STUDIES ON THE α-GALACTOSIDASES OF NORMAL AND FABRY PLASMA

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ABSTRACT

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By

Carol A. Mapes

Ceramide trihexosidase activity was discovered in normal human plasma. This enzymatic activity exhibited a bimodal pH optimum, suggesting that there might be two molecular species of ceramide trihexosidase in plasma. This possibility was confirmed when the ceramide trihexosidase activity with a pH optimum of 5.4 was separated from the activity with a pH optimum of 7.2 by low temperature ethanol fractionation.

The activity with a pH optimum of 5.4 (A form) could be further fractionated from Cohn fraction IV-1 and stabilized by a series of steps including ammonium sulfate precipitation of contaminating proteins, treatment with 5% butanol, acetone precipitation, and affinity chromatography. These procedures separated the A form of ceramide trihexosidase into two enzymatically active proteins (A-1 and A-2). The B form of ceramide trihexosidase was purified from Cohn fraction I using the same procedures and was separated into five enzymatically active proteins (B-I, B-II, B-III, B-IV, B-V) by isoelectric focusing.

The A-forms of ceramide trihexosidase appeared to be homogeneous as determined by a single band on polyacrylamide gel electrophoresis and by the presence of a single protein, coincident with enzymatic activity of constant specific activity, on isoelectric focusing, affinity chromatography, and sucrose density gradient centrifugation.

The A forms of the enzyme were activated by sodium taurocholate and sodium chloride. In the absence of these activators the enzymes displayed sigmoidal substrate saturation curves. The sigmoidality was eliminated by the addition of sodium taurocholate and sodium chloride to the incubation mixture. In addition, these enzymes demonstrated the anomalous characteristic of becoming inactive when concentrated. On the basis of inhibitor and activator studies a model for the mechanism of ceramide trihexosidase hydrolysis was presented. This model assumes that the enzyme expresses its optimum activity when complexed with a mixed substrate-cholate micelle and that excess enzyme causes formation of an inactive enzyme-micelle aggregate.

The A-1 form of ceramide trihexosidase was competitively inhibited by digalactosylceramide and the trisaccharide obtained by ozonolysis of GL-3. The A-2 form of enzymatic activity was inhibited by the products of the reaction, galactose and lactosylceramide. It was also competitively inhibited by digalactosylceramide, trisaccharide and

inositol. Under the conditions of these experiments neither of the enzymes was inhibited by the artificial substrate 4-methylumbelliferyl-α-galactoside.

The A forms of ceramide trihexosidase have molecular weights of approximately 95,000, are similar in their electrophoretic characteristics, and are specific for the lipid substrate.

Neuraminidase treatment of the A-1 form of ceramide trihexosidase converted it to an enzymatically active protein which was indistinguishable from the B-V form of ceramide trihexosidase on cellulose acetate electrophoresis. Both [^{14}C]sialic acid and [^{14}C]N-acetylglucosamine could be incorporated into the B-V form of the enzyme, forming several proteins. One of these proteins was electrophoretically indistinguishable from the A form of ceramide trihexosidase. Thus it was postulated that the two groups of ceramide trihexosidase are glycoproteins related to each other by their sialic acid content.

An α-galactosidase affinity column adsorbent was prepared by attaching p-aminophenylmelibioside to Affinose 202. This substituted agarose was used to study the enzymes of normal and Fabry plasma. Affinity chromatography of Fabry plasma revealed that the A forms of the enzyme were partially inactive, whereas all of the B forms of ceramide trihexosidase were absent. In addition, there was an accumulation of catalytically inactive proteins in Fabry

plasma. It was suggested that the catalytically inactive proteins were the B forms of ceramide trihexosidase which were converted to the A-1 form of the enzyme.

Affinity chromatography also revealed that six non-specific α-galactosidases were present in plasma. The specific activities of several of these α-galactosidases were altered in Fabry's disease. Unexpectedly, two of the enzymes hydrolyzing p-nitrophenyl-α-galactoside and 4-methylumbelliferyl-α-galactoside were activated by affinity chromatography. After affinity chromatography these enzymes had 98% of normal activity and could not be used to distinguish hemizygotes, heterozygotes, or normals.

A specific enzyme for the hydrolysis of digalactosyl-ceramide was discovered in normal plasma. This enzyme was separated into two active components by isoelectric focusing and cellulose acetate electrophoresis. In Fabry plasma only the protein of slower electrophoretic mobility was detectable.

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By

Carol A. Mapes

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INTRODUCTION

Thudichum published the first report on the occurrence of sphingolipids in brain in 1874 (1). Approximately 15 years later he summarized his life-work on the discovery and primary chemical characterization of these lipids which included cerebrosides, sphingomyelin, and sphingosine (2).

The second phase of sphingolipid work occurred during the first four decades of this century. These studies, carried out primarily by Thierfelder, Klenk, Levene and Rosenheim (3), were concerned mainly with the isolation and detailed structural characterization of the individual lipids. During this period the sulfatides were discovered by Blix (4) and a class of complex acidic glycosphingolipids, gangliosides, was reported by Klenk (5-7).

Experiments on the degradation of sphingolipids by tissue preparations were first reported by Thannhauser and Reichel (8,9). These as well as other investigators used crude preparations to degrade cerebrosides (8,10), sphingomyelin (9,11,12), sulfatides (13), and gangliosides (14). The first experiments with partially purified enzymes from mammalian tissues appeared in the 1960's and described

the hydrolysis of ceramide from brain and of sphingomyelin by enzymes from brain and liver (16,17). These studies were followed by reports on the isolation and properties of enzymes which catalyze the hydrolysis of ceramide (18,19), galactosylceramide (27-29), several glycosphingolipids having a terminal galactose residue (24, 30-32), N-acetyl-galactosamine (33, 34) and N-acetylneuraminic acid (35-37).

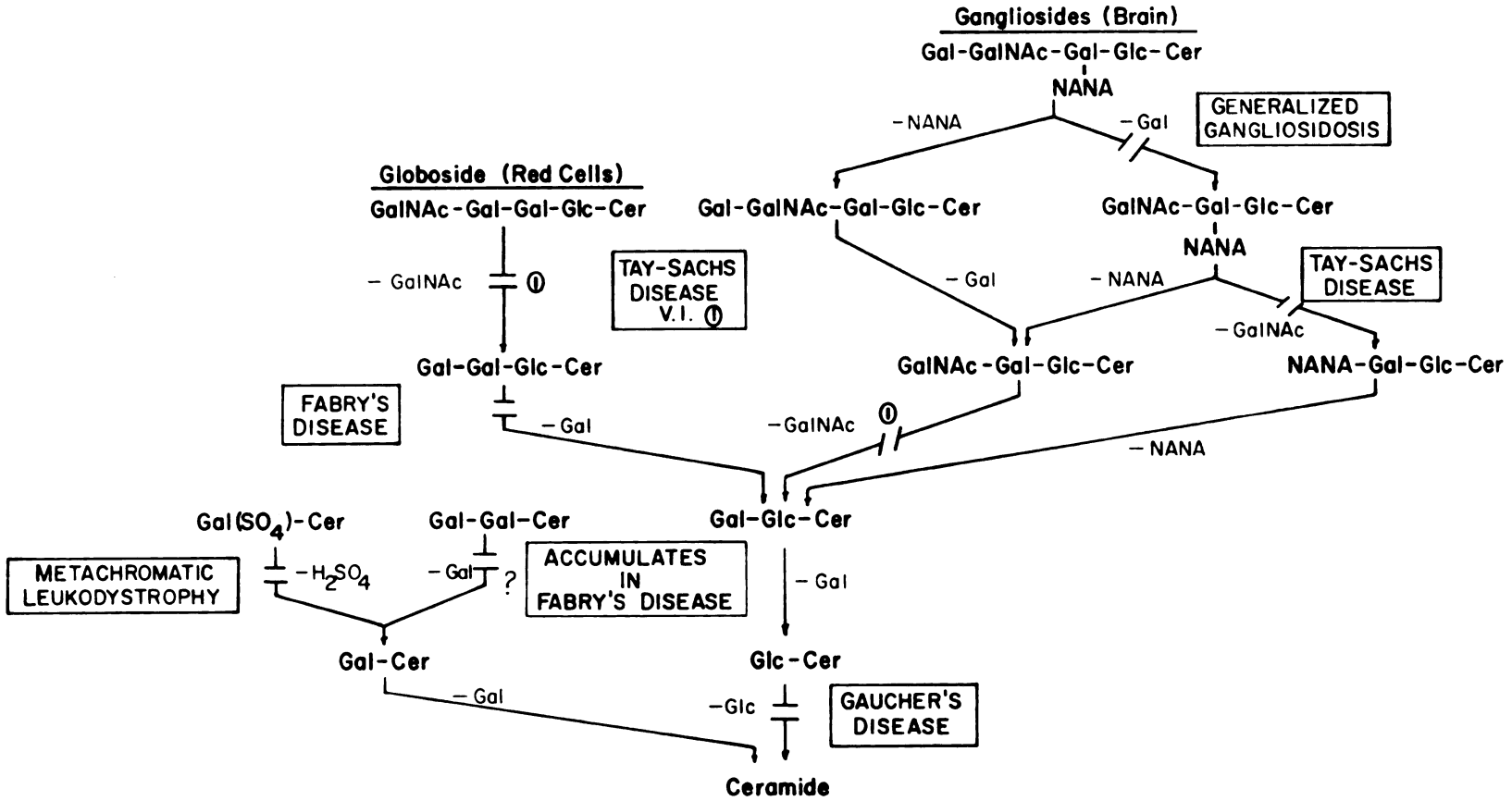
The cumulative results of these and other studies, as illustrated in Figure 1, were organized into a pattern consisting of a multi-step successive degradation of monosaccharide units which could account for the catabolism of glycosphingolipids to ceramide (N-acylsphingosine). The hydrolysis of ceramide to sphingosine and fatty acids, catalyzed by a ceramidase isolated from rat brain (38), results in the formation of sphingolipid bases which can be metabolized to either palmitic acid or pentadecanoic acid, both of which can be degraded by the β-oxidation and tricarboxylic acid systems. Thus a combination of these pathways may account for the total degradation and oxidation of most mammalian glycosphingolipids.

During the same periods of time when these lipids were first being discovered and characterized, reports of rare diseases, now know to be sphingolipidoses, were appearing in the literature. In the late 1800's Anderson (39) and Fabry (40) independently described the clinical symptoms of patients with angiokeratoma corporis diffusum (Fabry's disease). Reports on other patients having similar

Figure 1. The Current Concept of Glycosphingolipid Metabolism

The complex glycosphingolipids are degraded successively by the action of glycosidases. Sites at which there is a deficiency of a catabolic enzyme, resulting in one of the sphingolipidoses, are indicated by broken arrows.

Current Concept of Glycosphingolipid Metabolism



symptoms were also presented by Steiner and Voerner (41), Gunther (42), Weicksel (43), and Pompen (44,45).

Scriba was the first investigator to establish that the accumulated material in Fabry's disease was a lipid (46). Early, incomplete studies indicated that a phospholipid might be involved in the disease (46-48), but in 1963 Sweeley and Klionsky isolated the lipids from a formalin-fixed Fabry kidney and determined that the accumulated lipids were neutral glycosphingolipids--galactosylgalactosyl-glucosylceramide (GL-3) and galactosylgalactosylceramide (GL-2b) (49,50).

Brady *et al.* (51) discovered a GL-3 hydrolase, ceramide trihexosidase, in the small intestinal mucosa of normal persons which was absent in Fabry patients, thereby demonstrating that the accumulation of GL-3 in Fabry's disease is due to an enzyme deficiency. This work is supported by recent reports of an absence of ceramide trihexosidase activity in Fabry leukocytes (52-54), cultured skin fibroblasts (55-57), amniotic fluid (55), kidney (32,58), spleen (32), brain (32), and liver (32).

Since it has now been established that all of the known glycosphingolipidoses result from enzymatic defects, the emphasis in the sphingolipid field during the past 4-5 years has been on the purification of these enzymes for possible treatment of these metabolic disorders.

The primary emphasis of the present research was to determine whether the presumably lysosomal ceramide trihexosidase would have the ability to hydrolyze GL-3 *in vivo* if infused intravenously and to purify, stabilize, and study some of the properties of this enzyme in preparation for pilot studies to determine whether enzyme replacement on Fabry's disease might have therapeutic value. These studies resulted in several publications which are listed in the Appendix.

LITERATURE REVIEW

THE SPHINGOLIPID HYDROLASES

There has been a pattern for defining each of the sphingolipidoses which consists of three main subdivisions: 1) the reports giving the clinical, pathological and histochemical characteristics of the abnormality; 2) the identification of the accummulated products; and 3) the isolation and characterization of the individual enzymes. Information concerning the first two subdivisions is adequately summarized elsewhere (59) and will not be discussed in this paper. Primary emphasis will be placed on recent advances in characterization of the human sphingolipid hydrolases.

Investigation of these enzymes is usually centered around the following five topics: 1) detergent activation; 2) multiplicity of forms, at least one of which has an acidic pH optimum and is presumed to be a lysosomal hydrolase regardless of the tissue or biological fluid from which it is isolated; 3) classification of the protein, several of which appear to be glycoproteins; 4) substrate specificity; and 5) possible therapeutic utility of the enzyme. Table 1

Table 1. Nomenclature of the Sphingolipids and Sphingolipid Hydrolases

Disease	Accumulated Lipid(s)	Lipid Abbreviation	Catabolic Enzyme
Gaucher's	Glucosyl(β1→1)ceramide	GL-1a	β-Glucosidase
Krabbe's	Galactosyl(β1→1)ceramide	GL-1b	β-Galactosidase
Metachromatic Leukodystrophy	Galactosyl[3-sulfo]-(β1→1)ceramide	Sulfatide	Arylsulfatase A
Lactosyl-Ceramidosis	Galactosyl(β1→4)-glucosyl(β1→1)ceramide	GL-2a	Lactosylceramidase
Fabry's	Galactosyl(α1→4)-galactosyl(β1→1)ceramide	GL-2b	Unknown
	Galactosyl(α1→4)-galactosyl(β1→4)-glucosyl(β1→1)ceramide	GL-3	Ceramide Trihexosidase
Tay-Sach's	N-acetylgalactosaminyl-(β1→4)[N-acetylneuraminyl-(2→3)]galactosyl(β1→4)-glucosyl(β1→1)ceramide	G_{M2}	Hexosaminidase A
	N-acetylgalactosaminyl-(β1→4)galactosyl(β1→4)-glucosyl(β1→1)ceramide	Asialo G_{M2}	Hexosaminidases A and B
	N-acetylgalactosaminyl-(β1→3)galactosyl(α1→4)-galactosyl(β1→4)-glucosyl(β1→1)ceramide	Globoside	Hexosaminidases A and B

summarizes the nomenclature of the substrates and enzymes for each of the sphingolipidoses to facilitate the discussion of the individual enzymes.

β-GLYCOSIDASES

β-Galactosidase (Krabbe's Globoid Cell Leukodystrophy): Austin *et al.* reported that the activity of cerebroside-sulfatide sulfotransferase was deficient in the gray and white matter of brain and in the kidney of two patients with globoid cell leukodystrophy when compared to normal controls (60, 61). Cerebroside-sulfatide sulfotransferase is the enzyme which catalyzes the formation of sulfatide from cerebroside and active sulfate, phosphoadenosine phosphosulfate (62-64). Other laboratories originally were unable to confirm this finding (65, 66), but it now appears that cerebroside-sulfatide sulfotransferase is moderately depressed in globoid cell leukodystrophy although it is not the primary genetic defect (67).

A deficiency of galactocerebroside-β-galactosidase activity (pH optimum 4.5) has been demonstrated in the gray and white matter of brain, liver, kidney and spleen of Krabbe patients (67-69). Activity is also deficient in the peripheral leukocytes (70, 71), serum (70) and cultured fibroblasts (70) when measured with the natural lipid substrate [^{3}H]galactosylceramide.

It appeared that dogs of the Cairn and West Highland terrier family might serve as models for this disease, but their enzymes, at least in the serum, appear to be completely different from those of the human (72). In both the human and canine globoid cell leukodystrophy, there is no difference in the enzymatic activity of normals, heterozygotes, or affecteds when measured with the artificial substrate, 4-methylumbelliferyl-β-galactoside (72), thereby indicating that the enzyme is specific for the lipid substrate. The instability of this enzyme has prevented any further studies on its properties and has defeated all attempted isolations.

β-Galactosidase (Lactosylceramidosis): Dawson and Stein recently detected a previously unreported sphingolipidosis characterized by an elevation of GL-2a in erythrocytes, plasma, bone marrow, urinary sediment, liver biopsy, and brain biopsy (73). This paper also reported that lactosylceramide:galactosyl hydrolase activity (pH optimum 5.0) in the liver of this patient, measured with [^{3}H]GL-2a, was 15% of that of normal liver.

β-Glucosidase (Gaucher's Disease): In adult Gaucher's disease there is a deficiency of β-glucosidase activity which hydrolyzes GL-1a (glucocerebroside) in leukocytes (74,75), cultured skin fibroblasts (76,77), liver and spleen (78,79). These deficiencies have been detected using both natural and artificial substrates although there is some discrepancy in the results obtained with these substrates.

In the spleen the defect has been demonstrated by using glucocerebroside and p-nitrophenyl-β-glucoside as substrates (80,81). In the liver however, the two activities do not parallel each other since the p-nitrophenyl-β-glucosidase activity is increased while that of glucocerebrosidase is decreased (81). In contrast to these findings it was reported that liver homogenate from a patient with Gaucher's disease was inactive in the hydrolysis of either 4-methylumbelliferyl-β-glucoside or GL-1a (82).

Recent studies comparing the reactivity of β-glucosidase with natural and artificial substrates have proved that, at least in leukocytes, the enzyme specifically hydrolyzes only GL-1a (83). This conclusion was based on the results of leukocyte assays in two different patients. In one case an individual diagnosed as a Gaucher on the basis of histochemical studies was found to have normal levels of enzymatic activity when measured with the artificial substrate, but incubation of the leukocytes with [^{14}C]glucocerebroside revealed depressed β-glucosidase activity. In the second case, diagnosed as a Gaucher on the basis of decreased activity toward 4-methylumbelliferyl-β-glucoside, the patient was normal when diagnosed with radioactive lipid substrate. Kanfer and associates (83) summarized their conclusions on artificial substrate assays as follows:

> "The assay of β-glucosidase either as the 4-methylumbelliferyl- or p-nitrophenyl-glucoside derivative usually presents difficulties. All determinations were routinely carried out in triplicate in this laboratory. The problem has been that in 5 consecutive attempts to assay a sample, 300% variation can occur in the triplicates. The sixth attempt may be satisfactory..... It should be mentioned that one accurate diagnosis has been obtained with 4-methyl-umbelliferyl-β-glucoside."

Studies were recently reported by Ho *et al.* (78) on some properties of normal spleen β-glucosidase and reconstitution of β-glucosidase activity with two factors, one derived from normal spleen and one from Gaucher spleen.

Normal spleen β-glucosidase activity, measured with 4-methylumbelliferyl-β-glucoside at pH 4.0 to 4.3, was stimulated about 40% by the addition of 0.02% Triton X-100. Gel filtration on Sephadex G-50 separated the β-glucosidase activity into two proteins. One of these proteins, active at pH 4.0, showed 100% stimulation with Triton X-100, whereas the second protein, enzymatically active between pH 5.0 and 7.0, showed no appreciable detergent activation.

The β-glucosidases prepared in an identical manner from Gaucher spleen were also separated into acidic and neutral proteins which differed from the normal enzymes in both pH optima and a lack of stimulation by Triton X-100. The profile of enzymatic activity from the Sephadex column appeared to separate the acidic protein into two enzymes both having negligible activity toward 4-methylumbelliferyl-β-glucoside,

whereas the neutral glycosidase, reportedly uneffected in Gaucher's disease, appeared to have approximately 50% normal activity. Neither the quantity of protein nor the weight of the spleens from which these proteins were prepared was reported, so the apparent deviations from normal values may represent differences in protein quantity rather than in enzymatic activity.

When homogenates prepared from normal and Gaucher spleens were mixed there was an enhancement of β-glucosidase activity 2-3 times greater than expected from theoretical calculations. This "reconstitution" of β-glucosidase activity was attributed to two factors referred to as P, an acid glycoprotein derived from Gaucher spleen, and C, a low molecular weight, particulate, thermolabile substance derived from normal spleen.

"Purified" factor P (Gaucher), having no enzymatic activity, was mixed with a crude preparation of factor C (normal) which had negligible β-glucosidase activity. Following the incubation there was reconstitution of β-glucosidase activity. This phenomenon did not occur when factor P (Gaucher) was incubated with factor C (Gaucher) that was prepared in the same way as factor C from normal spleen.

During the incubation the disappearance of factor P (factors P and C are separated by centrifugation; factor C sediments) indicated that it was incorporated in some form into the defective Gaucher β-glucosidase causing

reconstitution of normal enzymatic activity. It was concluded that Gaucher's disease may involve the deficiency of factor C which is involved in the formation of acid β-glucosidase in association with another protein, factor P. The presence of factor P in normal spleen may indicate that a reaction between the two factors occurs under normal physiological conditions.

The reconstituted β-glucosidase hydrolyzed artificial substrates, but its activity against glucocerebroside has not been tested.

ARYLSULFATASES (METACHROMATIC LEUKODYSTROPHY)

Three forms of arylsulfatase, designated as A, B and C, have been reported (84). Only arylsulfatase A has been purified in substantial amounts, and it is the only one for which at least one natural substrate (cerebroside sulfate; sulfatide) has been identified (85). Arylsulfatase A in combination with a heat-stable complementary factor cleaves sulfate from sulfatide (86,87).

Arylsulfatase A has been partially purified from ox liver (88), ox brain (89), pig kidney (90), human brain (91,92), and human liver (93). Recently Breslow and Sloan purified arylsulfatase A 175-fold from human urine using an affinity column adsorbent with a substrate analogue, psychosine sulfate, as the ligand (94). This protein, which appeared as a single band on polyacrylamide electrophoresis, was assumed to have a monomeric molecular weight of 110,000 from gel filtration studies on Sephadex G-200. The amino

acid composition of the protein was reported with a large portion of the residues being glutamic acid, proline and glycine, while methionine was the only sulfur-containing amino acid present. There was no indication by these authors that arylsulfatase A is a glycoprotein, although earlier work by Goldstone *et al.* showed that neuraminidase treatment of arylsulfatase A resulted in a protein of reduced electrophoretic mobility which reacted as arylsulfatase B on biochemical analysis (95).

Antibody to human arylsulfatase A was prepared by injecting highly purified enzyme into rabbits, then rendering it monospecific by absorption with liver fractions which were isoelectrically focused next to the enzyme (93). This antibody increased the rate at which the normal enzyme hydrolyzed the artificial substrate (p-nitrocatechol sulfate) and stabilized it against heat inactivation.

Inactive human liver arylsulfatase A, indistinguishable from the normal enzyme by immunodiffusion and immunoelectrophoresis, combined with this antibody to form an enzymatically active protein (93). A similarly prepared goat antibody to human arylsulfatase A was found to inhibit enzymatic activity at high concentrations but not at low concentrations (96).

Many of the classical antibody experiments were performed on arylsulfatase A derived from ox (97) but this enzyme is substantially different from that of the human in

electrophoretic characteristics (93,98) and inhibition (99). Goat and rabbit antibodies to the human arylsulfatase A cross-react with monkey and dog but not with sheep, ox or mouse enzymes (96).

The possibility was raised that the arylsulfatase A deficiency in metachromatic leukodystrophy might be caused by a deficiency of a sialyltransferase (95). This was based on studies with rat sulfatases which suggested that sulfatase B might be sulfatase A minus sialic acid. However there is no cross-reaction between human sulfatases A and B (96). The question of whether sialic acid residues would account for this immunological difference has not been investigated.

THE HEXOSAMINIDASES (TAY-SACH'S DISEASE)

Tay-Sach's disease shows an accumulation of G_{M2} ganglioside (100,101) and its sialic acid-free component, asialo G_{M2} ganglioside (102,103), also commonly referred to as trihexosylceramide. In most sphingolipidoses the accumulation of a specific sphingolipid is caused by a deficiency of an enzyme for catabolism of the accumulated lipid. By analogy, Tay-Sach's disease should be caused by a deficiency of a catabolic enzyme. Early studies with extracts from calf brain proved that N-acetyl-β-hexosaminidase, possessing both N-acetyl-β-glucosaminidase and N-acetyl-β-galactosaminidase activity, hydrolyzed these gangliosides (34). Thus it might be assumed that

hexosaminidase is altered in cases of Tay-Sach's disease, however a complete deficiency of this enzyme is found in only one type of the disease which is characterized by the visceral storage of an additional lipid, globoside (104).

Hexosaminidase activity consists of two major components designated A and B (105) having isoelectric points of 5.0 and 7.3, respectively (106). Both enzymes A and B degrade asialo G_{M2} ganglioside and globoside whereas only component A degrades the main storage compound, G_{M2} ganglioside (107).

Investigation of the enzymatic activity in autopsied brain tissue led to the recent discovery of three variants of Tay-Sach's disease (108). Variant O is characterized by an absence of both hexosaminidases A and B (108). Variant B (classical Tay-Sach's disease) is characterized by an absence of hexosaminidase A (109,110). Variant AB is characterized by an enhancement of both hexosaminidases A and B in brain extracts (108).

Several investigators have demonstrated a deficiency of hexosaminidase A by starch gel electrophoresis (109), isoelectric focusing (108,110) and cellulose acetate electrophoresis (11). In addition the absence of enzymatic activity for hydrolysis of the N-acetylgalactosyaminyl moiety was demonstrated through the use of specifically labeled G_{M2} ganglioside (112). Hexosaminidase A activity was found to be completely deficient in cultured skin fibroblasts, peripheral leukocytes and serum of patients with Tay-Sach's

disease, as measured for N-acetyl-β-glucosaminidase activity (111). O'Brien *et al.* reported similar results using an enzyme assay based on the thermal lability of hexosaminidase A (113).

Sandhoff *et al.* (114) have reported the following characteristics for the hexosaminidases: 1) both hexosaminidases A and B have a similar pH dependency with optimal activity around pH 4.5; 2) both enzymes have a molecular weight of 130,000 as determined by gel filtration on Sephadex G-150; 3) the B form is stable for 30 minutes at 50° C in 1 M acetate buffer, pH 5.0, whereas the A form loses 60% of its activity under these conditions; 4) sodium chloride and crude sodium taurocholate activate the enzymes, while purified sodium taurocholate, Triton X-100 and Cutscum are not stimulatory; 5) the K_m's for hydrolysis of p-nitrophenyl-N-acetyl-β-glucosamine, p-nitrophenyl-N-acetyl-β-galactosamine and asialo G_{M2} ganglioside are 0.67 mM, 0.16 mM and 0.2 mM, respectively. These data were collected using 4000-fold purified hexosaminidase A and 2000-fold purified hexoaminidase B.

In contrast to these results, Brady reported that the K_m for the hexosaminidases, purified 6000-fold, was one order of magnitude higher for asialo G_{M2} ganglioside than for the artificial substrates (115).

Although hexosaminidase activity consists of two primary components, Sandhoff reported that both impure and purified

preparations of N-acetylhexosaminidase from pig kidney, calf brain, rat brain, and human brain could be resolved into at least four components by isoelectric focusing (106). This observation was supported by Young *et al.*, who resolved human brain hexosaminidase into multiple forms by chromatography on DEAE-cellulose (116).

There is evidence that hexosaminidase A from human spleen (105) and human kidney (117) is an acidic glycoprotein which owes much of its charge to sialic acid residues. The effect of neuraminidase on "purified" hexosaminidase A was to produce a number of forms, each showing a stepwise decrease in anodic mobility. If sufficient neuraminidase was added the conversion of Form A into Form B took place without the intermediate stages being detectable. The change in electrophoretic mobility was roughly the same at each successive step, and on this basis it would require at least 12 molecules of sialic acid to be removed to explain the transformation of hexosaminidase A into the basic form B (105). Similar results have been reported by Goldstone *et al.* (95).

Since no evidence has been found to indicate any significant difference in molecular weight of Forms A and B, Form A could be envisaged as a glycoprotein consisting of Form B with up to 12 short carbohydrate side chains, each terminating with a sialic acid residue.

Purification of β-N-Acetylglucosaminidases from Beef Spleen: Two enzymes designated A and B, each with N-acetylglucosaminidase and N-acetylgalactosaminidase activity, were purified from beef spleen extracts (118). Homogeneity of the enzymes was confirmed by gel electrophoresis and ultracentrifugation.

The authors reported these enzymes to have the following characteristics: 1) only enzyme A was found by DEAE-cellulose at pH 7.0; 2) the enzymes had identical weight-average and z-average molecular weights; 3) the z-average molecular weight was unchanged by addition of guanidine hydrochloride or dithiothreitol, but decreased when both were present, indicating more than one peptide chain in each enzyme; 4) amino acid compositions were similar, but more sialic acid and neutral carbohydrates were present in A than in B; 5) K_m values were identical but V_{max} was slightly greater for Form A; 6) changes in pH affected the K_m and V_{max} of both enzymes; 7) the two p-nitrophenyl substrates competed for the active sites on both enzymes; 8) stimulation by bovine serum albumin enhanced V_{max} but did not affect K_m; 9) both enzymes were inhibited by Ag^{+}, Hg^{+2}, Fe^{+2} and Fe^{+3} in a noncompetitive manner; 10) incubation of the enzymes with low concentrations of dithiothreitol reduced enzymatic activity; 11) N-ethylmaleimide, iodoacetamide and iodoacetate did not affect activity but p-chloromercuribenzoate was an inhibitor; and 12) EDTA did not cause a significant change in activity.

Sialidase Hydrolyzing G_{M2} Ganglioside: A particulate enzyme that catalyzes the hydrolysis of N-acetylneuraminic acid from G_{M2} ganglioside has been found in various tissues of the rat (119). Preparations of enzyme from the small intestine were used to delineate the following properties of this enzyme: 1) optimal enzymatic activity occurs at pH 5.0; 2) enzymatic activity is inhibited by sodium taurocholate, Triton X-100, p-chloromercuribenzenesulfonate and cations including Zn^{+2}, Ca^{+2}, Cu^{+2}, and Fe^{+3}; 3) the K_m for G_{M2} ganglioside was 0.53×10^{-4}M; and 5) the time course for the sialidase reaction proceeded with a lag phase which could not be removed by prior incubation of the enzyme in the absence of substrate or explained by a two-step sequence in which the N-acetylgalactosaminyl residue was cleaved prior to release of sialic acid.

Previously described ganglioside sialidases obtained from rat liver (37) and brain (36,120) were ineffective in catalyzing the hydrolysis of G_{M2} ganglioside. It now appears that enzymatic hydrolysis of this lipid could theoretically proceed via the removal of the N-acetylgalactosaminyl moiety or, alternatively, by the sialidase reaction. The lipid product of the alternative reaction would be G_{M3} ganglioside. Frohwein and Gatt (34) described an enzyme in calf brain which catalyzes this reaction.

The physiological significance of the two alternative pathways, the N-acetylhexosaminidase and sialidase reaction, for catabolism of G_{M2} ganglioside in Tay-Sach's disease have

been discussed by Brady (119) but there is not evidence that G_{M3} ganglioside accumulates in this sphingolipidosis.

α-GALACTOSIDASES

Digalactosylceramidase: Although digalactosylceramide (GL-2b) accumulates in Fabry's disease (49, 50) there has been no evidence that there is an existing α-galactosidase specific for its hydrolysis. There has also been no evidence that ceramide trihexosidase hydrolyzes GL-2b.

Ceramide Trihexosidase (Fabry's Disease): Brady *et al.* discovered ceramide trihexosidase in normal small intestinal mucosa and demonstrated its absence in tissue from Fabry patients (51), thereby showing that the accumulation of GL-3 in Fabry's disease is due to an enzyme deficiency. This work is supported by several recent reports demonstrating an absence of ceramide trihexosidase activity in Fabry leukocytes (52-54), cultured skin fibroblasts (55-57), amniotic fluid (55), kidney (32, 58), spleen (32), brain (32), and liver (32).

Relatively few reports concerning the characteristics of ceramide trihexosidase have been published. The enzyme, originally isolated from rat intestinal tissue by Brady *et al.*, occurred as a single protein having optimal activity at pH 5.0, as measured with [^{3}H]GL-3 (32). The addition of sodium cholate (2 mg/ml) enhanced enzymatic activity which occurred with a linear rate of hydrolysis. Half-maximal

velocity for the rat ceramide trihexosidase was 3.7×10^{-4} M. The same assay conditions were used by Brady to detect human ceramide trihexosidase activity (51). The rate of hydrolysis was nonlinear with the human enzyme but the addition of beef spleen extract (unknown composition) corrected the non-linearity.

More recent investigations, using 4-methylumbelliferyl-α-galactoside as substrate, have demonstrated that ceramide trihexosidase consists of two components (121,122). Beutler and Kuhl studied the α-galactosidase activity of fibroblasts and leukocytes from both normal and Fabry patients and found the normal α-galactosidase activity to be composed of two isozymes. A thermolabile, low K_m component, electrophoretically rapid at pH 7.0, was designated as α-galactosidase A and a second, smaller fraction designated α-galactosidase B, had a higher K_m, greater thermal stability and slower electrophoretic mobility. In Fabry's disease, only the B isozyme could be detected and it was indistinguishable from the normal B isozyme (121).

Crawhall and Banfalvi, also using 4-methylumbelliferyl-α-galactoside as substrate, concluded that in normal cultured skin fibroblasts there are two α-galactosidases (122). This conclusion was based on the fact that *myo*-inositol appeared to be a competitive inhibitor of normal but not of the residual activity (15-20% normal) in fibroblasts obtained from patients with Fabry's disease, thereby suggesting that

normal fibroblasts contain two α-galactosidases, only one of which is present in cells from patients with Fabry's disease.

The α-galactosidase activity in normal cell lines was rapidly inactivated at 51° over a period of 60 minutes. After longer periods of heating, the rate of heat inactivation was much slower and closely paralleled that found for the residual enzyme activity in the cell strains from patients with Fabry's disease.

Myo-inositol appeared to be a competitive inhibitor of the normal enzyme, whereas enzymatic activity present in cell extracts from patients with Fabry's disease was not inhibited by *myo*-inositol, but was mildly stimulated by its addition. The residual activity in Fabry cells had a K_m of 14-29 mM, while the K_m for normal enzymatic activity was 3-4 mM.

A third report on α-galactosidase activity purified 500-fold from placenta concerned the kinetic properties and enzymatic alterations in Fabry's disease (123). The following kinetic properties were reported: 1) hydrolysis of [^{3}H]GL-3 exhibited a sigmoidal substrate saturation curve and was competitively inhibited by lactosylceramide (GL-2a); 2) a mixed type of inhibition was observed in the presence of the synthetic substrate, 4-methylumbelliferyl-α-galactoside; and 3) digalactosylceramide (GL-2b) was stimulatory at low substrate concentrations and inhibitory at high substrate concentrations.

To explain these observations, the author proposed that this α-galactosidase is an enzyme with an effector site

accessible to glycolipids only and a catalytic site accessible to a wide variety of galactosides.

INTERACTION OF ENZYMES WITH LIPID SUBSTRATES

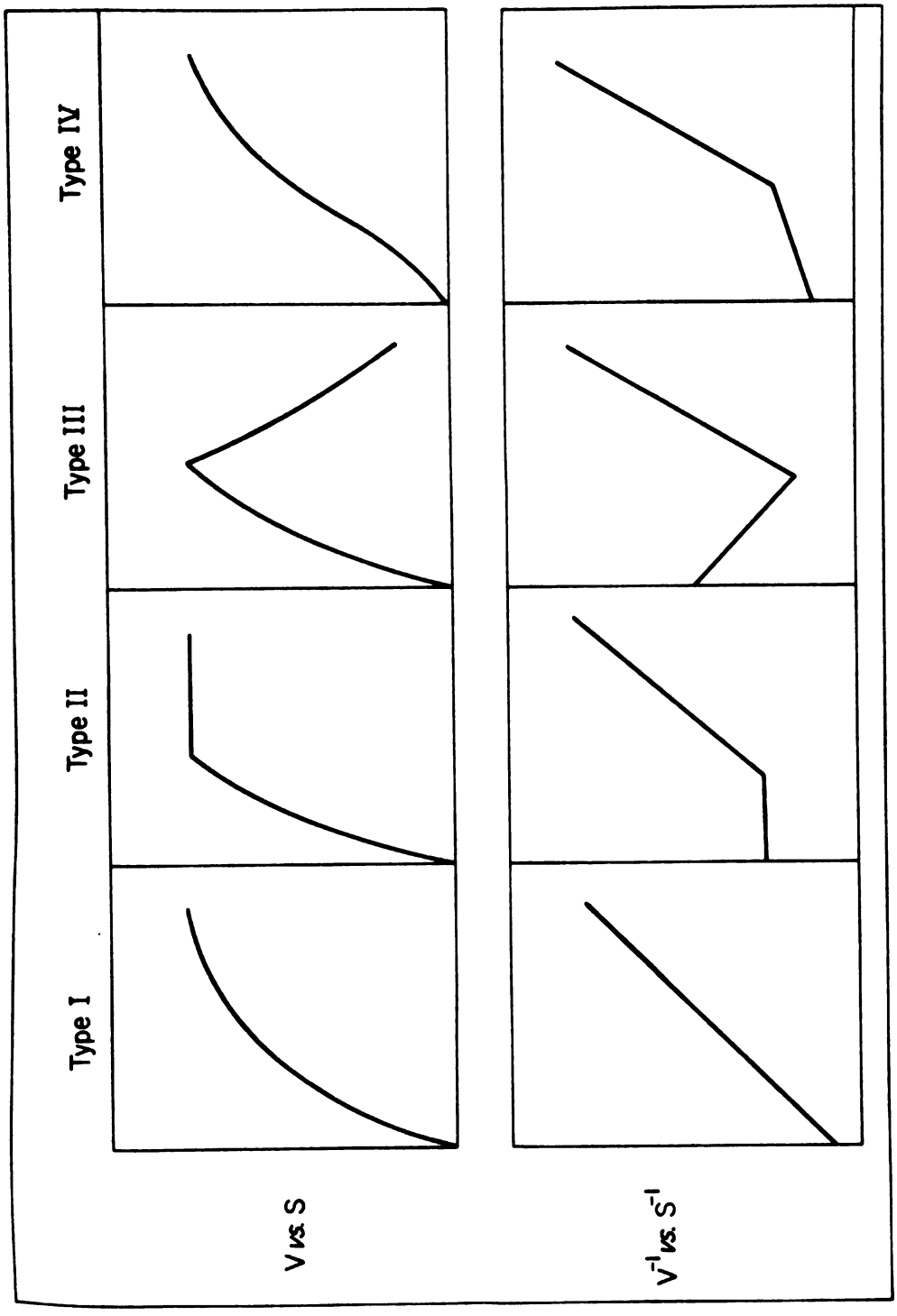
Information on the mechanism of action of the sphingolipid hydrolases is negligible. Most kinetic studies have been limited to determinations of K_m and V_{max}. Gatt has investigated the interaction of lipid enzymes with pure substrates and classified these interactions into four main types (124). Figure 2 shows the typical kinetic curves obtained for each of the four classifications.

