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# Application of lectin histochemistry and carbohydrate analysis to the characterization of lysosomal storage diseases*,†

Joseph Alroy‡,
*Department of Pathology, Tufts University Schools of Medicine and Veterinary Medicine, Boston, Massachusetts, 02111 (U.S.A.)*

Rita De Gasperi, and Christopher D. Warren
*Laboratory for Carbohydrate Research, Massachusetts General Hospital and Departments of Biological Chemistry and Medicine, Harvard Medical School, Boston, Massachusetts, 02114 (U.S.A.)*

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ABSTRACT

In lysosomal storage diseases that involve a defect in the catabolism of glycoconjugates, lectin histochemistry adds a new dimension to the characterization of stored carbohydrates as it identifies sugar residues *in situ* in the affected cells and, thus, determines which cell types are affected by storage. It may be combined with chemical and biochemical analysis by h.p.l.c. The present review summarizes recent results for a variety of storage diseases and presents new data for $G_{M1}$-gangliosidosis.

## INTRODUCTION

Lysosomal storage diseases comprise a large group of genetically determined as well as acquired heterogeneous metabolic disorders affecting humans[1] and various animal species[2]. Biochemically, they are characterized by the deficient activity of specific lysosomal hydrolases and the intralysosomal accumulation of one or more substrates in affected cells. Initially, the diseases were grouped according to their clinical characteristics and hereditary pattern. The identification of the major storage substrate or the deficient hydrolase served as the basis for the classical division of the hereditary lysosomal storage disorders into glycoprotein-storage disorders, mucolipidoses, mucopolysaccharidoses, and sphingolipidoses. Insights concerning the synthetic pathway of lysosomal enzymes, their functions, and the role of activator proteins, protein stabilizers, enzyme trafficking, inhibition of enzymes, and transport of end products led to a reclassification of these diseases[1,3,4].

In the past, the application of standard biochemical, histochemical, and morphological methods resulted in an incomplete understanding of the nature of the stored

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‡ To whom correspondence should be addressed at Tufts University Schools of Medicine and Veterinary Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111, U.S.A.

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compounds as well as the biology of these diseases. With the introduction of lectin histochemistry and electron microscopy, and various biochemical methods for studying the stored compounds, it became apparent that the nature of the undegraded stored material is complex and that, in the case of diseases involving aberrations of glycoprotein breakdown, the composition and structures of stored carbohydrates cannot necessarily be predicted on the basis of knowledge of the primary enzymic defect[5]. This suggested that the storage must be determined not only by the nature of the mutation, but also by other factors, such as the amount or type of substrates available to be catabolized by the cells. We discuss herein the usefulness of lectin histochemistry for studying lysosomal storage diseases and stress the interdependence of lectin histochemistry and biochemical analysis. Past studies are reviewed and some recent findings on gangliosidosis are presented.

## ANALYSIS OF STORED CARBOHYDRATES: A REVIEW

*Lectin histochemistry of stored carbohydrates.* — Lectins are proteins and glycoproteins that have known sugar-binding specificities[6,7] (see Table I). When they are conjugated directly or indirectly to a "visualant" (*i.e.*, enzymes, electron-dense metals, or fluorescent compounds), they are useful as histochemical probes for identifying *in situ* the presence of specific carbohydrate residues[8–10]. These properties have enabled us to examine specimens from a variety of lysosomal storage diseases, and to determine not only the nature of the stored compounds, but also in which cell types the compounds are stored. The correct interpretation of lectin-staining data depends, in our experience, on a number of factors that will be discussed below.

Positive or negative staining results may be influenced by whether or not the tissue was "fixed", how it was fixed, and whether or not lipids were extracted from the tissue. The results may also be influenced by the nature of the "visualant" and whether the staining was by a direct or indirect method[9]. Because some stored lipids are autofluorescent, it is imperative not to use fluorochrome as the visualant. Also, since the strength of lectin binding is dependent on the concentrations of both lectin and carbohydrate, and on the duration of the incubation, we established optimum, standardized conditions for the staining procedure.

The successful application of lectin histochemistry is also dependent on an appreciation of basic factors that affect lectin-carbohydrate binding. The lectin-binding determinants of glycoconjugates often consist of extended sites involving several hexose units, the relative orientations of which define a three-dimensional surface, complementary to that of the sugar-binding site. Thus, the avidity of lectin binding to a given carbohydrate determinant may be influenced by other sugar units adjacent to the binding site[11], and it may be advantageous to employ various tissue treatments, such as enzymic unmasking of penultimate residues, or mild periodate oxidation, prior to staining with lectins[12,13].

One of the most successful applications of lectin histochemistry has been for the *in situ* characterization of metabolites accumulated in cells or tissues of humans and

TABLE I

Lectins used in our laboratory for identifying carbohydrate units

| *Lectin origin* | *Common name* | *Acronym* | *Concentration (μg/mL)* | *Major sugar specificity* | *Binding inhibitor* |
|---|---|---|---|---|---|
| *Arachis hypogea* | Peanut | PNA | 20 | β-D-Gal*p*-(1→3)-D-Gal-NAc | Lactose |
| *Concanavalia ensiformis* | Jack bean | Con A | 10 | α-D-Glc, α-D-Man | α-D-Man*p*OMe |
| *Datura stramonium* | Jimsonweed | DSA | 10 | [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)]$_n$ | (β-D-Glc*p*NAc)$_{2\text{-}3}$ |
| *Dolichos biflorus* | Horse gram | DBA | 10 | α-D-GalNAc | α-D-GalNAc |
| *Glycine max* | Soybean | SBA | 10 | α-D-GalNAc, α-D-Gal | α-D-GalNAc |
| *Griffonia simplicifolia* | Bandeirea | GS-I | 50 | α-D-Gal | Lactose |
| *Lens culinaris* | Common lentil | LCA | 10 | α-D-Glc, α-D-Man | α-D-Man*p*OMe |
| *Ricinus communis* | Castor bean | RCA-I | 50 | β-D-Gal | Lactose |
| *Triticum vulgaris* | Wheat germ | WGA | 50 | [β-(1→4)-D-Glc*p*NAc]$_2$, NeuNAc*p* | NeuNAc |
| | Succinyl-WGA | S-WGA | 10 | [β-(1→4)-D-Glc*p*NAc]$_2$ | β-D-GlcNAc |
| *Ulex europaeus* | Gorse | UEA-I | 10 | α-L-Fucose | α-L-Fucose |

animals with lysosomal storage diseases. The studies have been performed on cultured dermal fibroblasts[14,15] and cultured lymphoid cell lines[16], on frozen and paraffin sections[9,17–22], or paraffin sections only[5,23–34]. Ideally, the affected tissue should be studied in both frozen and paraffin sections. Lectin staining of stored glycoconjugates in paraffin sections is limited to the identification of carbohydrate units in glycoproteins and oligosaccharides that are retained in the tissue section after paraffin processing. In frozen sections, lectins may identify glycoconjugates retained in paraffin sections, as well as additional glycolipids and low-molecular-weight oligosaccharides, which are usually extracted by paraffin processing[9,17]. Some glycoconjugates found in affected cells are masked by lipids in frozen sections; they are stained by their corresponding specific lectins only after delipidation with xylene or chloroform–methanol, or by paraffin processing of the tissue sections[9,17,20,21,27].

Finally, lectin histochemistry is particularly useful for studying tissue sections obtained from autopsy of patients in which the initial diagnosis of a lysosomal storage disease followed histologic examination of affected tissues[26,35]. Formalin-fixed, paraffin-embedded tissue sections, commonly preserved after surgery or autopsy, may serve as a valuable but as yet underused source for the diagnosis of storage diseases[26,32,35] and for studying the nature of accumulated carbohydrates. This is best illustrated by the following example. In 1984, we used lectin histochemistry on paraffin sections to diagnose α-D-mannosidosis in an 11-week old kitten which died in 1974. The autopsy done at the time suggested a possible storage disease, but specimens for biochemical diagnosis were lacking. The information from lectin histochemistry enabled us to confirm the nature of the disease, to identify seven presumptive carriers, and eventually reproduce the disease[35].

*Chemical analysis of stored carbohydrates.* — For the study of glycoprotein glycans or of oligosaccharides derived from them *in vivo*, it is necessary to release the compounds from their cellular locations, and to separate them from other materials, *e.g.*, proteins and lipids, that would interfere with subsequent analysis. For stored oligosaccharides, extraction of a tissue homogenate with 1:1 ethanol–water, followed by gel filtration and de-ionization, yields an oligosaccharide mixture suitable for analysis by h.p.l.c. For oligosaccharides in body fluids, only gel filtration and de-ionization are necessary. H.p.l.c. performed either with or without precolumn derivatization[36] may be employed to (a) identify stored oligosaccharides by comparison of retention times with those of reference compounds, (b) compare the concentrations of oligosaccharides in different samples, or (c) generate a "profile", *e.g.*, for comparison of the pattern of oligosaccharide storage in two or more different tissues or body fluids, or to compare tissues from normal, affected, and heterozygous individuals.

In our laboratory, reference compounds for h.p.l.c. were generated by the isolation and purification of oligosaccharides from humans or animals afflicted with α-D-mannosidosis[37,38], followed by structure determination using chromatographic, chemical, enzymic, and spectroscopic procedures[38]. To generate a greater variety of "standards", the mannosidosis oligosaccharides were modified when necessary, by chemical synthesis[39] or digestion with endo-*N*-acetyl-β-D-glucosamidases D and H[38]. Because

their substrate-specificities are well-established, the latter enzymes were also very useful as "structure-probes" for oligosaccharides stored in tissues or body fluids, particularly when this application was combined with h.p.l.c. performed before and after the digestions[38,40].

