lipids. The upper phase was concentrated in vacuo and dialyzed for 48 hrs against running tap water at 4° for the determination of the ganglioside NANA concentration.

Lipid Composition of Human Kidney

Specimen	Water Content %	Total Lipids mg/g dry wt.	Glycolipid Hexose mg/g dry wt.	Lipid Phosphorus mg/g dry wt.	Ganglioside NANA* µg/g dry wt.
Controls					
1	81.4	185.2	1.43	2.60	88.3
2	80.9	189.6	1.29	2.73	64.8
Fabry's					
A.H.	70.5	261.5	19.6	1.67	100.9
A.H. (Graft)	87.7	136.8	1.89	3.15	64.6

*NANA = N-acetylneuraminic acid

the control kidneys were obtained from patients who had died at over 65 years of age. Martensson (59) has shown that in humans the glycosphingolipid concentration of the kidney decreases with advancing age.

Efforts are now being made to quantitate all the glycolipids in the pathological and normal tissues with particular attention directed towards the identification and measurement of digalactosyldiglycerid , which contains a terminal α-D-galactopyranosyl residue (315), and digalactosylceramide, which probably contains a similar α-galactosyl residue.

5. Effects of peritoneal dialysis, hemodialysis and renal homotransplantation on plasma and urinary glycosphingolipids

The effects of peritoneal dialysis, hemodialysis and renal transplantation on plasma glycosphingolipids are shown in Table XIII and Figure 28. Peritoneal dialysis had no effect; hemodialysis appeared to cause a decrease in plasma CTH levels on one occasion, but not on another. The effects of bilateral nephrectomies and renal transplantation are complex. Both operations were followed by reductions in CTH levels followed by moderately rapid increases to higher than preoperative levels. These findings indicate that many factors, including simple laparotomy, may affect the plasma levels of CTH. Studies designed to evaluate this possibility are planned.

Whereas renal transplantation appears to have no long-term effect on plasma levels of CTH, the levels in the urine dropped precipitously, but remained 4-8 times normal levels (Figure 29). The urinary excretion of

Figure 28. The effects of bilateral nephrectomies and renal homotransplantation on the plasma CTH concentration in Fabry's disease.