Type I has a classical Michaelis-Menton type V/S curve. It is obtained in cases of enzymes acting on lipid substrates where, under the experimental conditions used, there is no monomer-micelle transition. This type of interaction occurs with many enzymes acting on lipid substrates solubilized in detergents. This type of curve may also be obtained when the critical micellar concentration (CMC) of the substrate is low enough that the assay procedure does not detect deviations from the hyperbolic shape below the CMC.

Type II has a V/S curve which is hyperbolic to a certain substrate concentration, then breaks and becomes parallel to the abscissa. Similarly, a presentation of the data in the form of a double reciprocal plot has a straight line parallel to the abscissa and a second straight line with a upward slope. This is the case where the enzyme utilizes monomeric but not micellar forms of the substrate. The curve becomes

Figure 2. Possible Kinetic Curves for Enzyme Interactions with Lipid Substrates

These figures were taken directly from Gatt's work (124) and are discussed in the text.


Type I
Type II
Type III
Type IV
V vs. S
V⁻¹ vs. S⁻¹

parallel since the concentration of monomer above the CMC is constant. This monomeric concentration remains constant and equals the CMC even when substrate molecules are removed by the action of the enzyme due to a rapid disaggregation of the micelles to maintain an equilibrium between monomeric and micellar forms. If the "activity" of one micelle equals that of a monomer, this type of interaction would be indistinguishable from Type I.

Type III has a V/S curve which is hyperbolic to a certain concentration, then breaks and shows a decreased rate of hydrolysis. In the double reciprocal plot the straight line breaks and is succeeded by a second straight line with an upward slope. This is the case where the enzyme utilizes monomers but probably not micelles. This type occurs when the micelles inhibit the enzyme or interfere with the action of the enzyme on the substrate monomer.

Type IV has a V/S curve which is sigmoidal although the sigmoid is usually not symmetrical. This is the case where the enzyme utilizes micelles but not monomer.

When considering these classifications two factors should be considered: 1) Below the CMC, values on the abscissa are correctly presented, since the concentration of the substrate available for interaction with the enzyme equals the number of monomeric molecules in the solution. 2) Above the CMC, where part of the lipid is present as

micelles rather than as monomers, the values on the X-axis, while equalling the total concentration, do not represent the true concentration of the substrate as related to its interaction with the enzyme.

MULTIPLICITY OF PROTEINS

The term isozyme, as loosely defined, refers to different molecular forms of an enzyme serving the same or a closely related function (125). This definition is general and connotes no specific type of structural relationship between the protein species which may be observed to have similar enzymatic activities. The methods by which isozymes may be generated have been classified into three convenient categories by Harris (126).

Multiple gene loci coding structurally distinct polypeptide chains of a protein. In one case nonidentical polypeptides may be associated in various members of a set of isozymes, so that the individual isozymes vary in the combination of polypeptides they possess. One example of this is the five lactic dehydrogenase isozymes in which the A and B polypeptides form a tetramer series (127). Alternatively, polypeptide products of different loci may separately form the various members of a set of isozymes. This appears to be the case with phosphoglucomutase, for which there are three distinct loci, at each of which multiple alleles occur, giving rise to a series of isozymes and extensive heterogeneity of the protein (128,129).

Multiple alleles at a single locus. Since heterozygotes carry two different alleles they may be expected to show a more complex pattern of isozymes than homozygotes. An example of this is again seen with phosphoglucomutase, in which individuals homozygous at all three loci show at least eight isozymes, whereas heterozygosity at all three loci can lead to as many as fifteen recognizable forms (130).

Secondary modifications of protein structure. The first two categories are further complicated by structural changes which modify the assembled protein. These may involve deamination of glutamine or asparagine, phosphorylation of serine residues, addition of carbohydrate groups, or removal of components from the protein by proteolytic enzymes. Structural variants of this nature have been observed with phosphoglucomutase (130), adenylate kinase (131), adenosine deaminase (129), and peptidase B (129).

Four compilations (132-135) of proteins which have been detected in multiple forms in man have been published. The primary proteins, which occur as multiple forms in leukocytes, erythrocytes or plasma, have been summarized in Table 2 using these primary sources. This table is far from complete and does not include many of the blood proteins and clotting factors in plasma which have been found to occur in more than one form.

When the Jacob-Monod model of regulation of gene activity in the *lac* system of *E. coli* was first proposed there

Table 2. Enzymes Having Multiple Forms in Human Blood

Protein	No. of Forms
Haptoglobin	
α-chain	10
β-chain	3
Transferrin	18
Pseudocholinesterase	5
Albumin	7
Glucose-6-phosphate dehydrogenase	25
Carbonic anhydrase	5
Erythrocyte esterase	2
Acid phosphatase	5
Catalase	3
6-Phosphate gluconate dehydrogenase	7
Myoglobin	2
Phosphoglucomutase	
PGM I	8
PGM II	5
PGM III	2
Fibrinogen	3
Adenylate kinase	4
Lactate dehydrogenase	
A subunit	5
B subunit	1
Amylase	4
α_1-Acid glycoprotein	2
β-Lipoprotein	2
Galactose-1-phosphate uridyl transferase	2
Malate dehydrogenase (soluble)	2
Malate dehydrogenase (mito.)	2
α_1 Antitrypsine	3
Glutathione reductase	2
Ceruloplasmin	3
Peptidase A	4
Peptidase B	4
Hypoxanthine-guanine phosphoribosyl transferase	6
Glutamic oxalacetic transaminase	2
NADH diaphorase	2
Phosphohexose isomerase	10

were several applications of this concept to human structural and control genes in an attempt to explain the multiplicity of observed proteins (136-139). However in most cases in which this hypothesis has been tested it has been found to be fallacious (132,140-144). Data on the primary structure of the human enzymes are not obtainable in most cases, thereby making it impossible to make a distinction between allelism and genes at different loci.

MATERIALS AND METHODS

MATERIALS

Sources of Enzymatic Activity

Human Plasma: Blood from voluntary donors was collected in either heparin or EDTA containing a sodium cation. The unrefrigerated blood was centrifuged at full speed in an IEC clinical centrifuge and the plasma was decanted and stored at 4°. Alternatively, out-dated plasma, containing acid-citrate-dextrose (ACD), was obtained from the American Red Cross (Lansing, Mich.).

Fabry plasmas, obtained from blood collected in either heparin or EDTA with a sodium cation, were the generous gifts of Dr. William Krivit, Dr. Michel Philippart and Dr. Matthew Spence.

Human Kidney: Human kidney, excised from a 15-year old male accident victim approximately 2 hr following death, was obtained through the courtesy of Dr. William Walker of the Pathology Department of St. Lawrence Hospital (Lansing, Mich.).

Human Urine: Urine was collected without refrigeration and concentrated 15-fold using an Amicon hollow fiber ultrafiltration system having a molecular weight cut-off of 50,000.

Cohn Fractions: Cohn fractions IV-1 and I were obtained through the courtesy of Dr. James Sgouris of the Michigan Department of Health (Lansing, Mich.). Alternatively ceramide trihexosidase (Form A) was purified from an acetone precipitate of Cohn fraction IV-1 prepared by the National Red Cross (Bethesda, Md.).

Reagents

The common reagents used were all of reagent-grade quality. The special reagents used are listed below.

Solvents

General Solvents	All solvents were redistilled by constant flow rotary evaporation.
Dry Methanol	Methanol was dried by distillation from magnesium turnings containing a catalytic amount of iodine and was stored over molecular sieves.
Dry Pyridine	Pyridine was dried by distillation from barium oxide after refluxing 4 hr and was maintained over Drierite.

Resins

Affinose 201, Affinose 202, and Bio•Gel A-5m	Bio•Rad Laboratories, Richmond, Ca.
Silicic Acid (Unisil)	Clarkson Chemical Co., Williamsport, Pa.
Sephadex G-10	Pharmacia, Uppsala, Sweden
Amberlite's CG-120, CG-400, and IR-120	Mallinckrodt, McGraw Park, Il.

Dowex 50W-X8	J. T. Baker, Phillipsburg, N.J.
Darco G-60	Fisher Scientific Co., Fair Lawn, N.J.
Celite	Johns-Manville, Denver, Co.

Chromatography Supplies

Silica Gel-G Plates	Quantum Industries, Fairfield, N.J.
Kieselguhr G	Macherey, Nagel and Co., Duren, Germany
OV-101 and SE-30	Applied Science Laboratories, Inc., State College, Pa.

Silylating Reagents

Bis(trimethylsilyl)-trifluoroacetamide	Regis Chemical Co., Chicago, Il.
Pyridine-Hexamethyl-disilazane-Trimethyl-chlorosilane	Prepared according to Sweeley *et al.* (145).

Electrophoretic Supplies

Ampholine Carrier Ampholytes	LKB, Rockville, Md.
Sepraphore III Polyacetate strips	Gelman Instrument Co., Ann Arbor, Mi.
High Resolution Buffer (Tris-barbital-sodium barbital)	Gelman Instrument Co., Ann Arbor, Mi.
Coomassie Blue, Ponceau S, Nitro Blue Tetrazolium, and Phenazine Methosulphate	Sigma Chemical Co., St. Louis, Mo.
Schiff's Reagent	Scientific Products (Harleco), Detroit, Mi.

Detergents

Triton X-100	Rohm & Haas Philadelphia, Pa.
Bile Salt Detergents	Sigma Chemical Co., St. Louis, Mo.

Enzymes

Galactose Dehydrogenase	Boehringer Mannheim, New York, N.Y.
Galactose Oxidase and Catalase	Worthington Biochemical Corp., Freehold, N.J.
Phospholipase A^2, Invertase, and Neuraminidase	Sigma Chemical Co., St. Louis, Mo.

Scintillation Fluid

DPO Toluene	Prepared by dissolving 50 mg POPOP and 4.0 gm PPO per liter of toluene. POPOP and PPO are supplied by Packard Instrument Company, Inc., Downers Grove, Il.
Aquasol	New England Nuclear, Boston, Ma.

Radioactive Chemicals

[1-^{14}C]Stearic Acid (54 mCi/mmole)	New England Nuclear Boston, Ma.
[^{14}C]UDP-N-Acetyl-glucosamine (40 mCi/mmole)	New England Nuclear Boston, Ma.
[^{14}C]CMP-Sialic Acid (1.3×10^6 dpm/μmole)	Dr. Saul Roseman, Johns-Hopkins University

Miscellaneous Chemicals

Phospholipids	Serdary Research Laboratories, London, Ontario
N-(p-Nitrophenyl)-oxamic Acid	K & K Laboratories, Jamaica, N.Y.
p-Nitrophenyl-α-galactoside	Pierce Chemical Co., Rockford, Il.
4-Methylumbelliferyl-α-galactoside	Koch-Light Laboratories Ltd., Colnbrook, England
1-Ethyl-3(3-dimethyl-aminopropyl)carbodiimide	Ott Chemical Co., Muskegon, Mi.
p-Nitrophenol, Sodium hydrosulfite, Sodium methylate, and Anthrone	Fisher Scientific Co., Pittsburgh, Pa.
Hydrogen Bromide	Matheson Gas Products, Chicago, Il.
Polyethylene Glycol 6000	Matheson, Coleman & Bell Detroit, Mi.
Melibiose, Stachyose, and Tyrosine	Sigma Chemical Co., St. Louis, Mo.

All other miscellaneous cofactors, enzymes and reagents were purchased from Sigma Chemical Co.

METHODS

Purification of Lipids

Isolation of GL-3 and GL-2b: GL-3 and GL-2b were isolated from formalin-fixed Fabry kidneys. Total lipids were extracted using a modification of the original Folch Procedure (146). The minced kidney tissue was homogenized with seven volumes of methanol (w/v) in a Sorvall Omni-Mixer at room temperature. Sufficient chloroform was added to make the solvent ratio of chloroform-methanol 2:1, after which the mixture was homogenized a second time. The cellular debris was removed by filtration using a Buchner funnel containing coarse-grade solvent-washed filter paper. The residue was extracted with 10 volumes (based on the original weight) of chloroform-methanol (2:1). A volume of 0.1 M KCl equivalent to one-fifth that of the final volume of the combined extracts was added. The solvents were mixed and allowed to stand at 4° until the two phases completely separated.

The washed lower phase from the Folch extraction was evaporated to dryness under reduced pressure and the residue was extracted with 200 ml of acetone. The precipitate was removed by filtration and washed with 200 ml of diethyl ether. The residue of crude glycolipids obtained from the acetone and ether extracts was subjected to mild alkaline hydrolysis according to the method of Vance and Sweeley (147). The residue obtained from mild alkaline hydrolysis was taken up in chloroform-methanol (19:1) and applied to a 250 gm silicic

acid column prepared in chloroform-methanol (19:1). The individual glycolipids were eluted stepwise with chloroform-methanol solutions consisting of 12%, 14%, 16%, 20%, 30%, and 50% methanol. Each solvent was applied until the Land's spot test was negative (148).

The volume of each fraction was reduced *in vacuo* by constant flow rotary evaporation and an aliquot was streaked on a pre-coated, non-heat activated silica gel G plate and chromatographed in chloroform-methanol-water (100:42:6). Fractions containing pure glycolipids were evaporated to dryness. The residue was dissolved in benzene-chloroform (2:1, v/v), frozen in liquid nitrogen and lyophilized into a white powder. The purity of the glycolipids obtained by this method was assessed by gas-liquid chromatography, following methanolysis, as described by Vance and Sweeley (147).

Isolation of GL-2a: Lactosylceramide was isolated from porcine erythrocyte stroma. The stroma were prepared from 20 liters of porcine blood, laked by addition of glacial acetic acid to a final concentration of 5%, as described by Yamakawa (149). The extraction and isolation of lipids from the stroma followed the procedure outlined above.

Preparation of Phospholipids: Commercially prepared phospholipids were tested for purity using the two dimensional thin-layer system of Rouser *et al.* (150). Phospholipid (20 µg) was applied to a non-heat-activated silica gel G plate and chromatographed in chloroform-methanol-28% aqueous

ammonia (65:25:5). The plate was air-dried for 10 min and chromatographed in chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5). A single spot was taken as an indication that the lipid was at least 95% pure and no further purification was performed. When several lipids were present in a sample, the desired phospholipid was purified from the mixture using silicic acid chromatography as outlined by Sweeley (151).

Chemical Modification of GL-3

Preparation of [^{3}H]GL-3: GL-3 (10 μmole) was suspended in 10 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.02 M sodium cholate. Galactose oxidase (2 mg) and catalase (1 mg) were added and the solution was incubated at 37° overnight. Sodium borotritiide (20 μmole; 140 μCi/μmole) was added slowly at room temperature and allowed to stand with occasional mixing for 8 hr. The mixture was evaporated to dryness under nitrogen and the residue was taken up in chloroform-methanol (19:1). The sample was applied to a silicic acid column and eluted with 12% methanol in chloroform followed by 25% methanol in chloroform. The residue obtained by evaporation of the 25% eluate was dissolved in chloroform-methanol (2:1, v/v) and continuously dialyzed against running tap water for 6 days to remove exchangeable hydrogen atoms.

Preparation of Galactosyl(α1→4)galactosyl(β1→4)-glucopyranose: Trisaccharide was obtained from GL-3 by a modification of the Wiegandt procedure (152). GL-3 (100 mg) was dissolved in 25 ml of dry methanol. Ozone, produced by a Welsbach ozonator, was introduced at room temperature until ozone consumption ceased, as determined by a positive reaction when a filter paper saturated with a KI-starch solution was introduced into the flask. The solution was evaporated to dryness under reduced pressure at 60°. The residue was dissolved in 10 ml of 0.2 M sodium carbonate and allowed to react for 12 hr at 20°. After neutralizing the solution with Dowex 50W-X8 (H^+), it was extracted three times with 10 ml portions of hexane to remove the fatty acids. The trisaccharide was obtained by lyophilization of the lower aqueous phase obtained from the hexane extractions.

Preparation of GL-2a by Chemical Degradation of GL-3: GL-2a was prepared by a modification of Taketomi's procedure for preparation of lysohematoside (153). GL-3 (100 mg) was added to 10 ml of 90% aqueous butanol containing a final concentration of 1 N KOH. The mixture was placed in a sealed tube and heated at 80° for 8 hr. After cooling to room temperature, the reaction mixture was dialyzed against constant running tap water for 2 days. The glycolipid mixture was evaporated to dryness under reduced pressure at 80°. The residue was dissolved in chloroform and applied to a silicic acid column prepared in chloroform.

Lactosylceramide was eluted with 12% methanol in chloroform.

Enzymatic Syntheses

Preparation of Galactosyl(α1→6)galactosyl(α1→6)-glucopyranose: Stachyose (2.5 gm) was dissolved in 100 ml of 0.01 M sodium acetate buffer, pH 4.8. Invertase (5 mg) was added and the mixture was incubated at 27° for 12 hr. The concentrated reaction mixture (10 ml) was applied to a column (5.3 i.d. x 45 cm) composed of a prewashed mixture of Darco G-60 and Celite (equal parts by weight). Monosaccharide, trisaccharide and unreacted stachyose were sequentially eluted from the column with water, 15% ethanol and 20% ethanol, respectively, as described by Whistler and Durso (154). Ethanol was removed from the 15% eluate by rotary evaporation and the trisaccharide was obtained from the water solution by lyophilization.

Preparation of Lysolecithin: Lysolecithin was prepared enzymatically by the action of phospholipase A^2, from *Crotalus adamanteus* venom, on lecithin as described by Wells and Hanahan (155). Lysolecithin was separated from unreacted lecithin by silicic acid chromatography.

Preparation of [^{14}C]GL-3

Preparation of Trihexosylsphingosine: [^{14}C]GL-3 was prepared by condensation of N-dichloroacetyl-3-O-benzoyl-sphingosine with acetobromotrisaccharide to form trihexosyl-sphingosine. ^{14}C-Labeled fatty acids were coupled to the

trihexosylsphingosine using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide.

Trisaccharide (50 mg), obtained by ozonolysis of GL-3, was added to 4 ml acetic anhydride-dry pyridine (1:1, v/v). The suspension was heated at 80° until the carbohydrate dissolved, then allowed to stand at room temperature for 12 hr. Dry methanol (5 ml) was added and the mixture was allowed to stand for 2 hr. The solution was evaporated to dryness under reduced pressure at 60° and the product was dried *in vacuo* over potassium hydroxide pellets.

The acetylated trisaccharide was brominated by a modification of the procedure outlined by Wolfrom and Thompson (156). The trisaccharide (35 mg) was dissolved in 10 ml of a freshly prepared solution of acetic anhydride containing 40% hydrogen bromide. The mixture was allowed to stand at room temperature for 20 min before extraction with two 10 ml portions of chloroform. The combined chloroform extracts were evaporated to dryness under reduced pressure at 30°. Final traces of acetic acid were removed by drying *in vacuo* over potassium hydroxide pellets.

N-Dichloroacetyl-3-O-benzoylsphingosine was prepared and coupled to the acetobromotrisaccharide (25 mg) using mercuric cyanide as outlined by Shapiro *et al.* (157). The protecting groups were removed by 0.5% sodium methoxide treatment followed by 5% barium hydroxide at 80° for 1 hr as described by Shapiro *et al.* (157). The reaction mixture

was dialyzed against constant running tap water overnight and the dialyzed solution was evaporated to dryness *in vacuo*.

Coupling of Trihexosylsphingosine and [^{14}C]Fatty Acids: Trihexosylsphingosine (5 mg), dissolved in 1 ml dichloromethane-methanol (1:1, v/v), was added to a solution containing 5.0 mg of [1-^{14}C]stearic acid (54 mCi/mmole) and 5 mg 1-ethyl-3(3-dimethylaminopropyl)carbodiimide dissolved in 1 ml dichloromethane-methanol (1:1). Acetonitrile (1 ml) was added and the mixture was incubated at 40° for 12 hr. The incubation mixture was evaporated to dryness *in vacuo* and the residue was dissolved in 5 ml of chloroform. The chloroform solution was washed successively with two 1 ml portions of 0.1 M sodium bicarbonate, 0.1 N HCl, and water, then dialyzed against constant running tap water for 8 hr. The [^{14}C]GL-3 was evaporated to dryness under reduced pressure and the residue was dissolved in water containing 0.03 M sodium taurocholate.

Preparation of α-Galactosidase Affinity Column Adsorbent

Melibiose Octaacetate: elibiose octaacetate was prepared and brominated by modification of the procedures described by Wolfrom and Thompson (156). Acetic anhydride (50 ml) and dry pyridine (50 ml) were added to 5 gm of melibiose. The suspension was heated at 80° until the carbohydrate dissolved, then allowed tc stand at room temperature for 12 hr. Dry methanol (100 ml) was added slowly

with stirring and allowed to stand for 3 hr. The solution was evaporated to dryness under reduced pressure at 60° and the product was dried *in vacuo* over potassium hydroxide pellets.

Acetobromomelibiose: Melibiose octaacetate (8.8 gm) was dissolved in 50 ml of a freshly prepared solution of acetic anhydride containing 40% hydrogen bromide. The mixture was allowed to stand 45 min at room temperature, then was filtered through a sintered glass funnel into 150 ml chloroform. The chloroform solution was washed twice with 70 ml portions of water, dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure at 30° until the chloroform was removed, then at 60° to remove acetic acid. Final traces of acetic acid were removed by drying *in vacuo* over potassium hydroxide pellets.

p-Nitrophenylacetomelibioside: Coupling of p-nitrophenol to acetobromomelibiose was based on the Koenigs-Knorr reaction for oligosaccharide synthesis (158). Acetobromomelibiose (4.8 gm) was dissolved in acetone (25 ml) and added to 2.0 gm p-nitrophenol dissolved in 25 ml 1.0 M sodium carbonate. The mixture stood at room temperature overnight and was evaporated to dryness under reduced pressure at 60°. The residue was taken up in chloroform (400 ml) and extracted with 100 ml portions of 0.25 M glycine-carbonate buffer, pH 9.5, until no yellow color was obtained in the aqueous phase. The chloroform layer was evaporated under reduced pressure at 30°.

Deacetylation: The product of the coupling reaction (4.2 gm) was suspended in 300 ml dry methanol containing 0.5% sodium methylate (w/v). Following gentle stirring at room temperature for 26 hr, the precipitated materials were removed by filtration and discarded. The supernate was neutralized by stirring with Amberlite IR-120 (H^+) and the neutralized solution was evaporated under reduced pressure at 60°.

Reduction of p-Nitrophenylmelibioside: p-Nitrophenylmelibioside (1.6 gm) was dissolved with sonication in 100 ml of distilled water containing 5% methanol. The pH was adjusted to 7.4 with 0.01 N sodium hydroxide after which sodium hydrosulfite (5 gm) was added with stirring. The solution was stirred for 10 min, desalted by passage through a column of Sephadex G-10, and evaporated to dryness under reduced pressure at 60°.

Coupling of p-Aminophenylmelibioside to Succinoylaminoalkyl-Agarose based on the Method of Cuatrecasas (159): Affinose 202 (10 ml packed bed) was washed with 20 bed volumes of 0.1 M sodium chloride, pH 6.0. p-Aminophenylmelibioside (1.6 gm), dissolved in 150 ml of 40% dimethylformamide, was added to the washed resin suspended in 50 ml of 40% dimethylformamide. After adjusting to pH 5.0 with 0.1 N HCl, a solution of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (1 gm) in 8 ml of water was added over a 10 min period. The reaction was allowed to proceed at room temperature for 24 hr

with occasional shaking. The product of the coupling reaction was packed into a column and washed successively with 40% dimethylformamide, water and 0.001 M MES buffer, pH 5.4, until UV-absorbing materials and carbohydrate [as measured by the anthrone reaction (160)] were absent from the eluate.

Thin-layer Chromatography of Carbohydrate Derivatives: Kieselguhr G plates of 250 μ thickness were prepared by the method of Lewis and Smith (161). The plates were heat-activated at 80° for 2 hr prior to use in either of the following solvent systems:

Solvent I: Benzene-ethyl acetate (1:1, v/v)

Solvent II: Butanol-pyridine-water (75:15:10, v/v)

Melibiose octaacetate, acetobromomelibiose and p-nitrophenyl-acetomelibioside were chromatographed in Solvent I using paper-lined tanks which were pre-equilibrated for a minimum of 6 hr. p-Nitrophenylmelibioside and p-aminophenylmelibioside were chromatographed in Solvent II using paper-lined tanks which were pre-equilibrated for a minimum of 12 hr.

Identification of Products by GLC: An aliquot (50 μl) of the solution obtained from Sephadex chromatography of p-aminophenylmelibioside was evaporated to dryness under nitrogen. The residue was silylated with 20 μl of bis(trimethylsilyl)trifluoroacetamide-dimethylformamide (1:1, v/v) and analyzed with a Perkin-Elmer 9000 gas chromatograph equipped with a 3 ft x 1/8 in i.d. column, packed with 0.05%

OV-101 on textured glass beads (Corning Glass). The oven temperature was increased linearly from 140° to 300° at 10°/min.

The residue from a second 50 µl aliquot was dissolved in 3 ml of 0.5 N methanolic HCl and heated at 80° for 24 hr. The methyl glycosides were silylated with 20 µl of pyridine-hexamethyldisilazane-trimethylchlorosilane (10:4:2) (145) and analyzed with a Hewlett-Packard 402 gas chromatograph equipped with a 6 ft x 1/8 in i.d., 3% SE-30 column, maintained at 165°.

Operating Conditions: A standard procedure was used for affinity chromatography of all samples. After equilibrating the column (0.6 cm i.d. x 6.5 cm) and sample to the optimal pH for a specific enzyme, the sample, not exceeding 100 ml in volume, was percolated through the column until it reached the top of the resin, then the flow was stopped for 30 min. The column was eluted with the buffer used for sample application until the non-adsorbed proteins were eluted (20-30 fractions depending upon the sample volume); then 0.1% Triton X-100 (v/v) was added to the eluting buffer. All fractions (1 ml) were eluted at 4° using the maximum flow rate of the column, kept constant by use of an LKB peristaltic pump.

Specific samples were prepared for affinity chromatography as follows. Urine and plasma were adjusted to pH 5.4, using 0.1 M citric acid, prior to chromatography. Partially purified ceramide trihexosidases, Form A, from

plasma and kidney were dialyzed against 0.001 M MES buffer, pH 5.4, containing 5% butanol. Partially purified ceramide trihexosidases, Form B, were dialyzed against 0.01 M sodium phosphate buffer, pH 7.2, containing 5% butanol. Ficin was dissolved in 0.05 M sodium acetate buffer, pH 4.5, and applied to the affinity column after it had been equilibrated with the same buffer. Proteins without α-galactosidase activity were dissolved in 0.001 M MES buffer and chromatographed in the same manner as the ceramide trihexosidases.

Preparation of Neuraminidase Affinity Column

Coupling of Tyrosine to Affinose 201: Affinose 201 (10 ml packed bed) was washed with 20 bed volumes of 0.1 M sodium chloride, pH 6.0. The washed resin was suspended in 200 ml of 40% dimethylformamide containing 30 mg of tyrosine. After adjusting the solution to pH 5.0, with 0.1 N HCl, 2 gm of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, dissolved in 10 ml of water, was added with gentle stirring over a 10 min period. The reaction was allowed to proceed at room temperature for 24 hr with occasional shaking. The product of the coupling reaction was packed into a column and washed with distilled water until uncoupled tyrosine was absent from the eluate, as determined by spectrophotometric measurements at 280 nm.

Reduction of N-(p-Nitrophenyl)oxamic acid: N-(p-Nitrophenyl)oxamic acid (5 mg) was dispersed in 100 ml of 5% methanol by sonication. After adjusting to pH 7.4 with 0.01 N sodium hydroxide, 5 gm of sodium hydrosulfite was added with rapid stirring. The solution was stirred for 10 min, acidified with 0.1 N HCl, filtered, and evaporated to dryness.

Coupling of p-Aminophenyloxamic Acid to Tyr-Succinoyl-aminoalkyl-Agarose as Described as Cuatrecasas and Illiano (162): p-Aminophenyloxamic acid (3 mg) was dissolved in 5 ml of cold 0.4 N HCl, and 30 mg of sodium nitrite, dissolved in 1 ml of water, was added with striring over a one-minute period. After stirring an additional 5 min, the mixture was added to the tyr-Affinose 201, suspended in 25 ml of 0.5 M sodium bicarbonate buffer, pH 8.9. The pH was readjusted to 8.8 and the reaction was allowed to proceed for 8 hr at room temperature with gentle stirring. The coupled resin was washed with 4 liters of 0.1 N sodium chloride.

Operating Conditions: Commercially prepared *Clostridium perfringens* neuraminidase, Type VI, was chromatographed essentially as described by Cuatrecasas and Illiano (162). Neuraminidase (30 mg) was dissolved in 7 ml of 0.05 M sodium acetate buffer, pH 5.5, and dialyzed for 12 hr at 4° against four liters of 0.05 M sodium acetate buffer, pH 5.5, containing 2 mM $CaCl_2$ and 0.2 mM EDTA. The dialyzed material was applied to the column (0.6 x 5 cm), equilibrated with the same buffer. The column was eluted with 0.1 M $NaHCO_3$, pH 9.1.

The eluted fractions (1 ml) were adjusted to pH 6.0 with 1 N NaOH and tested for neuraminidase activity by incubation with porcine submaxillary mucin.

Assays for α-Galactosidase Activity

Assay for Ceramide Trihexosidase Activity: Method 1 (Incubation of GL-3 with Crude Enzyme Preparations): Crude preparations include whole plasma, concentrated urine, and all samples obtained from steps prior to affinity chromatography. With these samples the incubation consisted of the following: 0.5 ml of crude enzyme preparation; 0.1 ml bovine serum albumin (5 mg); 0.2 μmole of GL-3 dissolved in 0.1 ml of water containing 0.96 mg sodium cholate; and buffer to a final volume of 1.0 ml. Assays for ceramide trihexosidase, Form A activity, were buffered with 0.2 M citrate-phosphate (sodium cation), pH 5.4, whereas those for ceramide trihexosidase, Form B activity, were buffered with 0.2 M sodium phosphate buffer, pH 7.2. Incubations without GL-3 served as controls.

The mixture was incubated for 4 hr at 23°, and the reaction was then terminated by addition of 2 ml of chloroform-methanol (2:1, v/v). The solution was vortexed and centrifuged at full speed in an IEC clinical centrifuge to separate the two phases. The upper phase was removed and clarified by boiling for 10 min, cooling in an ice bucket and recentrifuging. Enzymatic activity was detected by assaying the upper phase for liberated galactose.

Method 2 (Incubation of GL-3 with Purified Enzyme Preparations): The incubation consisted of the following: 1.68×10^{-7} M ceramide trihexosidase Form A-1; 0.2 μmole GL-3 dissolved in 0.1 ml of water containing 0.96 mg sodium cholate; 0.03 M sodium taurocholate (0.04 M sodium taurocholate if assaying ceramide trihexosidases, Form A-2); 0.15 M sodium chloride; and either citrate-phosphate or sodium phosphate buffer to a total volume of 0.5 ml. Assays with boiled ceramide trihexosidase served as controls. After 4 hr incubation at 23° the reaction was terminated by addition of 1 ml of chloroform-methanol (2:1). The upper phase was separated and clarified as described in Method 1.

Method 3 (Incubation of Purified Ceramide Trihexosidase with Radioactive Substrate): The incubation consisted of the following: 1.68×10^{-7} M ceramide trihexosidase Form A-1; 0.2 μmole unlabeled GL-3 dissolved in 0.1 ml of water containing 0.96 mg sodium cholate; 1.4×10^{-4} μmole of [^{14}C]GL-3 (10,000 cpm) or 2.7×10^{-3} μmole [^{3}H]GL-3 (22,000 cpm); 0.03 M sodium taurocholate (0.04 M for ceramide trihexosidase, Form A-2); 0.15 M sodium chloride; and either citrate-phosphate or sodium phosphate buffer to a total volume of 0.5 ml. Assays with boiled ceramide trihexosidase served as controls. After 1-4 hr incubation at 23° the reaction was terminated as described in Method 2. Radioactivity in [^{14}C]lactosylceramide or [^{3}H]galactose was determined as described in the following section.

Method 4 (Assay in Butanol): A solution of ceramide trihexosidase, Form A-1, (1 volume) was mixed with one volume of butanol. After thorough mixing, the butanol phase was decanted and the aqueous phase was washed with 1 volume of butanol. The butanol extracts were combined and an aliquot (1 ml) was removed for determination of enzymatic activity.

Incubations contained 1.4×10^{-4} μmole of [^{14}C]GL-3, 0.2 μmole GL-3, 0.03 M sodium taurocholate, and 0.1 N NaOH to pH 5.4, in a total volume of 1.5 ml. Assays with boiled ceramide trihexosidase served as controls. The mixture was incubated 4 hr at 23° then terminated by boiling for 30 min. After centrifugation the reaction mixture was evaporated to dryness. The residue was taken up in chloroform-methanol (2:1) and dialyzed overnight against constant running tap water. [^{14}C]GL-2a was determined as described in the following section.

Method 5 (Incubation of Trisaccharide with Ceramide Trihexosidase): The incubation consisted of the following: 1.68×10^{-7} M ceramide trihexosidase; 0.2 μmole trisaccharide [galactosyl(α1→4)galactosyl(β1→4)glucose]; 4 mg lecithin; 5 mg bovine serum albumin; and citrate-phosphate buffer, pH 5.4, to a final volume of 0.5 ml. After 2 hr incubation at 23° the reaction was terminated by addition of 1 ml of chloroform-methanol (2:1). The upper phase was separated and clarified as described in Method 1.

Methods for Detecting the Hydrolysis Products: [Spectrophotometric Quantitation of Liberated Galactose]: Galactose liberated from unlabeled GL-3 was determined with an end-point assay consisting of 77 µl of 0.1 M tris buffer, pH 8.6; 20 µl NAD^+, (10 mg/ml) Sigma Grade V; 100 µl of upper phase; and 3 µl of galactose dehydrogenase added in 1 M $(NH_4)_2SO_4$; in a final volume of 0.2 ml. The increase in absorbance at 340 nm was measured with a Gilford 2400 Model recording spectrophotometer thermostated at 30°. An absorbance change of 0.01 was equivalent to 0.32 nmoles of galactose. From the observed absorbance change, corrected for the control without added GL-3, galactose in the entire upper phase from the incubation was calculated.

Quantitation of Liberated Galactose by Gas-Liquid Chromatography: Alternatively, liberated galactose was quantitated by gas-liquid chromatography. The upper phase obtained from the enzyme incubation (Method 1) was passed through a column (0.5 cm i.d. x 4 cm) of mixed bed resin composed of equal quantities of Amberlites CG-120 (H^+) and CG-400 (OH^-). Sugars were eluted from the column with 5 ml of methanol-water (1:1). The eluates were evaporated to dryness and the residue was dissolved in approximately 100 µl of methanol-water (9:1). These solutions, alternated with standard galactose solutions, were applied to Whatman No. 1 chromatography paper. The carbohydrates were separated by chromatography in isopropanol-acetic acid-water (3:1:1)

(163). The sections spotted with standard galactose were removed from the chromatogram and visualized with aniline-acid-oxalate (163). The areas adjacent to the standard galactose were eluted from the chromatogram in the following manner: the paper was placed in 2 ml of methanol-water (1:1) and allowed to stand at room temperature for 1 hr, after which the solution was heated to boiling, cooled, and the paper removed and rinsed with 1 ml of methanol-water (1:1).

Mannitol (2.8×10^{-3} - 1.12×10^{-2} μmole, based on the quantity of residue obtained from the column eluates) was added to these solutions as an internal standard. The samples were evaporated to dryness and 5 μl of bis(trimethylsilyl)trifluoroacetamide-dimethylformamide (1:1, v/v) was added to the residue. After standing for one-half hour at 80° the samples were analyzed with a Hewlett-Packard 402 gas chromatograph equipped with a 6 ft x 1/8 in i.d. column packed with 3% SE-30 and maintained at 170°.

The yield of galactose (in nmoles) was calculated from the GLC data by the method of Vance and Sweeley (147), using a correction factor of 1.2 to account for the differences in the relative molar response of the detector to TMSi mannitol and TMSi galactose. The data obtained from a method control and the enzyme incubation controls were used to correct this value.