For the analysis of stored glycoproteins, a proteolytic digestion provides glycopeptides that are fractionated by gel filtration (see discussions below of Gaucher's disease and $G_{M1}$ gangliosidosis). The presence in the fractions of specific classes of glycans may be probed by digestion with endoglycosidases of known substrate specificity.

*Chemical analysis and lectin histochemistry are complementary.* — Whereas lectin histochemistry employs very small samples of tissue and is rapid and convenient, chemical and biochemical analyses require several grams of tissue and are time- and labor-intensive. Also, whereas the lectin histochemical procedure is standardized, the biochemical analysis must be carefully tailored to the problem at hand. Most importantly, the two approaches are complementary in the sense that they provide different information about the stored material. Thus, lectin histochemistry identifies carbohydrate residues *in situ*, so that based on known lectin-binding specificities, classes of glycans can be localized to specific-cell types. Lastly, whereas the lectin procedure can be applied to any class of glycoconjugate with suitably exposed sugar units, for chemical and biochemical analysis the glycoconjugate must be amenable to analysis by the available methods. On the other hand, chemical and biochemical procedures can provide a wealth of compositional, structural, and quantitative data that lectin histochemistry cannot provide. For example, detailed chromatographic profiles, derived in parallel with enzymic treatments as discussed earlier, can facilitate detailed comparisons of tissues and fluids from affected animals and controls, *e.g.*, for early or prenatal diagnosis of a storage disease[41–44], or to examine the storage process in different tissues, organs, and body fluids[45]. Furthermore, the determination of the structures of the oligosaccharides that correspond to the peaks in the chromatogram, and the determination of their relative abundance at different time points, can reveal pathways for the catabolic breakdown of the glycans of glycoproteins[46].

*Glycoprotein-storage diseases.* — These are disorders resulting from an inborn deficiency of one or more of the lysosomal hydrolases that degrade *O*- or *N*-linked glycans[47–49]. In addition, an acquired disorder (*i.e.*, swainsonine toxicosis) results from ingestion by grazing animals of a plant alkaloid, swainsonine, found in locoweeds such as *Astragalus lentiginosus* and *Swainsona galegifolia*[50,51]. Because the nature of the carbohydrate stored in each disease is a reflection of the identity of the missing enzyme, lectin histochemistry can easily identify α-D-mannosidosis, α-L-fucosidosis, and sialidosis, as well as conditions caused by defective, carrier-mediated transport of sialic acid[33].

In α-D-mannosidosis, which has been identified in humans, cattle, and cats, the deficiency of lysosomal α-D-mannosidase (EC 3.2.1.24) causes an accumulation of oligosaccharides containing α-D-mannosyl nonreducing, terminal groups and one or two 2-acetamido-2-deoxy-β-D-glucose residues at the reducing end[26,37]. Cells of patients

or animals affected by α-D-mannosidosis stain with *Concanavalia ensiformis* agglutinin (Con A), wheat germ agglutinin (WGA), and succinylated-WGA (S-WGA), but not with *Ulex europaeus* agglutinin-I (UEA-I). Similar staining was observed in cells of cats, pigs, or sheep affected by swainsonine toxicosis[25,26]. In order to characterize further the stored oligosaccharides, we have analyzed them by chemical methods. For human α-D-mannosidosis, h.p.l.c. was found to be an excellent procedure for separating the oligosaccharides excreted in urine[37]. A similar h.p.l.c. analysis of the oligosaccharides in urine of swaisonine-poisoned sheep showed that this toxicosis is indeed an induced α-D-mannosidosis[52], and that it provides a reversible model for the human genetic disease[40]. By combining h.p.l.c. analysis with digestions involving exo- and endo-glycosidases, chemical analysis, and n.m.r. spectroscopy, the structures of eleven oligosaccharides found in the urine of swainsonine-intoxicated sheep were elucidated[38], and a further study showed that the same oligosaccharides were accumulated in the tissues of locoweed-fed ewes and their fetuses[45]. In this study, it was found that the pattern and levels of oligosaccharides varied from tissue to tissue, and between ewe and fetus for the same tissue. Oligosaccharide concentrations varied markedly from one gestation period to another, and were highest in fetal kidney. In other studies, the patterns of urine oligosaccharide excretion were compared for genetic bovine α-D-mannosidosis and the swainsonine-induced ovine α-D-mannosidosis[46]. More recently, a different study compared oligosaccharides accumulated in the tissues of calves affected by the genetic or swainsonine-induced disease[53].

The oligosaccharides accumulated in a variety of tissues and body fluids of cats affected by α-D-mannosidosis[54], including amniotic fluid and placenta[41,42] were analyzed in detail. For the latter tissue, this analysis provided a novel procedure for early diagnosis of the disease in the feline model[41,42], and it was an important aspect of a study to compare biochemical and morphological features of placentas from normal, heterozygous, and affected kittens[42].

In contrast to α-D-mannosidosis, cells of goats affected with β-D-mannosidosis did not stain with Con A, WGA, or S-WGA[26]. However, the comparison of oligosaccharide h.p.l.c. profiles for amniotic and allantoic fluids from normal, heterozygous, and affected goats was shown to be important for prenatal diagnosis[43,44]. Unexpectedly, the studies of allantoic fluid of a goat afflicted with β-D-mannosidosis and of placental extracts from cats afflicted with α-D-mannosidosis revealed low levels of mannosidosis oligosaccharides from heterozygous animals[41,44].

When lysosomal storage disorders result in storage of sialo-oligosaccharides or free sialic acid, affected cells are stained positively with WGA, whereas the staining with S-WGA is negative[26,33].

Differences in lectin staining may occur in similar storage diseases in various species. For example, human cells affected by α-L-fucosidosis are stained with UEA-I[26,29,30], but those in canine α-L-fucosidosis are not[29]. The difference in staining is attributed to the differing location of the unhydrolyzed α-L-fucosyl group linked in the stored compounds. In the human disorder, the accumulated compounds contain the "H determinant" which interacts with UEA-I, whereas in canine α-L-fucosidosis the storage

compounds contain the "X antigenic determinant" which does not interact with UEA-I[29].

*Mucolipidosis.* — I-cell disease (*i.e.*, mucolipidosis II) is characterized by deficient activity of *N*-acetylglucosamine phosphotransferase (EC 2.7.8.17), resulting in the absence of D-mannose 6-phosphate units, a "recognition marker" on newly synthesized lysosomal hydrolases, which is required for their delivery to lysosomes, and its deficiency results in individuals having multiple deficiencies of these hydrolases[55]. Nine different sialylated, *N*-linked oligosaccharides have been isolated and characterized in urine of patients affected by I-cell disease[56], but little was known about the nature of stored oligosaccharides in affected kidneys. Studies on paraffin sections of kidneys from patients affected with I-cell disease revealed that affected cells stained with Con A, WGA, and S-WGA, suggesting storage of sialylated, *N*-linked oligosaccharides[30].

*Mucopolysaccharidoses (MPS)**. — This group of disorders results from the deficient activity of lysosomal hydrolases needed for the degradation of glycosaminoglycans (proteoglycans). To date, ten different enzyme deficiencies have been recognized in the human[57]. The major stored compounds for these disorders include chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate. Other storage compounds that have been demonstrated ultrastructurally, such as lipids[5], have not been studied chemically.

So far, there is only a single report regarding the lectin-staining pattern from MPS-I (*i.e.*, Hurler's disease) in two patients[31]. In this disorder, deficient activity of lysosomal α-L-iduronidase (EC 3.2.1.76) results in the accumulation of dermatan and heparan sulfate[57]. Formalin-fixed, paraffin-embedded sections revealed staining with *Lotus tetragonolobus* agglutinin (LTA), peanut agglutinin (PNA), soybean agglutinin (SBA), and WGA of reticuloendothelial (RE) cells in liver and spleen, and neurons in brain[31]. The staining of neurons with LTA indicated the storage of compounds containing terminal α-L-fucosyl groups on Type 2 oligosaccharides[9]. Recently, we observed[58] the similar presence of α-L-fucosyl groups, part of the "H determinant" in neurons of a patient with MPS-II (*i.e.*, Hunter's disease), a condition characterized by deficient activity of lysosomal L-iduronate sulfatase (EC 3.1.6.13) and accumulation of dermatan and heparan sulfate[57]. In MPS-III A, (*i.e.*, Sanfilippo A) deficient activity of heparan *N*-sulfatase (EC 3.10.1.1) results in profound mental deterioration[57]. In these patients, intense Con A staining of neurons was noted on sections of brains embedded in paraffin[22]. Differences were noted in lectin staining of affected glomeruli in cats affected by MPS-I and by MPS-VI. In MPS-I, the glomerular epithelium stained with *Ricinus communis* agglutinin-I (RCA-I) and S-WGA, indicating the storage of compounds with β-D-galactosyl and 2-acetamido-2-deoxy-β-D-glucosyl groups in these cells, but not in MPS-VI[5]. In MPS-VI (*i.e.*, Maroteaux–Lamy syndrome), there is deficient activity of lysosomal *N*-acetyl-D-galactosamine 4-sulfatase (EC 3.1.6.12) (*i.e.*, arylsulfatase B) and, unlike in MPS-I, only dermatan sulfate is stored[53].