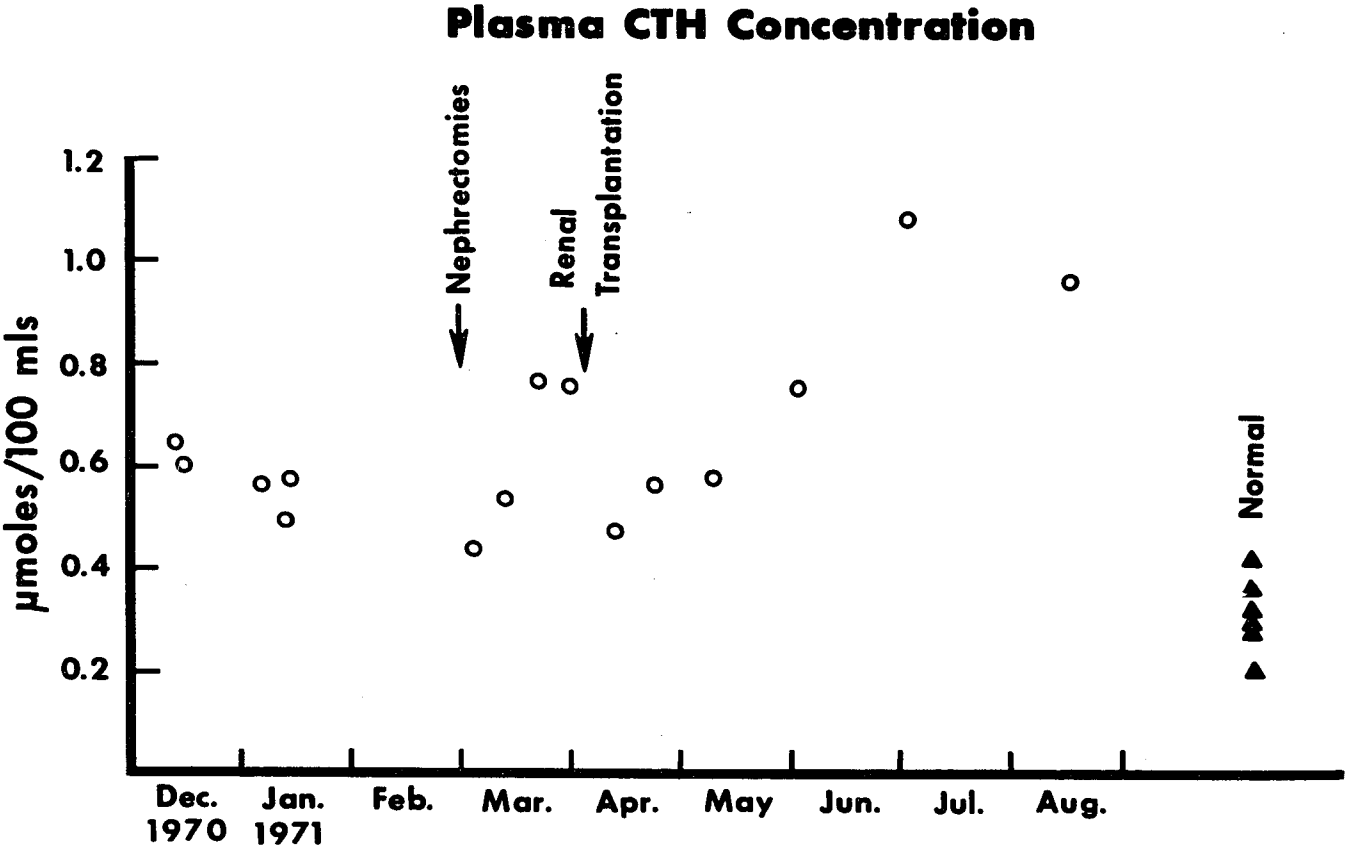
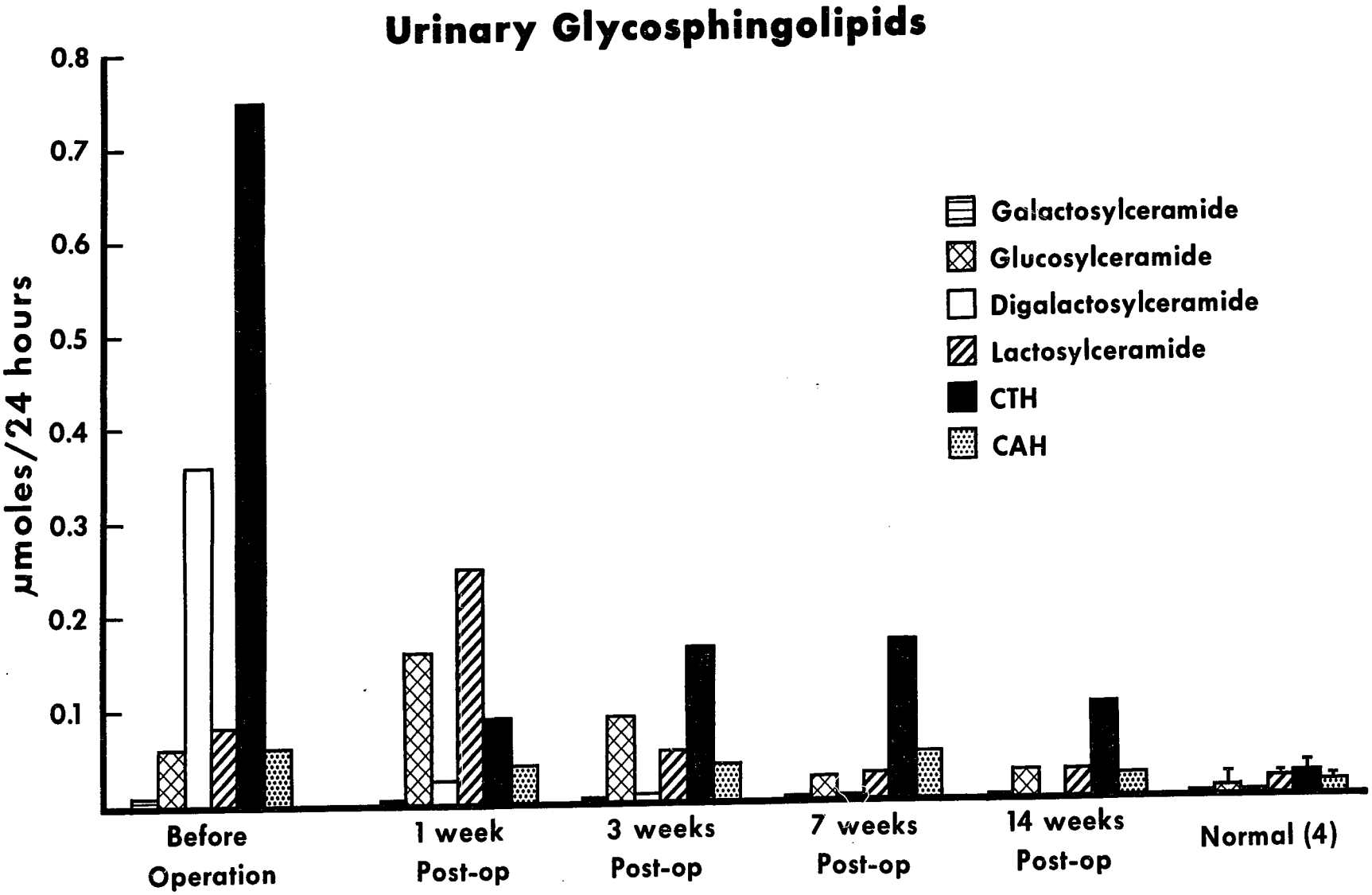