Detection of [6-^{3}H]Galactose: The reaction was terminated with chloroform-methanol and separated into two phases as previously described. After removing the upper phase, the lower phase was washed twice with 1 ml portions of theoretical upper phase. The combined upper phases were washed once with 1 ml of lower phase. The upper phases were desalted as described in the preceding section and reduced to a constant volume of 0.5 ml. An aliquot of this solution (100 µl) was spotted on Whatman #540 filter paper (2.1 cm circle), the paper was dried, and placed in 10 ml of DPO toluene. Alternatively the entire 0.5 ml sample was added to 15 ml of Aquasol for counting. Radioactivity in these samples was monitored in a Beckman LS-150 liquid scintillation counter. Controls with boiled ceramide trihexosidase were treated in an identical manner.

Detection of Liberated [^{14}C]GL-2a: The incubation was terminated by vortexing with 1 ml of chloroform-methanol (2:1). After separating the two phases by centrifugation, the upper phase was washed twice with 1 ml of chloroform. The combined lower phases were evaporated under nitrogen, the residue was dissolved in approximately 0.2 ml of chloroform-methanol (2:1), and the solutions were transferred to pre-coated silica gel G plates. The glycolipids were separated by chromatography in chloroform-methanol-water (100:42:6). Chromatography tanks were paper-lined and equilibrated for a minimum of 8 hr prior to use. Tanks were

used to develop two plates simultaneously and discarded. The area of [^{14}C]GL-2a corresponding to standard lactosyl-ceramide was scraped directly into 10 ml of DPO toluene and counted in a Beckman LS-150 liquid scintillation counter. Controls containing boiled ceramide trihexosidase were treated in an identical manner.

Assay for Digalactosylceramide : Galactosyl Hydrolase: The standard reaction mixture for the assay of digalactosyl-ceramidase (in the dialyzed acetone precipitate obtained from Cohn fraction IV-1) consisted of 0.5 ml of enzyme solution, 0.2 µmole of GL-2b dissolved in 0.5 mg aqueous sodium cholate, 0.03 M sodium taurocholate, 0.15 M sodium chloride and citrate-phosphate buffer, pH 5.5, in a final volume of 1.0 ml. Following partial purification by affinity chromatography or isoelectric focusing, 0.2 ml of enzyme solution was incubated as above in a total volume of 0.5 ml. Incubations containing boiled enzyme served as controls.

The reaction was terminated with chloroform-methanol (2x the volume of the incubation) and the two phases were separated as previously described. Liberated galactose was determined spectrophotometrically, using galactose dehydrogenase, as described in the preceding section (Method 1).

Assays for Non-specific α-Galactosidases

4-Methylumbelliferyl-α-galactoside as Substrate: The standard reaction mixture contained 100 µl of enzyme solution, 2.2 µmoles of substrate and 0.1 M citrate-phosphate buffer,

pH 4.5, to a constant volume of 0.5 ml. After incubation at 37° for 2 hr the reaction was terminated by addition of 2.5 ml of 0.1 M ethylenediamine buffer, pH 11.2. The mixture was centrifuged and the fluorescence of the liberated methylumbelliferone was measured using a Turner fluorimeter with 365 nm excitation and 450 nm fluorescence filters. Boiled enzyme controls were used.

p-Nitrophenyl-α-Galactoside as Substrate: Enzyme solution (100 µl) was added to 0.5 ml of 0.1 M citrate-phosphate buffer, pH 3.0, containing 2.0 µmoles of substrate. After 2 hrs at 37° the reaction was terminated by addition of 0.1 ml of 0.01 N sodium hydroxide and 0.5 ml of 0.2 M glycine-carbonate buffer, pH 9.5. The absorbance was read at 420 nm using a Gilford 2400 Model spectrophotometer. Boiled enzyme controls were used.

Plasma Infusions

Plasma for infusion was obtained by plasmapheresis of freshly drawn, heparinized blood from cross-matched normal donors whose previously assayed plasma had normal concentrations of ceramide trihexosidase activity (Method 1). A 17-year old hemizygote received 550 ml of plasma (2145 units)* over a 30 min period. A 31-year old hemizygote received 600 ml of plasma (4680 units)* over a 30 min period.

*A unit of ceramide trihexosidase activity is defined as the amount that liberates 1 nanomole of galactose per hour at pH 7.2 and 23°C.

In a third experiment blood was drawn from two normal donors and processed through normal blood bank procedure at the American Red Cross. The heparinized plasma (540 ml containing 2584 units)* was administered to a 30-year old hemizygote over a 1 hr period.

At two hour intervals post-infusion blood was obtained by venepuncture or through the aid of a heparin lock for enzymatic and/or substrate determinations.

Isolation of Ceramide Trihexosidases

Purification of Human Plasma Ceramide Trihexosidases, Form A Extraction: The enzymes were isolated from Cohn fraction IV-1, prepared by Method 6 of the low temperature ethanol precipitation described by Cohn *et al.* (164). Enzymatic activity was preferentially extracted from Cohn fraction IV-1 by dispersing the frozen protein paste (200 gm) in 600 ml 0.001 M MES buffer, pH 5.4, using a Waring blendor at low speed. After removal of the undissolved material by centrifugation at 16,000 g for 30 min at 4°, the supernatant solution was adjusted to pH 7.0 with 0.01 N sodium hydroxide.

Ammonium Sulfate Treatment: To the 750 ml of buffered supernate, maintained at 4°, 487 gm of ammonium sulfate (0 to 80% saturation) was added with stirring over a period

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*A unit of ceramide trihexosidase activity is defined as the amount that liberates 1 nanomole of galactose per hour at pH 7.2 and 23°C.

of 1.5 to 2 hr. After 30 min of additional stirring, the suspension was centrifuged for 30 min at 16,000 g and the precipitate was discarded.

Butanol Treatment and Acetone Precipitation: To 990 ml of the 80% ammonium sulfate supernate, maintained at 4°, 52 ml of n-butanol was added slowly with stirring over a 1 hr period as described by Morton (165). The 5% butanol solution was stirred for an additional hour at 4° then transported to a -20° room. Following the method of Askonas (166), 1,563 ml of acetone, chilled to -20°, was added slowly with stirring so as to make the final concentration 60% acetone. The protein-ammonium sulfate precipitate was removed immediately from the acetone solution by suction filtration. The precipitate was slurried with about 30 ml of 0.001 M MES buffer, pH 5.4, containing 5% butanol and dialyzed against 100 volumes of the same solution for 8 to 12 hours.

Affinity Chromatography: Following dialysis, the purification was completed by affinity chromatography as previously described.

Pilot-Scale Isolation of Ceramide Trihexosidases, Form A: The following protocol was submitted to the National Red Cross for preparation of ceramide trihexosidases, Form A:

Dissolving the material (pH 7.0; temperature should not exceed 4°): Cohn fraction IV-1 (10 kg) is dissolved in 9 volumes (90 l) of 0.25 M sodium phosphate buffer, pH 7.0. Any undissolved material should be removed by centrifugation at 700 g or above.

Ammonium sulfate treatment (pH 7.0; temperature should not exceed 4°): The supernate is adjusted to 80% saturation with solid ammonium sulfate (50.6 kg or 111 lbs). Precipitated proteins are removed by centrifugation (10,400 g) and discarded.

Butanol treatment (pH is unadjusted; temperature should not exceed 4°): Butanol (5 l) is added to the 80% ammonium sulfate supernate with stirring. It is desirable to stir the solution for at least one hour following the addition of butanol.

Acetone precipitation (pH is unadjusted; temperature is -20°): Acetone (158 l) is added with stirring until the solution contains 60% acetone by volume. The resulting precipitate, consisting of a protein-ammonium sulfate sludge, is removed by either filtration or centrifugation immediately following the addition of acetone. The precipitate is stored at 0° or below.

Isolation of Ceramide Trihexosidase, Form B

Extraction: Enzymatic activity was extracted from Cohn fraction I, prepared by Method 6 of the low temperature ethanol precipitation described by Cohn (164). The enzymes were extracted from frozen Cohn fraction I by slowly stirring the protein paste in six volumes (w/v) of 0.01 M sodium phosphate buffer, pH 7.2, at room temperature for 30 minutes. The undissolved fibrinogen was removed by centrifugation at 16,000 g at 4° for 30 minutes. Removal of contaminating proteins by ammonium sulfate precipitation, butanol treatment, acetone precipitation, and affinity chromatography were performed as previously described for Forms A. The separation of the multiple forms of ceramide trihexosidase, Form B, was completed by isoelectric focusing.

Isolation of Ceramide Trihexosidase from Human Kidney

Extraction: The kidney of a 15-year old male accident victim was excised approximately two hours following death. The outer capsule was removed and the tissue was minced by passage through a meat grinder. The minced tissue (58.7 gm) was suspended in 150 ml of 0.25 M sucrose. Homogenization was performed in a Waring blendor for six 30-sec periods at full speed, with intermittent cooling in an ice bath for 3-5 minutes. The homogenate was centrifuged at 600 g for 30 min at 4° and the precipitate discarded. The supernate was poured through cheesecloth to remove floating fat and centrifuged at 16,000 g for 30 minutes.

Release of Enzymatic Activity from the Particulate Fraction: The crude lysosomal-mitochondrial pellet (16,000 g precipitate) was suspended in 100 ml water (one-half the original volume of supernate) and treated with sodium cholate (10 mg/ml) overnight, as described by Brady *et al.* (32). The sodium cholate-treated solution was then centrifuged at 100,000 g for 90 min and the precipitate discarded.

Concentration of the Enzymatic Activity: To the 110 ml of 100,000 g supernate, 56.8 gm of ammonium sulfate (0 to 80% saturation) was added with stirring over a 1 hr period. Stirring was continued for an additional 30 min and the precipitated proteins were removed by centrifugation at 16,000 g for 30 min and discarded. To the 136 ml of 80% ammonium sulfate supernate, maintained at 4°, 7 ml of butanol was added slowly with stirring over a 1 hr period as described by Morton (165). Stirring was continued at 4° overnight. Ceramide trihexosidase activity was precipitated from the butanol solution by addition of acetone to a final concentration of 60% as described for the plasma enzymes. The ammonium sulfate-protein precipitate was dialyzed and chromatographed on an affinity column in the manner described for the plasma ceramide trihexosidases, Form A.

Miscellaneous Assays

Protein Determinations: Protein was determined during enzyme purifications by the method of Lowry *et al.* (167) as modified by Hess and Lewin (168). For affinity column profiles protein was measured with commercial Biuret reagent as follows: protein solution (0.1 ml) and 0.2 ml of Biuret reagent were mixed and allowed to stand at room temperature for 30 min. The absorbance was read at 540 nm using a Gilford 2400 Model spectrophotometer. Bovine serum albumin was used to prepare standard curves.

Sialic Acid Determinations: Free sialic acid was determined by the periodate-resorcinol method of Jourdian *et al.* (169). N-Acetylneuraminic acid was used to prepare a standard curve.

Molecular Weight Determinations

Sucrose Density Gradient Centrifugation: Sucrose density gradient studies were conducted by the method of Martin and Ames (170), using a Beckman SW 50 rotor at 50,000 rpm at 0° for 14 hr. Linear gradients from 5-20% sucrose without added preservatives were employed. The protein (8 µg) was applied to the gradient in 0.2 ml of 0.001 M MES buffer. Fractions (0.15 ml) were collected and assayed for ceramide trihexosidase activity, while the positions of the marker enzymes, cytochrome c and hemoglobin, were determined by spectrophotometric measurements at 550 nm.

Gel Chromatography: The ceramide trihexosidases were individually chromatographed on a Bio-Gel A-5m column (3 x 95 cm) along with cytochrome c, β-lactoglobulin, leucine aminopeptidase, hemoglobin, and glyceraldehyde-3-phosphate dehydrogenase (1 mg each) as standards (171). The column was equilibrated with 0.01 M MES buffer, pH 5.4, and the proteins were applied and eluted with the same buffer. Eluted proteins were detected by spectrophotometric measurements at 280 nm.

The same series of proteins was chromatographed on another column of Bio-Gel A-5m (3 x 95 cm) equilibrated with 0.01 M tris-chloride, 1% SDS, pH 8.0 and 2% 2-mercaptoethanol (172). The proteins were dissolved in 5 ml of this solvent, sonicated briefly, and heated at 80° for 30 min. The proteins (1 mg each) were applied to the column and eluted with the same buffer. Eluted proteins were detected in the following manner (168): an aliquot (4 ml) of each fraction was mixed with 4 ml of 5% TCA and allowed to stand for 15 min at 0° when centrifuged. The aqueous solution was removed and 0.2 ml of 0.5 N NaOH was added to the sample. This sample was mixed with 0.4 ml of commercial Biuret reagent and protein was determined as previously described.

Electrophoretic Methods

Isoelectric Focusing: Isoelectric Focusing was performed essentially as described by Vesterberg and Svensson (173). The sucrose density gradient, with 50% sucrose (w/v) as the

densest solution, was prepared and layered into a 110 ml LKB electrolysis column, as suggested by the manufacturer. The column was maintained at 1° with a thermostated Lauda K-2R water bath. Wide pH ranges were equilibrated for 48 hr with a maximum potential of 600 V, whereas narrow pH ranges were equilibrated for 72 hr with a maximum potential of 700 V. After focusing of the carrier ampholytes, fractions (0.5 or 2.0 ml) were eluted from the column keeping the flow rate constant with a peristaltic pump. The pH of the individual fractions was determined using a Corning Model 12 pH meter with the electrode equilibrated at 20°. The fractions were assayed for enzymatic activity and/or protein without removal of the ampholytes.

Polyacrylamide Electrophoresis: Polyacrylamide electrophoresis in 9% gels was performed by the method of Fairbanks *et al.* (174). The gels were scanned at 280 nm, then stained with Coomassie Blue. Following destaining with 10% TCA-33% methanol by the method of Johnson (175) the gels were scanned at 550 nm.

Cellulose Acetate Electrophoresis: Protein solution (2 µl containing 1-4 µg protein) was streaked onto a buffer-dampened Sepraphore III polyacetate strip. Electrophoresis was carried out in 0.05 M tris barbital-sodium barbital buffer, pH 8.8, with a current of 0.5 mA/cm strip width for 30-40 min.

Staining of Activity: The cellulose strip was overlaid with a filter paper saturated with a solution containing 4 x 10^{-4} M unlabeled substrate (GL-3 or GL-2b), 0.03 M sodium taurocholate and 0.15 M sodium chloride dissolved in either 0.001 M MES buffer, pH 5.4, or 0.01 M sodium phosphate buffer, pH 7.2. This reaction was kept moist for 4 hr at 37°. When Form A and Form B proteins were on the same strip, it was stained for 2 hr at pH 7.2 and for 2 hr at pH 5.4. The strip was then sprayed three times, at five minute intervals, with a fine mist of a solution containing 20 µl of galactose dehydrogenase and 5 mg NAD^+ in 1.0 ml tris buffer, pH 8.6. The strip was then overlaid with a filter paper saturated with tris buffer, pH 9.2, containing 6 x 10^{-5} M Nitro Blue Tetrazolium and 3.3 x 10^{-5} M phenazine methosulphate. Staining was carried out at 37° for 1 hr in the dark.

Staining of Glycoproteins: The cellulose strips were immersed in 5% TCA for 2-3 min to precipitate the proteins. They were then rinsed in tap water and transferred to Muller's colloidal iron oxide reagent (176) for 60 min, washed in 3% acetic acid until excess ferric chloride was removed, as determined by an absence of color upon addition of potassium ferrocyanide, then immersed in a 2% solution of potassium ferrocyanide in 2% HCl for 30 min. The strip was washed with distilled water and transferred to 1% periodate for 30 min, then to commercial Schiff's reagent for 30-60 min, following thorough rinsing with distilled

water. Following the development of reddish-purple bands, the strips were washed in three changes of 0.5% sodium metabisulfite (5 min each) and dried.

Ponceau S Staining for Quantitation of Proteins: The cellulose strips were stained with 0.2% Ponceau S made in 3% aqueous trichloroacetic acid for 20-30 min then destained in 5% acetic acid. The strips were sectioned between protein bands and the individual segments were added to 0.1 N NaOH (0.5-1.0 ml depending upon the intensity of the stain) and the dye was eluted by vortexing for about 2 min. The absorbance at 565 nm, read against a blank prepared from an unstained section of the strip, was measured with a Gilford 2400 spectrophotometer. The protein level was determined from the absorbance by comparison with a standard curve obtained by electrophoresis, staining and elution of the dye from 1-10 μg of albumin.

Interconversion of Ceramide Trihexosidases, Forms A and B

Neuraminidase Treatment of Partially Purified Ceramide Trihexosidases, Form A: An extract (40 ml) of Cohn fraction IV-1, containing 7 mg of protein per ml, was made in 0.5 M citrate-phosphate buffer, pH 5.4. Commercially prepared neuraminidase (14 mg) was added and the mixture was incubated for 3 hr at 37^{0}. Following incubation, samples (1 ml) were assayed for ceramide trihexosidase activity over the pH range 4.6 to 8.0

In a second experiment whole plasma (35 ml) was adjusted to pH 5.4 with 0.5 M citrate-phosphate buffer, pH 3.0. The diluted plasma, containing 70 mg of protein per ml, was mixed with 125 mg of neuraminidase and incubated at 23° for 4 hr. At one-hour intervals samples (1 ml) were assayed for enzymatic activity at pH 5.4 and 7.2, and separate samples (0.5 ml) were used to determine sialic acid liberated. Controls, in which neuraminidase was omitted, were treated under identical conditions.

Treatment of Purified Plasma Ceramide Trihexosidase (Form A-1) with Neuraminidase: Purified plasma ceramide trihexosidase, Form A-1, 300 µg in 4 ml of 0.001 M MES buffer, pH 5.4, was added to 16 ml of 0.2 M citrate-phosphate buffer, pH 5.4. Purified *Cl. perfringens* neuraminidase, Type VI (50 units)* was added and the mixture was incubated in a Dubnoff shaking incubator at 37°. Aliquots (5 ml) were removed after 1 and 4 hr and were concentrated by dialysis against polyethylene glycol 6000 in preparation for cellulose acetate electrophoresis. After 2 hr incubation 10 ml was removed and dialyzed against 0.001 M MES buffer, then concentrated by dialysis against polyethylene glycol 6000. This sample was used for isoelectric focusing in the pH range 3-10. Two controls were run, one in which the

* A unit of neuraminidase is defined as that quantity which will liberate 1.0 µmole of N-acetylneuraminic acid per minute at pH 5.4 and 37° using porcine submaxillary mucin as substrate.

neuraminidase was omitted from the incubation and one in which the incubation containing neuraminidase was maintained at 0^0 for 4 hr.

Incorporation of [^{14}C]UDP-N-Acetylglucosamine into Ceramide Trihexosidase, Form B: Ceramide trihexosidase, Form B-V (pI 8.4), was partially purified from Cohn fraction I and was separated from the other B forms of the enzyme by a combination of affinity chromatography and isoelectric focusing. Enzyme B-V (600 µg) in 5 ml of 0.001 M MES buffer, pH 5.4, was added with gentle stirring to 50 mg of porcine kidney homogenized in 20 ml of 0.25 M sucrose, pH 7.0. [^{14}C]UDP-N-Acetylglucosamine (10 µmole), 20 µmole ATP, 20 µmole phosphoenolpyruvate, and 20 µmole CTP were added and the mixture was incubated in a Dubnoff shaking incubator at 37^0. After 4 hr incubation, the reaction mixture was centrifuged at 12,000 g for 30 min. The precipitate was washed twice with 5 ml portions of 0.001 M MES buffer, pH 5.4, containing 15% butanol. These extracts were combined with the 12,000 g supernate and concentrated by dialysis against polyethylene glycol 6000. The concentrated protein solutions (285 µg) were divided into 3 equal aliquots and electrophoresed on cellulose acetate strips.

The cellulose strips were treated as follows: one was stained with Ponceau S and used for protein determinations; one was stained for ceramide trihexosidase

activity; and one was sectioned and added directly to 10 ml of DPO toluene for counting in a Beckman LS-150 liquid scintillation counter.

Three controls were run, one in which the basic protein was eliminated from the incubation mixture; one in which [^{14}C]UDP-N-acetylglucosamine was omitted from the incubation; and one in which [^{14}C]UDP-N-acetylglucosamine, ATP, CTP, phosphoenolpyruvate, and ceramide trihexosidase were incubated in the absence of kidney homogenate.

Incorporation of [^{14}C]CMP-Sialic Acid into Ceramide Trihexosidase, Form B-V: The above experiment was repeated using [^{14}C]CMP-sialic acid instead of [^{14}C]UDP-N-acetyl-glucosamine. The only exceptions in the procedure were the following: 1) the [^{14}C]CMP-sialic acid (10 μmole) was added in 4 aliquots at one hour intervals and 2) the cofactors ATP, phosphoenolpyruvate and CTP were eliminated.

RESULTS

IDENTIFICATION OF RADIOACTIVE SUBSTRATES

Preparation of [^{3}H]GL-3

GL-3 purified from Fabry kidney was treated with galactose oxidase from *Dactylium dendroides* to oxidize the terminal galactose residue to galactohexodialdose (177). Following sodium borotritiide reduction and silicic acid chromatography the product was chromatographed on a silica gel G plate in chloroform-methanol-water (100:42:6). The plate was scanned for radioactivity after which the standard GL-3 was located by staining the lipid with iodine vapors. As shown in Figure 3, the majority of the radioactivity co-chromatographed with standard GL-3, although a small amount of radioactive material migrated slightly ahead of GL-3.

The radioactive compound was eluted from the silica gel and added to carrier GL-3. After methanolysis and conversion of the carbohydrates to trimethylsilyl derivatives, GLC analysis, shown in Figure 4, proved that the compound contained galactose and glucose in a molar ratio of 2:1.

The carbohydrate, fatty acid and sphingosine moieties obtained by methanolysis of [^{3}H]GL-3 were counted and found to contain 75.8%, 11.1% and 13.3%, respectively, of the total

Figure 3. Strip Scan of [^{3}H]GL-3

[^{3}H]GL-3 was chromatographed on a silica gel G plate using chloroform-methanol-water (100:42:6). The blueprint of the TLC plate showing the position of standard GL-3 was aligned with the strip scan by the marker (dye containing [^{14}C]sucrose) spotted on each end of the plate.

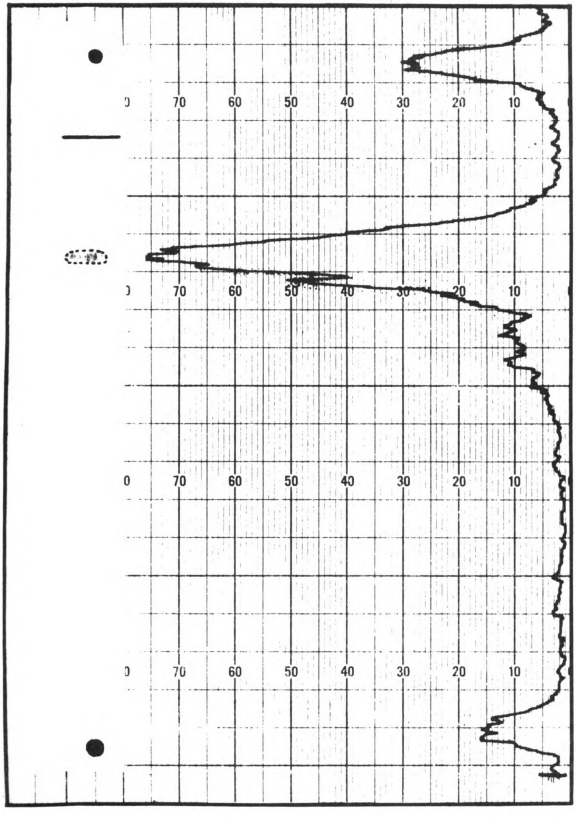
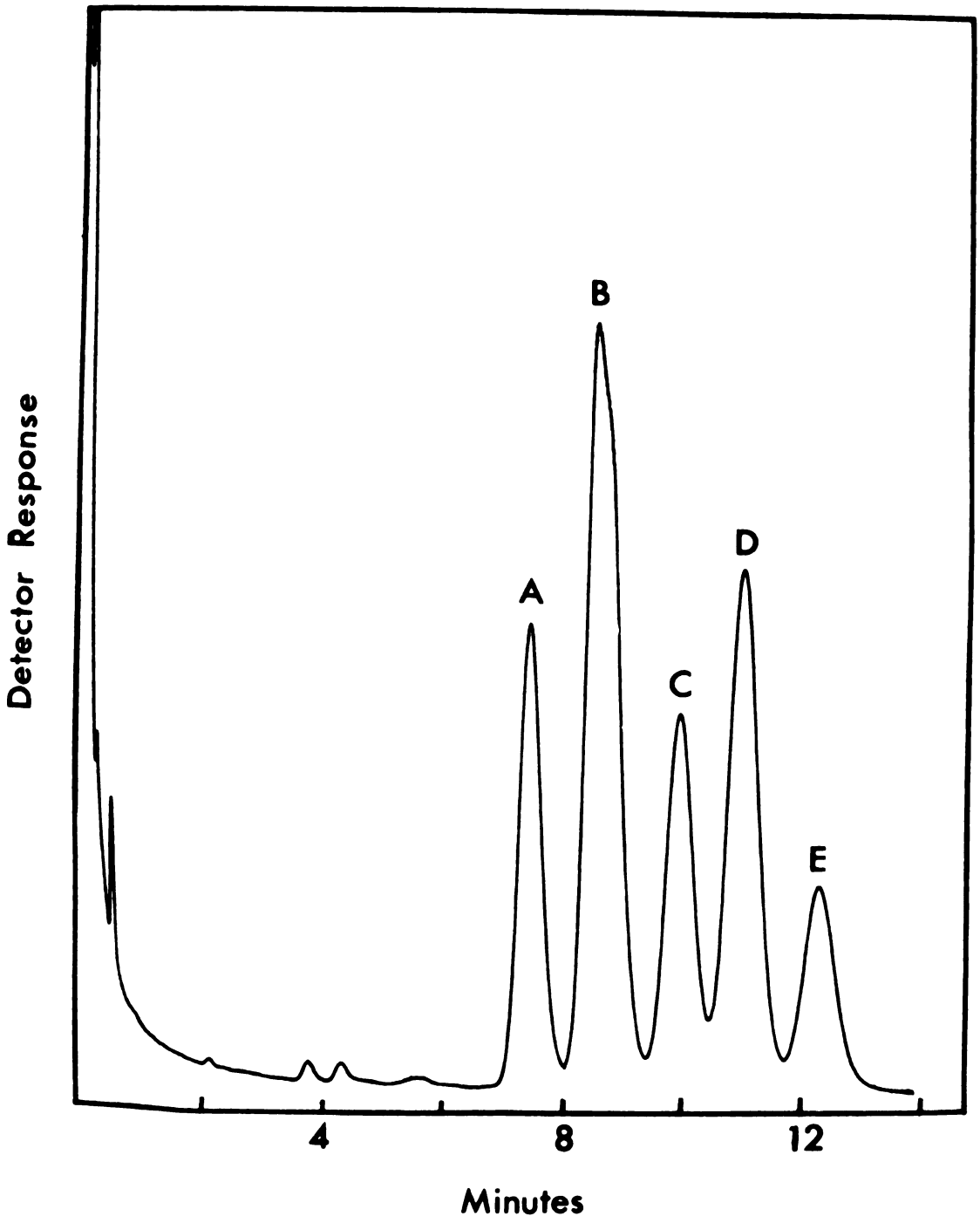

70
60
50
40
30
20
10

Figure 4. GLC of TMSi Methyl Glycosides from [^{3}H]GL-3

The methyl glycosides were silylated with 20 µl of pyridine-hexamethyldisilazane-trimethylchlorosilane (10:4:2) and analyzed with a Hewlett-Packard 402 gas chromatograph equipped with a 6 foot 3% SE-30 column maintained at 165°.


Detector Response
A
B
C
D
E
4
8
12
Minutes

radioactivity. Glucose contained 3.4% of the radioactivity associated with the carbohydrate, as determined by counting the methyl glycosides recovered from the flame detector during GLC analysis. The [^{3}H]GL-3, obtained in 85% yield, contained 22,000 cpm/2.7 x 10^{-3} μmole.

Preparation of [^{14}C]GL-3

Attempts were made to prepare trihexosylsphingosine by deacylating GL-3 using the alkaline hydrolysis procedures of Taketomi (153) and Carter (178). The Carter method, requiring a 12 hour reflux in 50% aqueous dioxane containing 5% $Ba(OH)_2$, produced nine unidentified degradation products and no detectable trihexosylsphingosine. The milder procedure of Taketomi, which requires heating the lipid in 90% aqueous butanol containing 1 N KOH, produced 70% GL-2a and 0.5% trihexosylsphingosine. Therefore it was necessary to synthesize trihexosylsphingosine using Shapiro's method for preparation of psychosine (157).

Trihexosylsphingosine was identified by thin-layer chromatography and GLC analysis. The product chromatographed with an R_f of 0.10-0.13 on silica gel G plates developed in chloroform-methanol-4N ammonium hydroxide (60:40:9). After methanolysis of this compound, the mixture of carbohydrates was converted to trimethylsilyl derivatives and analyzed by gas chromatography. The results afforded evidence for the presence of galactose and glucose in a molar ratio of 2:1.

The product obtained by coupling of [^{14}C]stearic acid and trihexosylsphingosine was chromatographed on a silica gel G plate using chloroform-methanol-water (100:42:6) and the plate was scanned for radioactivity after drying. The majority of the radioactivity co-chromatographed with standard GL-3, as shown in Figure 5. [^{14}C]GL-3, having 10,000 cpm/1.4 x 10^{-4} μmole, was obtained in 5% yield, based on the quantity of sphingosine used as starting material.

PREPARATION OF α-GALACTOSIDASE AFFINITY COLUMN

Choice of Carbohydrate for Coupling to Affinose 202

Since it was not feasible to synthesize enough trihexosylsphingosine to be used to prepare an affinity column having a sphingosine-containing ligand, analogous to those prepared by Sloan *et al.* (179) for the purification of sphingomyelinase and glucosyl ceramide hydrolase, a substrate analog of GL-3 was required.

The ability of enzymes normally hydrolyzing substrates solubilized in micelles to cleave water-soluble analogs of the lipophilic molecule in a "pseudo-micellar" system was recently reported by Gatt (124). Thus the ability of the ceramide trihexosidases to cleave oligosaccharides was investigated. As shown in Figure 6 the partially purified ceramide trihexosidases, Form A, hydrolyzed a tetrasaccharide (stachyose) and several trisaccharides [galactosyl(α1→6)galactosyl(α1→6)-glucose; galactosyl(α1→4)-

Figure 5. Strip Scan of [^{14}C]GL-3

[^{14}C]GL-3 was chromatographed on a silica gel G plate using chloroform-methanol-water (100:42:6). The blueprint of the TLC plate showing the position of standard GL-3 was aligned with the strip scan by the marker spotted on each end of the plate.

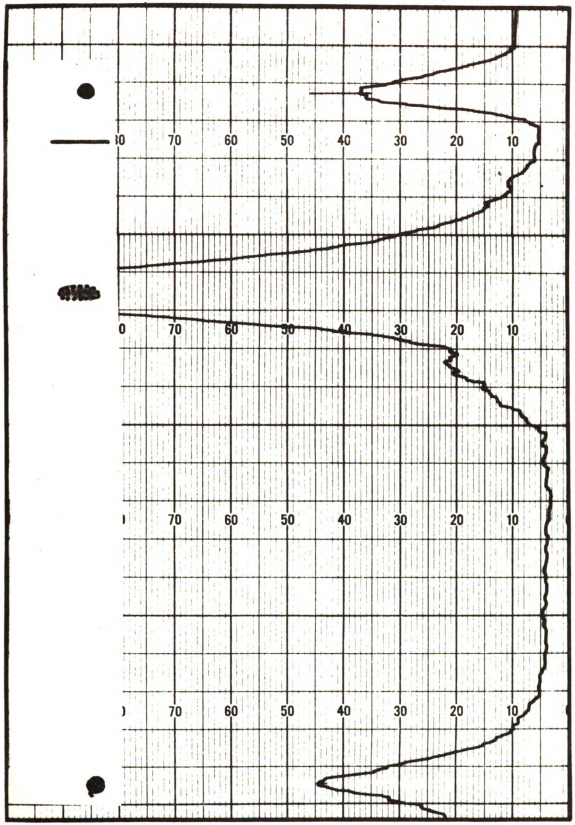
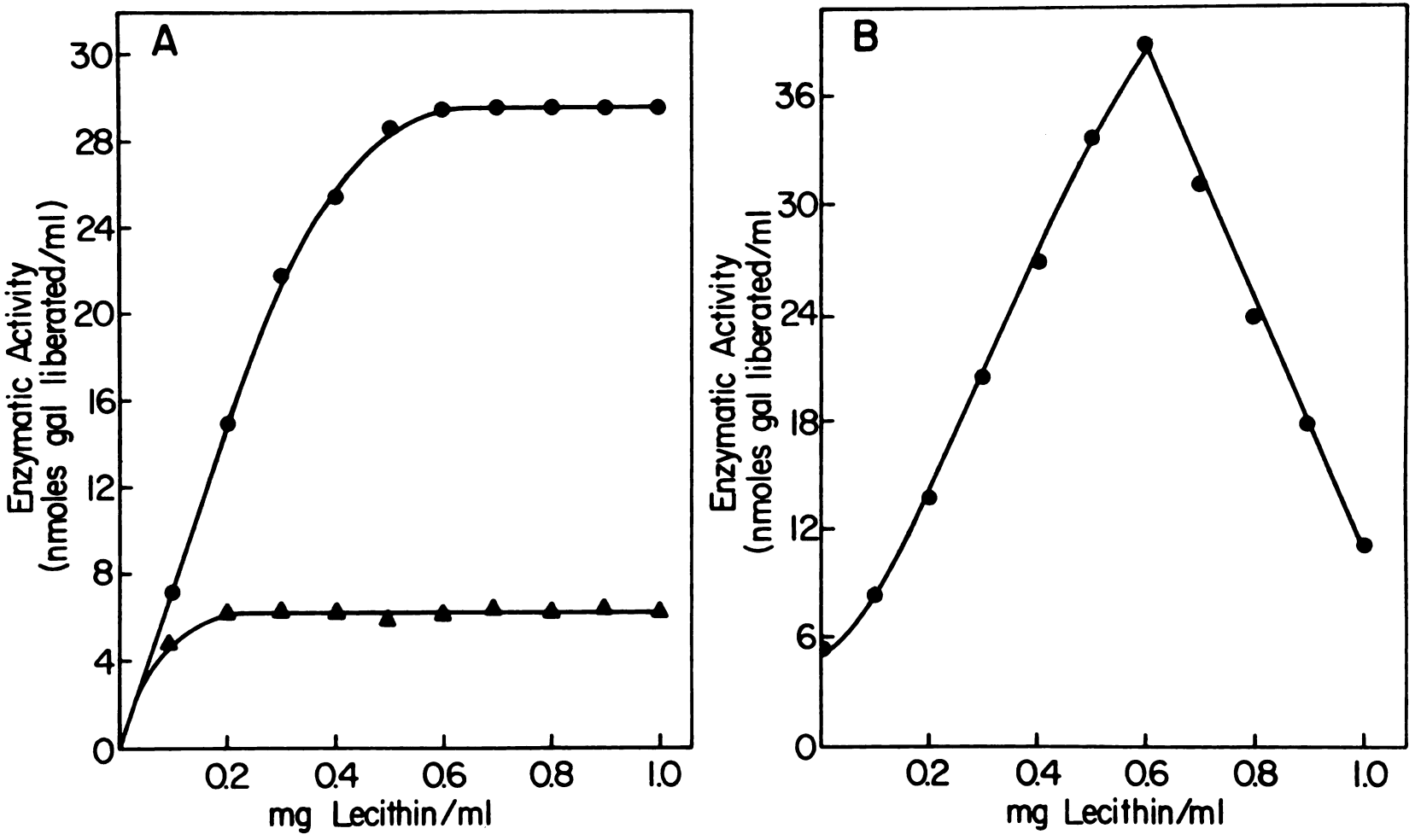

70
60
50
40
30
20
10

Figure 6. Enzymatic Hydrolysis of Oligosaccharide Substrates

(A) Hydrolysis of galactosyl(α1→4)galactosyl(β1→4)glucose (-●-) and melibiose (-▲-) by the plasma ceramide trihexosidases, Form A. Identical results were obtained for trisaccharide hydrolysis using either galactosyl(α1→6)galactosyl(α1→6)-glucose or galactosyl(α1→4)galactosyl(β1→4)sorbitol as substrate.

(B) Enzymatic hydrolysis of stachyose by the plasma ceramide trihexosidases, Form A.


A
B
Enzymatic Activity
(nmoles gal liberated/ml)
mg Lecithin/ml
0
4
8
12
16
20
24
28
30
6
18
36
0.2
0.4
0.6
0.8
1.0

galactosyl(β1→4)-glucose; and galactosyl(α1→4)galactosyl-(β1→4)-sorbitol] equally well at optimal concentrations of phosphatidylcholine, while hydrolysis of the disaccharide (melibiose) was negligible.

Although melibiose was not a good substrate for the enzyme, the coupling of p-nitrophenol from which an amine could be produced for attaching the carbohydrate to Affinose 202 would result in a compound having approximately the same size as a trisaccharide. In addition, the 14 Å spacer attached to the Affinose 202 might confer some of the properties of GL-3 to this product. Therefore melibiose was chosen as the carbohydrate for coupling to the affinity column.