* Although the term "mucopolysaccharide" is not accepted in present terminology because it was applied, in the past, to very different high-mol.wt. compounds containing amino sugars, the term "mucopolysaccharidosis" is used, in medicine, for diseases affecting what are presently known as "proteoglycans" (Editor).

*Neuronal ceroid–lipofuscinosis (NCL).* — This is a group of disorders classified into four types by age of onset and clinical manifestation. It is characterized by lysosomal storage of ultrastructurally distinct curvilinear bodies[58]. However, the nature of the basic biochemical defect is unknown. A disease condition has been identified in dogs and sheep that has been suggested to serve as a model for the human disease[59,60]. Lectin staining of fresh-frozen brain sections from patients affected by juvenile NCL revealed intense staining with Con A[22]. When sections of brain embedded in paraffin were employed, neurons from brains of patients affected by infantile, juvenile, or adult forms of NCL stained with Con A, but the staining of paraffin sections from the ovine and the canine models was poor or absent[22]. In our studies of brains from patients affected by juvenile NCL and from the ovine model of NCL, Con A did not stain the affected neurons. This suggested either that stored compounds differ between species, or that the materials stored in the animals are not well retained on paraffin section[61]. The recent identification of dolichyl disaphosphate oligosaccharides in the storage cytosomes of sheep affected by NCL[62] supports the conclusion[22] that Con A staining originated from Con A binding to oligomannosyl components of the dolichol-linked oligosaccharides.

*Sphingolipidoses.* — In this group of diseases, deficiency of enzyme activity or of activator protein results in aberrant catabolism of sphingolipids, and lysosomal storage of lipids, glycolipids, glycoproteins, and oligosaccharides. In Fabry disease, the deficient activity of lysosomal α-D-galactosidase A (EC 3.2.1.22) results in storage of glycosphingolipids having nonreducing, terminal α-D-galactosyl groups, preponderantly globotriosylceramide [α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→O)-Cer] and, to a lesser extent, galactobiosylceramide [α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→O)-Cer] and compounds containing structures corresponding to blood group isoantigen B[63]. The affected cells stained with *Griffonia simplicifolia*-I (GS-I)[16,18], PNA[16,18,19], RCA-I (18), and SBA (16), only in unfixed frozen sections. These histochemical findings confirmed the presence of glycolipids having a nonreducing, terminal α-D-galactosyl group, which was lost during delipidation of the paraffin section with xylene. Lack of staining on paraffin sections also showed that no other classes of glycoconjugates were stored. Cultured fibroblasts from patients afflicted with Schindler disease[63], deficient in activity of *N*-acetyl-α-D-galactosaminidase (EC 3.2.1.97), stained intensely with *Helix pomatia* agglutinin (HPA), which specifically binds to nonreducing, terminal 2-acetamido-2-deoxy-α-D-galactopyranosyl groups[15].

In galactosylceramide lipidosis (*i.e.*, Krabbe's disease, or globoid cell leukodystrophy), deficient activity of lysosomal galactosylcerebroside β-D-galactosidase (EC 3.2.1.46) results in the accumulation of galactosylsphingosine (*i.e.*, psychosine)[64]. This disorder affects humans, cats, dogs, mice, monkeys, and sheep. In Twitcher mice, an animal model for this condition, the globoid cells stained with GS-I, PNA, and RCA-I in formalin fixed, frozen sections, whereas paraffin sections or frozen sections, which were pretreated with chloroform–methanol or xylene, also stained with Con A, WGA, and S-WGA[17]. These findings indicated not only the presence of the expected glycolipids with terminal, nonreducing D-galactosyl groups, but also the presence of additional

compounds containing α-D-mannosyl, 2-acetamido-2-deoxy-β-D-glucosyl, and possibly sialyl residues, the accumulation of which was revealed by delipidation. On formalin-fixed, paraffin-embedded tissue sections, the globoid cells stained positively with S-WGA in affected humans, cats, dogs, and mice. GS-I stained the globoid cells in mice, cats, and dogs, but not in the human or monkey. Con A, WGA, and RCA-I stained the globoid cells in mouse, cats, dogs, and monkey tissue, but not in all of the human cases[28,65,66]. In this disease, the explanation for the staining with S-WGA could not be based on the nature of the nonreducing, terminal group, because S-WGA stains nonreducing, terminal 2-acetamido-2-deoxy-D-glycosyl and not β-D-galactosyl groups. Therefore, our results showed that, beside the storage predicted on the basis of the primary defect, other glycoconjugates are accumulated that warrant a more thorough chemical analysis. Comparison of the pattern of lectin staining for various animal species revealed variable staining affinity for globoid cells that appeared to be both species and individually determined[28].

In glucosylceramide lipidosis (*i.e.*, Gaucher's disease), deficient activity of lysosomal β-D-glucocerebrosidase (EC 3.2.1.45) causes the affected cells to accumulate undegraded glucocerebroside[67]. On frozen sections, Gaucher's cells are stained intensely with the periodic acid–Schiff reagent (PAS), and PAS following diastase[68], indicating the accumulation of carbohydrates that contain vicinal glycol structures[69]. Frozen sections of formalin-fixed spleens from patients afflicted with Gaucher disease did not stain with several different lectins, including Con A, *Datura stramonium* agglutinin (DSA), *Lens culinaris* agglutinin (LCA), RCA-I, and WGA[9,70]. In contrast, corresponding paraffin sections, or frozen sections pretreated with either chloroform–methanol or xylene, did stain with all the aforementioned lectins. These observations suggested that during delipidation there is unmasking of carbohydrates which are stored in Gaucher cells, the nature of which is not related in any obvious way to the primary enzymic defect. Because the results of lectin staining, particularly for DSA, suggested that the stored materials might consist of glycoproteins, extracts of livers and spleens from patients afflicted with Gaucher's disease were subjected to proteolysis, and the resulting glycopeptides were fractionated and analyzed for their content of specific sugars. In the high-mol.-wt. fractions, levels of galactose and *N*-acetylglucosamine were significantly elevated over those in controls. This finding, and the DSA staining, suggested the occurrence in the glycopeptides of poly(lactosamino)glycans, a subclass of *N*-linked glycans that contain repeating units having the structure[70], β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3). Therefore, the glycopeptides were treated with endo-β-D-galactosidase (EC 3.2.1.102, 103)[71] and the resulting oligosaccharides analyzed by t.l.c.[70]. This analysis confirmed that the Gaucher tissues did contain glycoproteins with poly(lactosamino)glycan chains. This was an unexpected result because patients afflicted with Gaucher's disease are not deficient in β-D-galactosidase or *N*-acetyl-β-D-glucosaminidase, so degradation of the glycans would have been expected to proceed normally.

Sphingomyelin–cholesterol lipidoses (*i.e.*, Niemann–Pick group of diseases) are divided into two broad types based on the etiology. Each type is subdivided into three classes based on the clinical manifestations. In type I [Niemann–Pick type A, type B, and

type E (NP-A, NP-B, and NP-E)], there is a primary deficiency of sphingomyelinase (EC 3.1.4.41) activity. Type II [Niemann–Pick type C, type D, and type E (NP-C, NP-D, and NP-E)] is a sphingomyelinase-nondeficient group[72], which is thought to be due to a genetic deficiency in the mechanism of cholesterol egress from lysosomes[72]. In both types, neurovisceral and reticuloendothelial (RE) cells are affected[73]. Frozen sections obtained from patients affected by NP-A, NP-B, and NP-C revealed various degrees of staining of the RE cells with Con A, GS-I, PNA, WGA, and S-WGA[19]. Paraffin sections from spleen and liver of a patient affected by NP-C stained with Con A, DSA, LCA, RCA-I, WGA, and S-WGA[70]. These observations are similar to those noted in the corresponding animal model (*i.e.*, MCTR-Balb/c mice) in which the RE cells stained intensely with Con A, GS-I, LCA, RCA-I, WGA, and S-WGA[74]. These findings indicated that in NP-C and its murine model the RE cells stored compounds which are not found in other visceral cells. Furthermore, these compounds interact with lectins which bind carbohydrate residues that are characteristic of sialylated, *N*-linked oligosaccharides which contain an L-fucosyl group adjacent to the *N*-glycosyl linkage. Fresh-frozen brain section from these mice did not stain with any of the nine lectins that were used, whereas in corresponding frozen sections the enlarged neurons stained positively with Con A and S-WGA only after lipid extraction with chloroform–methanol or in paraffin sections[21].