Plasma CTH Concentration
Nephrectomies
Renal Transplantation
µmoles/100 mls
1.2
1.0
0.8
0.6
0.4
0.2
Normal
Dec. 1970
Jan. 1971
Feb.
Mar.
Apr.
May
Jun.
Jul.
Aug.

Figure 29. The effect of renal homotransplantation on the urinary neutral glycosphingolipids in Fabry's disease.


Urinary Glycosphingolipids
µmoles/24 hours
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Galactosylceramide
Glucosylceramide
Digalactosylceramide
Lactosylceramide
CTH
CAH
Before Operation
1 week Post-op
3 weeks Post-op
7 weeks Post-op
14 weeks Post-op
Normal (4)

digalactosylceramide, which is derived entirely from the kidney, fell to negligible levels. On the basis of this observation, it was felt that the CTH in the urinary sediment after renal transplantation was derived principally from the patient's own collecting system, which was preserved during the operative procedures.

Immediately postoperatively the urinary levels of lactosylceramide were very high. This correlated with a mild pyelonephritis during which the urinary sediment contained large numbers of leukocytes. Lactosylceramide accounts for almost 95 percent of the glycolipid hexose in leukocytes in man (289). As the pyelonephritis and pyuria remitted, the urinary lactosylceramide levels fell rapidly to normal.

IV. DISCUSSION

A. Structure of CTH from Human Kidney

Despite the variations in fatty acid and long-chain base composition, the marked difference in the specific optical rotations of CTH and lactosylceramide was the first indication that the terminal galactosidic linkage of CTH was of the α-stereoconfiguration. The observed molecular rotations of CTH were, in fact, almost identical to that calculated for a trihexosylceramide containing a single α-D-galactopyranosyl residue. Some published optical rotations of comparable materials are shown in Table XVI. Although CTH has been consistently shown to be dextrorotatory, Kawanami (106) was the first to draw attention to the significance of this seemingly paradoxical finding in terms of the stereochemistry of the oligosaccharide. Nevertheless, since the lipid contains an optically active aglycone, ceramide, which is different in naturally occurring CTH and lactosylceramide, the interpretation of optical rotatory data must be undertaken with care. Kawanami eliminated the confusion caused by the contribution of the aglycone by comparing the rotation of CTH with that of the lactosylceramide formed by the partial hydrolysis of CTH.

Many infrared spectra of various glycosphingolipids and derivatives have been published. They were used largely to "fingerprint" the lipids, rule out the presence of various types of esters, and to determine the configuration of the double bond in the long-chain base. They can also be used, however, to determine the configuration of the anomeric protons in oligosaccharides. Absorption at 850 cm^{-1} in the spectra of glycosides is