Product Identification

The chemical synthesis of the affinity column adsorbent, the structure of which is shown in Figure 7, was followed primarily by thin-layer chromatography. The R_f value for the product of each reaction is shown in Table 3. In addition, melibiose octaacetate was identified by mass spectrometry using an LKB 9000 mass spectrometer and the p-nitrophenyl and p-aminophenyl derivatives were analyzed by GLC both before and after hydrolysis with methanolic HCl. Prior to hydrolysis the trimethylsilyl derivatives of p-nitrophenylmelibioside and p-aminophenylmelibioside had the same retention time as a standard trisaccharide [galactosyl-(α1→6)-galactosyl(α1→6)-glucose] when analyzed by gas

Figure 7. α-Galactosidase Affinity Column Adsorbent

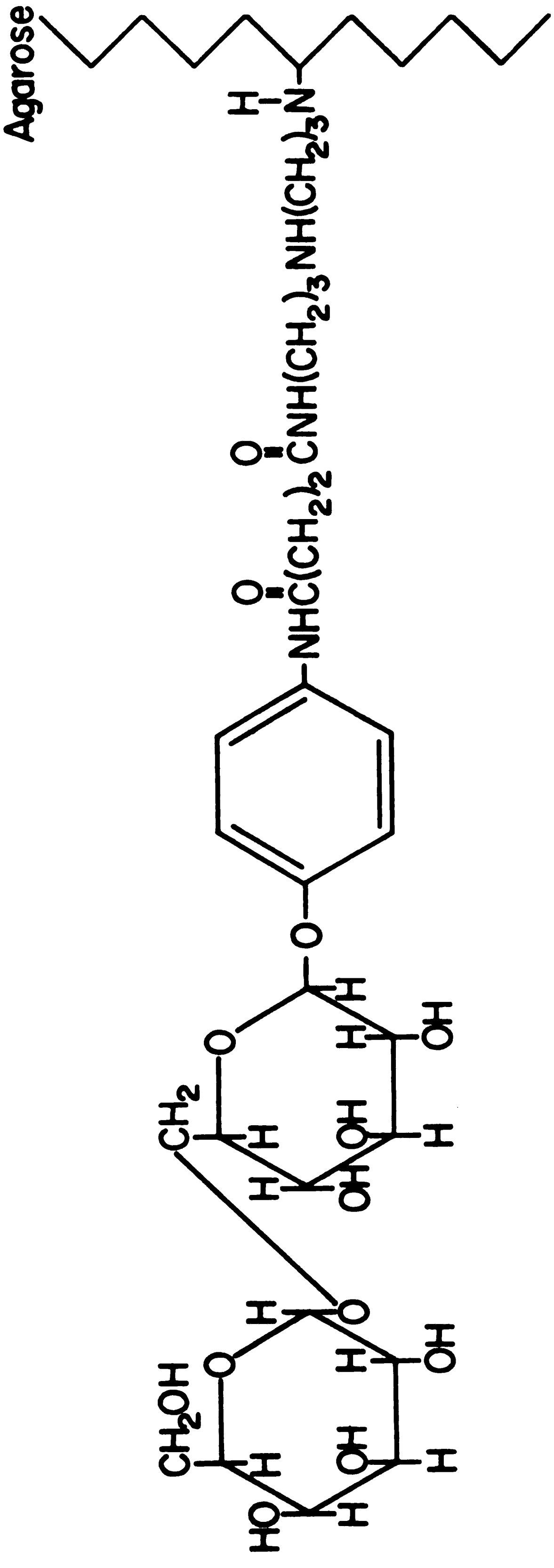


Table 3. R_f of Carbohydrate Derivatives

All carbohydrate derivatives were chromatographed on Kieselguhr G plates heat-activated at 80° for 2 hr prior to use. Solvent I is benzene-ethyl acetate (1:1, v/v) and Solvent II is butanol-pyridine-water (75:15:10, v/v).

Compound	Solvent	R_f
Melibiose Octaacetate	I	0.51
Acetobromomelibiose	I	0.63
p-Nitrophenylacetomelibioside	II	0.71
p-Nitrophenylmelibioside	II	0.44
p-Aminophenylmelibioside	II	0.60

chromatography on a 0.05% OV-101 column. Following acid hydrolysis and conversion of the carbohydrate mixture to trimethylsilyl derivatives, GLC analysis proved that the molar ratio of glucose and galactose was 1:1.

The attachment of the p-aminophenylmelibioside to Affinose 202, containing 8 μmole carboxyl group content per ml packed bed, was estimated to be 6-7 μmoles per ml of packed resin, from the amount of uncoupled material obtained from the coupling reaction and by thorough washing of the packed resin. The adsorbent prepared by this method can be used repeatedly without apparent loss of binding capacity. However the flow rate decreased after 45-50 runs and could not be improved by repacking the resin.

Specificity for α-Galactosidases

Investigations were carried out to determine if the substituted Affinose 202 selectively adsorbed only α-galactosidases. The proteins selected for this purpose were bovine serum albumin, β-lactoglobulin, mucin, transferrin, and liver β-galactosidase. These studies, summarized in Table 4, proved that the substituted Affinose was not a non-specific protein adsorbent.

Adsorption of Ceramide Trihexosidase

To insure that the attached carbohydrate was responsible for adsorption of the ceramide trihexosidases, a partially purified fraction of ceramide trihexosidases, Form A, isolated as described in the following section, was

Table 4. Specificity of α-Galactosidase Affinity Column

All proteins were applied to the affinity column in 10 ml of 0.001 M MES buffer, pH 5.4. Chromatography was performed using the conditions described in Materials and Methods.

Protein	Mg Applied	Mg Recovered as Non-adsorbed Protein
Bovine Serum Albumin	100	99.0
β-Lactoglobulin	100	98.0
Mucin	100	98.7
Transferrin	100	100.0
β-Galactosidase (liver)	100	99.0

chromatographed on a column of unsubstituted Affinose 202. Both the protein and enzymatic activity emerged together in the break-through protein as shown in Figure 8. No additional activity or protein could be eluted from the unsubstituted resin when detergent was added to the buffer. The recovery of enzymatic activity from the unsubstituted Affinose was consistently in excess of 97%.

AFFINITY CHROMATOGRAPHY OF NEURAMINIDASE

Synthesis of Affinity Column Adsorbent

The affinity column adsorbent for purification of neuraminidase, shown in Figure 9, was analogous to that described by Cautrecasas (162), differing only in the spacer used for attaching the ligand to the matrix backbone. The coupling of tyrosine to Affinose 201, having 8 μmole carboxyl group content and an 8 Å spacer, was estimated to be 7-8 μmoles per ml of packed bed, while the coupling of N-(p-nitrophenyl)-oxamic acid was estimated to be 6 μmoles per ml of packed bed. Both estimates were based on the amount of unreacted material recovered from the coupling reaction. The adsorbent prepared by this method was unstable, lasting for approximately 5 runs.

Purification of Neuraminidase

Affinity chromatography of commercially purified *Clostridium perfringens* neuraminidase consistently gave 40-45 fold purification of the enzyme. Rechromatography

Figure 8. Affinity Chromatography of the A Forms of Ceramide Trihexosidase on Unsubstituted Affinose 202

The dialyzed acetone precipitate prepared from Cohn fraction IV-1 was chromatographed as described in Materials and Methods. Each of the 1 ml fractions was assayed for protein and ceramide trihexosidase activity.

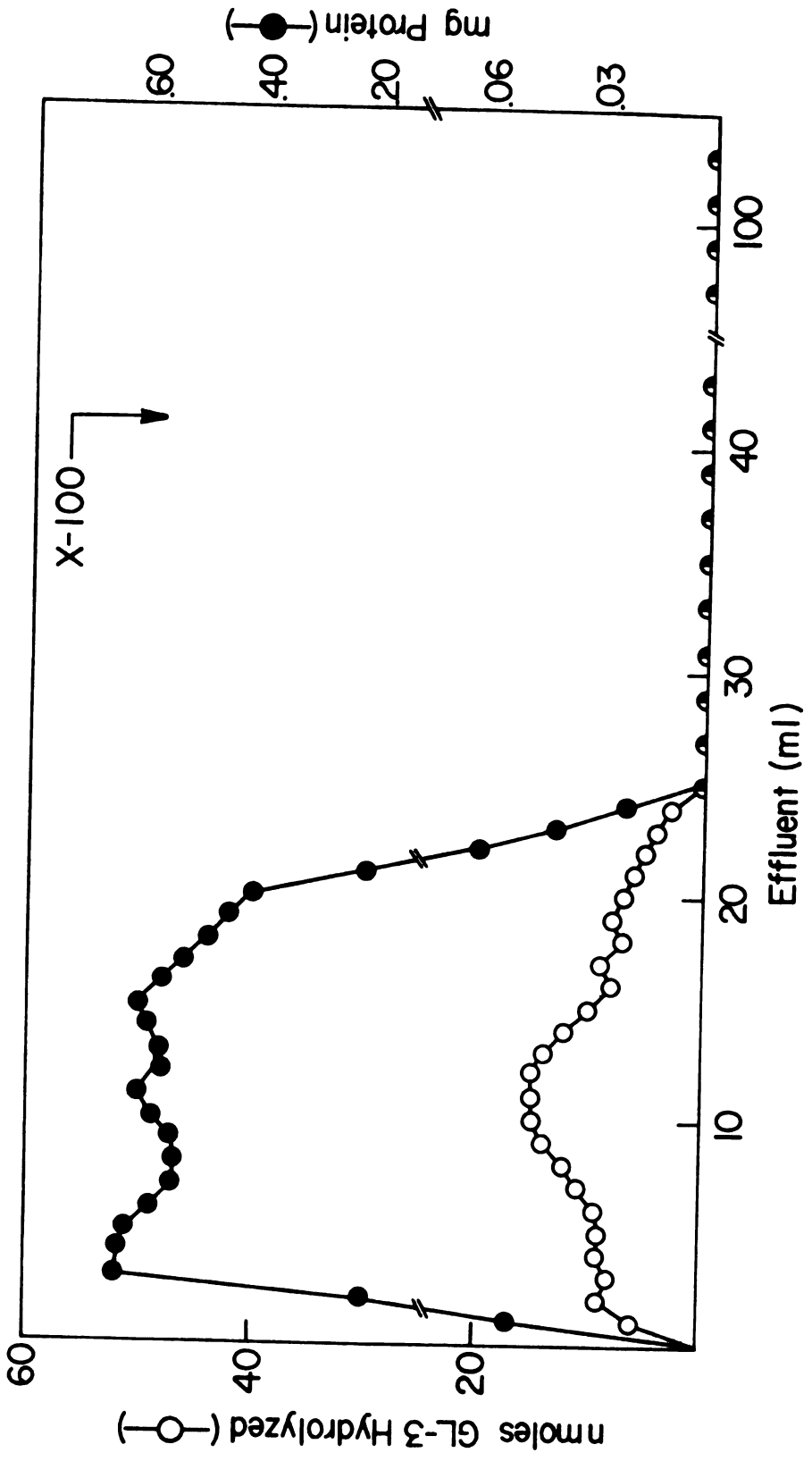
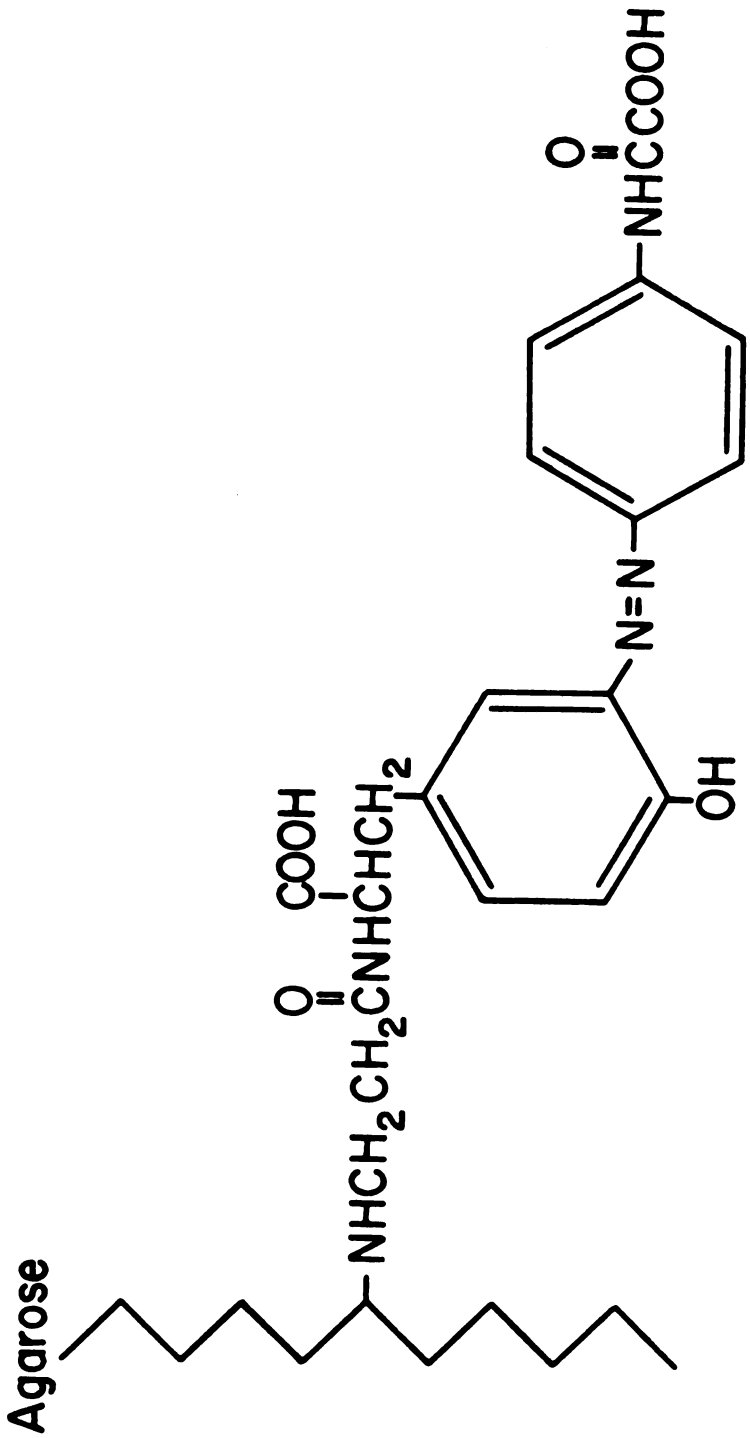

nmoles GL-3 Hydrolyzed (—○—)
mg Protein (—●—)
Effluent (ml)
X-100
60
40
20
.60
.40
.20
.06
.03
10
20
30
40
100

Figure 9. Neuraminidase Affinity Column Adsorbent


Agarose
NHCH2CH2CNHCHCH2
O
COOH
OH
N=N
O
NHCCOOH

of the purified enzyme did not result in either increased purification or loss of specific activity. A typical purification is summarized in Table 5.

DETERMINATION OF PRODUCTS LIBERATED BY ENZYMATIC HYDROLYSIS OF GL-3

Enzymatic Determination of Liberated Galactose

Ceramide trihexosidase activity was determined routinely by quantitation of the galactose liberated, using an end-point assay with galactose dehydrogenase (Method 1). This assay was linear over the range of 0.56 nmole-560 nmoles of galactose, using a total assay volume of 0.2 ml. The reproducibility of the assay for 0.56-5.6 nmoles of galactose was ±2.5%.

Determination of Radioactive Hydrolysis Products

[^{3}H]GL-3 and [^{14}C]GL-3 were prepared to enable rapid determination of the liberated hydrolysis products by a method which would be less affected by organic solvents and other possible inhibitors than the galactose dehydrogenase assay. The determination of radioactive products released by ceramide trihexosidase (Method 3) cannot be used with crude preparations which have a high enough protein concentration to form a protein pellet between the chloroform and aqueous phases upon termination of the incubation. In this case 75-90% of the radioactivity, depending on the quantity of protein, was bound to the protein pellet and the results were not reproducible. In addition [^{3}H]GL-3 had a

Table 5. Purification of *Clostridium perfringens* Neuraminidase

Neuraminidase (30 mg) was taken up in 7 ml of 0.05 M sodium acetate buffer, pH 5.5, and dialyzed for 12 hr at 4° against 0.05 M sodium acetate buffer, pH 5.5, containing 2 mM $CaCl_2$ and 0.2 mM EDTA. The dialyzed material was applied to the affinity column.(0.6 x 5 cm) equilibrated with the same buffer. Enzymatic activity was measured using porcine submaxillary mucin as substrate.

Purification Step	
Sample applied on column	
Protein (mg)	30
Volume (ml)	7
Specific activity*	3.6
Sample eluted from the column	
Protein (mg)	0.65
Specific activity*	160
Yield of Activity (%)	99
Fold Purification	44

* Specific activity = μmoles N-acetylneuraminic acid liberated per minute per mg of protein.

tendency to equilibrate between the aqueous and chloroform phases, necessitating extreme care to insure that all of the radioactivity in the upper phase was actually [^{3}H]-galactose.

OCCURRENCE OF CERAMIDE TRIHEXOSIDASE IN HUMAN PLASMA

Presence of Ceramide Trihexosidase Activity in Normal Plasma

Early attempts to determine whether added lysosomal ceramide trihexosidase from porcine liver and kidney would be active in human plasma at pH 7.0 led to the discovery of ceramide trihexosidase activity in normal human plasma. Aliquots (0.5 ml) of plasma were incubated for 4 hr with 100 nmoles of unlabeled GL-3, and liberated galactose was determined by an end-point assay with galactose dehydrogenase (Method 1). A bimodal curve of enzymatic activity, shown in Figure 10, indicated that there might be two forms of ceramide trihexosidase in plasma, with pH optima at 5.4 and 7.2. Normal levels of enzymatic activity, summarized in Table 6, were about 8 nmoles of galactose liberated per ml of plasma per hour at pH 5.4 and 17 nmoles per ml of plasma per hour at pH 7.2.

Ceramide Trihexosidase Activity in the Sphingolipidoses

Fabry plasma was assayed to determine whether this glycosidase, presumably responsible for the enzymatic hydrolysis of GL-3, would be detectable. Plasma obtained

Figure 10. Effect of pH on Ceramide Trihexosidase Activity in Normal Plasma

The curve of pH optima was the same using either whole plasma or the fraction obtained after $(NH_4)_2SO_4$ precipitation of ceramide trihexosidase from whole plasma. The pH was adjusted with 0.1 M citrate-phosphate buffer, pH 3.0, between pH 4.6 and 6.2, whereas 0.1 M MES buffer, pH 4.5, was used between pH 6.2 and 7.0. Tris buffer, pH 8.6, was used to adjust pH's above 7.0.

The bimodal curve could also be demonstrated with citrate-phosphate buffer for assays between pH 4.0 and 7.2.

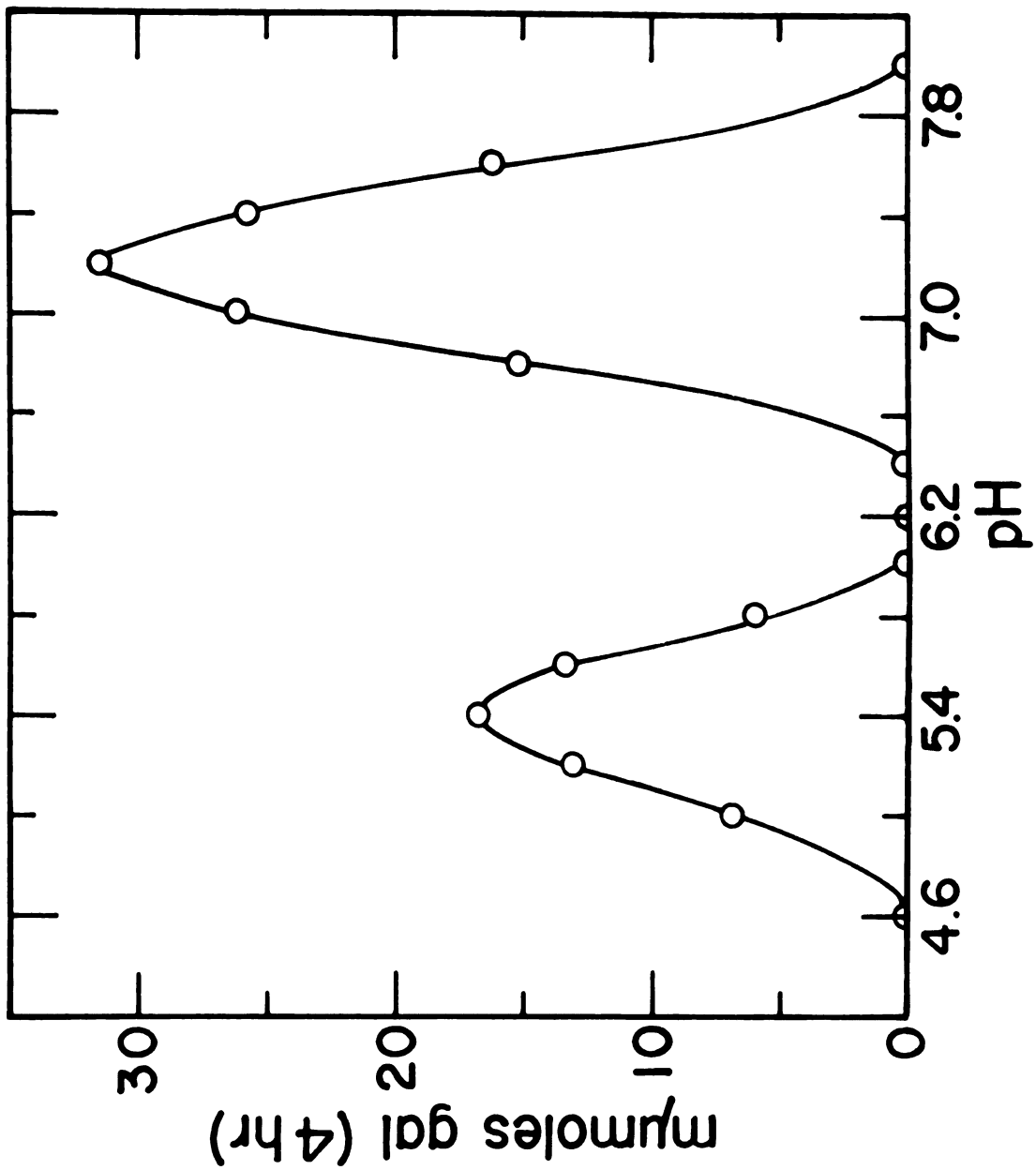

μmoles gal (4 hr)
0
10
20
30
4.6
5.4
6.2
7.0
7.8
pH

Table 6. Ceramide Trihexosidase Activity in Human Plasma

Enzymatic activity was determined using Method 1. Total units is expressed as nanomoles of galactose liberated per 4 hr per 0.5 ml plasma. Specific activity (S.A.) is expressed as nanomoles of galactose liberated per hr per mg of protein.

Subject	pH 5.4		pH 7.2	
	Total Units	S.A.	Total Units	S.A.
Fabry Hemizygotes				
A. G.	<1.5	<0.01	<1.5	<0.01
Ro. Lu.	<1.5	<0.01	<1.5	<0.01
Ri. Lu.	<1.5	<0.01	<1.5	<0.01
D. Lu.	<1.5	<0.01	<1.5	<0.01
C. Lu	<1.5	<0.01	<1.5	<0.01
C. Br.	<1.5	<0.01	<1.5	<0.01
M. C.	<1.5	<0.01	<1.5	<0.01
F. L.	<1.5	<0.01	<1.5	<0.01
F. B.	<1.5	<0.01	<1.5	<0.01
R. B.	<1.5	<0.01	<1.5	<0.01
R. Ca.	<1.5	<0.01	<1.5	<0.01
Fabry Heterozygotes				
S. C.	7.6	0.06	<1.5	<0.01
S. Ca.	8.3	0.06	<1.5	<0.01
H. L.	8.0	0.06	<1.5	<0.01
C. L.	9.8	0.07	<1.5	<0.01
L. F.	8.2	0.06	<1.5	<0.01
P. B.	7.4	0.06	<1.5	<0.01
M. B.	7.5	0.06	<1.5	<0.01
T. Le.	11.8	0.08	<1.5	<0.01
H. O.	12.4	0.08	<1.5	<0.01
Normals				
Ki. M. (MLD)*	11.1	0.08	26.0	0.19
Ke. M. (MLD)*	16.9	0.12	28.6	0.21
J. H. (Tay-Sach's)	18.3	0.12	44.1	0.30
R. B. (Gaucher)	15.1	0.11	30.5	0.23
Females (n=20)	18.0±4.0	0.11±0.03	36.0±4.0	0.20±0.10
Males (n=25)	16.0±3.0	0.11±0.04	30.0±5.0	0.20±0.10

* (MLD) = metachromatic leukodystrophy

fr

wi

of

th

nc

ac

ph

be

ha

le

th

ge

sp

tr

ta

N

se

fr

co

sp

a

s

from Fabry patients had no detectable enzymatic activity, within the limits of the method of assay, even when 1 ml of plasma was used in the incubation (Table 6). In contrast, the Fabry heterozygote, expected to have from 50-90% of normal enzymatic activity, had no detectable enzymatic activity at pH 7.2, but from 50-80% of normal activity at pH 5.4 (Table 6).

Since the terminal linkage of GL-3 had incorrectly been assigned a β-configuration (180), plasma from persons having Gaucher's disease, Tay-Sach's disease and metachromatic leukodystrophy was assayed for enzymatic activity to insure that the absence of ceramide trihexosidase activity was not a generalized glycosidase deficiency occurring in all the sphingolipidoses. As can be seen from Table 6 ceramide trihexosidase activity in the sphingolipidoses, other than Fabry's disease, was within the normal range.

REPLACEMENT OF DEFICIENT CERAMIDE TRIHEXOSIDASE ACTIVITY IN FABRY PLASMA

Substrate and Enzyme Levels Following Plasma Infusion

Since Fabry plasma showed a complete deficiency of ceramide trihexosidase activity, replacement of the enzymatic activity in the plasma of these persons might produce a decrease in their elevated plasma substrate concentration and lower the rate of accumulation of glycosphingolipid (GL-3) in their tissues. Since pure enzyme was not available, whole plasma was used as a source of enzymatic activity for these investigations.

The hemizygous patients selected for this investigation had the characteristic clinical symptoms of Fabry's disease, as well as consistently high concentrations of substrate in their plasma (181) and urinary sediment (182), which chemically confirmed the clinical diagnosis. Ceramide trihexosidase activity was not detectable at either pH optimum in the plasma of these patients.

Plasma for infusion was obtained by plasmapheresis of freshly drawn, heparinized blood from cross-matched normal donors whose previously assayed plasma had normal concentrations of ceramide trihexosidase activity. Prior to infusion, enzymatic activity in the normal plasma had decreased to one-fourth to one-half that in plasma from freshly drawn blood due to routine handling procedures. Plasma infusion in the recipients was completed within 30 minutes after venesection. A 17-year old hemizygote (D.L.) received 550 ml of plasma containing 2145 units of activity and a 31-year old hemizygote received 600 ml of plasma containing 4680 units of enzymatic activity.

Blood from each patient was assayed periodically for ceramide trihexosidase activity and for the concentration of GL-3 for 10 days after plasma infusion. Enzymatic activity was measured as previously described using galactose dehydrogenase for an end-point assay of galactose liberated from the substrate. The concentration of GL-3 in the plasma was determined by the method of Vance and Sweeley (147). These values were not corrected for manipulative losses

since the variation in triplicate analyses of GL-3 in control plasma was less than 5 percent.

The results of analyses for ceramide trihexosidase activity in the plasma of the recipients are shown in Figure 11. Unexpectedly, the enzymatic activity increased beyond that anticipated. Maxium activity at pH 7.2 occurred at 6 hours post-infusion in both patients, and was 28.7 (A.G.) and 20.8 (D.L.) units/ml. These values were 22- and 35-fold greater, respectively, than would be predicted from the volume and enzymatic activity of the infused plasma and were about 150 percent of the activity in normal plasma. The enzymatic activity decreased rapidly from 6-12 hours and then slowly until activity could no longer be detected after 7 days. Similar results were obtained with both patients.

The concentration of substrate in the plasma of each recipient, summarized in Table 7, decreased at a time just after the peak of maximum ceramide trihexosidase activity and then increased beyond initial levels, coincident with the rapid decline in enzymatic activity. From about 30 hours after infusion, there was a gradual decline in the concentration of substrate in the plasma, and after 10 days, it had decreased in both patients to about 50% of the initial concentration.

The enzymatic activity at pH 5.4 was also followed in recipient D.L. Enzymatic activity at this pH, as shown in Figure 12, was optimal at 1 and 8 hours post-infusion, which

Figure 11. Levels of Enzymatic Activity and Substrate Concentration in Fabry Plasma Following Infusion of Whole Plasma

(Top) Plasma ceramide trihexosidase activity in two hemizygous male patients with Fabry's disease after infusion of normal plasma (t_o on the graph is at the end of the infusion). Enzymatic activity is expressed as nmoles of galactose released from GL-3 per hour per milliliter of plasma at pH 7.2.

(Bottom) GL-3 levels in plasma during the same period as above in the same patients after infusion of normal plasma.

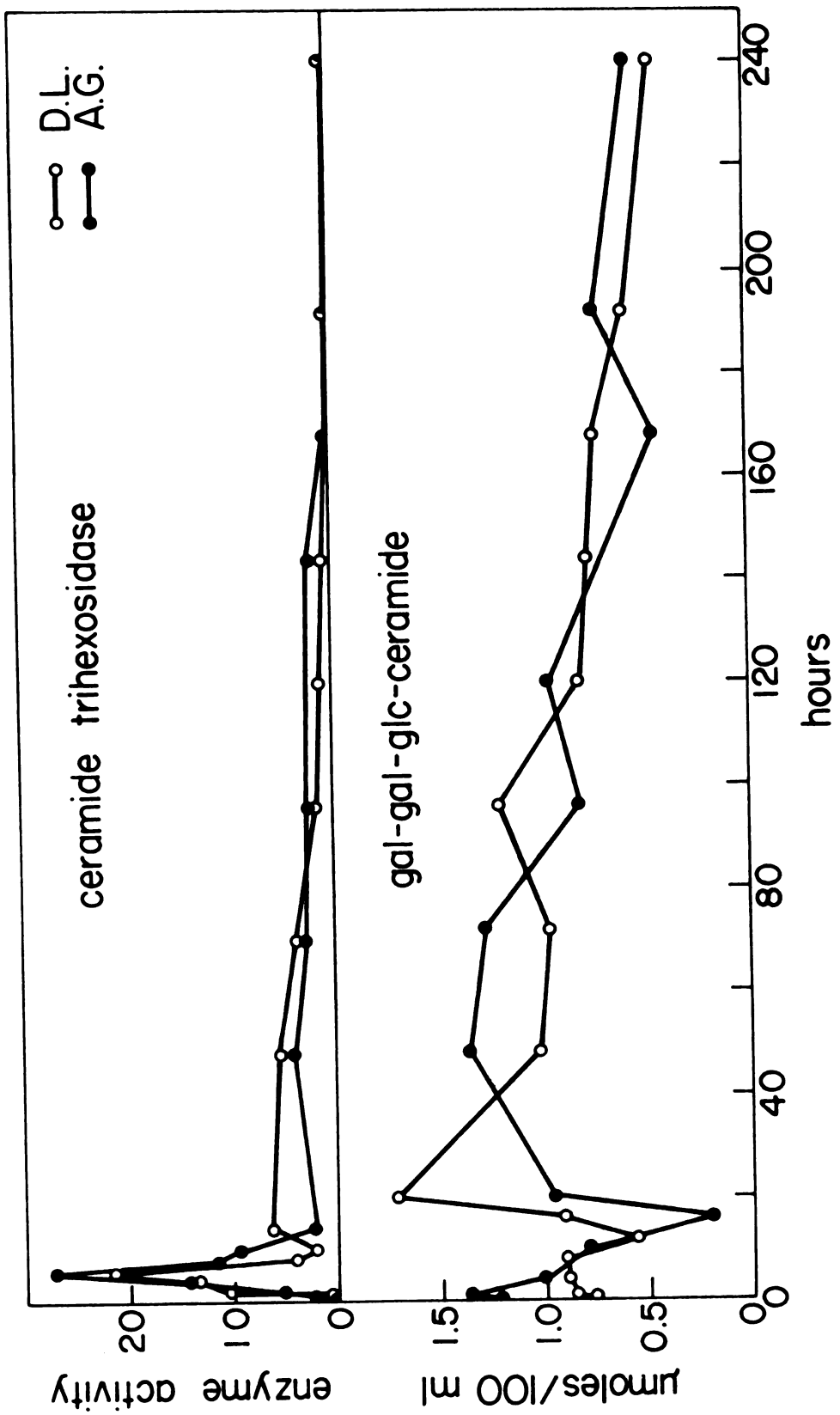

D.L.
A.G.
ceramide trihexosidase
enzyme activity
20
10
0
gal-gal-glc-ceramide
μmoles/100 ml
1.5
1.0
0.5
0.0
40
80
120
160
200
240
hours

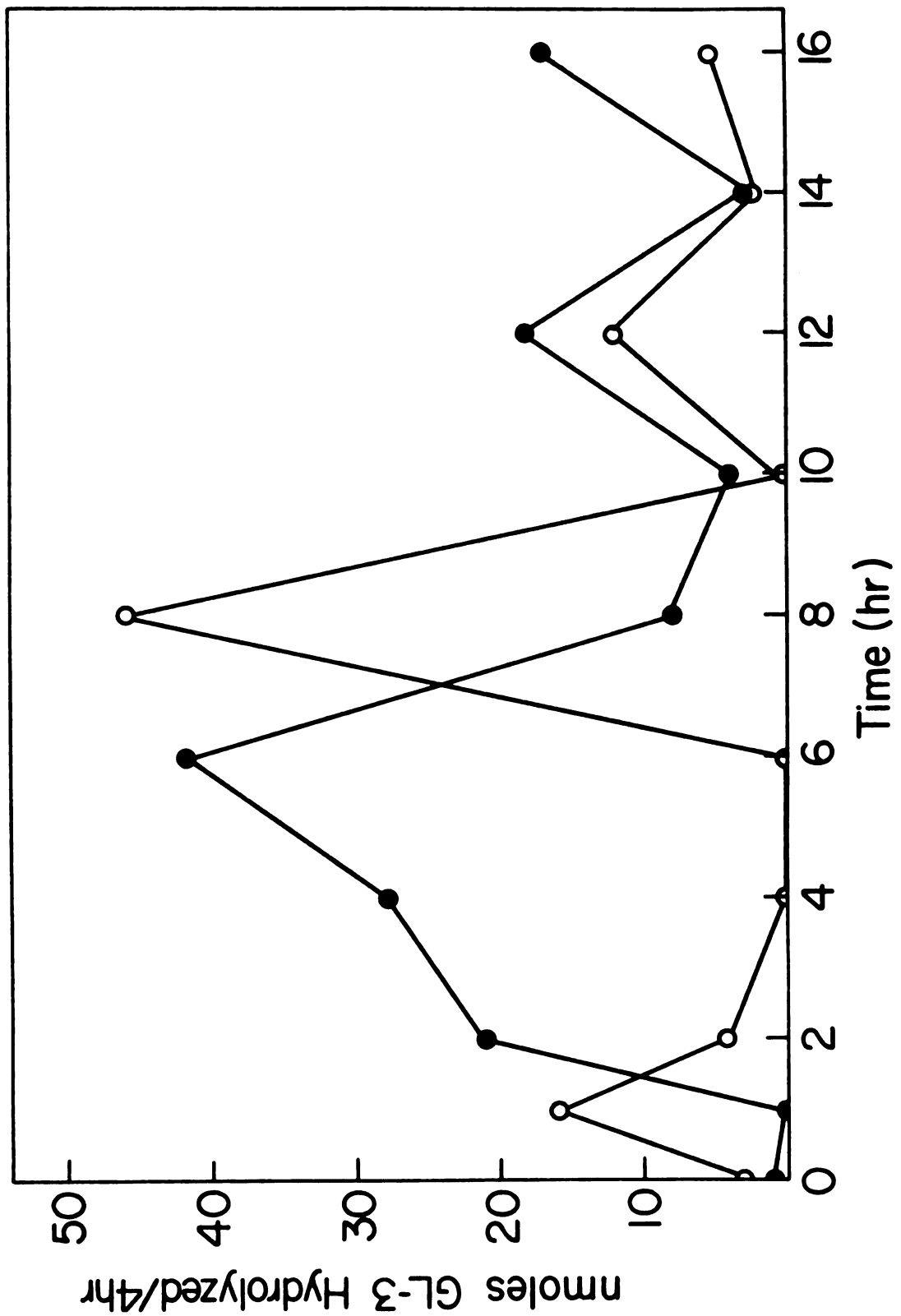
Table 7. Concentration of Glycolipids in Fabry Plasma Following Infusion of Normal Plasma

Glycolipids were quantitated by the method of Vance and Sweeley (147).

Time(hr)	Volume (ml)	GL-1	GL-2	GL-3	GL-4
			µmoles/100 ml		
A.G.					
0	19.4	1.420	0.980	1.208	0.419
1	22.7	0.62	1.446	1.362	0.576
4	16.5	0.907	0.831	1.002	0.341
10	33.5	0.956	0.834	0.791	0.364
16.5	36.0	0.516	0.476	0.178	0.396
20	34.0	1.145	0.732	0.965	0.234
48	31.0	1.249	0.889	1.354	0.334
72	35.0	1.059	0.929	1.276	0.593
96	35.0	1.002	0.948	0.816	0.495
120	35.0	1.256	1.090	0.968	0.467
168	41.0	1.097	0.620	0.444	0.563
192	39.5	1.270	1.389	0.725	0.430
216	35.2	0.840	0.463	0.747	0.281
240	41.0	0.714	0.500	0.590	0.253
D.L.					
0	28.7	0.767	0.553	0.768	0.553
1	30.9	0.898	0.801	0.849	0.740
4	29.4	0.862	0.771	0.894	0.635
8	32.8	1.778	0.848	0.889	0.883
12	29.8	0.748	0.628	0.546	0.532
16	15.1	1.000	1.007	0.923	0.802
20.5	28.4	0.820	0.674	1.727	0.684
48	36.2	1.084	0.856	1.012	0.618
71.5	34.6	1.107	0.791	0.962	0.276
96	19.5	0.999	0.970	1.195	0.719
120	39.0	0.934	0.691	0.818	0.533
144	39.0	0.883	0.661	0.756	0.403
167.5	44.3	0.832	0.705	0.726	0.573
192.5	37.4	0.775	0.612	0.583	0.610
217	46.5	0.771	0.593	0.569	0.384
239.5	44.5	0.965	0.519	0.441	0.164
263.5	40.6	0.514	0.423	0.437	0.227

Figure 12. Comparison of Enzymatic Activity at pH 5.4 and pH 7.2 in Fabry Plasma Following Infusion of Normal Plasma

Ceramide trihexosidase activity at pH 7.2 (-●-) in the hemizygous Fabry D.L. is compared with enzymatic activity at pH 5.4 (-o-) following plasma infusion (t_o is at the end of the infusion). Both enzymatic activities are expressed as nmoles of GL-3 hydrolyzed per hour per milliliter of plasma.


nmoles GL-3 Hydrolyzed/4hr
50
40
30
20
10
0
2
4
6
8
10
12
14
16
Time (hr)

was coincident with the time periods of decreased ceramide trihexosidase activity at pH 7.2. Both enzymatic activities were slightly increased at 12 hours and their decay curves over the following 7 days were approximately parallel. Although ceramide trihexosidase activity appeared to be sporadic at both pH optima, summation of the individual activities, indicated that there were actually two increments of enzymatic activity without any periods of inactivation.