Defects of ganglioside degradation are divided into two major groups according to the nature of the primary defect, the $G_{M1}$-gangliosidoses and the $G_{M2}$-gangliosidoses. Similar to the findings in galactosylceramide and glucosylceramide lipidoses, and

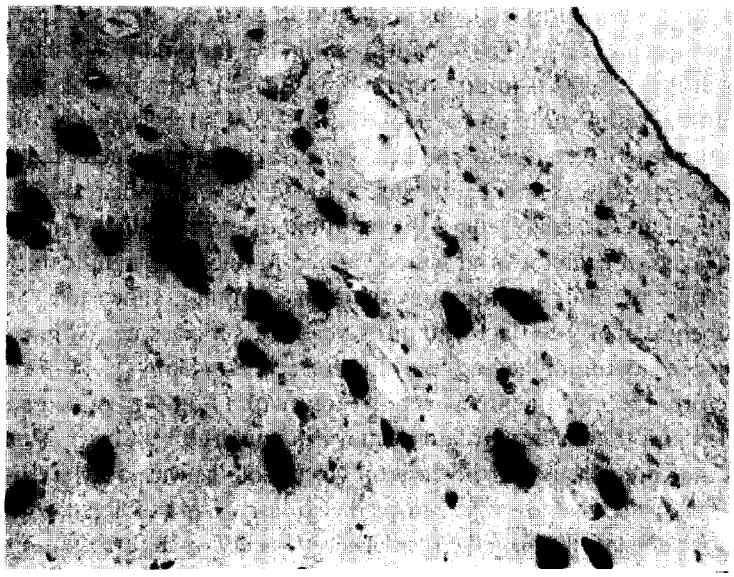

Fig. 1. Section through the midbrain of a 3-year-old boy afflicted with Tay–Sachs disease, stained with DBA. The enlarged neurons stained intensely with DBA, but not the adjacent endothelial cells, erythrocytes, or glial cells. The staining of the perikaryae of the neurons with DBA is indicative of the abnormal presence of nonreducing, terminal 2-acetamido-2-deoxy-α-D-galactopyranosyl groups in the affected neurons. × 256.

TABLE II

Pattern of lectin staining of neuronal perikaryon on paraffin sections from patients affected by gangliosidosis

| | *Lectin*[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Disease* | *Con A* | *DBA* | *GS-I* | *LCA* | *PNA* | *RCA-I* | *SBA* | *UEA-I* | *WGA* | *SWGA* |
| Human Type I $G_{M1}$ | 3/6[b] | 2/6 | 1/6 | [b] | 1/6 | 4/6 | 1/6 | 3/6 | 1/6 | 0/6 |
| Human Type II $G_{M1}$ | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 0/1 | 1/1 | 1/1 | 1/1 | 1/1 |
| Human adult $G_{M1}$ | 1/1 | 0/1 | 0/1 | [b] | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Bovine $G_{M1}$ | 2/2 | 2/2 | 2/2 | [b] | 0/2 | 0/2 | 2/2 | 2/2 | 0/2 | 1/2 |
| Canine infantile $G_{M1}$ | 1/1 | 1/1 | 1/1 | [b] | 0/1 | 0/1 | 1/1 | 1/1 | 0/1 | 0/1 |
| Canine juvenile $G_{M1}$ | 1/1 | 0/1 | 0/1 | [b] | 1/1 | 1/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Feline $G_{M1}$ | 3/3 | 3/3 | 3/3 | [b] | 1/3 | 2/3 | 0/3 | 1/3 | 1/3 | 3/3 |
| Human $G_{M2}$ Tay–Sachs | 9/9 | 5/9 | 8/9 | 2/4 | 3/9 | 2/9 | 4/9 | 8/9 | 5/9 | 2/9 |
| Juvenile Tay–Sachs | 1/1 | 1/1 | 1/1 | [b] | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 0/1 |
| Non-Jewish Tay–Sachs | 0/1 | 0/1 | 1/1 | [b] | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Sandhoff's disease | 12/12 | 8/12 | 11/12 | 9/11 | 8/12 | 4/12 | 6/12 | 12/12 | 9/12 | 8/12 |
| AB Variant | 3/3 | 3/3 | 3/3 | [b] | 2/3 | 2/3 | 2/3 | 3/3 | 2/3 | 2/3 |
| Canine $G_{M2}$ | 3/3 | 3/3 | 3/3 | [b] | 2/3 | 2/3 | 2/3 | 2/3 | 0/3 | 2/3 |
| Feline $G_{M2}$ | 3/3 | 1/3 | 1/3 | [b] | 1/3 | 2/3 | 3/3 | 3/3 | 2/3 | 2/3 |
| Porcine $G_{M2}$ | 1/3 | 3/3 | 2/3 | [b] | 1/3 | 0/3 | 2/3 | 1/3 | 0/3 | 0/3 |

[a] For explanation of abbreviations, see Table I. The fractions indicate the number of cases stained positive over the total number of cases tested. [b] Not done.

sphingomyelin-cholesterol lipidoses, in fresh-frozen tissue sections from dogs[17,27] and sheep[20] affected by $G_{M1}$-gangliosidosis, the cells stained with fewer lectins than the corresponding paraffin sections[17,20]. Our observations on $G_{M1}$-gangliosidosis will be discussed at the end of this paper.

$G_{M2}$-gangliosidoses are a group of heritable disorders caused by mutations in the $\alpha$ and $\beta$ subunits of $\beta$-D-hexosaminidase, as well as in a protein activator[76]. Seven different mutations of the $\alpha$ subunit are known[77]. The onset of the resulting diseases can occur early in life, as in "Tay–Sachs disease", in adolescence, or in adulthood[77,78]. In Tay–Sachs disease (*i.e.*, variant B), a defect in the hexosaminidase $\alpha$ subunit affects the

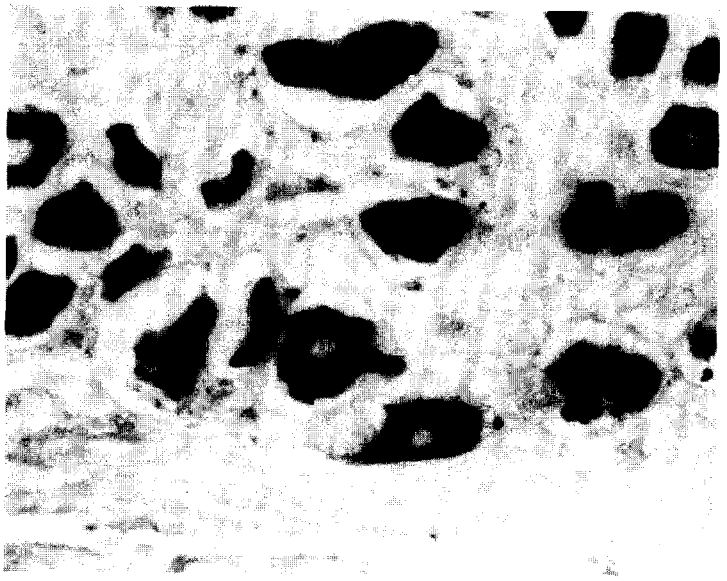

Fig. 2. Section through pancreatic plexus of a 4-year-old boy afflicted with juvenile, non-Jewish Tay–Sachs disease (Ref. 79, case 1). The perikaryae of the enlarged neurons are heavily stained with PNA, indicating the probable presence of nonreducing, terminal β-D-Gal*p*-(1→3)-D-GalNAc groups. × 395.

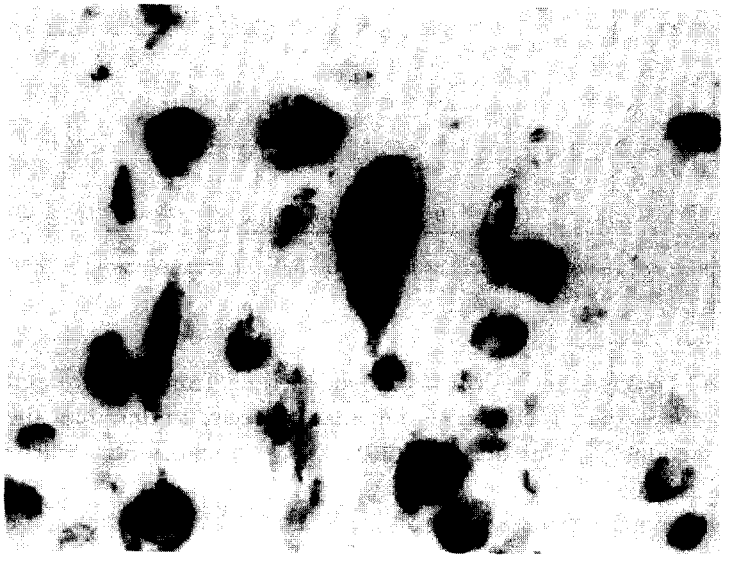

Fig. 3. Section through the cerebral cortex of a 4-year 8-month-old boy afflicted with AB-variant of $G_{M2}$-gangliosidosis (Ref. 80, case 1). The enlarged perikaryae of affected neurons are intensely stained with S-WGA, indicating the presence of nonreducing, terminal 2-acetamido-2-deoxy-β-D-glucopyranosyl groups. × 395.

activity of hexosaminidase A, but not B (EC 3.2.1.52). In Sandhoff disease (*i.e.*, O variant), a defect in $\beta$ subunit affects the activity of both hexosaminidases A and B. When deficiency of $G_{M2}$ activator protein (*i.e.*, variant AB) occurs, even though both hexosaminidase isozymes are unaffected, the degradation by hexosaminidase A still does not occur[76]. In Sandhoff disease, storage of glycolipids, oligosaccharides, and glycosaminoglycans occurs in various tissues and cell types[76]. Both lectin histochemistry[23,76], and chemical and biochemical analysis have contributed to an understanding of this disease. Recently, we observed on formalin-fixed, paraffin-embedded tissue sections that neurons from patients affected by Tay–Sachs disease stained with various lectins (Fig. 1) (see Table II)[62]. Although the concentrations of hexosamine and hexose in dialyzable glycopeptides, which do not contain sialic acid, were elevated in the brain of a Tay-Sachs patient[78], little is known about the nature of these glycopeptides. Similarly, our studies on atypical juvenile Tay–Sachs disease (Fig. 2) and on the AB-variant (Fig. 3) demonstrated lectin binding to enlarged affected neurons, which indicated the abnormal presence of carbohydrate units[58]. For these two conditions, the results of lectin staining have revealed the need for biochemical studies to confirm these observations and determine the nature of the stored carbohydrates.