Table XVI: Some published optical rotations of CTH and lactosylceramide

Lipid	Source	$[\alpha]_D$ in pyridine	Reference
CTH	Human kidney	+26.1	101
		+25.4	59
		+23	present work (110)
	Partial hydrolysis of erythrocyte globoside	+23	117
	Fabry kidney	+24.2	93
		+21	present work (110)
	Fabry lymph node	+20.6	93
Lactosylceramide			
	Human erythrocyte	-9.7	321
	Human spleen	-10.31	100
	Partial hydrolysis of erythrocyte globoside	-14	117
	Partial hydrolysis of brain ganglioside	-12	present work (110)

commonly attributed to the C-H deformation of equatorial, viz. α-anomeric, protons. On the other hand, a peak at 885 cm^{-1} is due to the C-H deformation of axial, or β-anomeric, protons. Kawanami (106) and more recently Hakomori et al (111) demonstrated the presence of α-anomeric protons in CTH from a murine sarcoma and human erythrocytes, respectively, by this method. The spectra of CTH from normal and Fabry's kidney reported in this Thesis contain peaks at 885 cm^{-1} but none at 850 cm^{-1}. They are, moreover, almost identical to spectra of CTH from serum, liver and spleen reported by Svennerholm and Svennerholm (98,99). Initially, this was interpreted as presumptive evidence for the existence of two species of CTH, one containing all β-glycosidic linkages and another containing a single α-glycosidic linkage. The inferior quality of the spectra, however, tempered such a conclusion particularly in light of the optical rotatory data. Indeed a re-examination of the excellent spectra of Fabry's CTH published by Miyatake (93) revealed the presence of a small but distinct peak at 850 cm^{-1}. Miyatake, however, did not comment on this region of the spectrum, and hence overlooked the possibility of the presence of an α-galactosidic linkage in the lipid.

By far the most powerful tool for determining the configuration of the anomeric centers in oligosaccharides is nuclear magnetic resonance spectrometry. The spectra of CTH and other glycosphingolipids and derivatives reported in this Thesis demonstrate irrefutably that normal human renal CTH and Fabry's CTH contain an α-D-galactopyranosyl residue. Moreover, the ratio of the areas under the three doublets attributable to the anomeric protons is almost unity. This indicates that, in terms of the

stereoconfiguration of the oligosaccharide, there is only one species of CTH in human kidney. In contrast to these findings, Sweeley, Snyder and Griffin (92) deduced from nmr spectral data that the interglycosidic linkages in Fabry's CTH were all of the β-stereoconfiguration. The spectrum in the paper by Sweeley et al (92) is of the TMSi derivative of the intact lipid recorded in $CDCl_3$. It contains doublets in the region 3.8 to 4.3 ppm which correspond to those we observe at 4.74 and 4.94 ppm for unmodified CTH and lactosylceramide. There is also a broad singlet at 4.62 ppm which these authors attributed to the presence of an impurity. This peak almost certainly corresponds to the narrow doublet we have assigned to the anomeric proton of the α-D-galactopyranosyl residue.

Although the optical rotatory and nmr data show the presence of an α-D-galactopyranosyl residue in CTH, they do not indicate whether it is in the terminal or subterminal position. The acid lability of the terminal galactosidic linkage of CTH is highly suggestive that the α-D-galactopyranosyl residue is terminal. The results of the hydrolysis of the lipid with coffee bean α-galactosidase provided the conclusive proof. Over 65 percent hydrolysis of the terminal galactosidic linkage of CTH was obtained using a crude α-galactosidase preparation. Because of the presence of residual β-galactosidase activity in the preparation, the experiments were repeated using purified enzyme. Considerable difficulty was experienced reproducing Courtois and Petek's (304) purification. Although the α- and β-galactosidases were reproducibly separable, the α-galactosidase activity could not be resolved into two enzymes with different pH optima. What is more, attempts to concentrate the purified enzyme resulted in prohibitive

losses of activity. Hence, the final comparison of the hydrolysis of CTH[^{3}H] and ^{3}H-lactosylceramide by α-galactosidase was done using a very dilute enzyme system. Nevertheless, significant hydrolysis (approx. 15 percent) of the CTH[^{3}H] was achieved whereas ^{3}H-lactosylceramide was virtually unaffected. The products of the reaction of CTH with crude α-galactosidase were shown by tlc to be only unreacted CTH and a lipid which co-chromatographed with lactosylceramide. These data prove the presence of an α-D-galactopyranosyl residue in CTH and, in addition, demonstrate conclusively that it is at the nonreducing terminal of the oligosaccharide of the lipid.

Shortly after these data had been submitted for publication, Bensaude et al (108) and Li and Li (109) arrived at the same conclusion through their studies using an α-galactosidase purified from ficin, a fig protein. Although a systematic comparison has not been done, fig α-galactosidase appears to be superior to the coffee bean enzyme for structural studies of glycosphingolipids.