In Vitro Studies: The apparent interconversion of the two forms of ceramide trihexosidase activity following plasma infusion was not an *in vitro* occurrence, since it could not be demonstrated by mixing donor plasma and Fabry plasma. Normal and Fabry plasma mixed in equal volumes and assayed without pre-incubation gave one-half the normal activity as expected. Pre-incubation of normal plasma containing 15.7 units of activity at pH 7.2 and 7.9 units of activity at pH 5.4 with an equal volume of Fabry plasma for 4 hours prior to the incubation for determination of enzymatic activity (Method 1) resulted in 26.7 units of activity at pH 7.2 and no detectable activity at pH 5.4, thus suggesting that at least *in vitro*, the enzymatic activity at pH 5.4 could be converted to that at pH 7.2 but not *vice versa* as suggested by the *in vivo* studies.

Comparison of Enzymatic Activity Determined by Artificial and Lipid Substrates Following Plasma Infusion

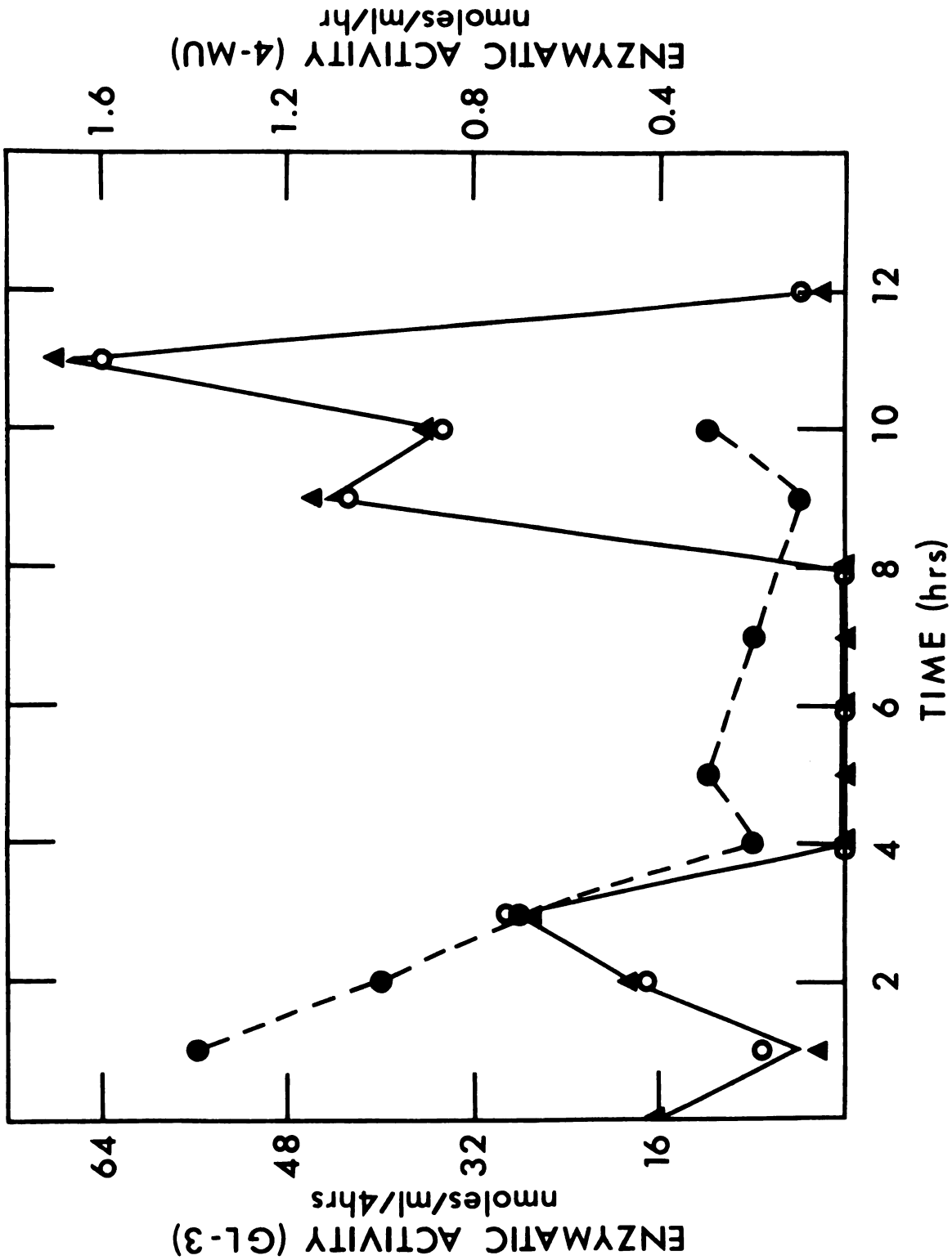
A third patient was infused with normal plasma to study the difference in enzymatic activity toward artificial substrate and lipid substrate, as well as to insure that the enhancement of enzymatic activity following plasma infusion was not an artifact.

A 30-year old hemizygote (R.R.) received 540 ml of freshly prepared, heparinized plasma, containing 2584 units of enzymatic activity, over a 1 hour period. Enzymatic activity was determined by measuring the galactose liberated from GL-3 by both GLC and galactose dehydrogenase; an enhancement of ceramide trihexosidase activity occurred 3 and 8-12 hours post-infusion, as shown in Figure 13. The peak of optimal enzymatic activity occurred 6 hours post-infusion in the previous infusion experiments.

Enzymatic activity measured with the artificial substrate, 4-methylumbelliferyl-α-galactoside, shown in Figure 13, was completely inconsistent with the activity measured using the lipid substrate. With 4-methyl-umbelliferyl-α-galactoside there was an enhanced level of enzymatic activity immediately following infusion which sharply declined to pre-infusion levels within two hours post-infusion.

Figure 13. Plasma α-Galactosidase Activity in a Patient with Fabry's Disease after Infusion of Normal Plasma

After incubation of 0.5 ml aliquots of plasma with GL-3, liberated galactose was quantitated by galactose dehydrogenase (-▲-) and by gas-liquid chromatography (-o-); assays were also made on separate aliquots with 4-methylumbelliferyl-α-galactoside (-●-).


ENZYMATIC ACTIVITY (GL-3)
nmoles/ml/4hrs
64
48
32
16
ENZYMATIC ACTIVITY (4-MU)
nmoles/ml/hr
1.6
1.2
0.8
0.4
2
4
6
8
10
12
TIME (hrs)

ISOLATION AND PURIFICATION OF CERAMIDE TRIHEXOSIDASES

The results of plasma infusions suggested that enzyme replacement might have some therapeutic value in the treatment of Fabry's disease. Thus purification of ceramide trihexosidase was undertaken to make this enzyme available in a stable, concentrated form without large quantities of contaminants. Attempts were made to isolate ceramide trihexosidase activity from whole plasma, but the apparent instability of the enzyme and the problem of acquiring fresh starting material resulted in negligible success.

Separation of Plasma Ceramide Trihexosidase into Two Enzymatically Active Forms

Out-dated plasma collected in 13% acid-citrate-dextrose (ACD) contained no enzymatic activity when assayed directly by the galactose dehydrogenase assay (Method 1). Protein concentrates from out-dated ACD plasma were prepared by the low temperature ethanol fractionation procedure described by Cohn *et al.* (164) and were tested for enzymatic activity. These fractions were active.

As summarized in Table 8 Cohn fractionation of out-dated whole plasma separated the ceramide trihexosidase activity into two components. The component designated ceramide trihexosidase, Form A, had optimal activity at pH 5.4 and the second component, designated as ceramide trihexosidase, Form B, had optimal activity at pH 7.2. Most of the Form A activity occurred in Fraction IV-1 (by-product fraction) and

Table 8. Ceramide Trihexosidase Activity in Cohn Fractions

The individual Cohn fractions (25 mg) were dissolved in 50 ml of glass-distilled water. Each fraction (1 ml) was assayed for enzymatic activity using Method 1. The supernate of Fraction V was concentrated 50-fold by ultrafiltration prior to assaying for enzymatic activity.

Fraction	% of Total Plasma Protein	Specific Activity* pH 5.4	Specific Activity* pH 7.2	Units/liter Plasma pH 5.4	Units/liter Plasma pH 7.2
II	5.1	<0.01	0.80	-----	2,320
II + III	26.7	<0.01	<0.01	-----	-----
IV - I	5.8	1.10	<0.01	3,520	-----
IV - 4	6.6	<0.01	<0.01	-----	-----
V	53.8	0.023	0.016	699	486
super. V	2.0	<0.01	<0.01	-----	-----

* Sp. Act. = nanomoles galactose liberated per mg protein per 4 hrs.

most of the Form B activity was recovered in Fraction I (fibrinogen fraction). Variable amounts of both forms of ceramide trihexosidase were found in Fraction V (serum albumin fraction), accounting for 15-20% of the total enzymatic activity. Similar binding of enzymatic activity could be demonstrated by adding bovine serum albumin to either Cohn fraction I or IV-1, thereby indicating that the enzymatic activities in Fraction V did not represent additional forms of ceramide trihexosidase.

Isolation of Ceramide Trihexosidases

Efforts at enzyme purification and characterization were concentrated on plasma ceramide trihexosidase, Form A, since it appeared to be metabolically the more significant of the two activities in view of the finding that the Fabry heterozygote retained this form of activity. Although primary emphasis was placed on the purification and characterization of plasma ceramide trihexosidase, Form A, the B form was also partially purified to investigate the relationship between the two forms of enzymatic activity. Kidney ceramide trihexosidase was partially purified for comparison with the plasma enzymes. Tables 9, 10 and 11 give the results for a typical purification of plasma Form A, plasma Form B, and kidney ceramide trihexosidases, respectively. The results are reproducible only if the conditions of the isolation and enzyme assays are stringently controlled, due to the anomalous effect of

Table 9. Purification of Human Plasma Ceramide Trihexosidases, Form A

The ceramide trihexosidases become inactive in the presence of more than 50% ammonium sulfate and are only partially stable to dialysis prior to acetone precipitation

Step	Protein (mg)	Activity (nmoles/hr)	Specific Activity (nmoles/mg/hr)	Purification (-fold)	Recovery (%)
I. Cohn Fraction IV-1	7,480	524	0.07	1	100
II. Subfractionation of IV-1					
a. 80% $(NH_4)_2SO_4$ supernatant	37.4	inactive			
b. 5% butanol treated	37.4	inactive			
c. 60% acetone precipitate	14.5	1,030	71	1,015	197
d. dialysis *vs* 5% butanol	13.9	1,020	73	1,048	195
III. Affinity Chromatography					
a. Form A-1	0.17	375	2,206	31,513	72
b. Form A-2	0.25	623	2,492	35,600	119
Total for A Forms	0.42	998	2,376	33,943	190

Table 10. Purification of Human Plasma Ceramide Trihexosidase, Form B

The ceramide trihexosidases become inactive in the presence of more than 50% ammonium sulfate and are only partially stable to dialysis prior to acetone precipitation.

	Step	Protein (mg)	Activity (nmoles/hr)	Specific Activity (nmoles/mg/hr)	Recovery (%)	Purification (-fold)
I.	Cohn fraction I	4872	244	0.05	100	1
II.	Subfractionation of I					
	a. 80% $(NH_4)_2SO_4$ supernate	28.7	inactive			
	b. 5% butanol	28.7	inactive			
	c. 60% acetone precipitate dialyzed *vs.* 5% butanol	11.1	1,887	170	773	3,400
III.	Affinity Chromatography	0.48	2,050	4,270.8	840	85,416
	a. Form B-1	0.17	760	4,470.6		
	b. Form B-2	0.15	560	3,733.3		
	c. Form B-3	0.16	730	4,562.5		
IV.	Isoelectric focusing	0.48	2,050	4,270.8	840	85,416
	a. Form B-I	0.11	509	4,627.3		
	b. Form B-II	0.06	251	4,183.3		
	c. Form B-III	0.07	252	3,600.0		
	d. Form B-IV	0.08	308	3,850.0		
	e. Form B-V	0.16	730	4,562.5		

Table 11. Purification of Human Kidney Ceramide Trihexosidase, Form A

	Step	Protein (mg)	Activity (nmoles/hr)	Specific Activity (nmoles/mg/hr)	Recovery (%)	Purification (-fold)
I.	600 g Supernate	2268	45.4	0.02	100	1
II.	16,000 g Precipitate	1156	92.5	0.08	204	4
III.	100,000 g Supernate	401	120.0	0.30	264	15
IV.	Subfractionation of III	103				
	a. 80% $(NH_4)_2SO_4$ super.	103	124.0	1.20	273	60
	b. 5% butanol treated	103	824.0	8.0	1815	400
	c. 60% acetone ppt. dialyzed *vs.* 5% butanol	4	640.0	160.0	1410	8000
V.	Affinity Chromatography	0.14	640.0	4571.0	1410	228,550

detergents, salt and enzyme concentration on the activity of the ceramide trihexosidases, as discussed in the following sections.

Purification Steps

Extraction of Ceramide Trihexosidase, Form A: The enzymatic activity was extracted from Cohn fraction IV-1 using 0.001 M MES buffer, pH 5.4. It was also possible to extract the enzymes without loss of activity using buffer containing 5% butanol, however the butanol interfered with the ammonium sulfate step.

The laboratory-scale procedure for selectively extracting ceramide trihexosidase activity was altered for the pilot-scale preparation. The Cohn fraction was completely dissolved in 9 volumes (minimum volume for dissolution) of 0.25 M sodium phosphate buffer, pH 7.0, to eliminate the necessity of removing a gelatinous precipitate and adjusting the pH prior to the ammonium sulfate treatment. The enzymes obtained by this procedure had no detectable differences from those isolated on a small scale.

Extraction of Ceramide Trihexosidase, Form B: The ceramide trihexosidases, Form B, were extracted from Cohn fraction I by dissolving the frozen protein paste in sodium phosphate buffer at room temperature. Dissolving Cohn fraction I at 4° resulted in complete inactivation of the enzymatic acitivty. Otherwise the ceramide trihexosidases, Form B, were not noticeably cold-labile.

Extraction of Kidney Ceramide Trihexosidase: The time in performing the initial steps was critical since prolonged homogenization released Form A into the 16,000 g supernate where it has a half-life of 2-3 hours. The whole homogenate from human kidney contained approximately equal levels of the A and B forms of ceramide trihexosidase. During the preparation of the 16,000 g precipitate and sodium cholate treatment to release the enzymes from the membrane the activity of the B form disappeared and was accompanied by a concomitant increase in activity of Form A.

Stabilization and Precipitation of the Enzymes: It was consistently observed that the addition of butanol stabilized both the A and B forms of the enzyme. Commonly employed stabilizers including 2-mercaptoethanol, Cleland's reagent, inert atmosphere and metal ions had no effect on the stability of the enzymes, while glycerol caused immediate inactivation of enzymatic activity. The temperature at which the butanol step is performed is not critical since addition of butanol to a final concentration of 5% without cooling does not depress the specific activity of the enzymes.

The proteins were precipitated from the butanol solution by the addition of acetone to a final concentration of 60% following the low temperature method described by Askonas (166). The addition of acetone at 4° decreased the specific activity by one-half. After this step, which yields an ammonium sulfate-protein sludge containing 0.2% protein,

the enzymes can be stored as a frozen paste for at least six months. Although freezing causes total inactivation of the ceramide trihexosidases, enzymatic activity is completely restored by dialyzing the proteins overnight against 0.001 M MES buffer, pH 5.4, containing 5% butanol.

After dialysis the ceramide trihexosidase, Form A, can be stored at 4° in 0.001 M MES buffer containing 5% butanol for one month without loss of activity, whereas Form B can be stored for two weeks without loss of activity under these conditions. The gradual loss of activity which occurs after this time is accompanied by the formation of a protein precipitate. Redissolving the precipitated protein restores 90% of Form A activity and 70% of Form B activity.

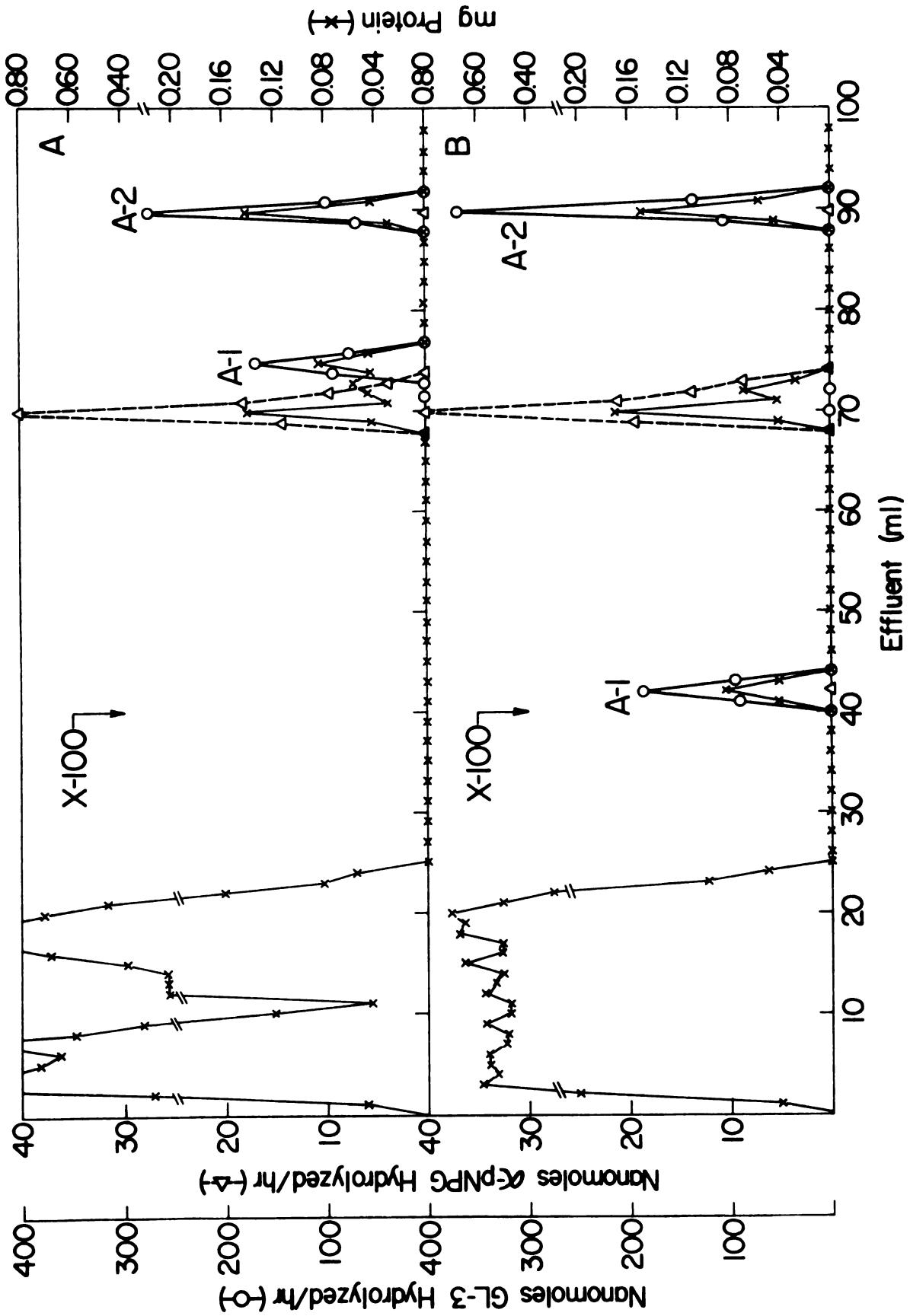
Affinity Chromatography of Ceramide Trihexosidase, Form A: Since it had been determined that the ceramide trihexosidases hydrolyzed oligosaccharides in the presence of optimal concentrations of phosphatidylcholine (lecithin), the possibility that lecithin would be required for the enzyme to bind to the affinity column was investigated. As shown in Figure 14, affinity chromatography of the dialyzed acetone precipitate prepared from Cohn fraction IV-1 separated ceramide trihexosidase, Form A activity, into two protein peaks. These peaks were designated by their order of elution as A-1 and A-2. Ceramide trihexosidase, Form A-2, binds to the column equally well in the presence and

Figure 14. Affinity Chromatography of Plasma Ceramide Trihexosidase, Form A

The acetone precipitate prepared from 500 gm of Cohn fraction IV-1 was dialyzed against 0.001 M MES buffer, pH 5.4, containing 5% butanol, then divided into two equal parts.

(A) Lecithin (8 mg/ml) was added to the dialyzed acetone precipitate and incubated for 0.5 hour prior to affinity chromatography. The eluting buffer also contained 8 mg/ml of licithin.

(B) The dialyzed acetone precipitate was chromatography without addition of lecithin.


A
B
A-1
A-2
X-100
mg Protein (×)
Nanomoles GL-3 Hydrolyzed/hr (○)
Nanomoles α-pNPG Hydrolyzed/hr (△)
Effluent (ml)

absence of lecithin, whereas Form A-1 is retarded but not adsorbed to the column in the absence of lecithin. This property was selectively used for complete separation of the A-1 form from other proteins in the preparation.

The purity of enzyme A-1 was 31,513-fold and that of enzyme A-2 was 35,600-fold greater than that of the initial extract. This fold-purification varied by as much as 4,000 depending upon the degree of contamination in the starting material. No interconversion of Form A-1 and A-2 was observed upon rechromatography of each fraction. The percentage of the total activity associated with each peak was consistent for a given preparation but not within different preparations.

Affinity Chromatography of Ceramide Trihexosidase, Form B: Affinity chromatography of the dialyzed acetone precipitate prepared from Cohn fraction I, shown in Figure 15, separated ceramide trihexosidase, Form B activity, into three protein peaks, designated by their order of elution as B-1, B-2 and B-3. If the individual peaks were allowed to stand in solution for 8-12 hours, rechromatography showed that peak B-1 was partially converted to peak B-2, and peak B-2 was partially converted to peak B-3. There was no detectable conversion of peak B-3 to either B-1 or B-2.

Cellulose acetate electrophoresis of the pooled protein peaks, shown in Figure 16, separated these ceramide trihexosidases into five protein bands which could be stained for both activity and glycoprotein. Isoelectric focusing of the

Figure 15. Affinity Chromatography of Plasma Ceramide Trihexosidase, Form B

The acetone precipitate prepared from 500 gm of Cohn fraction I was dialyzed against 0.01 M sodium phosphate buffer, pH 7.2, containing 5% butanol. The sample was applied to the pre-equilibrated column in a total volume of 40 ml.

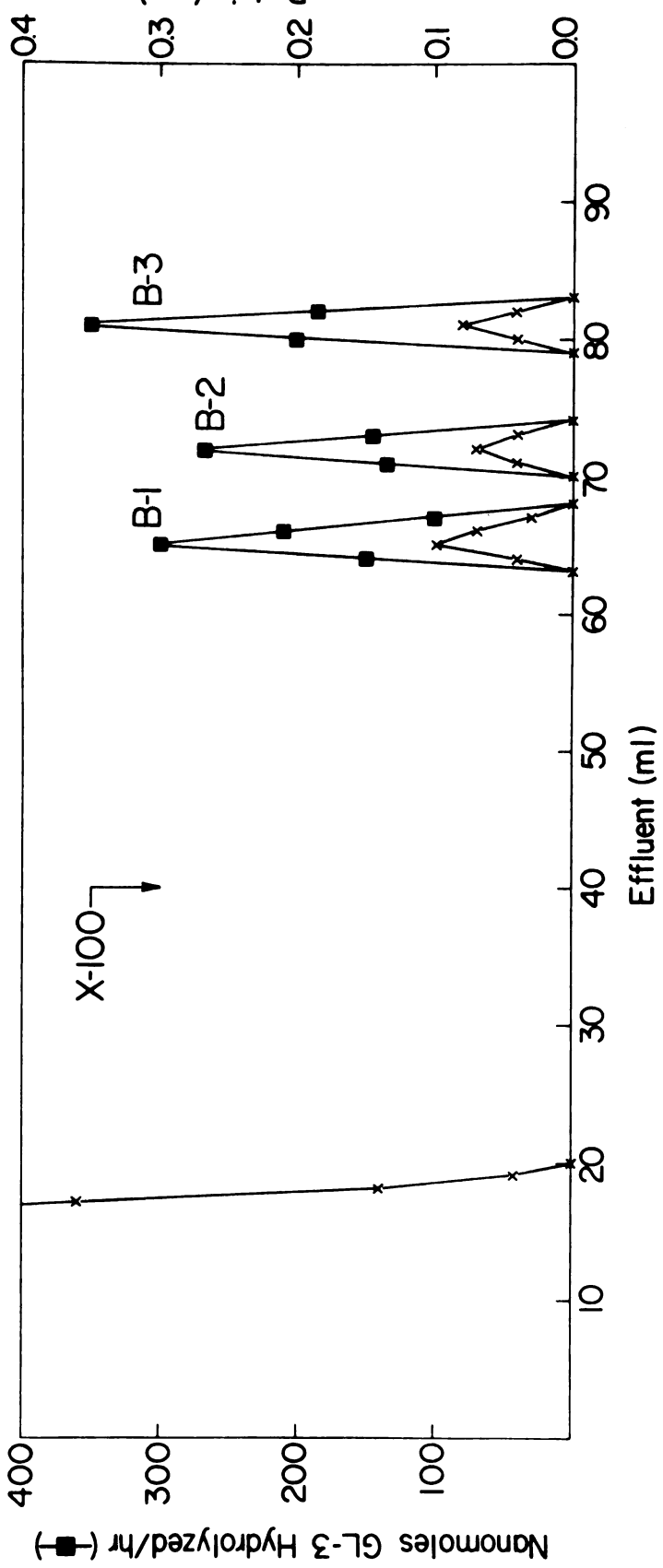
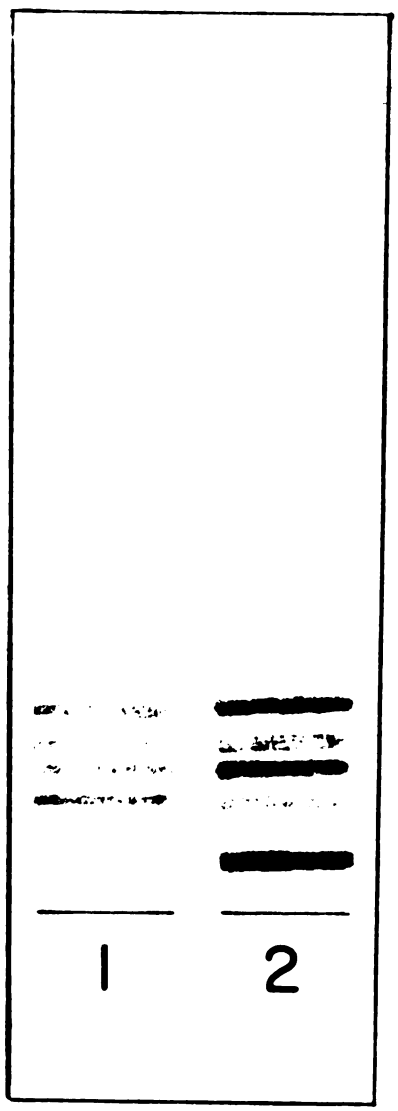

Nanomoles GL-3 Hydrolyzed/hr (■)
mg Protein (×)
Effluent (ml)
X-100
B-1
B-2
B-3
400
300
200
100
0.4
0.3
0.2
0.1
0.0
10
20
30
40
50
60
70
80
90

Figure 16. Cellulose Acetate Electrophoresis of Plasma Ceramide Trihexosidase, Form B

The protein peaks obtained from affinity chromatography of the dialyzed acetone precipitate prepared from Cohn fraction I were pooled and concentrated against polyethylene glycol 6000. Protein (16 μg of each peak) was applied to the cellulose acetate strips and electrophoresed at room temperature. Strip (1) was stained for activity and strip (2) was stained for glycoprotein. The strips were redrawn for purposes of photography.


1
2

pooled protein peaks also separated the proteins into five enzymatically active peaks, as shown in Figure 17. Isoelectric focusing of the individual protein peaks obtained by affinity chromatography revealed that peak B-1 was separated into peaks B-I and B-II, peak B-2 was separated into peaks B-III and B-IV, and peak B-3 remained as a single component, B-V.

A combination of isoelectric focusing and affinity chromatography resulted in 85,416-fold purification of ceramide trihexosidase, Form B-V.

Affinity Chromatography of Kidney Ceramide Trihexosidase: Affinity chromatography of the dialyzed acetone precipitate prepared from human kidney, shown in Figure 18, revealed the presence of only one form of ceramide trihexosidase. This protein had the same binding capacity as plasma ceramide trihexosidase, Form A-2, and was indistinguishable from either of the A form of plasma enzymes by cellulose acetate electrophoresis as shown in Figure 19. SDS polyacrylamide gel electrophoresis of this enzyme indicated that it was approximately 90% pure.

Other Purification Procedures Evaluated

Prior to the development of a suitable scheme for purification of the ceramide trihexosidases, Form A, several methods commonly used for fractionation of plasma proteins were investigated. These included pH precipitation, fractionation with polyethylene glycol, ethanol fractionation,

Figure 17. Isoelectric Focusing of Plasma Ceramide Trihexosidases, Form B

The protein peaks, obtained from affinity chromatography of the dialyzed acetone precipitate prepared from Cohn fraction I, were focused in a supportive sucrose gradient for 48 hours at 1°. Fractions (0.5 ml) were eluted with the aid of a peristaltic pump and assayed for enzymatic activity without removal of the carrier ampholytes.

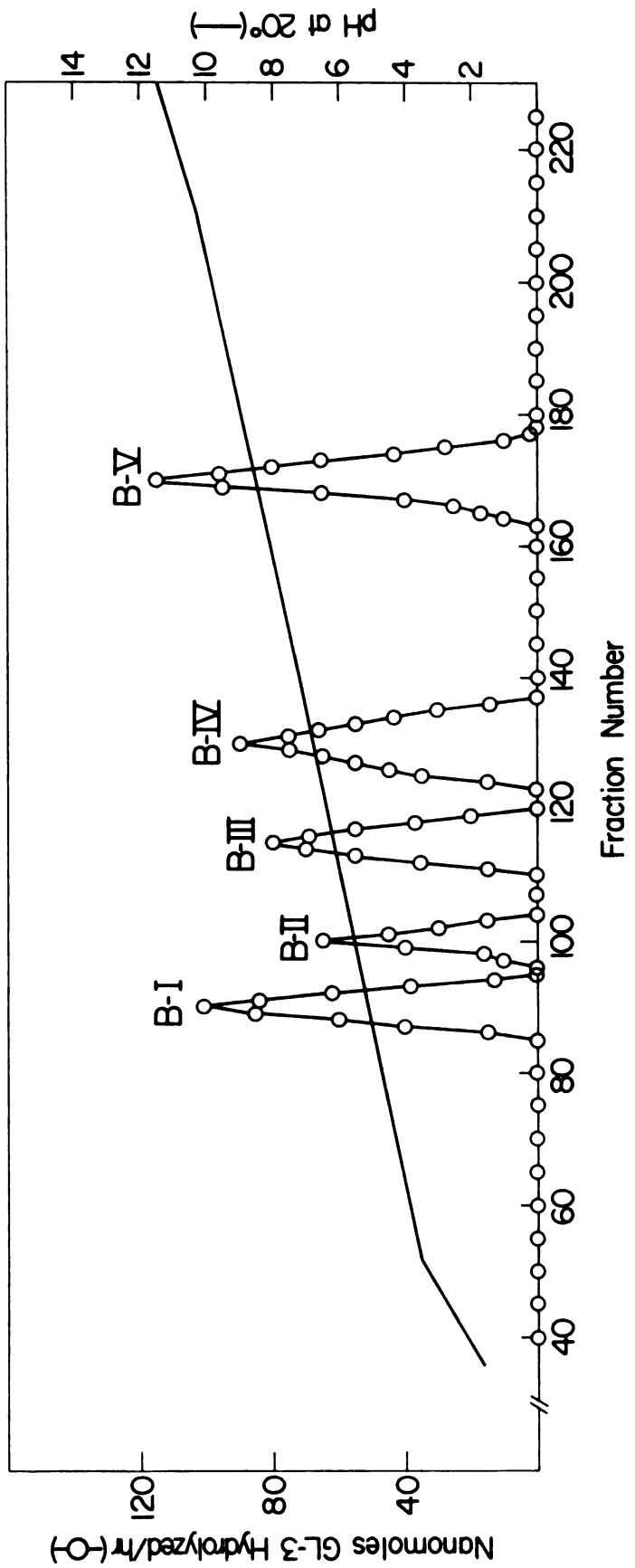

Nanomoles GL-3 Hydrolyzed/hr (-O-)
120
80
40
pH at 20° (—)
14
12
10
8
6
4
2
40
60
80
100
120
140
160
180
200
220
Fraction Number
B-I
B-II
B-III
B-IV
B-V

Figure 18. Affinity Chromatography of Human Kidney Ceramide Trihexosidase

The dialyzed acetone precipitate was applied to the affinity column equilibrated with 0.001 M MES buffer, pH 5.4. Aliquots (0.1 ml) of each 1 ml fraction were assayed for ceramide trihexosidase activity, Forms A and B, and for non-specific α-galactosidase activity.

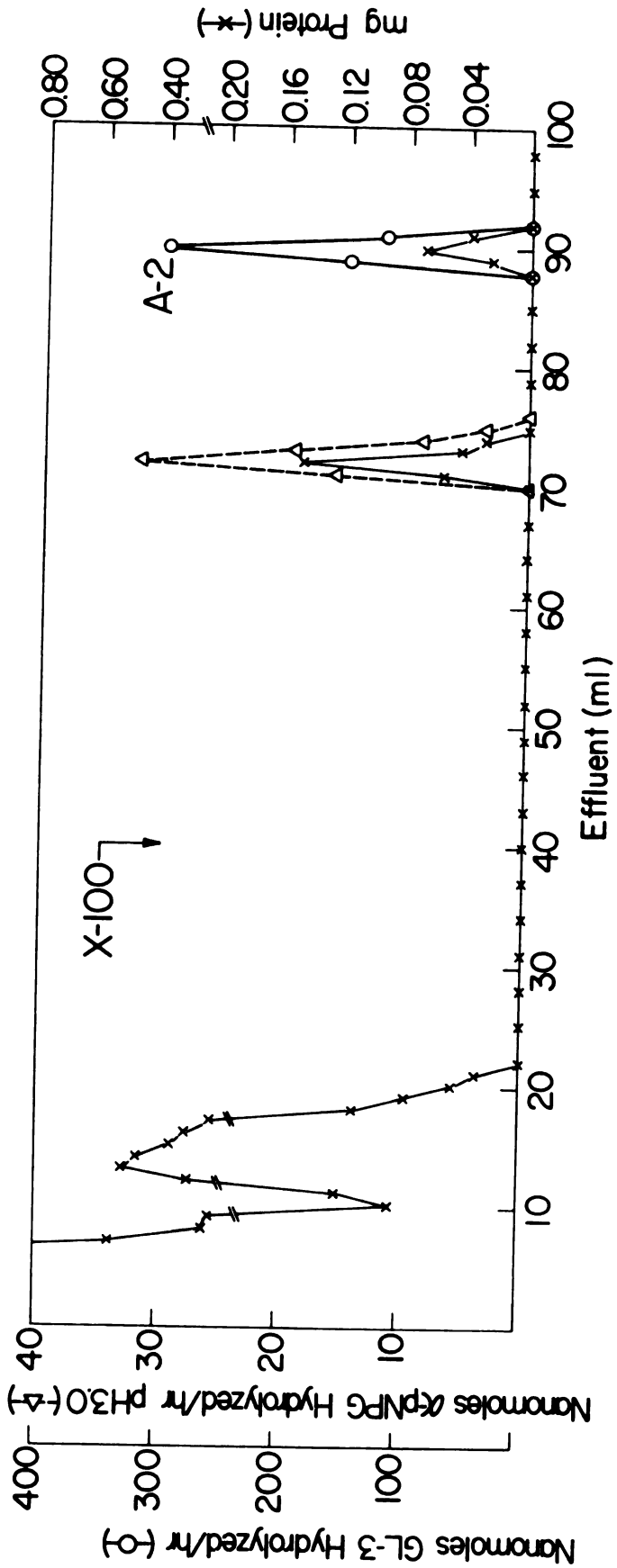
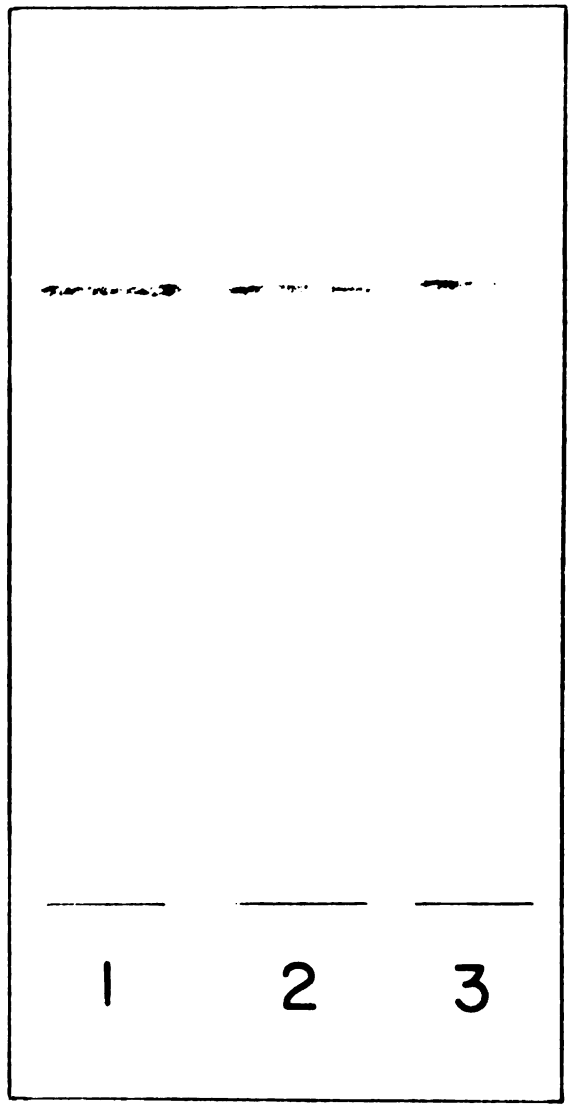

Nanomoles GL-3 Hydrolyzed/hr (○)
Nanomoles αGpNPG Hydrolyzed/hr pH3.0 (-△-)
mg Protein (-×-)
Effluent (ml)
X-100
A-2
400
300
200
100
40
30
20
10
0.80
0.60
0.40
0.20
0.16
0.12
0.08
0.04
10
20
30
40
50
60
70
80
90
100

Figure 19. Comparison of Kidney and Plasma Ceramide Trihexosidases, Form A, by Cellulose Acetate Electrophoresis

The ceramide trihexosidases obtained from the affinity column were concentrated against polyethylene glycol 6000. Protein (8 μg) was applied to the strips which were stained for activity. Electrophoresis was carried out at room temperature. The strips are (1) plasma A-1, (2) plasma A-2, and (3) kidney. The strips were redrawn for purposes of photography.