## APPLICATION OF LECTIN HISTOCHEMISTRY AND CHEMICAL ANALYSIS TO $G_{M1}$-GANGLIOSIDOSIS

### RESULTS AND DISCUSSION

*Lectin histochemistry.* — In $G_{M1}$-gangliosidosis, deficient activity of lysosomal acid $\beta$-D-galactosidase (EC 3.2.1.23) results in storage of structurally-related glycolipids and oligosaccharides in multiple tissues and cell types[81]. The oligosaccharides, which contain nonreducing terminal $\beta$-D-galactosyl groups, originate from the incomplete catabolism of complex glycans of *N*-glycoproteins. The English Springer Spaniel (ESS) is an animal model for the infantile form of this disease[27], whereas the Portuguese Water Dog (PWD) is an animal model with features of both infantile and juvenile conditions[82]. The affected neurons from both mutants stained only with RCA-I in formalin-fixed, frozen sections, and did not stain in delipidated, frozen sections or paraffin sections. These findings indicated the storage of glycolipids having terminal nonreducing $\beta$-D-galactosyl groups[17,27]. On the other hand, in paraffin sections of brain from both mutants, the neurons stained with Con A, and UEA-I but not with RCA-I. Furthermore, in the ESS mutant, the neurons stained with *Dolichos biflorus* agglutinin (DBA) and soybean agglutinin (SBA), but this was not observed in the PWD mutant. These findings indicated that the neurons of these dogs accumulate glycoconjugates that are unmasked during delipidation of tissue sections[17]. In addition, the lectin staining demonstrated that the two mutants differ with regard to the composition of the compounds stored.

In paraffin brain sections obtained from human patients afflicted with the in-

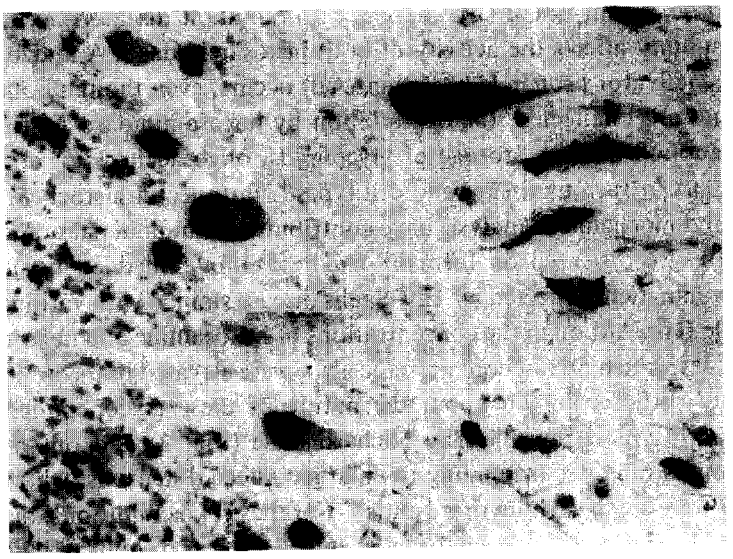

Fig. 4. Section through the cerebellum of a 7-month-old white boy affected with $G_{M1}$-gangliosidosis (Ref. 84, case No 4). The perikaryae and swollen axons of Purkinje cells are positively stained with UEA-I, but not the perikaryae of the neurons in the granular cell layer. This observation indicates the presence of terminal nonreducing α-L-fucopyranosyl groups in a structure containing the "H-isoantigen determinant". × 395.

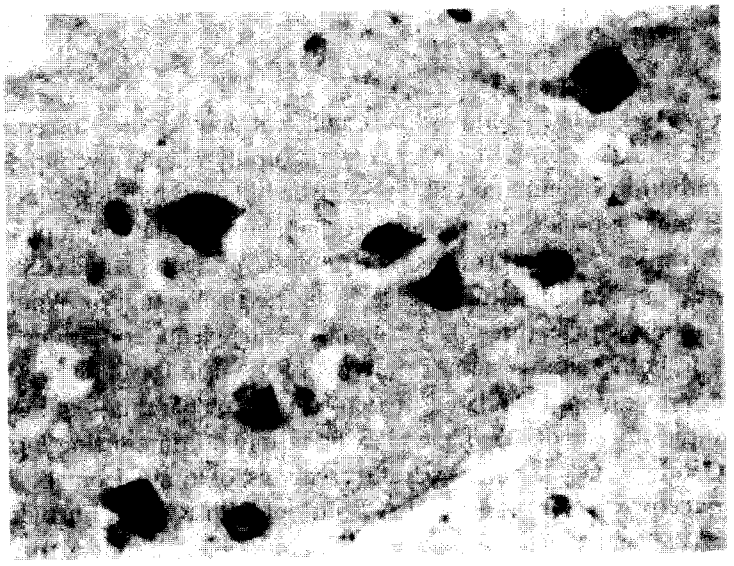

Fig. 5. Section through the cerebrum of a 27-year-old man afflicted with adult (type III, chronic) $G_{M1}$-gangliosidosis (Refs. 83, 85). The perikaryae of the enlarged neurons are heavily stained with Con A, but their axons, and the glial cells, are unstained. This finding indicates the presence of α-D-mannosyl groups in the perikaryae of the enlarged neurons. × 395.

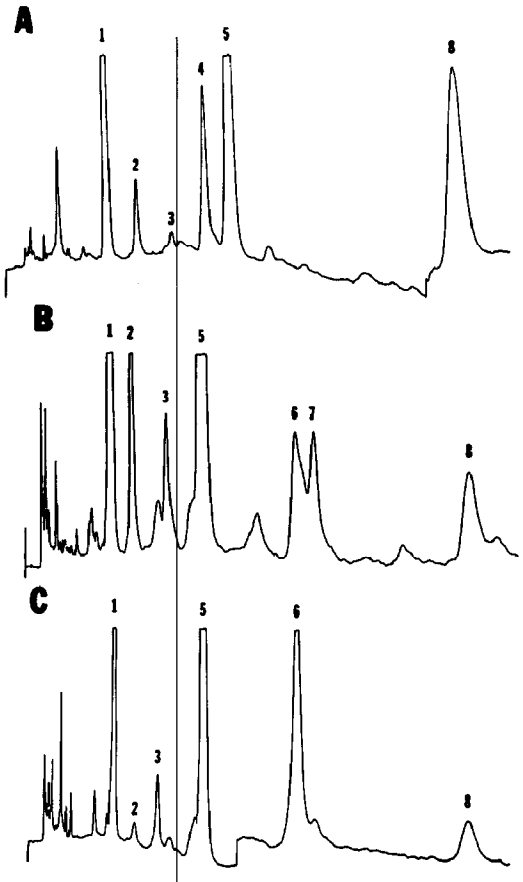


Fig. 6. H.p.l.c. of oligosaccharides extracted from tissues of Portuguese Water Dogs afflicted with $G_{M1}$-gangliosidosis: Panel A, kidney; Panel B, liver; and Panel C, pancreas. Extraction and sample preparation were performed as described in the Experimental section. Chromatography was on a Regis Hi-chrom reversible 5-$\mu$m Amino-Spherisorb column, with 7:3 acetonitrile–water at a flow rate of 2 mL/min, and detection by u.v. absorbance at 195 nm. The total elution time was 65 min for each run. The elution times of peaks 1 and 5 correspond to a hexasaccharide and a nonasaccharide, respectively.

fantile form of $G_{M1}$-gangliosidosis, the neurons in four out of six stained with RCA-I. In contrast, none of the patients afflicted with the juvenile or adult form did stain. For one unusual case of adult $G_{M1}$-gangliosidosis, in which examination of the cerebral cortex revealed a normal cytoarchitecture without neuron loss or gliosis[83], the perikaryae of some neurons, which contained meganeurites, stained with Con A (Fig. 4). This result showed that lectin histochemistry can uncover hidden abnormalities in such cases.

A peculiarity of the lectin-staining results from gangliosidosis is that in 38 out of 50 cases studied in humans and animals[21,22,25] (see Table II), the neurons stained positively with UEA-I (Fig. 5). This proportion is too high to be explained solely by the expression of the blood group O (*i.e.*, H) antigenic determinant. It is not obvious why glyconconjugates having nonreducing teminal $\alpha$-L-fucosyl groups should be accumulated in a disease in which the primary defect is the deficient activity of either $\beta$-D-galactosidase or *N*-acetyl $\beta$-D-hexosaminidase.