B. Enzymic Defect in Fabry's Disease

In 1963, Sweeley and Klionsky (91) revolutionized thinking on Fabry's disease by demonstrating vast accumulations of CTH and digalactosylceramide in the kidney of a patient who had died of the disease. Applying the same principles that they had used to advantage in studies of Gaucher's and Niemann-Pick diseases, Brady et al (234) showed that Fabry's disease was characterized by a defect in the hydrolysis of the terminal galactosidic linkage of CTH. Brady and his coworkers (233) extracted and partially

purified a CTH galactohydrolase from rat intestine and examined its properties. Its activity was enhanced by the presence of sodium cholate, and it exhibited a pH optimum of 5.0. The purified enzyme did not catalyze the hydrolysis of lactosylceramide, glucosylceramide, galactosylceramide, o-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside or p-nitrophenyl-2-acetamido-2-deoxy-β-D-galactopyranoside. With the exception of glucosylsphingosine and glucosylceramide, no inhibitors of the reaction could be found among a wide variety of compounds tested. Clearly the most significant negative result was the failure of O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-D-glucose to inhibit CTH hydrolysis. Unfortunately, no known α-D-galactosides were tested for substrate or inhibitory potential. CTH galactohydrolase was considered, on the basis of these studies, however, to be a highly specific glycolipid β-galactosidase.

As a result of Kawanami's demonstration of a terminal α-D-galactopyranosyl residue in CTH from Nakahara-Fukuoka sarcoma tissue, Kint (107), acting on the supposition that Fabry's CTH might have the same structure, tested the nonspecific α-galactosidase activity of leukocytes from patients with and carriers of the disease. His results provided strong evidence that Fabry's disease was due to an α-galactosidase deficiency, and that the CTH stored in the tissues of patients with the disease did, in fact, contain a terminal α-D-galactopyranosyl residue. Although others considered the enzyme deficiency a secondary phenomenon (92), the demonstration of a nonspecific α-galactosidase deficiency became accepted as diagnostic of Fabry's disease (261,275,277).

In this Thesis, preliminary studies of CTH galactohydrolase and nonspecific galactosidases in human tissues are described. Human renal CTH galactohydrolase, like the enzyme from rat intestine, is very labile. The pH optimum is however quite different (pH 3.6 vs pH 5.0 for the rat intestinal enzyme). The profile of the rat renal CTH galactohydrolase was, on the other hand, identical to that of the human renal enzyme (unreported results). In both cases, the pH profile of the CTH galactohydrolase was different from that of the nonspecific α-galactosidase. Moreover, the nonspecific α-galactosidase activity was stable to repeated freezing and thawing and storage for several weeks at -20°, conditions which destroyed CTH galactohydrolase activity in a few days. The difference in pH profiles is redolent of the differences of the effects of pH on the hydrolysis of melibiose and CTH ("natural" substrates) and p-nitrophenyl-α-D-galactopyranoside (an "artificial" substrate) by coffee bean α-galactosidase. This together with the difference in lability when the enzyme is measured with CTH or p-nitrophenyl-α-D-galactopyranoside suggest that the enzyme-substrate interaction is complex. Further speculation on the mechanism of the interaction between the enzyme and substrate is beyond the scope of this Thesis.

We were able to confirm the deficiency of nonspecific α-galactosidase in leukocytes from a number of patients with Fabry's disease, and succeeded in one patient to demonstrate concomitant deficiencies of CTH galactohydrolase and nonspecific α-galactosidase in renal tissue. In spite of Mapes et al (243) claim to have demonstrated CTH galactohydrolase activity in plasma, and its absence in Fabry's disease, we were unable, despite several

weeks of frustrating work, to reproduce their findings. At no time and under no conditions could we demonstrate CTH galactohydrolase activity in human plasma. It is clear that more work is required to resolve this inconsistency. Nevertheless, there is little doubt, in light of our present knowledge, that the nonspecific α-galactosidase deficiency observed in Fabry's disease is a direct reflection of the CTH galactohydrolase deficiency.

Continued studies are also necessary to explain the accumulation of digalactosylceramide in the kidneys of patients with Fabry's disease. The optical rotation of mixed lactosylceramide and digalactosylceramide from normal human kidney reported by Martensson (59), $[\alpha]^{23}_{587} = +15.0$, suggests that digalactosylceramide also contains a terminal α-D-galactopyranosyl residue. Studies are in progress to determine whether this is so. If it does contain a terminal α-D-galactopyranosyl residue, it would suggest that CTH galactohydrolase has two or perhaps more physiologic substrates.