1
2
3

barium acetate precipitation, and diaflo (183). In each case the proteins either were not precipitated or were inactivated. It was found that enzymatic activity could be precipitated from Cohn fraction IV-1 by 100% saturation with ammonium sulfate, but the enzymes obtained by this method were unstable, having half-lives of approximately 12 hours.

The ceramide trihexosidases, Form A, appeared to bind to Sephadex G-75 and a 2,000-fold purification was obtained by chromatographing Cohn fraction IV-1 on this gel. The enzymes were excluded in the void volume when chromatographed on Sephadex G-50. A combination of these two steps resulted in an impure enzyme preparation which was associated with lipoprotein, as judged by cellulose acetate electrophoresis of the protein followed by comparative staining for activity, protein and lipoprotein.

Ceramide trihexosidase, Form A, was separated into two enzymatically active fractions by elution of Cohn fraction IV-1 from DEAE-cellulose using a sodium chloride gradient (0.05-0.8 M). This procedure resulted in a 5% purification accompanied by loss of about 50% of the enzymatic activity.

CHARACTERIZATION OF THE CERAMIDE TRIHEXOSIDASES, FORM A

Substrate Specificity

The purification of α-galactosidases from Cohn fraction IV-1 was monitored with GL-3, 4-methylumbelliferyl-α-galactoside and p-nitrophenyl-α-galactoside as substrates.

The specific activities in the crude Cohn fraction and the dialyzed acetone precipitate, as determined with the various substrates, are shown in Table 12.

During the purification two discrepancies indicated that the glycolipid and artificial substrates were not measuring the same enzymatic activity. There was a partial loss of the α-galactosidase activity detected with the artificial substrates, whereas there was no decrease in the enzymatic activity measured with GL-3. Furthermore, the fold-purification measured with the artificial substrates could not be correlated with that obtained using GL-3 as substrate, even after taking into account the loss of non-specific α-galactosidase activity.

Figure 20 shows the elution profile of the dialyzed acetone precipitate from the affinity column. Two fractions of ceramide trihexosidase were obtained, neither of which had detectable activity toward the artificial substrates. In addition, a single fraction was obtained that hydrolyzed both 4-methylumbelliferyl-α-galactoside and p-nitrophenyl-α-galactoside, but had no detectable activity toward GL-3. Thus affinity chromatography resulted in complete separation of the GL-3-cleaving α-galactosidases from the enzymes hydrolyzing the artificial substrates.

Investigation of the Enzymes used in Sequencing GL-3

In view of the specificity for lipid substrates shown by the ceramide trihexosidases, it was of interest to determine whether α-galactosidases obtained from ficin,

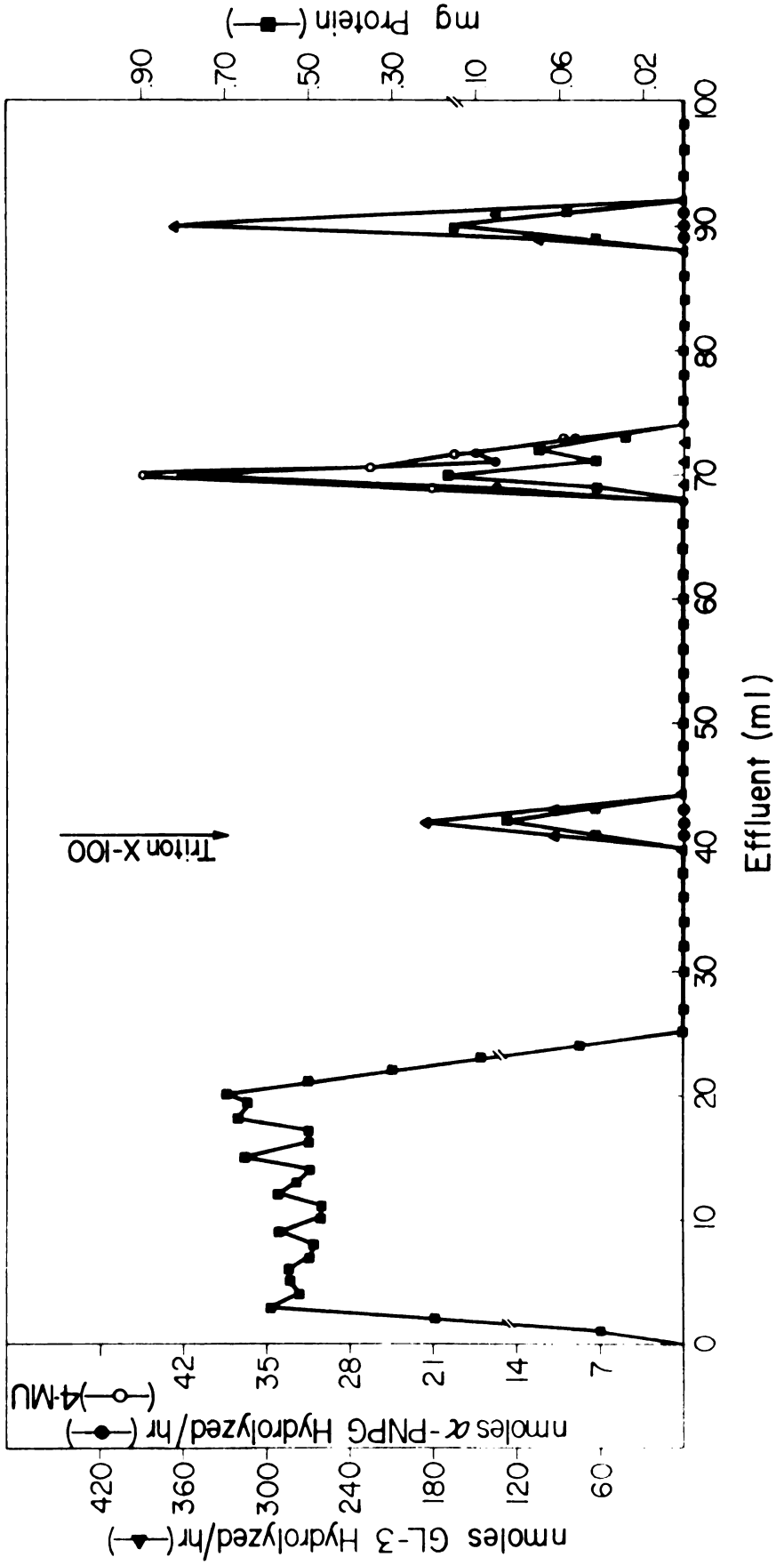
Table 12. Comparison of Enzymatic Activity using Natural and Artificial Substrates

The Cohn fraction and dialyzed acetone precipitate were assayed for enzymatic activity as described in Materials and Methods. In this case sodium chloride and sodium taurocholate were added to assay ceramide trihexosidase activity in the dialyzed acetone precipitate.

	Specific Activity nmoles (mg protein)$^{-1}$ (hr)$^{-1}$	
Substrate	Cohn frac. IV-1	Dialyzed Acetone Ppt.
CTH	0.07	138
α-4-MU	0.12	92
α-pNPG	0.10	76

Figure 20. Substrate Specificity of Plasma Ceramide Trihexosidases, Form A

Affinity chromatography of the dialyzed acetone precipitate prepared from 250 gm of Cohn fraction IV-1. Aliquots (0.1 ml) of each 1 ml fraction were assayed for enzymatic activity using both artificial and lipid substrates.


nmoles GL-3 Hydrolyzed/hr (—▲—)
nmoles α-PNPG Hydrolyzed/hr (—●—)
(—○—)4-MU
mg Protein (—■—)
Triton X-100
Effluent (ml)
420
360
300
240
180
120
60
42
35
28
21
14
7
.90
.70
.50
.30
.10
.06
.02
0
10
20
30
40
50
60
70
80
90
100

reportedly hydrolyzing GL-2b and GL-3 as well as non-specific α-galactoside substrates (184), might contain an enzyme specific for hydrolysis of GL-3. Affinity chromatography of ficin, shown in Figure 21, revealed three primary peaks of α-galactosidase activity. One of these hydrolyzed both lipid and non-specific α-galactoside substrates, one hydrolyzed only the artificial substrate, and one was specific for hydrolysis of GL-3.

Electrophoretic Properties

Electrophoretic investigation of the ceramide trihexosidases, Form A, was undertaken to obtain information regarding the purity of these enzymes as well as to delineate any differences between the two forms.

Isoelectric Focusing: Isoelectric focusing (Figure 22) of the ceramide trihexosidases, Form A, showed that both forms of the enzyme have a pI of 3.0, thereby indicating that both proteins have the same electrical charge. In both cases all of the detectable protein was associated with ceramide trihexosidase activity. The specific activity of both enzymes before and after isoelectric focusing was constant within experimental error and the same as that obtained following affinity chromatography.

Polyacrylamide Gel Electrophoresis: The proteins obtained by affinity chromatography were electrophoresed on 1% SDS, 9% polyacrylamide gels as described in Materials and

Figure 21. Affinity Chromatography of Ficin

Ficin (10 mg) was dissolved in 0.05 M sodium acetate buffer, pH 4.5, and applied to the affinity column which has been equilibrated with the same buffer. The column was eluted with buffer until no protein was detectable, then 0.1% Triton X-100 was added to the buffer and elution was continued.

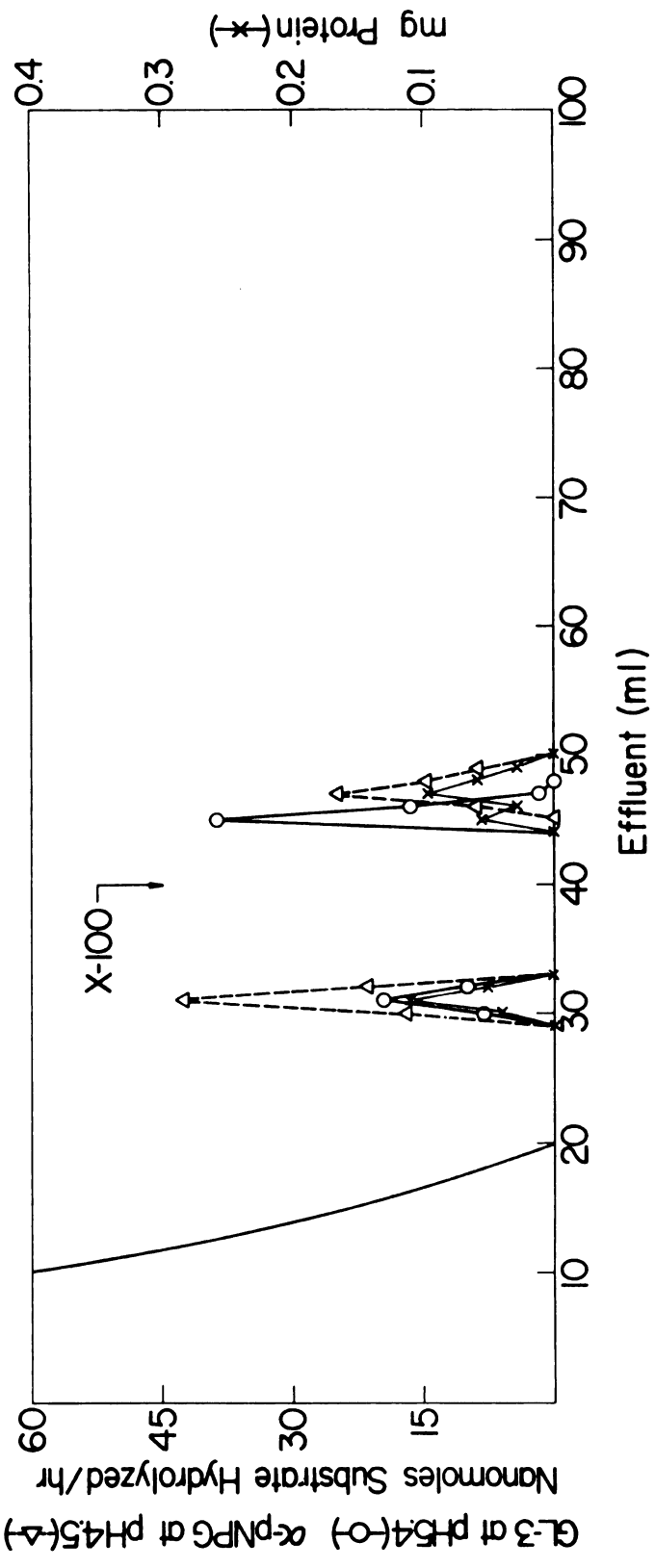
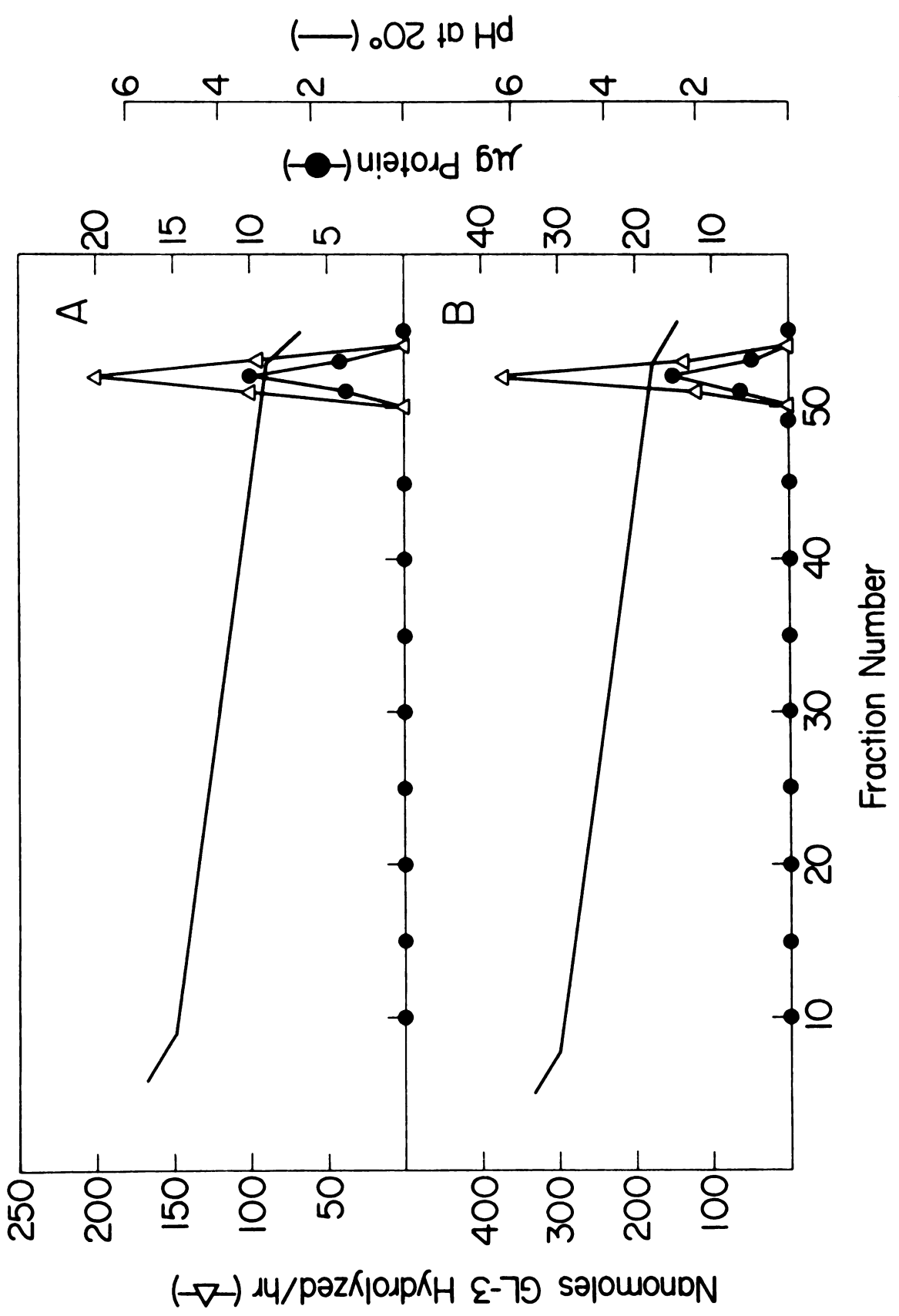

GL-3 at pH5.4(-○-) α-pNPG at pH4.5(-△-)
Nanomoles Substrate Hydrolyzed/hr
60
45
30
15
X-100
10
20
30
40
50
60
70
80
90
100
Effluent (ml)
mg Protein(-×-)
0.4
0.3
0.2
0.1

Figure 22. Isoelectric Focusing of the Plasma Ceramide Trihexosidases, Form A

Ceramide trihexosidase A-1 (A) and A-2 (B) obtained from the affinity column were focused in a supportive sucrose gradient for 72 hours at 1^{o}. The fractions (2 ml) were assayed for enzymatic activity without removal of the carrier ampholytes.


Nanomoles GL-3 Hydrolyzed/hr (—△—)
250
200
150
100
50
400
300
200
100
A
B
20
15
10
5
40
30
20
10
μg Protein (—●—)
pH at 20° (——)
6
4
2
6
4
2
10
20
30
40
50
Fraction Number

Methods. As shown in Figure 23, both proteins exhibited the same electrophoretic mobility. Only one protein band was detected for Form A-1 by developing with Coomassie Blue and scanning at 540 nm, whereas a minor impurity migrating slightly behind Form A-2 was detected. By this method the molecular weight of ceramide trihexosidase was approximately 15,000 as determined by comparison of its electrophoretic mobility with that of seven standard proteins.

Electrophoresis of the ceramide trihexosidases on native gels was not successful since the protein would not penetrate 5, 7, 9, or 15% gels.

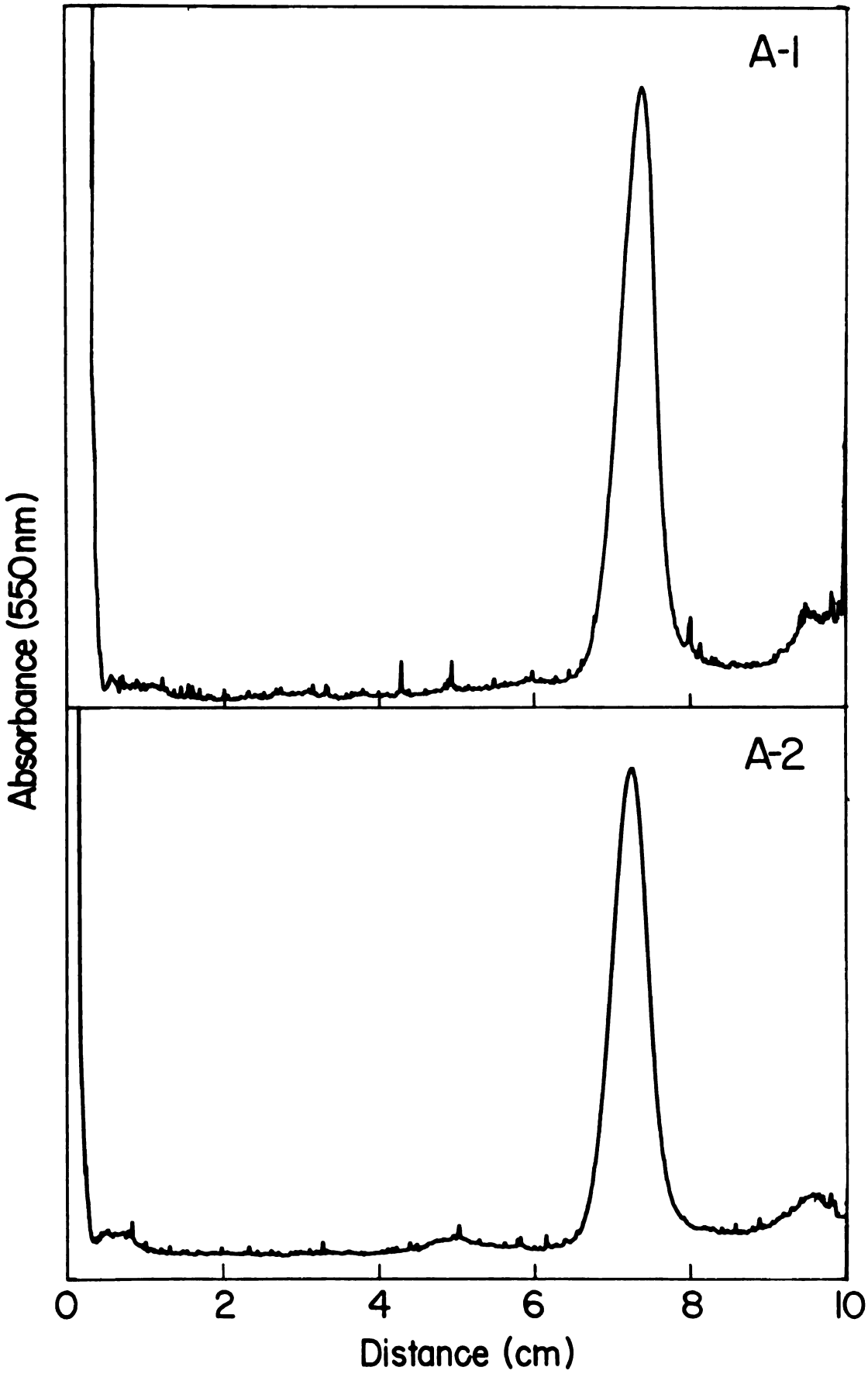
Estimation of Molecular Weight

Sucrose Density Gradient Centrifugation: Sucrose density gradient centrifugation was employed in order to obtain a minimum molecular weight for the ceramide trihexosidases and to gain further information regarding their purity. By sedimentation velocity both forms of the native protein had an apparent molecular weight of 95,000 and in each case all of the detectable protein not associated with the marker enzymes was coincident with ceramide trihexosidase activity.

Gel Filtration: Due to the discrepancy in molecular weights obtained by electrophoresis and sucrose gradient centrifugation, an independent determination was made by gel chromatography on Bio-Gel A-5m, using the method of

Figure 23. Scans from Polyacrylamide Gel Electrophoresis of Ceramide Trihexosidase A-1 and A-2

The 1% SDS, 9% acrylamide gels were pre-electrophoresed prior to applying the protein. The ceramide trihexosidases (50 μg) were dissolved in a 5% sucrose solution containing 1% SDS and 2% 2-mercaptoethanol, layered on the gel, and electrophoresed at 4°. The gels were then developed with Coomassie Blue and scanned at 550 nm in a Gilford spectrophotometer with a linear transport attachment.


A-1
A-2
Absorbance (550nm)
0
2
4
6
8
10
Distance (cm)

Fish (171). A molecular weight of 90,000, as shown in Figure 24C and 24D, was obtained for both forms of ceramide trihexosidase using this method. This value was in good agreement with that obtained by centrifugation and indicated that the value obtained by SDS gel electrophoresis was that of a subunit.

The molecular weights of the ceramide trihexosidases were determined by gel filtration in the presence of SDS and sulfhydryl reducing reagents using the method of Reynolds and Tanford (172). The molecular weights in each case, as shown in Figure 24A and 24B, were found to be 24,000. These results are consistent with a four-subunit protein having an approximate molecular weight of 95,000.

Heat Stability and Organic Solubility

The heat stability of the two forms of plasma ceramide trihexosidase is shown in Figure 25. Both forms of the enzyme exhibit slight heat stability. The most significant difference between the two enzymes is that Form A-1 retains 25% of its activity after boiling for 30 minutes; whereas Form A-2 activity is completely destroyed by the same boiling time.

Both of the ceramide trihexosidases, Form A, are active in various concentrations of butanol. Although their kinetic properties in this organic solvent have not been investigated, it is interesting to note that Form A-1 can be partitioned into butanol without loss of activity, whereas

Figure 24. Molecular Weight Determination by Gel Filtration

(A) and (B) Chromatography of the ceramide trihexosidases on a Bio Gel A-5m column (3 x 95 cm) in the presence of SDS and sulfhydryl reducing reagents.

(C) and (D) Chromatography of the ceramide trihexosidases on a Bio Gel A-5m column (3 x 95 cm).

K_{av} was calculated from the formula $K_{av} = (V_e - V_o)/(V_t - V_o)$ where V_o = the elution volume of Blue Dextran, V_e = elution volume of the proteins and V_t = column volume. The abbreviations are as follows: Cyto. c = cytochrome c; β-Lact. = β-lactoglobulin; Leu. Am. = leucine aminopeptidase; Hem. = hemoglobin; G-3-P Dehyd. = glyceraldehyde-3-phosphate dehydrogenase and CTHase = ceramide trihexosidase.

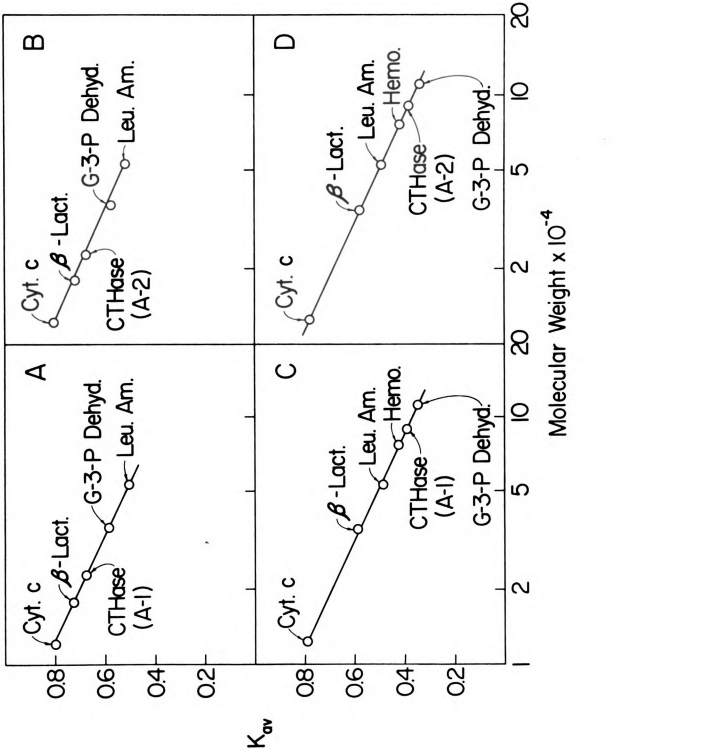
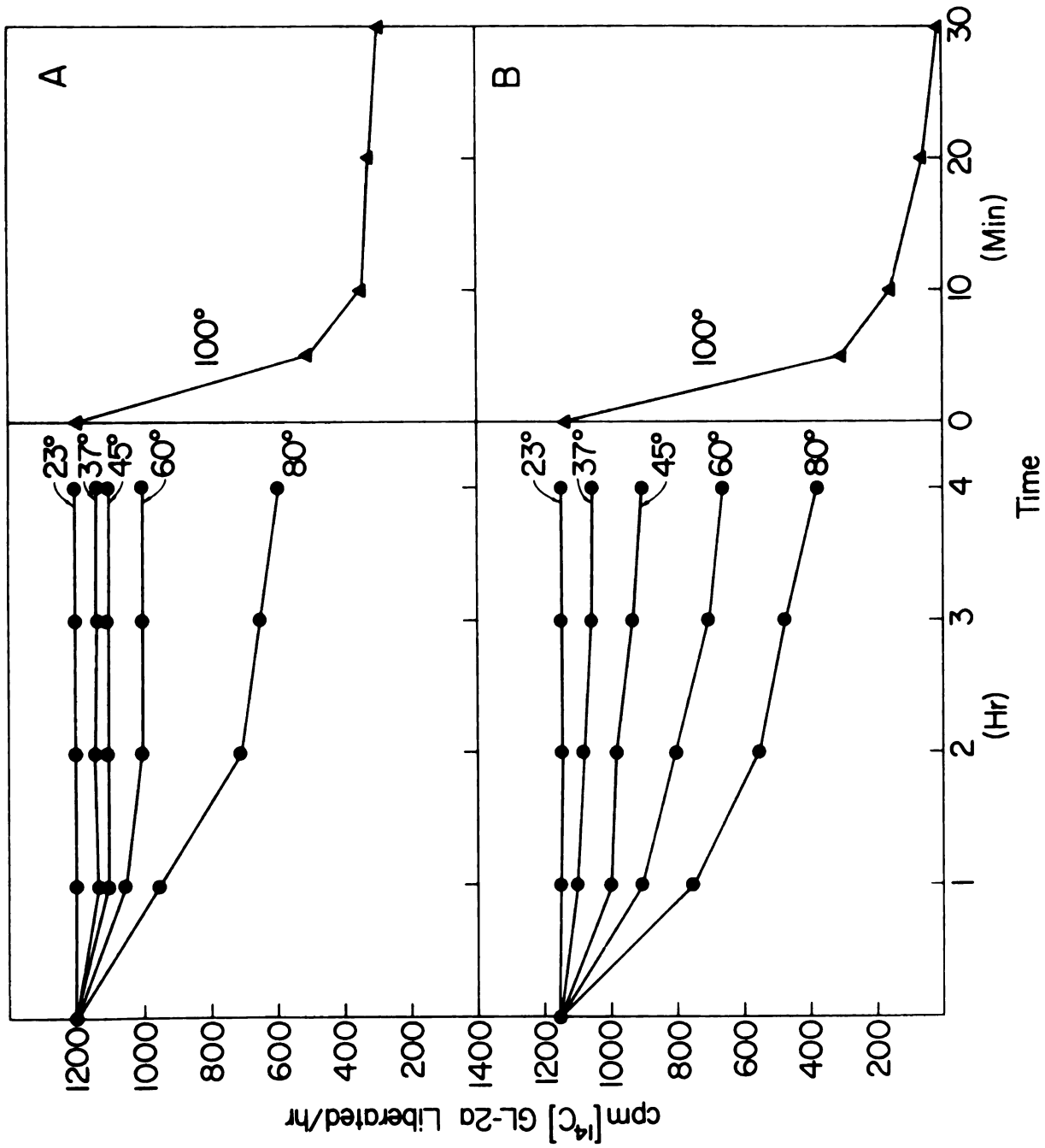

A
B
C
D
Cyt. c
β-Lact.
CTHase (A-1)
G-3-P Dehyd.
Leu. Am.
CTHase (A-2)
Hemo.
K_{av}
0.8
0.6
0.4
0.2
1
2
5
10
20
Molecular Weight x 10^{-4}

Figure 25. Heat Stability of the Ceramide Trihexosidases, Form A-1 (A) and Form A-2 (B)

Incubations were carried out with a mixture of [^{14}C]GL-3 and unlabelled GL-3, and assays were performed by Method 3.


A
B
100°
23°
37°
45°
60°
80°
cpm [14C] GL-2a Liberated/hr
Time
(Hr)
(Min)

Form A-2 shows no decrease in specific activity with up to 20% butanol. Above 20% butanol the mixture becomes biphasic and the enzyme, Form A-2, remains in the aqueous phase.

Effect of Detergents, Salts, and Phospholipids on Enzymatic Activity

Detergents: Sodium cholate is used routinely in the enzymatic assay to solubilize the glycosphingolipid substrate; the concentration of this bile acid was chosen to give optimal enzymatic activity. It is known, however, that lysosomal enzymes for glycosphingolipid metabolism may be activated by addition of a second detergent, usually sodium taurocholate (34, 114). The effect of several detergents, commonly used to solubilize glycosphingolipids, on ceramide trihexosidase activity are summarized in Table 13. These results indicate that a combination of the ionic detergents, sodium taurocholate and sodium chloride, are most effective in enhancing enzymatic activity whereas the non-ionic detergent, Triton X-100, decreased the enzymatic activity.

Salts: The enhancement of enzymatic activity by taurocholate was investigated in the presence of salts since they affect the critical micellar concentration (CMC) of bile salt solutions (185) and also enhance the specific activity of some glycosphingolipid hydrolases (114). In addition it had been noted that some commonly used salts such as

Table 13. Effect of Detergents on the Specific Activity of Ceramide Trihexosidase

The incubations contained 0.2 μmole GL-3 plus 10,000 cpm [^{14}C]GL-3 solubilized in a given detergent, 8 μg ceramide trihexosidase, and 0.1 M citrate-phosphate buffer, pH 5.4, in a constant volume of 0.5 ml. Boiled enzyme controls were treated in an identical manner.

Detergent	mg/ml	Specific Activity* Form A-1	Form A-2
Sodium Cholate	1	1895	1768
	2	2230	2500
	4	1600	1521
Sodium Taurocholate	5	2280	2180
	10	2641	2730
	12	2340	2240
Sodium Deoxycholate	2	1760	1781
	4	1849	2000
	6	1740	1692
Triton X-100	2	670	800
	4	240	650
	6	120	432
Sodium Cholate (2 mg) + Sodium Taurocholate	5	2600	2650
	10	3231	3420
	12	2380	2830
Sodium Cholate (2 mg) + Triton X-100	2	904	1200
	4	876	950
	6	740	875
Sodium Taurocholate (10 mg) + Triton X-100	2	1212	1435
	4	928	1280
	6	542	850

* Specific Activity = cpm [^{14}C]GL-2a liberated per hr per mg protein.

ammonium sulfate and potassium phosphate inactivated the ceramide trihexosidases at various points throughout the purification. The effect of salts on ceramide trihexosidase activity is summarized in Table 14. The enzymes show a strong cation effect in that they are completely inactivated by 0.05-0.10 M potassium, whereas they are uneffected by 0.85 M sodium. A combination of sodium and chloride ions activate the ceramide trihexosidases but neither the sodium nor the chloride ion is required for enzymatic activity.

Phospholipids: Since lecithin enhanced enzymatic hydrolysis of oligosaccharides, the possibility that phospholipids would also serve as activators for hydrolysis of GL-3 was investigated. The results summarized in Table 15 indicate that lecithin and lysolecithin are equally effective in slightly enhancing enzymatic activity, whereas the other phospholipids investigated have no effect on this reaction.

Kinetics

Kinetics of Stimulation of Ceramide Trihexosidase by Sodium Taurocholate and Sodium Chloride: In the absence of both sodium chloride and sodium taurocholate a plot of ceramide trihexosidase activity *vs*. GL-3 concentration gave a sigmoidal curve for both of the A forms, as illustrated by the double reciprocal plots shown in Figure 26. The addition of 0.15 M sodium chloride and increasing

Table 14. Effect of Salts on the Specific Activity of The Ceramide Trihexosidases

The incubations contained 0.2 μmole GL-3 plus 10,000 cpm [^{14}C]GL-3, 2 mg sodium cholate, 8 μg ceramide trihexosidase, a given salt concentration, and 0.1 M citrate-phosphate buffer, pH 5.4, in a constant volume of 0.5 ml. Boiled enzyme controls were used. Specific activity is expressed as cpm of [^{14}C]GL-2a liberated per hr per mg protein.

Salt	M	Specific Activity Form A-1	Specific Activity Form A-2
None		2230	2500
NaCl	0.05	2250	2540
	0.15	3500	3600
	0.85	2229	2485
Na_2SO_4	0.15	2239	2450
	0.20	2238	2500
	0.50	2229	2488
Na_2PO_4	0.10	2230	2500
	0.25	2248	2495
	0.50	2239	2490
$Na_3C_6H_5O_7$	0.10	2228	2500
	0.25	2239	2498
	0.50	2235	2495
KCl	0.01	450	500
	0.05	<0.01	<0.01
	0.10	<0.01	<0.01
K_2PO_4	0.01	460	480
	0.05	<0.01	<0.01
	0.20	<0.01	<0.01
K_2SO_4	0.10	449	479
	0.20	<0.01	<0.01
	0.40	<0.01	<0.01
NH_4Cl	0.05	1120	1600
	0.15	480	580
	0.85	<0.01	<0.01
$(NH_4)_2SO_4$	0.50	2229	2498
	1.00	1156	1200
	2.00	<0.01	<0.01

Table 15. Effect of Phospholipids on the Specific Activity of the Ceramide Trihexosidase (Form A)

The incubations contained 0.2 μmole GL-3 plus 10,000 cpm [^{14}C]GL-3, 2 mg sodium cholate, 8 μg ceramide trihexosidase, emulsified phospholipid, and 0.1 M citrate-phosphate buffer, pH 5.4, in a constant volume of 0.5 ml. Boiled enzyme controls were used. Specific activity is expressed as cpm of [^{14}C]GL-2a liberated per hr per mg protein.