*Oligosaccharides from different tissues.* — For dog mutant PWD affected by $G_{M1}$-gangliosidosis[82], we have investigated the pattern of oligosaccharide accumulation in a variety of tissues and body fluids. Because the level of residual activity of lysosomal β-D-galactosidase is not necessarily the same in all tissues, and because differences will also occur in the amounts and type of substrates to be degraded, each tissue is expected to show a characteristic pattern of storage. This was previously demonstrated for cats affected by α-D-mannosidosis[41,42,54]. To confirm similar variation in the $G_{M1}$-gangliosidosis dogs, samples of extracts from sixteen tissues were examined by h.p.l.c. The results for kidney, liver, and pancreas are shown in Fig. 6. Whereas certain prominent peaks (see Nos. 1, 2, and 5 in Fig. 6) were observed in all the chromatograms, no tissue gave exactly the same profile as any other, with differences apparent in the presence or absence of certain peaks and in their relative areas. In previous work, the oligosaccharides extracted from the liver of the dog mutant of $G_{M1}$-gangliosidosis, examined by Warner and O'Brien[86], showed a profile similar to that of the urine oligosaccharides of an ESS dog examined in our laboratory[27]. Because the peaks corresponding to peaks 1,

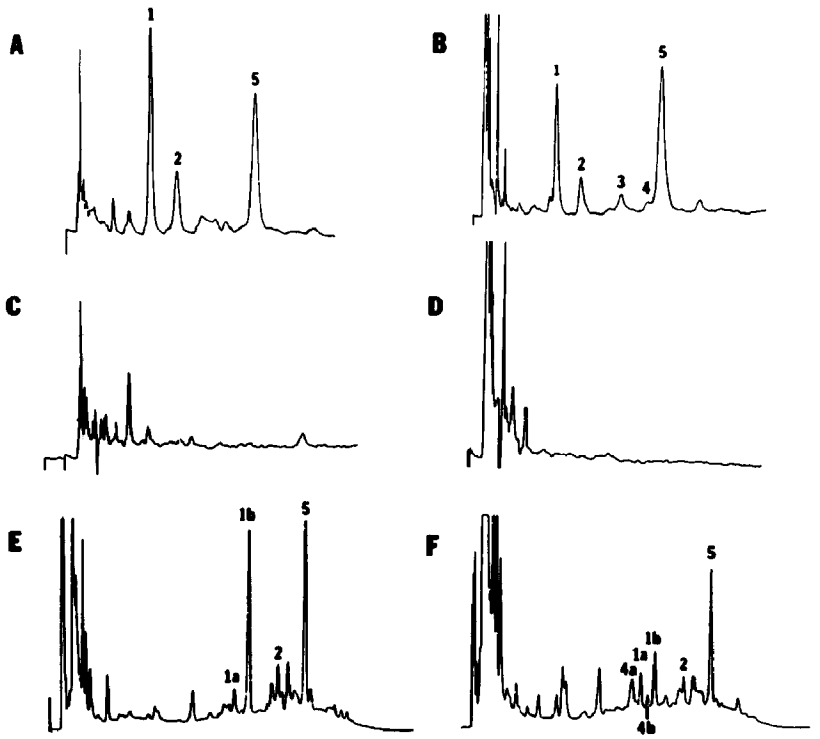


Fig. 7. H.p.l.c. of oligosaccharides extracted from affected placenta of Portuguese Water Dog with $G_{M1}$-gangliosidosis (Panels A and E), control placenta (Panel C), affected amniotic fluid (Panels B and F), and control amniotic fluid (panel D). Chromatography was performed on an Amino-Spherisorb normal-phase column (Panels A, B, C, and D) or on a C-8 reversed-phase column (Panels E and F); for details see the Experimental section. The duration of each h.p.l.c. experiment was 30 min. The peaks in Panels E and F were assigned by collection of pure samples from the normal-phase h.p.l.c. (see peaks numbered 1-5), per-*O*-benzoylation, and reversed-phase h.p.l.c.

2, and 5 in Fig. 6 comigrated with those of the liver extract, the oligosaccharides corresponding to these peaks, as determined by Warner and O'Brien[86], most probably correspond to the hexasaccharides, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc (peaks 1,2), and the nonasaccharide, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-β-D-Gal*p*-[(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc.

*Oligosaccharides from placenta and amniotic fluid.* — In order to determine whether the analysis of oligosaccharides stored in placenta and amniotic fluid could be employed to differentiate normal, affected, and heterozygous puppies born of parents affected by $G_{M1}$-gangliosidosis, as was previously shown for placentas of cats affected by α-D-mannosidosis[41,42], and allantoic fluid of goats affected by β-D-mannosidosis[43,44], oligosaccharides were extracted from 18 placentas, from three litters. When the oligosaccharides were analyzed by normal-phase h.p.l.c., those from three placentas having very low-β-D-galactosidase activity[87] showed several large peaks (Fig. 7, Panel A) with a profile similar to that seen in extracts from tissues of an affected adult dog (see Fig. 6) and with a resemblance to the profile for English Springer Spaniels affected by infantile $G_{M1}$-gangliosidosis[27]. In contrast, h.p.l.c. of extracts from placentas of puppies, shown to be presumptive heterozygotes or normal on the basis of β-D-galactosidase activity of placental extracts, revealed a randomized pattern of small peaks (Fig. 7, Panel C). Even when the chromatographic conditions were optimized with the best possible column and elution conditions, and the size of the injections increased, no consistent differences could be discerned between placentas from dogs having normal levels of β-D-galactosidase and those which were presumptive heterozygotes. This result differed from that obtained with placentas from cats affected by α-D-mannosidosis, where the heterozygotes were characterized by low levels of oligosaccharides with a diagnostic h.p.l.c. profile[41].

When oligosaccharides from the amniotic fluid were examined by normal-phase h.p.l.c., the result was the same as for the placentas, *i.e.*, only the fluids from affected puppies showed a profile typical for $G_{M1}$-gangliosidosis (Fig. 7, Panel B). In this case, the profiles from controls and heterozygotes showed an almost complete absence of peaks (Fig. 7, Panel D). Also, the levels of oligosaccharides in amniotic fluid were much lower than those in urine (results not shown).

To confirm that the peaks in the profiles from affected puppies corresponded to oligosaccharides, and to evaluate their homogeneity, a portion of each extract was per-*O*-benzoylated and chromatographed on a reversed-phase column (Fig. 7, Panels E and F). In order to cross-reference the peaks for the two types of h.p.l.c., the eluent corresponding to each numbered fraction from the normal-phase column was collected, and the components per-*O*-benzoylated separately. This showed that peaks 2 and 5 probably correspond to single compounds, whereas peaks 1 and 4 contain at least two components (1a,b; and 4a,b). Peak 3 is probably not a carbohydrate, as no per-*O*-benzoylated peak could be observed.

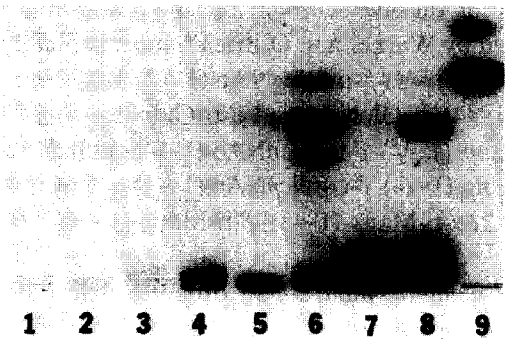


Fig. 8. T.l.c. analysis for the presence of poly(lactosamino)glycans in livers of control dog, and dog afflicted with $G_{M1}$-gangliosidosis. The glycopeptides were prepared by Pronase digestion of defatted livers from age-matched control Portuguese Water Dog (PWD) and from affected PWD. The glycopeptides were fractionated by chromatography on Sephadex G-50 into three fractions. Fractions I and II, of higher and intermediate molecular weight, respectively, were incubated with endo-β-D-galactosidase from *E. freundii* to probe for the presence of poly(lactosamino)glycans. Lanes are designated as follows: (1) Control fraction I; (2) Control(1) + endo-β-D-galactosidase; (3) Control fraction II; (4) 3 + endo-β-D-galactosidase-treated; (5) $G_{M1}$ fraction I; (6) 5 + endo-β-D-galactosidase; (7) $G_{M1}$ fraction II; (8) 7 + endo-β-galactosidase; (9) standards of galactose (top), and lactose (bottom).

Thus, the analysis of oligosaccharides from placentas provided a useful method for the early diagnosis of puppies affected by $G_{M1}$-gangliosidosis, and this procedure fully corroborated the results of β-D-galactosidase assay and the ultrastructural studies. Furthermore, the analysis of oligosaccharides in amniotic fluid offers an alternative procedure for prenatal diagnosis. In contrast to feline α-D-mannosidosis, the analysis of oligosaccharides from placentas or amniotic fluid could not be used to diagnose heterozygous puppies.

*Glycoprotein from brain and visceral organs.* — Because glycoproteins containing poly(lactosamino)glycans had been identified in brains of patients affected by $G_{M1}$-gangliosidosis[88], two dog models for this disease, PWD and ESS, were examined for accumulation of similar compounds. Brains and livers were homogenized, delipidated, and the defatted residues treated with Pronase. The resulting glycopeptides were examined by endo-β-D-galactosidase digestion and t.l.c., as described above for Gaucher's patients. The PWD liver contained poly(lactosamino)glycans having nonreducing, terminal β-D-galactosyl residues (Fig. 8), but the ESS liver did not. For brain, no accumulation was found in either dog. This result is consistent with the lack of staining by RCA-I in paraffin sections of brain. It also is surprising, because poly(lactosamino)-glycans containing a β-linked D-galactosyl group at their nonreducing terminus would be expected to accumulate in the absence of normal levels of lysosomal β-D-galactosidase activity. These results, and others based on lectin histochemistry[5,23] and ultrastructure[5], suggest that the mechanisms leading to carbohydrate accumulation in the tissues of patients and animals affected by lysosomal-storage diseases are more complex than previously imagined. Support for these conclusions is provided by the finding regarding the inhibition of lysosomal hydrolases by glycosaminoglycans[89] which may occur in mucopolysaccharidoses.