Blood group substance B (247,254) and digalactosyldiglyceride (315) also contain terminal α-D-galactopyranosyl residues. The first only occurs in a small percentage of the human population. The second is a minor constituent of brain, and would be hydrolyzed to water soluble fragments and lost in the usual procedures employed for the isolation of glycosphingolipids. In blood group substance B the galactose residue at the nonreducing end of the oligosaccharide is linked α1→3 to the subterminal galactose moiety (Figure 2). In digalactosyldiglyceride, on the other hand, the linkage is gal-(α1→6)-gal between the terminal and subterminal

sugars. Whether or not these substances are natural substrates of CTHase, which hydrolyzes the gal-(α1→4)-gal linkage of CTH, is unknown. Efforts are being made at present to determine whether digalactosyldiglyceride accumulates in the tissues of patients with Fabry's disease in an attempt to answer this question.

C. The Treatment of Fabry's Disease by Enzyme Replacement

Until recently, no specific treatment for Fabry's disease was known. In 1970, however, Mapes, Anderson and Sweeley (243) demonstrated the presence of a soluble CTH galactohydrolase in normal human plasma that was absent in patients with Fabry's disease. This opened the way to the possibility of treating the disease by enzyme replacement by way of plasma transfusions. Mapes and coworkers (316) subsequently reported the successful transfusion of CTH galactohydrolase from a normal donor to a patient with Fabry's disease and an improvement in some of the biochemical abnormalities characteristic of the disease. Among the improvements, the authors reported a marked increase in the activity of the CTH galactohydrolase in the patient's plasma. In fact, for some reason which remains unknown to the present, the increase in CTH galactohydrolase activity was many times the calculated amount of enzyme given in the transfusion. These results are still awaiting confirmation, and the results of long-term enzyme replacement by this technique remain unknown. Nevertheless, they mark the first reported attempt to treat a disorder of sphingolipid metabolism by enzyme replacement.

Organ transplantation offers another vehicle for enzyme replacement in genetic diseases. In 1971, Groth et al (317) reported the results of the treatment of advanced juvenile Gaucher's disease by splenic transplantation. The postoperative course was marred by severe bouts of incipient rejection which required aggressive immunosuppressive measures and multiple blood transfusions. There was a slight improvement in the concentration of glucocerebroside in the patient's plasma, but considering the variables, the results can at best be described as equivocal.

For a variety of reasons Fabry's disease appeared to be amenable to treatment by enzyme replacement through organ transplantation. First, the disease is very slowly progressive, suggesting that even the small amount of enzyme contributed by a grafted organ might be sufficient to metabolize the excess CTH which ordinarily accumulates in the patient's tissues. Secondly, the elevation of CTH levels in the plasma together with the distribution of accumulation of the lipid in the walls of blood vessels suggests that the deposition of the CTH occurs as a "metastatic" phenomenon. That is to say, the CTH derived from, for example, the catabolism of erythrocyte globoside, might enter the blood stream and, because its metabolism is blocked, would build up to plasma levels which might cause it to "precipitate" out in the walls of blood vessels. If this were the case, lowering the plasma levels of CTH would effectively arrest the disease. The third important fact is that the central nervous system involvement in Fabry's disease is minimal and is claimed by some (318) to be related simply to the accumulation of CTH in cerebral blood vessels rather than in the tissue itself. Finally, in the natural development of the disorder, patients

with Fabry's disease usually die of renal failure. Renal homotransplantation, the treatment these patients would ultimately receive in any case, is the most highly developed and successful form of parenchymatous organ transplantation available today.

In the patient (Case 2) who ultimately underwent renal transplantation because of progressive renal failure, we were concerned primarily with three questions: 1) Does peritoneal or hemodialysis affect plasma glycosphingolipid levels? 2) Does renal homotransplantation affect plasma CTH levels? 3) Does CTH accumulate in the donor kidney, viz. is the enzyme complement of the donor organ adequate to metabolize CTH brought to it in the circulation?

Peritoneal and hemodialysis did not have any consistent effect on plasma CTH levels. On the one occasion when hemodialysis appeared to reduce the plasma CTH concentration, the level before treatment was considerably higher than the patient's usual CTH levels and may have been in error.