Phospholipid	mg/ml	Specific Activity Form A-1	Specific Activity Form A-2
None		2238	2490
Lysophosphatidylcholine	4	2418	2600
	8	2800	2900
	10	2318	2500
Phosphatidylcholine	4	2630	2730
	8	3000	3100
	10	2421	2528
Phosphatidylethanolamine	4	2240	2500
	8	2237	2498
	10	2239	2496
Phosphatidylserine	4	2236	2490
	8	2240	2489
	10	2238	2491
Phosphatidylinositol	4	2236	2492
	8	2236	2490
	10	2238	2491
Sphingomyelin	4	2239	2490
	8	2237	2491
	10	2240	2490
Cardiolipin	4	2237	2490
	8	2236	2489
	10	2238	2490

Figure 26 (A). Lineweaver-Burk Plot Showing the Effect of Sodium Taurocholate on Ceramide Trihexosidase, Form A-1

The incubations contained 0.2 µmole GL-3 solubilized in 1 mg sodium cholate, 8 µg ceramide trihexosidase, 0.15 M sodium chloride, citrate-phosphate buffer, pH 5.4, and increasing concentrations of sodium taurocholate in a final volume of 0.5 ml. The sodium taurocholate concentrations are: no addition (-Δ-); 0.007 M (-x-); 0.022 M (-□-); and 0.03 M (-o-).

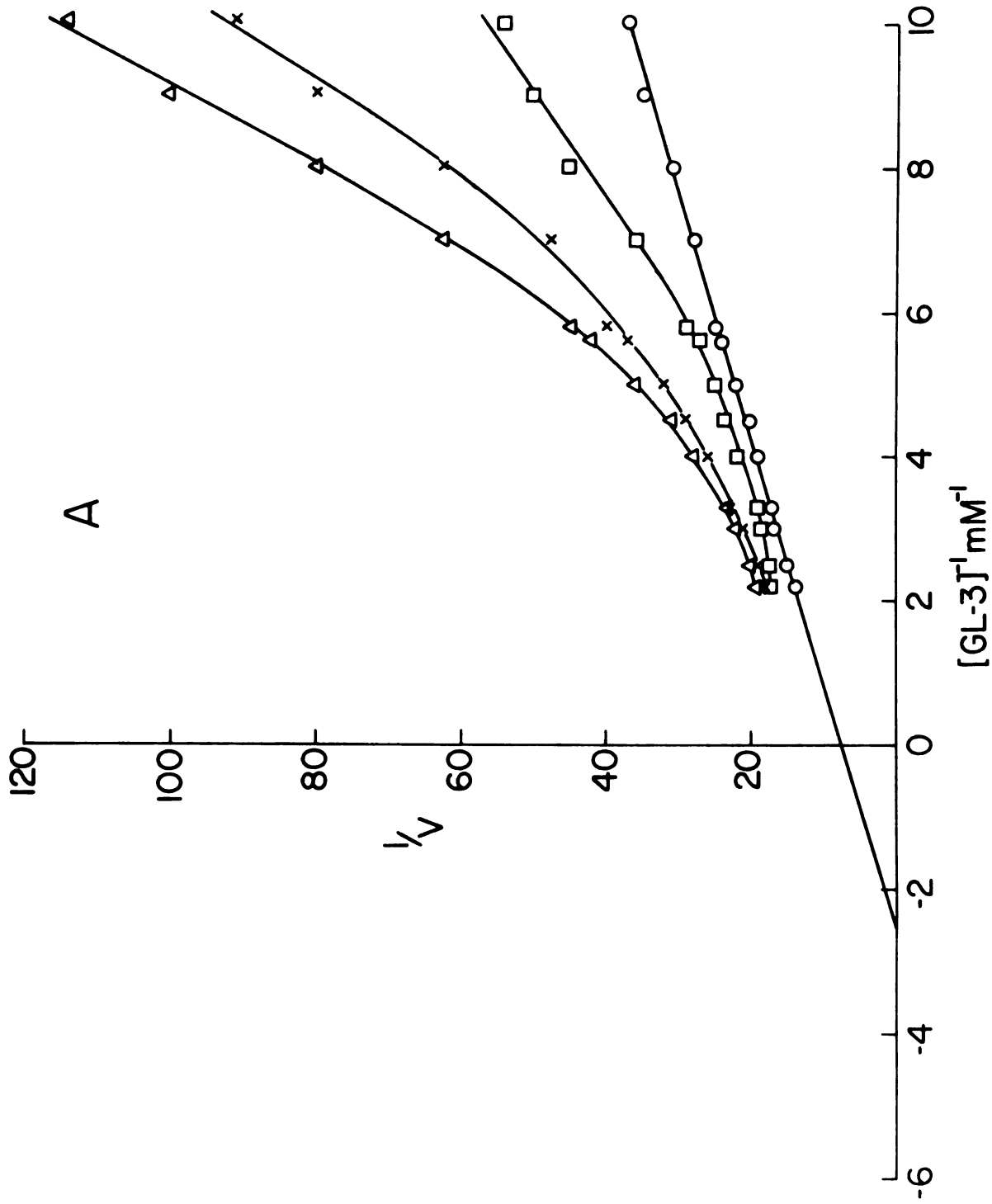
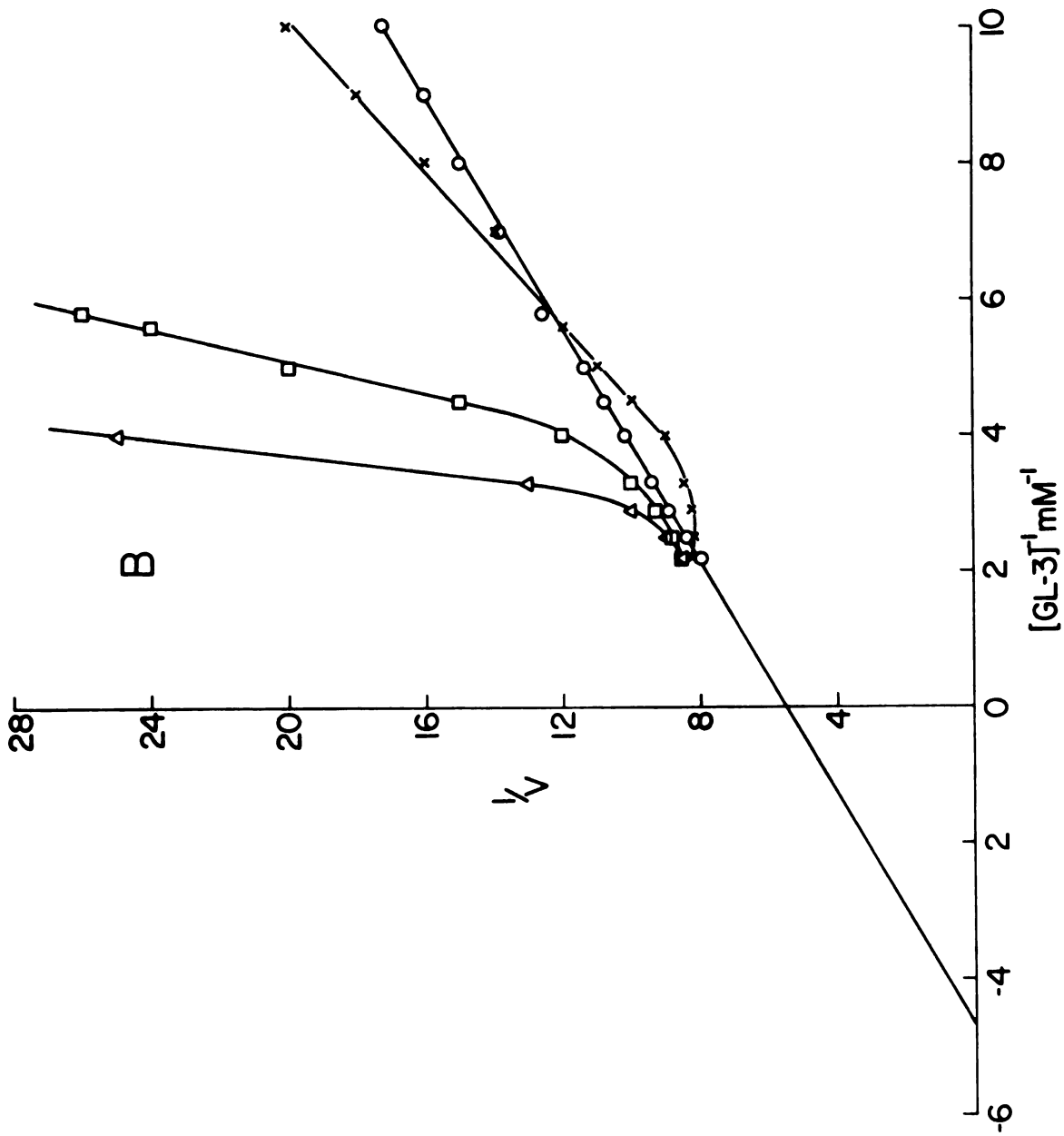

A
1/V
120
100
80
60
40
20
0
-2
-4
-6
2
4
6
8
10
[GL-3]⁻¹mM⁻¹

Figure 26 (B). Lineweaver-Burk Plot Showing the Effect of Sodium Taurocholate on Ceramide Trihexosidase, Form A-2

The concentrations of sodium taurocholate are: no addition (-Δ-); 0.14 M (-□-); 0.03 M (-x-); and 0.04 M (-o-).

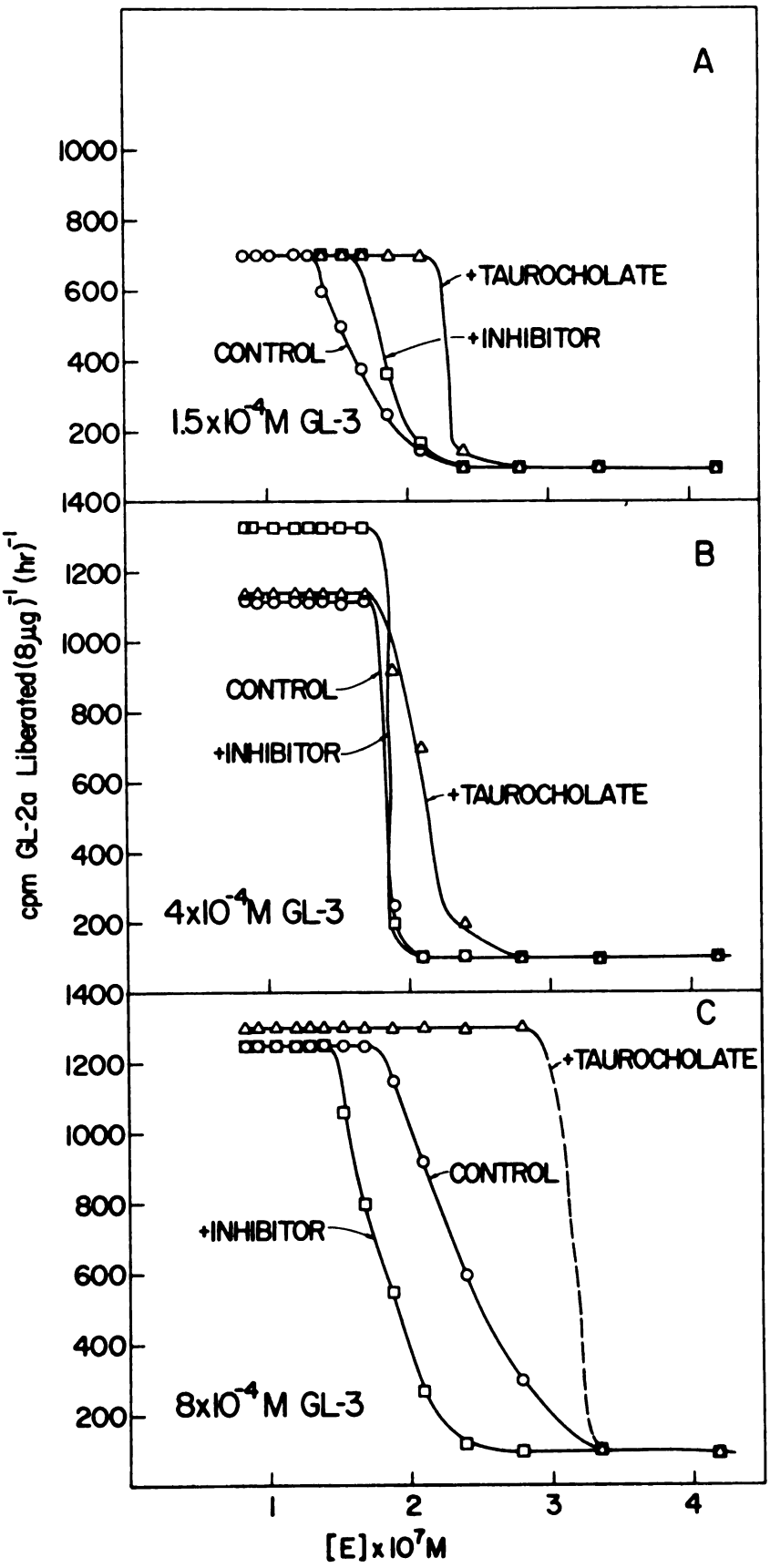

B
1/v
28
24
20
16
12
8
4
0
-4
-6
2
4
6
8
10
[GL-3]⁻¹ mM⁻¹

concentrations of sodium taurocholate decreased this sigmoidality but did not affect the maximal velocity. At optimal concentrations of sodium taurocholate (0.04 M for Form A-2 and 0.03 M for Form A-1) and 0.15 M sodium chloride the lag phase which occurs at low substrate concentrations was completely eliminated. Under these conditions, half-maximal velocity occurred at 4.5×10^{-4} M GL-3 for Form A-1 and at 5.0×10^{-4} M GL-3 for Form A-2. The K_m's for ceramide trihexosidases, Form A-1 and A-2, determined from the double reciprocal plot of velocity *vs.* substrate concentration, were 2.1×10^{-4} M and 2.2×10^{-4} M, respectively.

Effect of Enzyme Concentration on Hydrolysis of GL-3: The effect of ceramide trihexosidase, Form A-1, on the hydrolysis of GL-3 was compared in the presence and absence of both activators and inhibitors. The results of this experiment, shown in Figure 27, demonstrated that the enzymatic activity at a particular substrate concentration was dependent on both the enzyme concentration and the presence of activators or inhibitors. At a substrate concentration of 4×10^{-4} M or greater and in the absence of activators or inhibitors, the enzyme becomes inactive when its concentration exceeds 1.68×10^{-7} M, whereas with substrate concentrations of less than 4×10^{-4} M, the enzyme becomes inactive at a lower enzyme concentration.

Figure 27. Effect of Enzyme Concentration on the Hydrolysis of GL-3

The assays were carried out in volumes ranging from 0.2 ml to 1.0 ml, so that each incubation in a series contained a constant concentration of GL-3 as shown in the figure plus 10,000 cpm of [^{14}C]GL-3, 8 μg of ceramide trihexosidase, Form A-1, 1 mg of sodium cholate, 0.15 M sodium chloride, and sodium citrate-phosphate buffer, pH 5.4. Incubations with detergent contained 0.03 M sodium taurocholate; those with inhibitor contained 0.2 μmole of GL-2b. The concentrations of reagents were kept constant by serial dilution of concentrated solutions.


A
B
C
1000
800
600
400
200
1400
1200
1000
800
600
400
200
1400
1200
1000
800
600
400
200
+TAUROCHOLATE
+INHIBITOR
CONTROL
1.5x10⁻⁴M GL-3
CONTROL
+INHIBITOR
+TAUROCHOLATE
4x10⁻⁴M GL-3
+TAUROCHOLATE
CONTROL
+INHIBITOR
8x10⁻⁴M GL-3
cpm GL-2a Liberated(8µg)⁻¹(hr)⁻¹
1
2
3
4
[E]x10⁷M

The addition of sodium taurocholate and salt to the system results in loss of activity at higher enzyme concentrations. Although the point at which inactivation begins is not altered with a substrate concentration of 4×10^{-4} M, the inactivation occurs at a slower rate with increasing enzyme concentration.

As shown in Figure 28, the enzyme is activated at low substrate concentrations and inhibited at high substrate concentrations in the presence of an inhibitor (GL-2b) when sodium taurocholate is omitted from the incubation. From this data it was postulated that linear kinetics could be obtained in the presence of sodium taurocholate and sodium chloride in the range of substrate concentrations being studied, if the enzyme concentration was 1.68×10^{-7} M or less.

Inhibition Studies: The various inhibitors of each of the ceramide trihexosidases were compared under a single enzyme concentration of 1.68×10^{-7} M. All incubations, buffered at pH 5.4, contained the optimal concentrations of detergent and salt. Under these conditions, plasma ceramide trihexosidase, Form A-1, was competitively inhibited by trisaccharide [galactosyl(α1→4)galactosyl(β1→4)-glucose] and GL-2b, as shown in Figure 29.

As shown in Figure 30, ceramide trihexosidase, Form A-2, was inhibited by the products of the reaction, galactose and GL-2a. In addition it was competitively

Figure 28. The Effect of GL-2b on the Hydrolysis of GL-3

Enzymatic activity was measured using Method 3 with the omission of sodium chloride and sodium taurocholate from the incubation mixture. The incubations contained the following concentrations of GL-2b: no addition (-o-); 1 x 10^{-4} M (-●-); 2 x 10^{-4} M (-Δ-); and 4 x 10^{-4} M (-□-).

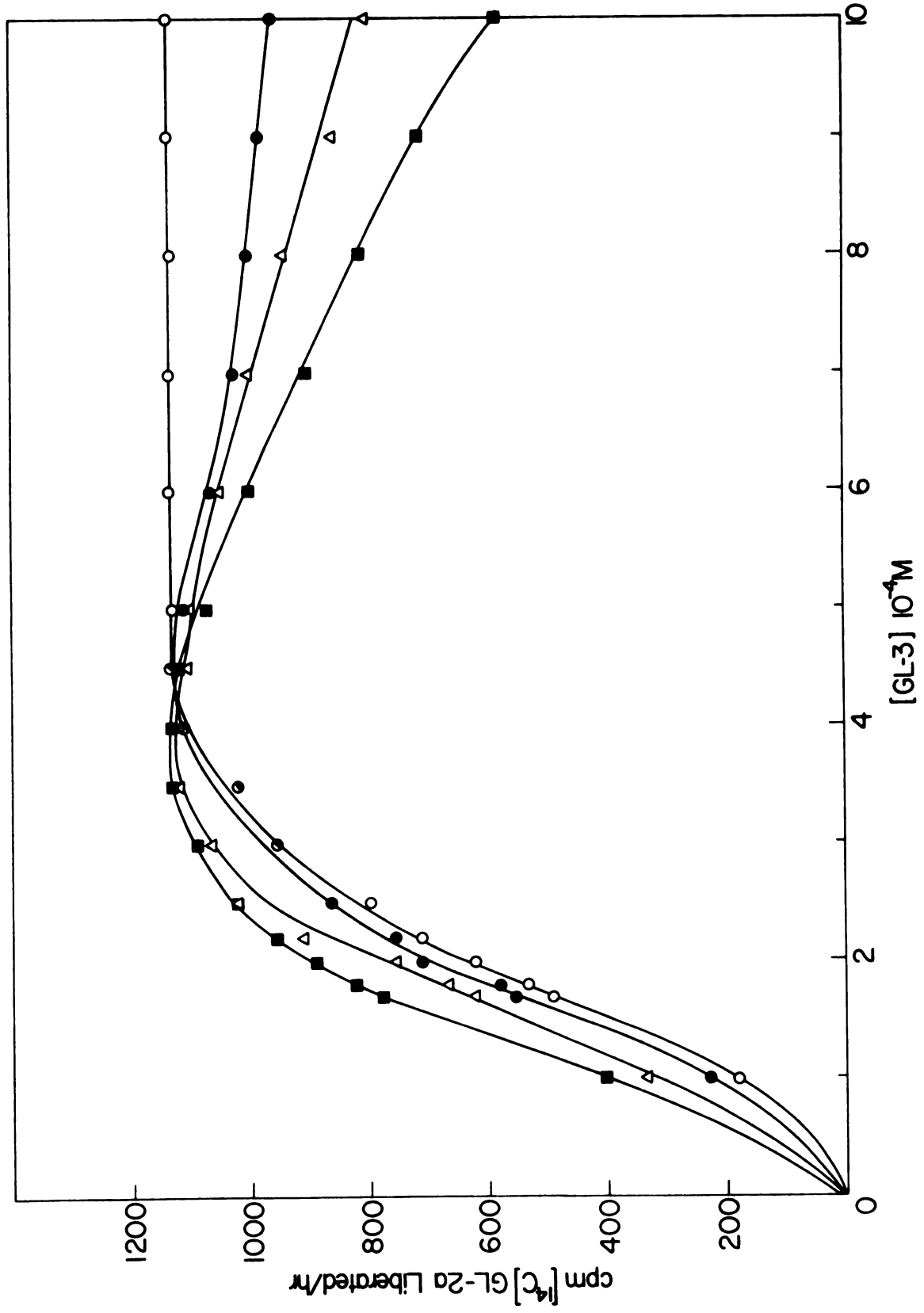

cpm [14C] GL-2a Liberated/hr
200
400
600
800
1000
1200
0
2
4
6
8
10
[GL-3] 10-4M

Figure 29 (A). Lineweaver-Burk Plot Showing the Inhibitory Effect of Trisaccharide on Ceramide Trihexosidase, Form A-1

Enzymatic activity was determined using Method 3. 1/V is expressed as [cpm [^{14}C]GL-2a liberated/hr]$^{-1}$ x 10^4. The inhibitor concentrations are 0.2 mM (-▲-) and 0.4 mM (-□-).

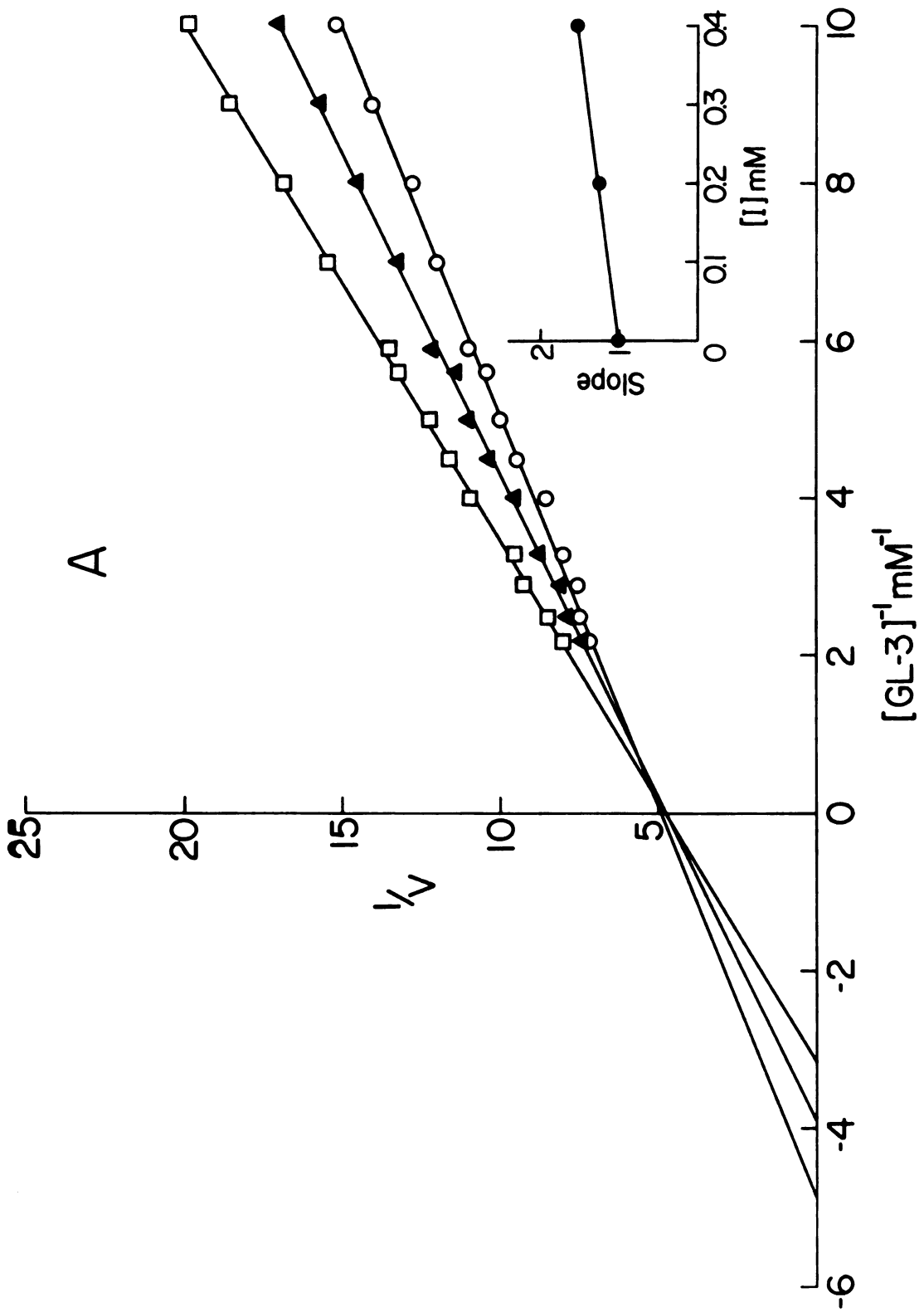

A
1/V
25
20
15
10
5
0
-2
-4
-6
2
4
6
8
10
[GL-3]⁻¹mM⁻¹
Slope
2
1
0
0.1
0.2
0.3
0.4
[I]mM

Figure 29 (B). Lineweaver-Burk Plot Showing the Inhibitory Effect of GL-2b on Ceramide Trihexosidase, Form A-1

Enzymatic activity was determined using Method 3. 1/V is expressed as [cpm [^{14}C]GL-2a liberated/hr]$^{-1}$ x 10^4. The inhibitor concentrations are 0.1 mM (- -); 0.2 mM(- -) and 0.4 mM (- -).

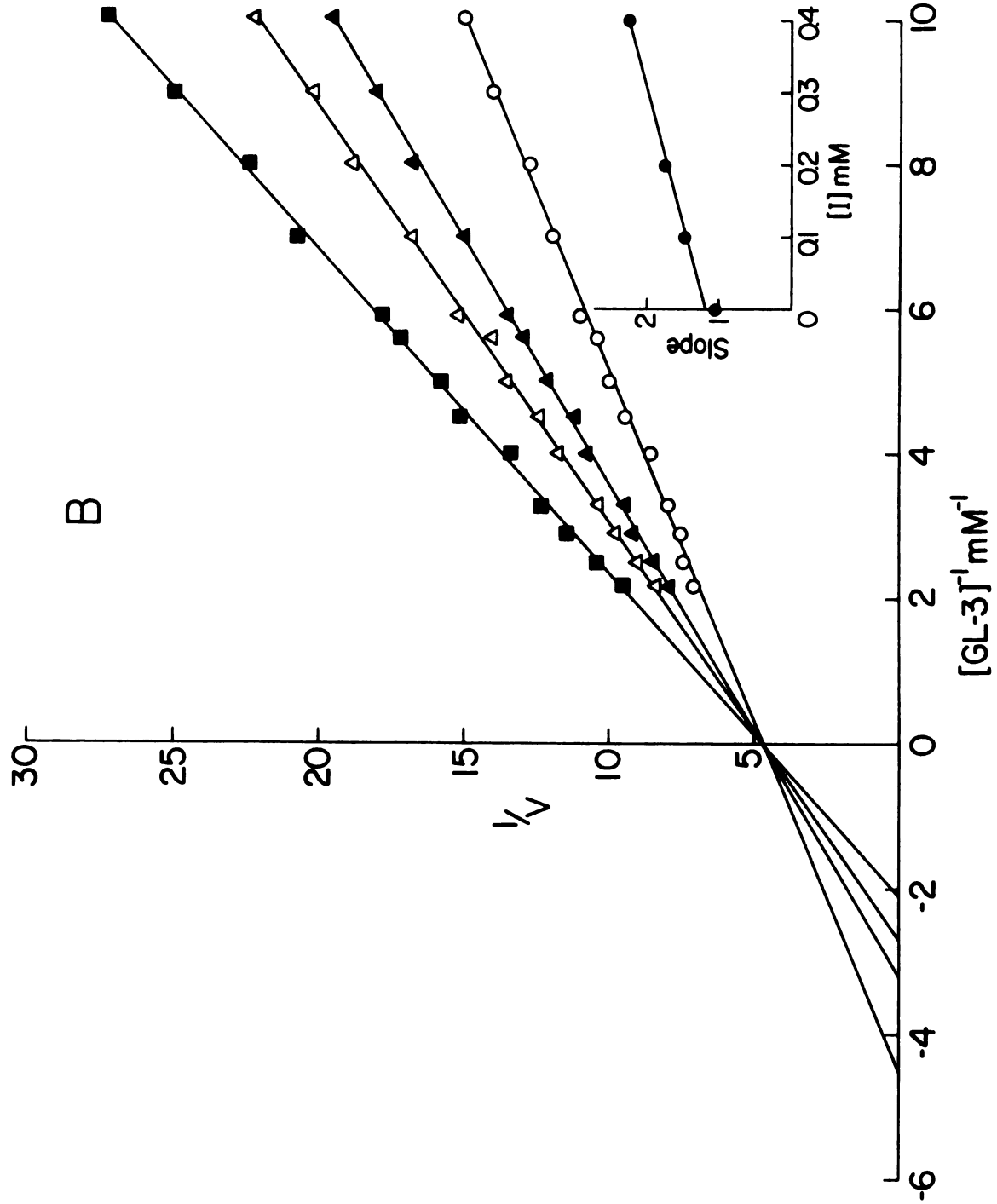

B
1/V
[GL-3]⁻¹ mM⁻¹
Slope
[I] mM

Figure 30 (A). Lineweaver-Burk Plot Showing the Inhibitory Effect of Galactose on Ceramide Trihexosidase, Form A-2

Enzymatic activity was determined using Method 3. 1/V is expressed as [cpm [^{14}C]GL-2a liberated/hr]$^{-1}$ x 10^{-4}. The inhibitor concentrations are 0.1 mM (-▲-), 0.2 mM (-Δ-) and 0.4 mM (- -).

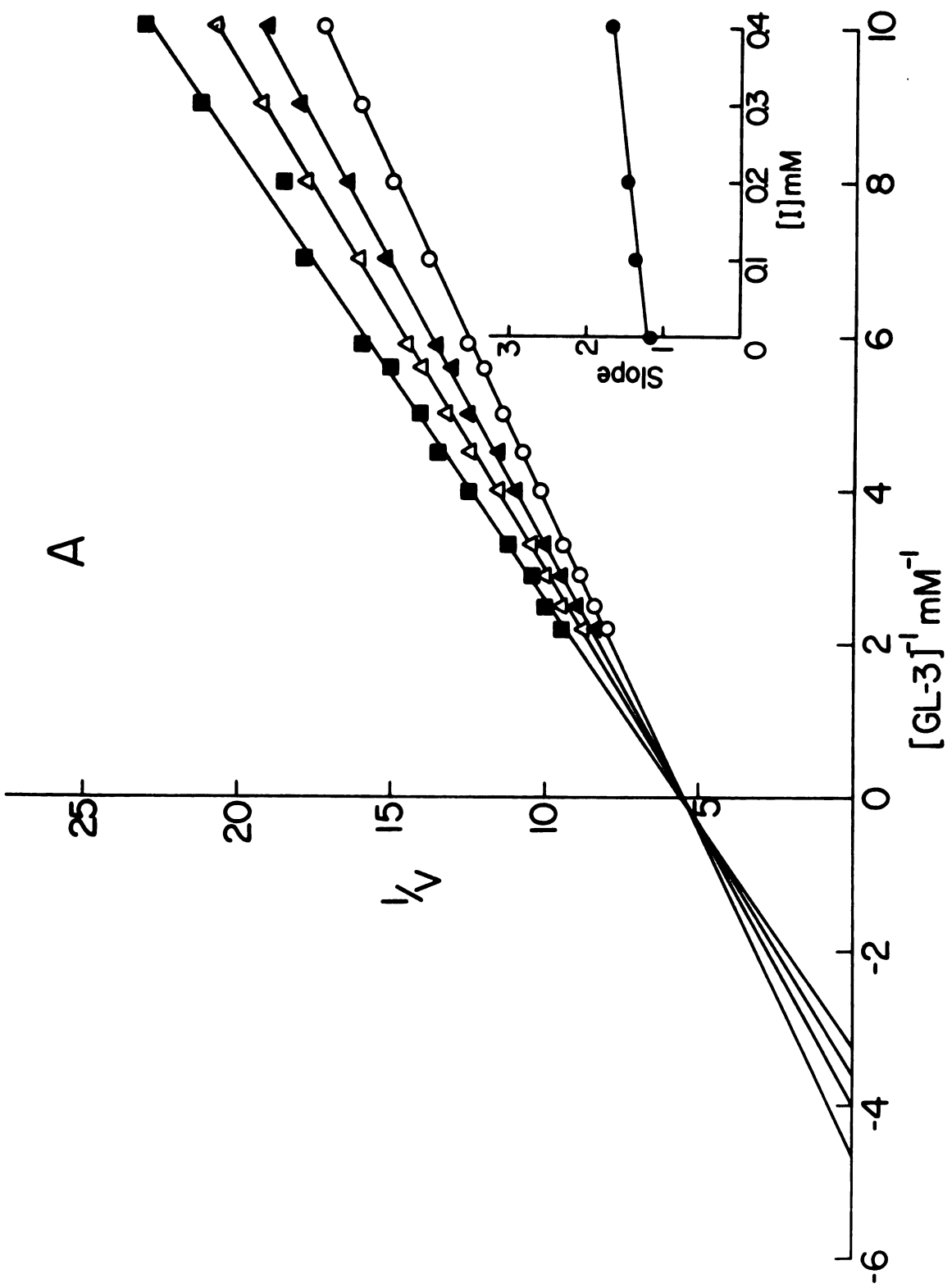

A
1/V
[GL-3]⁻¹ mM⁻¹
Slope
[I]mM

Figure 30 (B). Lineweaver-Burk Plot Showing the Inhibitory Effect of GL-2a on Ceramide Trihexosidase, Form A-2

The legend is the same as for part A.

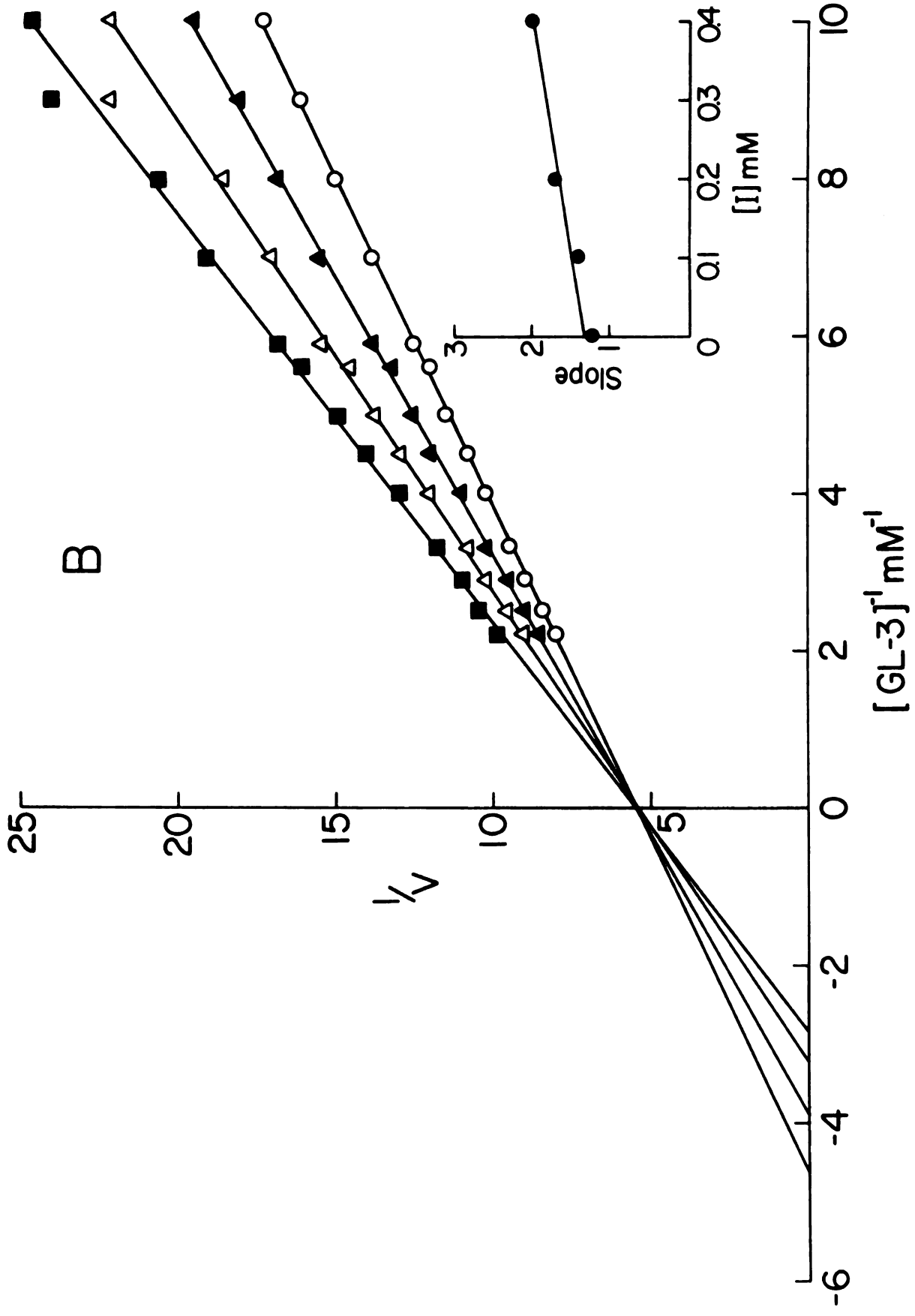

B
1/V
[GL-3]⁻¹mM⁻¹
Slope
[I]mM

Figure 30 (C). Lineweaver-Burk Plot Showing the Inhibitory Effect of Trisaccharide on Ceramide Trihexosidase, Form A-2

The legend is the same as for part A.