## EXPERIMENTAL

*Lectin histochemistry.* — Formalin-fixed, frozen, and formalin-fixed, paraffin-embedded sections of brain and various visceral organs were obtained from the archives of various departments of pathology (see Acknowledgments). Five-$\mu$m paraffin sections were deparaffinized by incubation in warm xylene (65°) and absolute ethanol, and hydrated. Afterwards, both frozen and paraffin sections were stained by the following procedure. The endogenous peroxidase in tissue sections was blocked by incubation with 2% $H_2O_2$. The sections were then covered with filtered mouse-liver powder solution, at a concentration of 100 $\mu$g/mL in phosphate-buffered saline solution (PBS), for 10 min. The excess solution was shaken off and the slide around the tissue was blotted. The sections were then incubated with one of the eleven different biotinylated lectins (Vector Laboratories, Burlingame, CA) for 30 min, washed three times in PBS, followed by incubation with avidin–biotin–peroxidase complex (ABC) (Vector Laboratories) for 30 min, and washed again three times in PBS. The visualant, horseradish peroxidase (EC 1.11.1.7), was activated by incubation for 8–10 min with PBS solution containing 3,3′-diaminobenzidine (DBA) and $H_2O_2$. The paraffin sections were washed in tap water for 5 min, counterstained with Methyl Green, dehydrated, and coverslipped with Permount. The frozen-brain sections were counterstained, washed in tap water, and coverslipped with Aquamount. Additional frozen sections were pretreated for 1 h in xylene or 2:1 (v/v) chloroform–methanol[17] and subsequently stained with lectins as described above. Incubation of lectins with 0.2M solutions of their corresponding sugar hapten, before application of the lectin solution to the tissue section, served as a control for binding specificity. Incubation of ABC or lectin solution alone served as a nonspecific negative control[8,17].

*H.p.l.c. analysis of oligosaccharides extracted from tissues and body fluids of Portuguese Water Dogs with $G_{M1}$-gangliosidosis.* — Tissues were homogenized and oligosaccharides extracted as described previously[41,54]. Sample preparation and normal-phase h.p.l.c. ("amino column") were performed as described[43,45]. Reversed-phase h.p.l.c. ("C-8 column") was performed as described by Warren *et al.*[54]. The size of the sample injected was varied to provide peaks of a suitable area to facilitate comparison of the profiles. The peaks in the profile for normal-phase h.p.l.c. of the kidney oligosaccharides were arbitrarily numbered 1–6, with elution times of 12.3, 16, 21, 24, 26.8, and 53 min, respectively.

Peaks in the reversed-phase h.p.l.c. profiles were assigned by collection of each peak from the amino column (for kidney sample), per-*O*-benzoylation[36], and h.p.l.c. on the C-8 column. Peaks 1 and 4 were found to contain two components, unresolved by the amino column. The major peaks in the reversed-phase profile were, therefore (in order of elution): 4a (12.7 min), Ia (13 min), 4b (13.8 min), Ib (14 min), 2 (16.25 min), and 5 (18.3 min). Peak 3 (amino column) gave no C-8 peak and is, therefore, not considered to be a carbohydrate. Peak 6 gave four peaks on the C-8 column with elution times between 17.1 and 19.2 min. The origin of this heterogeneity is not known. The profiles for placenta and amniotic fluid did not show significant peaks that were eluted later than

peak 5 (amino column). Therefore, only the profile for the first 30 min of the elution is shown in Fig. 6.

*Preparation and analysis of glycopeptides.* — Glycopeptides were prepared according to established procedures[88]. Digestion with endo-β-D-galactosidase and analysis of the products were performed as described[70,88].

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REFERENCES

1 J. Alroy, C. D. Warren, S. S. Raghavan, and E. H. Kolodny, *Human Pathol.*, 20 (1989) 823–826.
2 P. R. Dorling, in J. T. Dingle, R. T. Dean, and W. Sly (Eds.), *Lysosomes in Biology and Pathology*. Elsevier, Amsterdam, 1984, pp. 347–379.
3 S. Kornfeld, *J. Clin. Invest.*, 77 (1987) 1–6.
4 K. von Figura, A. Hasilik, and F. Steckel, in J. A. Barringer and R. D. Brady (Eds.), *Molecular Basis of Lysosomal Storage Disorders*, Academic Press, San Diego, 1984, pp. 133–146.
5 M. Castagnaro, J. Alroy, A. A. Ucci, and R. H. Glew, *Virchows Arch. B*, 54 (1987) 16–26.
6 I. J. Goldstein, R. C. Hughes, M. Monsigny, T. Osawa, and N. Sharon, *Nature (London)*, 286 (1980) 66.
7 I. J. Goldstein and R. D. Poretz, in I. E. Liener, N. Sharon, and I. J. Goldstein (Eds.), *Lectins, Properties, Functions and Applications in Biology and Medicine*, Academic Press, Orlando, 1968, pp. 33–247.
8 J. Alroy, A. A. Ucci, and M. E. A. Pereira, in R. A. DeLellis (Ed.), *Diagnostic Immunohistochemistry*, Vol. 2, Masson, New York, 1984, pp. 66–88.
9 J. Alroy, A. A. Ucci, and M. E. A. Pereira, in R. A. DeLellis (Ed.)., *Advances in Immunochemistry*, Raven Press, New York, 1988 pp. 93–131.
10 I. Damjanov, *Lab. Invest.*, 57 (1987) 5–20.
11 M. C. Glick and U. V. Santer, in G. Akoyunoglou, A. E. Evangelopoulos, J. Georgatsos, G. Palaidogos, A. Trakatellis, and C. P. Tsiganos (Eds.), *Cell Function and Differentiation, Part A*. Alan R. Liss, New York, 1982, pp. 371–383.
12 B. A. Schulte and S. S. Spicer. *J. Histochem. Cytochem.*, 33: (1985) 427–438.
13 T. Katsuyama and S. S. Spicer *J. Histochem. Cytochem.*, 26 (1978) 233–250.
14 I. Virtanen, P. Edblom, P. Laurila, S. Nordling, K. Raivo, and P. Aula, *Pediatr. Res.*, 14 (1980) 1199–1203.
15 O. P. van Diggelen, D. Schindler, R. Willemsen, M. Boer, W. J. Kleijer, J. G. M. Huijmans, W. Blom, and H. Galjaard, *J. Inher. Metab. Dis.*, 11 (1988) 349–357.
16 A. Lageron, A. Negre, and R. Salvayre. *J. Inher. Metab. Dis.*, 12, Suppl. 2 (1989) 75–378.
17 J. Alroy, A. A. Ucci, V. Goyal, and W. Woods. *J. Histochem. Cytochem.*, 34 (1986) 501–505.
18 T. Faraggiana, J. Churg, E. Grishman, L. Strauss, A. Prado, D. F. Bishop, E. Schuchman, and R. J. Desnick, *Am. J. Pathol.*, 103 (1981) 247–262.
19 A. Lageron, *Histochem. J.* 19 (1987) 419–425.
20 R. D. Murnane, A. Ahern-Rindell, and D. J. Prieur, *Am. J. Pathol.*, 135 (1989) 623–630.
21 H. Weintraub, E. Skutelsky, U. Sandbank, A. Abramovici, P. G. Pentchev, and J. Alroy, *Histochemistry*, 91 (1989) 339–344.
22 M. Elleder, *Histochemistry*, 93 (1989) 197–205.
23 J. Alroy, L. S. Adelman, and C. D. Warren, *Acta Neuropathol.*, 76 (1988) 359–365.
24 J. Alroy, V. Goyal, and C. D. Warren, *Acta Neuropathol.*, 76 (1988) 109–114.
25 J. Alroy, U. Orgad, A. A. Ucci, and V. E. Gavris, *Vet. Pathol.*, 22 (1985) 311–316.