Paradoxically, the effects of the bilateral nephrectomies and the renal transplantation were almost identical. Initial postoperative decreases in plasma CTH levels were followed by large increases to levels higher than preoperative concentrations. This suggested that simple laparotomy, with the attendant transient metabolic changes, might have an effect on plasma CTH levels. The therapeutic implications of this discovery, should it be confirmed, are obvious. It is also clear that care must be exercised in the interpretation of the effects of renal transplantation on CTH metabolism until the effects of laparotomy are understood.

The overall effect of renal transplantation was disappointing. If the accumulation of CTH in blood vessels is, in fact, a "metastatic" phenomenon due to increased plasma CTH levels, the process is not, on the basis of the results reported here, preventable by renal transplantation.

The analysis of urinary glycosphingolipids after renal transplantation was undertaken initially to determine if CTH was accumulating in the graft. The postoperative levels of CTH were elevated (4-8 times normal), but there was no sign of a trend to increasing levels. This, together with the observation that the lipid composition of the graft was virtually normal (Table XV), led us to conclude that the CTH in the urine postoperatively was derived principally from the patient's own urinary collecting system, not from the graft itself. Indeed, large amounts of glycolipid were demonstrable histochemically in a needle biopsy of the patient's prostate gland (322).

The source of the CTH stored in the tissues of patients with Fabry's disease is still unknown. Undoubtedly, some is derived from erythrocytes, and this may be the source of the major part of the CTH in the plasma. An equally plausible alternative is that the majority of the CTH stored in Fabry's disease is synthesized locally in the tissue in which the storage occurs. It is noteworthy, for example, that the accumulation of the lipid in blood vessels is mainly in the smooth muscle (263). The intima and subintimal regions closest to the circulation are less severely affected. Furthermore, Lou's (318) observations notwithstanding, Rahman and Lindenberg (319), in an exhaustive examination of the nervous system in Fabry's

disease, reported lipid storage in the neurones of peripheral autonomic ganglia and central autonomic nuclei. Lipid accumulates in the arrector pili muscles of the skin (261) in cultured fibroblasts (274), and probably in the cornea causing the characteristic corneal lesion. The accumulation of lipid in these tissues and the absence of accumulation in as highly vascular organs as the liver and spleen (263) is strong evidence that in Fabry's disease, CTH is stored in tissues which synthesize it. Hence, simply lowering plasma CTH levels alone, were it possible, is unlikely to affect the course of the disease substantially.

The possibility that organ transplantation may result in the distribution of donor enzyme throughout the recipient's tissues is currently under investigation by other groups (320). This represents an optimum type of therapy in which the patient's tissues are "repopulated" with normal CTH galactohydrolase and thus the accumulation of the lipid would be prevented or even reversed.

V. APPENDIX

Calculation of $[\alpha]_D$

$$\alpha_D = \frac{\frac{\alpha_{578}}{\alpha_{546}-\alpha_{578}}}{\frac{\alpha_{578}}{\alpha_{546}-\alpha_{578}} + 1.373} \times \alpha_{546}$$

$$[\alpha]_D = \frac{100 \times \alpha_D}{c \times l}$$

c = concentration in g/100 ml

l = length of light path in decimeters

SUMMARY

Fabry's disease is an X-linked inborn error of glycosphingolipid metabolism caused by a defect in the catabolism of galactosyl-(1→4)-galactosyl-(1→4)-glucosyl-(1→1')-ceramide (CTH). The resulting accumulation of this lipid in the walls of small arteries and in the kidneys usually causes death due to cerebrovascular disease or cardiac or renal failure before age fifty.

Two unrelated male patients are described who had increased levels of CTH in their plasma and urinary sediments and marked deficiencies of leukocytic α-galactosidase, but whose only clinical manifestation of Fabry's disease was moderately severe renal failure. Neither exhibited the pathognomonic skin and ocular lesions traditionally associated with the disease.

CTH extracted and purified from pooled normal human kidney tissue is shown to be virtually identical in terms of chromatographic properties, chemical composition (hexoses, fatty acids and long-chain bases), infrared spectra, mass spectra, optical rotations and nmr spectra to CTH purified from the kidney of a patient who died of Fabry's disease. The specific optical rotations of the lipids suggest the presence of an α-D-galactopyranosyl residue in the molecules. This is supported by nuclear magnetic resonance spectrometry of the intact lipids, and by the susceptibility of the compounds to hydrolysis by an α-galactosidase extracted from green coffee beans. The identification of the product of the enzymic hydrolysis of CTH as lactosylceramide indicates that the α-D-galactopyranosyl residue

is located at the nonreducing terminal of the oligosaccharide part of the molecule. These data show conclusively that CTH contains a terminal α-D-galactopyranosyl residue, and resolves the apparently paradoxical concomitant deficiencies of CTH galactohydrolase (hitherto considered a β-galactosidase) and nonspecific α-galactosidase in the tissues of patients with Fabry's disease.