26 J. Alroy, U. Orgad, A. A. Ucci, and M. E. A. Pereira, *J. Histochem. Cytochem.*, 32 (1984) 1280–1284.
27 J. Alroy, U. Orgad, A. A. Ucci, S. H. Schelling, K. L. Schunk, C. D. Warren, S. S. Raghavan, and E. H. Kolodny, *Science*, 229 (1985) 470–472.
28 J. Alroy, A. A. Ucci, V. Goyal, and A. Aurilio, *Acta Neuropathol.*, 71 (1986) 26–31.
29 J. Alroy, A. A. Ucci, and C. D. Warren. *Acta Neuropathol.*, 67 (1985) 265–271.
30 M. Castagnaro, J. Alroy, A. A. Ucci, and R. Jaffe, *Arch. Pathol. Lab. Med.*, 111 (1987) 285–290.
31 T. Faraggiana, S. Shen, C. Childs, L. Strauss, and J. Churg. *Histochem. J.*, 14 (1982) 655–664.
32 S. I. Ikeda, K. Kondo, K. Oguchi, N. Yanagisawa, R. Horigome, and F. Murata, *Neurology*, 34 (1984) 451–456.
33 S. M. Pueschel, P. A. O'Shea, J. Alroy, M. Ambler, F. Dangond, P. Daniel, and E. H. Kolodny, *Pediat. Neurol.*, 4 (1988) 207–212.
34 M. Ushiyama, S.-C. Ikedo, J. Nakayama, N. Yanagisawa, N. Hanyu, and T. Katsuyama, *J. Neurol. Sci.*, 71 (1985) 209–233.
35 J. Alroy, K. L. Schunk, S. S. Raghavan, C. D. Warren, P. F. Daniel, and E. H. Kolodny, in R. Salvayre, L. Douste-Blazy, and S. Gatt (Eds.), *Lipid Storage Disorders: Biological and Medical Aspects*, Plenum Press, New York, 1988, pp. 649–659.
36 P. F. Daniel, *Methods Enzymol.*, 138 (1987) 94–116.
37 C. D. Warren, A. S. Schmit, and R. W. Jeanloz, *Carbohydr. Res.*, 116 (1983) 171–182.
38 C. D. Warren, P. F. Daniel, B. Bugge, J. E. Evans, L. F. James, and R. W. Jeanloz, *J. Biol. Chem.*, 263 (1988) 15041–15049.
39 C. D. Warren, R. W. Jeanloz, and G. Strecker, *Carbohydrate. Res.*, 92 (1981) 85–101.
40 P. F. Daniel, C. D. Warren, and L. F. James, *Biochem. J.*, 221 (1984) 601–607.
41 C. D. Warren, J. Alroy, B. Bugge, P. F. Daniel, S. S. Raghavan, E. H. Kolodny, and R. W. Jeanloz, *FEBS Lett.*, 195 (1986) 247–252.
42 J. Alroy, C. D. Warren, S. S. Raghavan, P. F. Daniel, K. L. Schunk, and E. H. Kolodny, *Placenta*, 8 (1987) 545–553.
43 D. L. Dahl, C. D. Warren, E. J. S. Rathke, and M. Z. Jones, *J. Inher. Metab. Dis.*, 9 (1986) 93–98.
44 C. D. Warren, B. Bugge, K. Linsley, E. J. S. Rathke, M. Z. Jones, and R. W. Jeanloz, in L. F. James, A. D. Elbein, R. J. Molyneux, and C. D. Warren (Eds.), *Swainsoinine and Related Glycosidase Inhibitors*, University of Iowa Press, Ames, Iowa, 1989, pp. 304–315.
45 C. D. Warren, B. Bugge, K. Linsley, D. Daniels, P. F. Daniel, L. F. James, and R. W. Jeanloz, in L. F. James, A. D. Elbein, R. J. Molyneux, and C. D. Warren (Eds.), *Swainsonine and Related Glycosidase Inhibitors*, University of Iowa Press, Ames, Iowa, 1989, pp. 344–359.
46 P. F. Daniel, C. D. Warren, L. F. James, and R. D. Jolly, in A. A. Seawright, M. P. Hegarty, L. F. James, and R. F. Keller (Eds.), *Plant Toxicology*, The Queensland Poisonous Plants Committee, Yeerongpilly, Queensland, Australia, 1985, pp. 290–300.
47 A. L. Beaudet and G. H. Thomas, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw–Hill, New York, 1989, pp. 1603–1621.
48 G. Dawson and L. Hancock, in R. U. Margolis, and R. K. Margolis (Eds.), *Neurobiology of Glycoconjugates*, Plenum Press, New York, 1989, pp. 421–447.
49 T. G. Warner and J. S. O'Brien. *Annu. Rev. Genet.*, 17 (1983) 395–441.
50 P. R. Dorling, C. R. Huxtable, and S. M. Colgate, *Biochem. J.*, 191 (1980) 649–651.
51 R. J. Molyneux and L. F. James, *Science*, 216 (1982) 190–191.
52 C. D. Warren, S. Sadeh, P. F. Daniel, B. Bugge, L. F. James, and R. W. Jeanloz, *FEBS Lett.*, 163 (1983) 99–103.
53 P. F. Daniel, C. D. Warren, L. F. James, and R. D. Jolly, in L. F. James, A. D. Elbein, R. J. Molyneux, and C. D. Warren (Eds.), *Swainsonine and Related Glycosidase Inhibitors*, University of Iowa Press, Ames, Iowa 1989, pp. 331–343.
54 C. D. Warren, L. S. Azaroff, P. F. Daniel, J. Alroy, B. Bugge, and R. W. Jeanloz, *Carbohydr. Res.*, 180 (1988) 325–338.
55 C. M. Nolan and W. S. Sly, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw–Hill, New York, 1989, pp. 1589–1601.
56 G. Strecker, M. C. Feers, C. Michalski, T. Hondi-Assah, B. Fournet, G. Spik, J. Montreuil, J. P. Farriaux, P. Maroteaux, and P. Durand. *Eur. J. Biochem.*, 75 (1977) 391–403.
57 E. F. Neufeld and J. Muenzer, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw-Hill, New York, 1989, pp. 1565–1587.
58 R.-M. N. Boustany, J. Alroy, and E. H. Kolodny, *Am. J. Med. Genet. Supl.*, 5 (1988) 47–58.

59 R. D. Jolly, A. Shimada, A. S. Craig, K. B. Krikland, and D. N. Palmer, *Am. J. Med. Genet. Suppl.*, 5 (1988) 159–170.
60 N. Koppang. *Am. J. Med. Genet. Suppl.*, 5 (1988) 117–126.
61 J. Alroy, unpublished results.
62 N. A. Hall, R. D. Jolly, D. N. Palmer, B. D. Lake, and D. Patrick, *Biochim. Biophys. Acta*, 993 (1989) 245–251.
63 R. J. Desnick and D. F. Bishop, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw-Hill, New York, 1989, pp. 1751–1796.
64 K. Suzuki and Y. Suzuki, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw–Hill, New York, 1989, pp. 1699–1720.
65 G. Baskin, J. Alroy, Y. T. Li, Y. Dayal, S. S. Raghavan, and L. Sharer. *Lab. Invest.*, 60 (1989) 7A.
66 M. S. Pollanen and B. A. Brody, *Arch. Pathol. Lab. Med.*, 114 (1990) 213–216.
67 J. A. Barranger and E. I. Ginns, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw–Hill, New York, 1989, pp. 1677–1698.
68 F. T. Zugibe, *Am. J. Med. Genet. Suppl.*, 3 (1987) 221–226.
69 J. F. Lhotka, *Nature (London)*, 171 (1953) 1123–1124.
70 R. De Gasperi, J. Alroy, R. Richard, V. Goyal, U. Orgad, R. E. Lee, and C. D. Warren, *Lab. Invest.*, 63 (1990) 385–393.
71 H. Nakagawa, T. Yamada, J. L. Chien, A. Gardas, M. Kitamikado, S.-C. Li, and Y.-T. Li, *J. Biol. Chem.*, 255 (1980) 5955–5959.
72 M. W. Spence and J. W. Callahan, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*. 6th edn., McGraw–Hill, New York, 1989, pp. 1655–1698.
73 W. R. Den Tandt and F. van Hoof, *J. Inher. Metab. Dis.*, 12 (1989) 483–484.
74 H. Weintraub, R. De Gasperi, C. D. Warren, E. Skutelsky, and J. Alroy, unpublished results.
75 A. J. Ahern-Rindell, R. D. Murnane, and D. J. Prieur, *Somatic. Cell. mol. MD. Genet.*, 15 (1989) 525–533.
76 K. Sandhoff, E. Conzelmann, E. T. Neufeld, M. M. Kaback, and K. Suzuki, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw-Hill, New York, 1989, pp. 1807–1839.
77 E. F. Neufeld, *J. Biol, Chem.*, 264 (1989) 10927–10930.
78 E. G. Brunngraber, L. A. Witting, C. Haberland, and B. Brown, *Brain Res.*, 38 (1972) 151–162.
79 E. H. Kolodny and R. S. Williams, in P. Mittler (Ed.), *Research to Practice in Mental Retardation, Biomedical Aspects*, Vol. III, I.A.S.S.M.D., 1977, pp. 409–419.
80 T. Kobayashi and K. Suzuki, *Ann. Neurol.*, 9 (1981) 476–483.
81 J. S. O'Brien, in C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw–Hill, New York, 1989, pp. 1797–1806.
82 G. K. Saunders, P. A. Mood, R. K. Myers, L. F. Schell, and R. Carithers, *Vet. Pathol.*, 25 (1988) 265–269.
83 J. E. Goldman, D. Katz, I. Rapin, D. P. Purpura, and K. Suzuki, *Ann. Neurol.*, 8 (1981) 465–475.
84 B. H. Landing, F. N. Silverman, J. M. Craig, M. D. Jacoby, M. E. Lahey, and D. L. Chadwick, *Am. J. Dis. Child.*, 108 (1964) 503–522.
85 J. E. Goldman, T. Yamanaka, I. Rapin, M. Adachi, K. Suzuki, and K. Suzuki, *Acta Neuropathol.*, 52 (1980) 189–202.
86 T. G. Warner and J. S. O'Brien, *J. Biol. Chem.*, 257 (1982) 224–232.
87 J. Alroy, R. De Gasperi, S. S. Raghavan, and C. D. Warren, unpublished results.
88 B. Berra, R. De Gasperi, S. Rapelli, S. Okada, S.-C. Li, and Y.-T. Li, *Neurochem. Pathol.*, 4 (1986) 107–117.
89 J. L. Avila and J. Convit, *Biochem. J.*, 152 (1975) 57–64.