The results of preliminary studies of CTH galactohydrolase and nonspecific α-galactosidase activities in human kidney tissue indicate that the properties of the enzyme (pH profile, stability, and specific activity) are very different when CTH and p-nitrophenyl-α-D-galacto-pyranoside are used as substrates. This is tentatively interpreted as a manifestation of the complexity of the enzyme-substrate interaction in the course of the catabolism of CTH.

The results of studies on a patient with Fabry's disease who underwent bilateral nephrectomies and renal homotransplantation and subsequently died of septicemia are described. Neither peritoneal dialysis nor hemodialysis appeared to have any consistent effect on the concentration of CTH in the patient's plasma. Following renal transplantation the plasma CTH concentration fell transiently, but ultimately it returned to pre-transplantation levels. The same phenomenon was observed following the bilateral nephrectomies and therefore cannot be interpreted as due to the implantation of exogenous CTH galacto-hydrolase in the donor kidney. The results of the analyses of the patient's urine and the kidney graft indicate that there was no significant accumulation of CTH in the graft during the six months it was in place.

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CLAIMS TO ORIGINAL RESEARCH

1. Biochemical abnormalities characteristic of Fabry's disease (ceramide trihexosidosis) were demonstrated in two unrelated male patients whose only clinical manifestation of the disease was moderately severe proteinuria and, in one case, moderate renal failure. Neither exhibited the pathognomonic skin and ocular lesions of the disease. It was shown that both had increased concentrations of galactosylgalactosyl-glucosylceramide (CTH) in their plasma and excreted increased amounts of CTH and digalactosylceramide in their urinary sediment. It was also shown that they both exhibited marked deficiencies of α-galactosidase in their peripheral leukocytes.

2. CTH extracted from normal human kidneys was shown to be essentially identical with that purified from the kidney of a man who had died of Fabry's disease in terms of the fatty acid and long-chain base compositions and the structures of the oligosaccharides.

3. Nuclear magnetic resonance spectra of unusual clarity were obtained of galactosylceramide, hydrogenated galactosylceramide, lactosylceramide, CTH and human kidney globoside by recording them on the intact lipids dissolved in pyridine-$\underline{d}_5$. All the absorption bands in the region of the spectra where the anomeric protons absorb were identified.

4. CTH labelled with tritium in the C-6 position of the terminal galacto-pyranosyl residue was prepared by treating the purified lipid with D-galactose oxidase and reducing the resulting hexodialdose with sodium borotritiide.

5. The terminal galactosidic linkage of CTH, but not lactosylceramide, was shown to be susceptible to hydrolysis by a stereospecific α-galactosidase extracted and purified from green coffee beans.

6. Human renal CTH was shown to contain a terminal α-D-galactopyranosyl residue on the basis of optical rotations, nuclear magnetic resonance spectrometry, acid lability, and susceptibility to hydrolysis by a specific α-galactosidase.

7. CTH galactohydrolase and nonspecific α-galactosidase activities were both demonstrated in normal human kidney tissue. The pH optimum of the former was 3.6, and that of the latter was 5.2. Marked deficiencies of both enzyme activities were demonstrated in kidney tissue obtained at operation from a patient with Fabry's disease. No residual CTH galactohydrolase activity could be demonstrated. The pH optimum of the residual nonspecific α-galactosidase was the same as in normal human renal tissue.

8. Human renal nonspecific α-galactosidase was shown to be stimulated by low concentrations and inhibited by high concentrations of Mg^{++}. It was also shown to be inhibited by Hg^{++} and by melibiose, but not by myoinositol.

9. It was shown by analyses of the plasma neutral glycosphingolipids in a patient with Fabry's disease who was undergoing peritoneal dialysis and hemodialysis that neither procedure had any consistent effect on the plasma CTH concentration.

10. It was shown that following renal transplantation a transient decrease in the plasma CTH concentrations in a patient with Fabry's disease occurred. However, the effect was not significantly different from that observed following bilateral nephrectomies in the same patient.

11. The analysis of the lipids in the renal homograft after the patient's death showed that in the six months it was in place, no significant accumulation of neutral glycosphingolipid occurred.