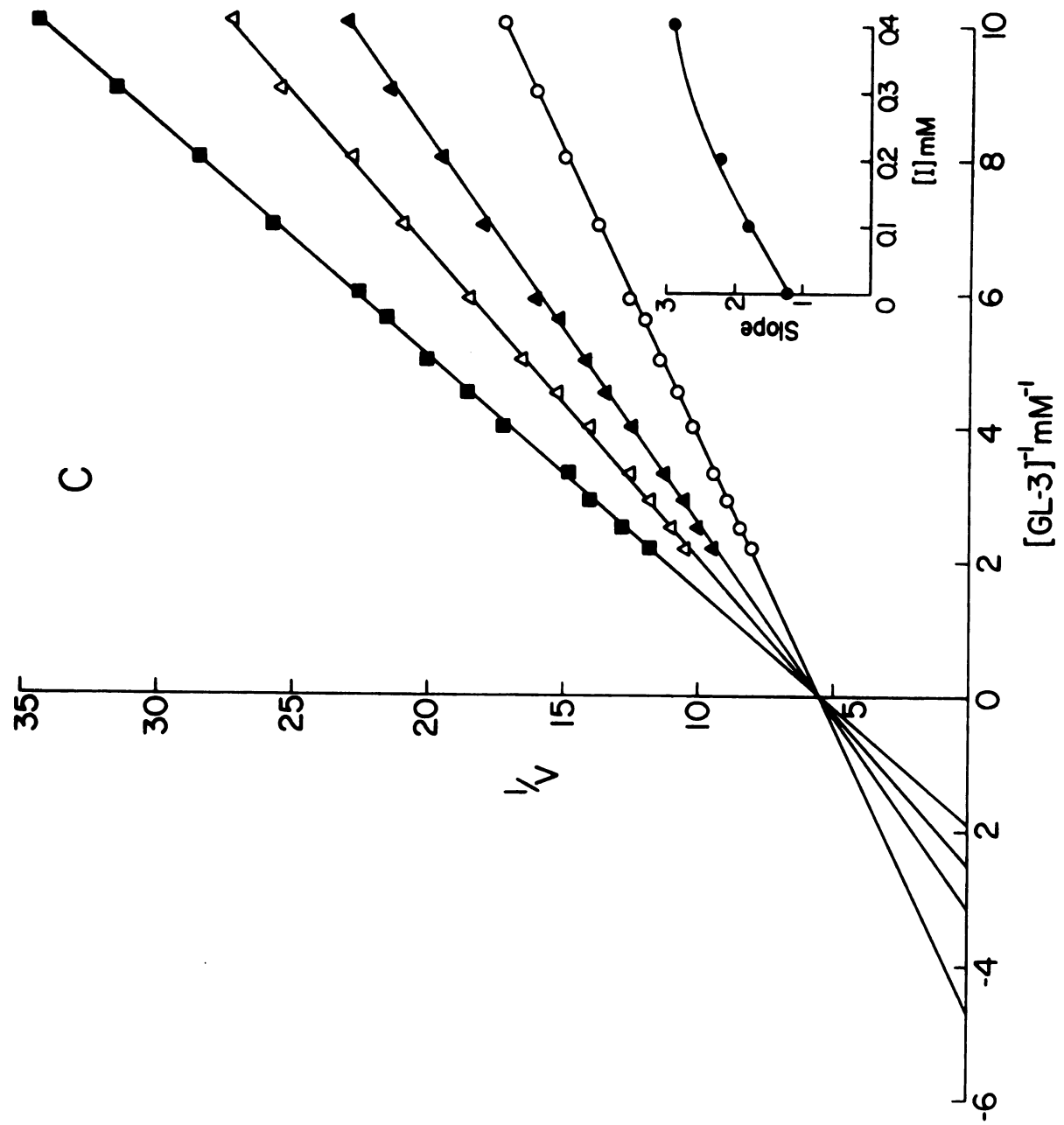

C
1/V
[GL-3]⁻¹mM⁻¹
Slope
[I]mM

Figure 30 (D). Lineweaver-Burk Plot Showing the Inhibitory Effect of GL-2b on Ceramide Trihexosidase, Form A-2

The legend is the same as for part A.

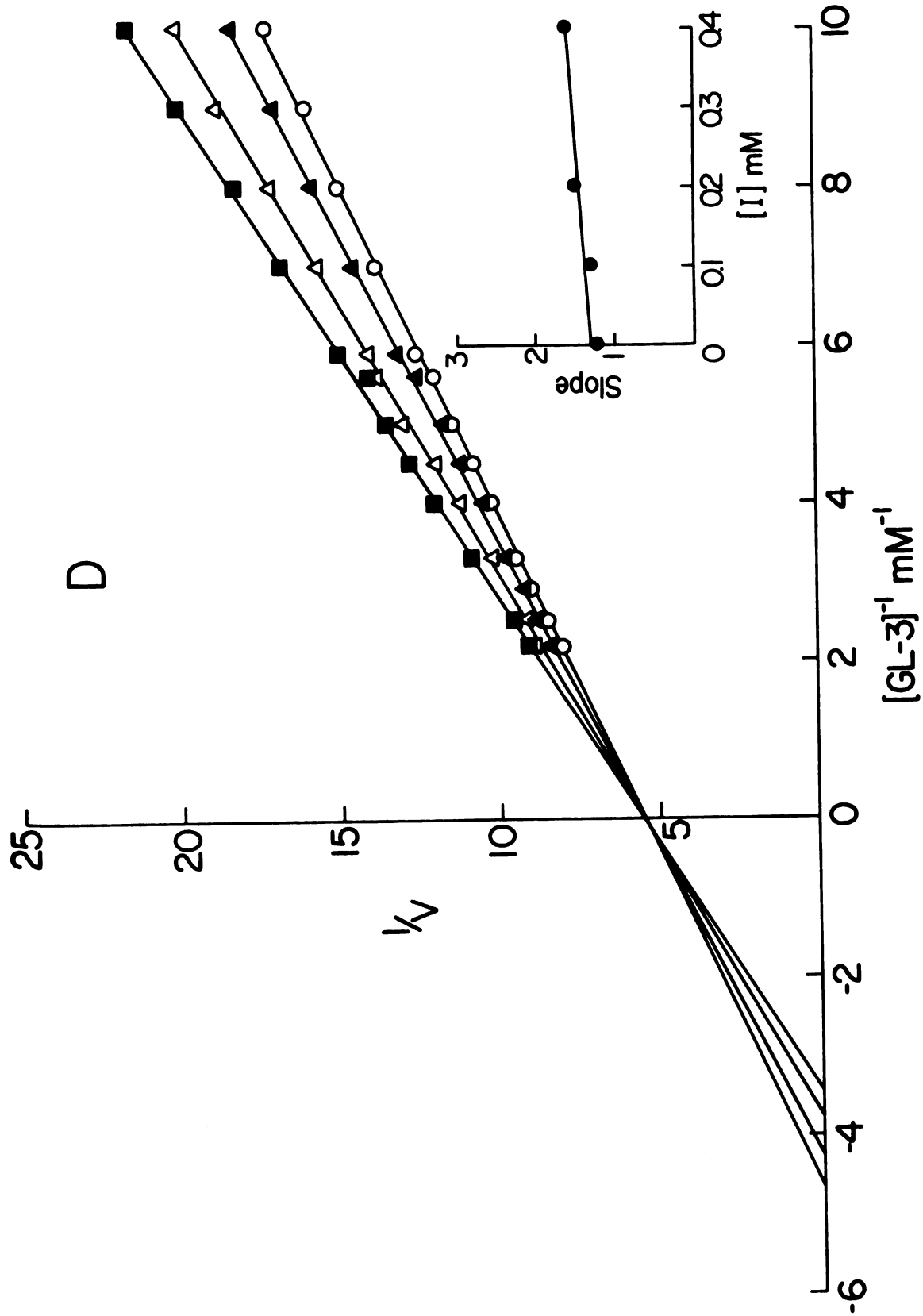
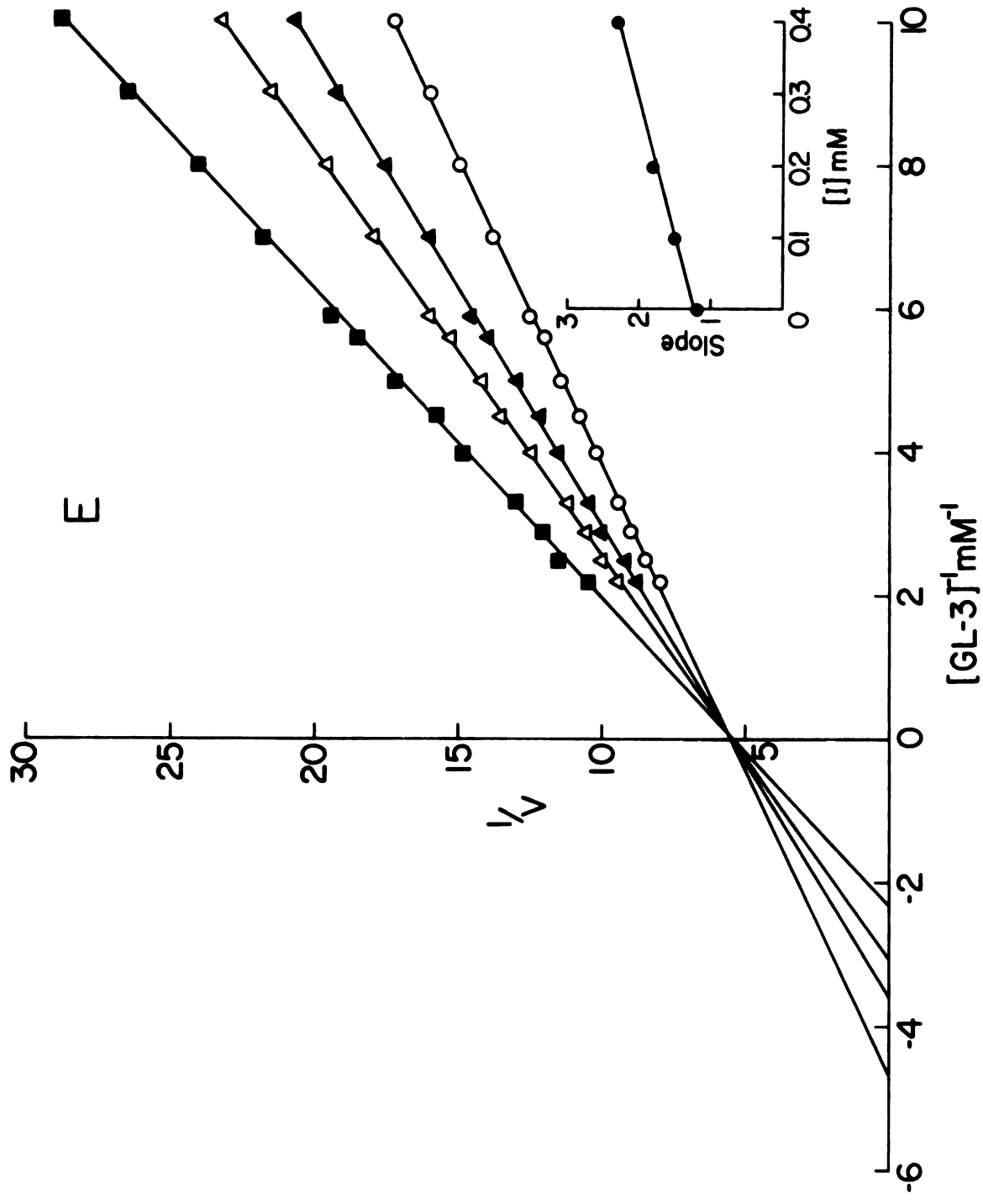

D
1/V
[GL-3]⁻¹ mM⁻¹
Slope
[I] mM

Figure 30 (E). Lineweaver-Burk Plot Showing the Inhibitory Effect of *myo*-Inositol on Ceramide Trihexosidase, Form A-2

The legend is the same as for part A.


E
1/v
[GL-3]⁻¹mM⁻¹
Slope
[I] mM

inhibited by GL-2b, trisaccharide and inositol. Under the conditions of this experiment neither form of the enzyme was inhibited by either p-nitrophenyl-α-galactoside or 4-methylumbelliferyl-α-galactoside as recently reported by Ho (123).

The kinetic parameters for the ceramide trihexosidases, as determined by computer analysis, are summarized in Table 16.

Classification by Gatt's Kinetic System: Bovine serum albumin and lecithin were used to form a pseudo-micellar system for hydrolysis of trisaccharide in the manner described in Materials and Methods (Method 5). The double reciprocal plots of velocity *vs.* trisaccharide concentration are shown in Figure 31.

Ceramide trihexosidases, Form A-1 has a non-symmetrical sigmoidal velocity *vs.* substrate saturation curve and according to Gatt's classification scheme is a Type IV sphingolipid hydrolase. This is the case in which the enzyme utilizes micelles but not monomers (124). Form A-2 has the classical Michaelis-Menton type V/S curve, under the conditions of this experiment, and is classified as a Type I enzyme. This type of curve is obtained when there is no monomer-micelle transition of the substrate (124).

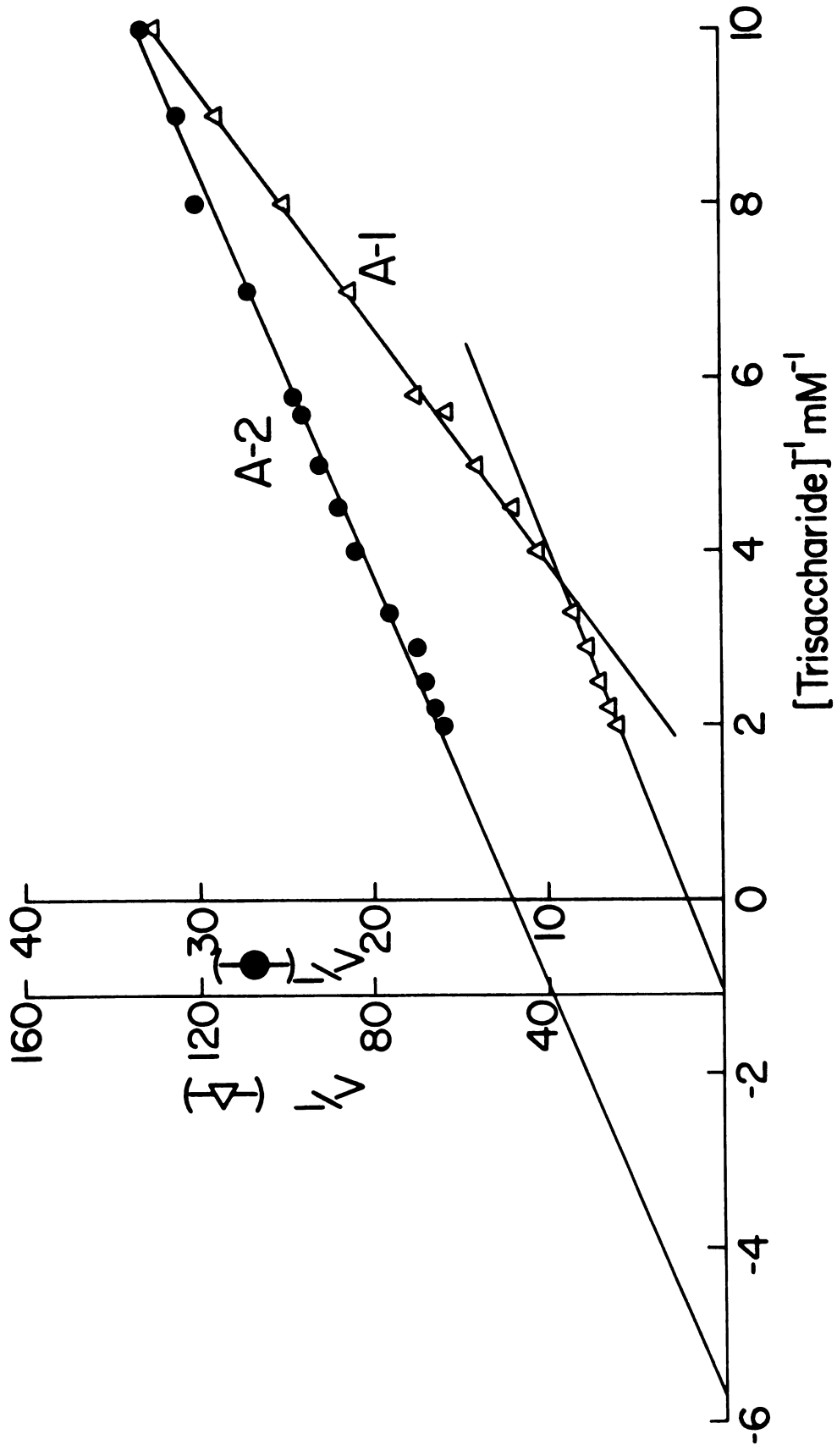
Table 16. Summary of Kinetic Parameters

Kinetic analyses were made using the ModHill computer program. K_i's were calculated from the intercept of the slope *vs.* intercept plots for the various inhibitors. In the cases where this plot was not a straight line the K_i was determined using the tangent to the curved line.

Ceramide Trihexosidase, Form A-1	
V_{max}	$= 4.5 \times 10^{-4}$ M
K_m	$= 2.3 \times 10^{-4}$ M
K_i (GL-2b)	$= 3.0 \times 10^{-4}$ M
K_i (Trisaccharide)	$= 8.3 \times 10^{-4}$ M
Ceramide Trihexosidase, Form A-2	
V_{max}	$= 5.0 \times 10^{-4}$ M
K_m	$= 2.5 \times 10^{-4}$ M
K_i (Galactose)	$= 7.0 \times 10^{-4}$ M
K_i (GL-2a)	$= 5.0 \times 10^{-4}$ M
K_i (*myo*-Inositol)	$= 4.3 \times 10^{-4}$ M
K_i (Trisaccharide)	$= 2.3 \times 10^{-4}$ M
K_i (GL-2b)	$= 1.2 \times 10^{-3}$ M

Figure 31. The Effect of Increasing Trisaccharide Concentration on Ceramide Trihexosidase Activity

Enzymatic activity was determined using Method 5. 1/V is expressed as [nmoles of galactose liberated/2 hr]$^{-1}$ x 10^3.


A-2
A-1
1/V
160
120
80
40
40
30
20
10
0
-2
-4
-6
2
4
6
8
10
[Trisaccharide]⁻¹ mM⁻¹

α-GALACTOSIDASES OF WHOLE PLASMA

Affinity Chromatography of Normal Plasma

Whole plasma was investigated by affinity chromatography to determine whether the ceramide trihexosidases could be studied without fractionation of plasma. As shown in Figure 32A all of the ceramide trihexosidases obtained by Cohn fractionation of plasma were adsorbed to the affinity column and eluted in nearly the same fractions as the partially purified enzymes. However it should be noted that in whole plasma the ceramide trihexosidases, Form A, are present in nearly equal quantities; whereas Cohn fraction IV-1 contains more Form A-2 than Form A-1.

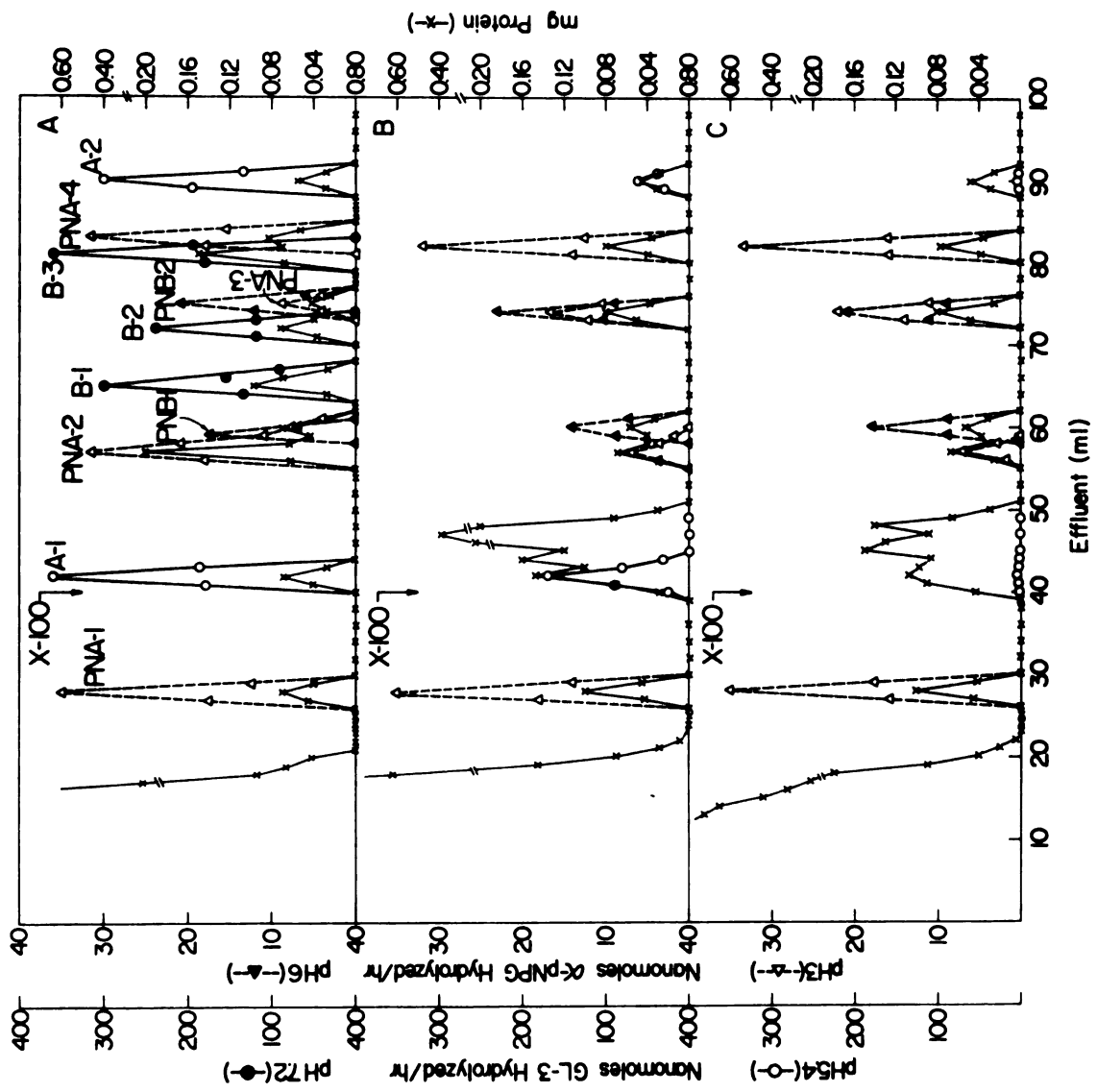
The fold-purification for the ceramide trihexosidases, Forms A and B, determined by comparison of the specific activity obtained after affinity chromatography with that obtained for whole plasma are 48,989 and 12,676, respectively. Polyacrylamide electrophoresis indicated that Form A-1 was about 90% pure and Form A-2 was about 50% pure.

In addition to the ceramide trihexosidases six non-specific α-galactosidases were obtained by affinity chromatography of whole plasma. The four α-galactosidases hydrolyzing p-nitrophenyl-α-galactoside at pH 3.0 were designated PN-A and were numbered in the order in which they were eluted; the two α-galactosidases, catalytically active at pH 6.0 were designated PN-B-1 and PN-B-2.

Figure 32. Affinity Chromatography of Whole Plasma

Whole plasma was adjusted to pH 5.4 with 0.1 M citric acid and percolated through the affinity column equilibrated with 0.001 M MES buffer, pH 5.4. (A) Affinity chromatography of normal plasma; (B) Affinity chromatography of heterozygous Fabry plasma and (C) Affinity chromatography of hemizygous Fabry plasma.

The data have been corrected to a constant volume of 100 ml of plasma. No correction was made for individual variations in plasma protein concentrations.


A
B
C
X-100
PNA-1
pA-1
PNA-2
B-1
PNB1
PNB2
B-2
PNA-3
B-3
PNA-4
A-2
pH72(-●-)
pH6(-▲-)
pH54(-○-)
pH3(-△-)
Nanomoles GL-3 Hydrolyzed/hr
Nanomoles α-pNPG Hydrolyzed/hr
mg Protein (-×-)
Effluent (ml)

Affinity Chromatography of Fabry Plasma

In an attempt to determine whether there was an actual deficiency of ceramide trihexosidase in Fabry's disease or rather a genetic alteration resulting in a catalytically inactive protein, the multiple forms of ceramide trihexosidase in both heterozygous and hemizygous Fabry plasma were investigated. Affinity chromatography, shown in Figure 32B and C revealed the presence of both of the ceramide trihexosidases, Form A, which occur in normal plasma. The total enzymatic activity of both of the A forms obtained from hemizygous Fabry plasma was 1-2% that of normal; whereas Form A-1, obtained from heterozygous Fabry plasma, had 50% of normal activity and Form A-2 had from 10-30% of normal activity.

In addition to the α-galactosidases Fabry plasma from heterozygotes and hemizygotes contained catalytically inactive proteins which were not observed in normal plasma. These proteins were associated with the A-1 form of ceramide trihexosidase, and cellulose acetate electrophoresis of this fraction from the affinity column (Fractions 39-50 in Figure 33) indicated that they were glycoproteins of similar electrophoretic mobilities.

In contrast to the Form A enzymes there was a total absence, in both hemizygous and heterozygous Fabry plasma, of all the proteins associated with the enzymatic activity of ceramide trihexosidase, Form B. This finding suggests

Figure 33. Comparison of Normal and Fabry Ceramide Trihexosidase by Cellulose Acetate Electrophoresis

The proteins obtained from the affinity column were concentrated against polyethylene glycol 6000. The cellulose acetate strips were electrophoresed at room temperature and stained for glycoprotein. The strips are (1) normal plasma, A-1; (2) heterozygous Fabry plasma, A-1; (3) hemizygous Fabry plasma, A-1; (4) normal plasma, A-2; (5) heterozygous Fabry plasma, A-2; and (6) hemizygous plasma, A-2.

umide

lumn were

The cellulose

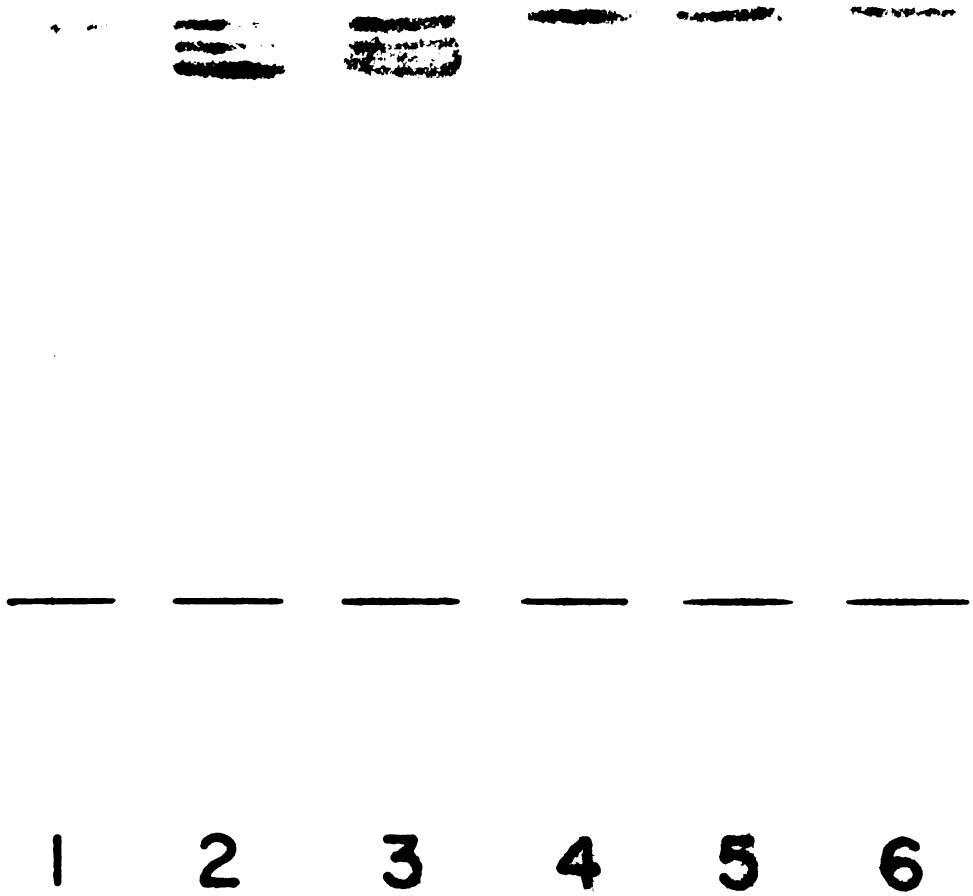
perature

(1) normal

;

lasma, A-2;

nizygous



that the ceramide trihexosidases, Form B, were converted to the catalytically inactive proteins which accumulate in Fabry plasma.

In addition to the alteration of the proteins having ceramide trihexosidase activity, the activity of the non-specific α-galactosidase, PN-A-2 was depressed in both hemizygous and heterozygous Fabry plasma, whereas the activity of PN-A-3 was elevated.

A consistent pattern of protein and enzymatic activity, summarized in Table 17, was obtained from the plasma of eight different Fabry's, although the ratio of the individual proteins and their activity showed some variations.

The finding that only one of the non-specific α-galactosidases in Fabry plasma had depressed activity is not compatible with the success of artificial substrates in diagnosing Fabry's disease. Therefore the α-galactosidase activity in whole plasma, as summarized in Table 18, was compared before and after affinity chromatography.

Prior to affinity chromatography the non-specific α galactosidase activity measured at pH 3.0 was depressed in Fabry plasma. Hemizygous plasma had approximately 20% of normal activity, while the activity in heterozygous Fabry plasma was from 70-90% that of normal. Following affinity chromatography, the combined activity of the PN-A α-galactosidases, obtained from both hemizygous and heterozygous Fabry plasma, was about 98% of that observed

Table 17. Summary of the Protein and Activity Obtained for the Individual α-Galactosidases Following Affinity Chromatography

	Mg Protein										
	mg Ceramide Trihexosidase					mg Non-specific α-Galactosidase					
Hemizygotes	A-1	A-2	B-1	B-2	B-3	PN-A-1	PN-A-2	PN-A-3	PN-A-4	PN-B-1	PN-B-2
Ri.L.	1.02	0.10	<0.01	<0.01	<0.01	0.19	0.13	0.15	0.15	0.12	0.15
Ro.L.	1.25	0.11	<0.01	<0.01	<0.01	0.12	0.13	0.14	0.15	0.13	0.16
D.L.	1.00	0.11	<0.01	<0.01	<0.01	0.18	0.12	0.17	0.16	0.13	0.16
C.L.	1.16	0.10	<0.01	<0.01	<0.01	0.18	0.13	0.15	0.13	0.12	0.15
M.C.	1.11	0.11	<0.01	<0.01	<0.01	0.13	0.15	0.17	0.14	0.13	0.14
F.L.	0.31	0.07	<0.01	<0.01	<0.01	0.14	0.12	0.14	0.14	0.14	0.15
Heterozygotes											
S.C.	1.24	0.11	<0.01	<0.01	<0.01	0.19	0.16	0.16	0.15	0.12	0.16
H.L.	1.49	0.12	<0.01	<0.01	<0.01	0.19	0.17	0.17	0.16	0.14	0.17
C.L.	1.45	0.10	<0.01	<0.01	<0.01	0.18	0.18	0.17	0.16	0.13	0.17
Normals (n=10)	0.14 ± 0.02	0.11 ± 0.01	0.14 ± 0.02	0.24 ± 0.01	0.29 ± 0.02	0.15 ± 0.02	0.32 ± 0.02	0.09 ± 0.02	0.20 ± 0.03	0.13 ± 0.03	0.09 ± 0.01

Total Activity

	nmoles GL-3 Hydrolyzed/hr					nmoles α-pNPG Hydrolyzed/hr					
Hemizygotes	A-1	A-2	B-1	B-2	B-3	PN-A-1	PN-A-2	PN-A-3	PN-A-4	PN-B-1	PN-B-2
Ri.L.	11.0	5.8	<1.0	<1.0	<1.0	68.4	11.6	47.0	65.1	34.7	40.9
Ro.L.	15.6	7.8	<1.0	<1.0	<1.0	70.0	10.5	46.4	67.4	34.9	43.8
D.L.	10.9	5.6	<1.0	<1.0	<1.0	65.9	12.3	45.9	66.2	36.7	49.7
C.L.	14.7	7.0	<1.0	<1.0	<1.0	72.1	14.2	48.0	59.8	30.2	50.0
M.C.	13.4	6.9	<1.0	<1.0	<1.0	64.3	9.8	47.2	63.5	40.1	38.6
F.L.	10.8	5.7	<1.0	<1.0	<1.0	67.2	14.3	46.3	65.0	32.6	47.2
Heterozygotes											
S.C.	321.6	126.4	<1.0	<1.0	<1.0	66.3	17.2	42.1	59.4	29.8	40.8
H.L.	398.4	133.0	<1.0	<1.0	<1.0	68.4	14.8	44.6	57.8	32.4	44.3
C.L.	392.8	130.1	<1.0	<1.0	<1.0	67.0	15.9	44.0	58.6	30.0	42.2
Normals (n=10)	728.0 ± 20.0	630.0 ± 20.0	684.0 ± 20.0	480.0 ± 20.0	738.0 ± 20.0	65.5 ± 10.5	93.5 ± 20.0	17.0 ± 10.0	64.9 ± 10.0	30.5 ± 10.0	44.3 ± 10.0

Table 18. Comparison of the Plasma α-Galactosidase Activity Before and After Affinity Chromatography

Duplicate assays utilizing p-nitrophenyl-α-galactoside as substrate were averaged. Duplicates varied by 5-10% using whole plasma at pH 3.0 and by 4-5% at pH 6.0. Following affinity chromatography duplicates varied by 0.5-1.0% at both pH optima.

Subject	Activity Before Affinity Chromatography				Activity Following Affinity Chromatography			
	α-pNPG		CTH		α-pNPG		CTH	
	pH 3.0	pH 6.0	pH 5.4	pH 7.2	pH 3.0	pH 6.0	pH 5.4	pH 7.2
	nmoles hydrolyzed $(hr)^{-1}$ $(100\ ml)^{-1}$							
Hemizygotes								
D.L.	39.8	40.1	<1.5	<1.5	190.3	86.4	16.5	<1.5
Ri.L.	33.2	43.2	<1.5	<1.5	192.1	75.6	16.8	<1.5
Ro.L.	42.4	41.0	<1.5	<1.5	194.3	78.7	23.4	<1.5
C.L.	35.0	38.9	<1.5	<1.5	194.1	80.2	21.7	<1.5
F.L.	68.0	50.6	<1.5	<1.5	192.8	79.8	16.5	<1.5
M.C.	74.0	48.4	<1.5	<1.5	184.8	78.7	20.3	<1.5
Heterozygotes								
S.C.	182.3	54.0	502.0	<1.5	185.0	70.6	448.0	<1.5
C.L.	168.4	43.6	480.0	<1.5	185.5	72.2	522.9	<1.5
H.L.	170.2	43.0	408.0	<1.5	185.6	76.7	531.4	<1.5
Normals (n=10)	257±30	49±20	800±20	1600±40	241±51	80±20	1358±40	1902±60

with normal plasma. These findings suggest that there is some type of inhibitor for the non-specific α-galactosidases in Fabry plasma which is removed by affinity chromatography.

Investigation of Digalactosylceramide:Galactosyl Hydrolase in Human Plasma

As shown in Figure 34, optimal hydrolysis of GL-2b occurred at pH 5.5. Since hydrolysis of GL-2b by the A and B forms of ceramide trihexosidase was negligible, the proteins obtained by affinity chromatography of whole plasma were assayed to determine whether one of the non-specific α-galactosidases coincided with an enzyme which would hydrolyze GL-2b (digalactosylceramide). The results of affinity chromatography of normal and Fabry plasma, illustrated in Figure 35, showed that the α-galactosidase hydrolyzing GL-2b in normal plasma is the same enzyme which shows depressed activity toward the artificial substrate, p-nitrophenyl-α-galactoside, in Fabry plasma.

Electrophoretic Investigation of Digalactosylceramide: Galactosyl Hydrolase: The enzymatic activity coincident with hydrolysis of both p-nitrophenyl-α-galactoside and GL-2b was investigated by isoelectric focusing. There were two primary protein peaks having pI's of 4.2 and 4.6, as shown in Figure 36. Both of these proteins were equally effective in cleaving either the artificial or natural substrates.

Figure 34. Effect of pH on the Hydrolysis of GL-2b

The curve of pH optimum was obtained using a solution of Cohn fraction IV-1. The pH was adjusted with 0.1 M citrate-phosphate buffer, pH 3.0

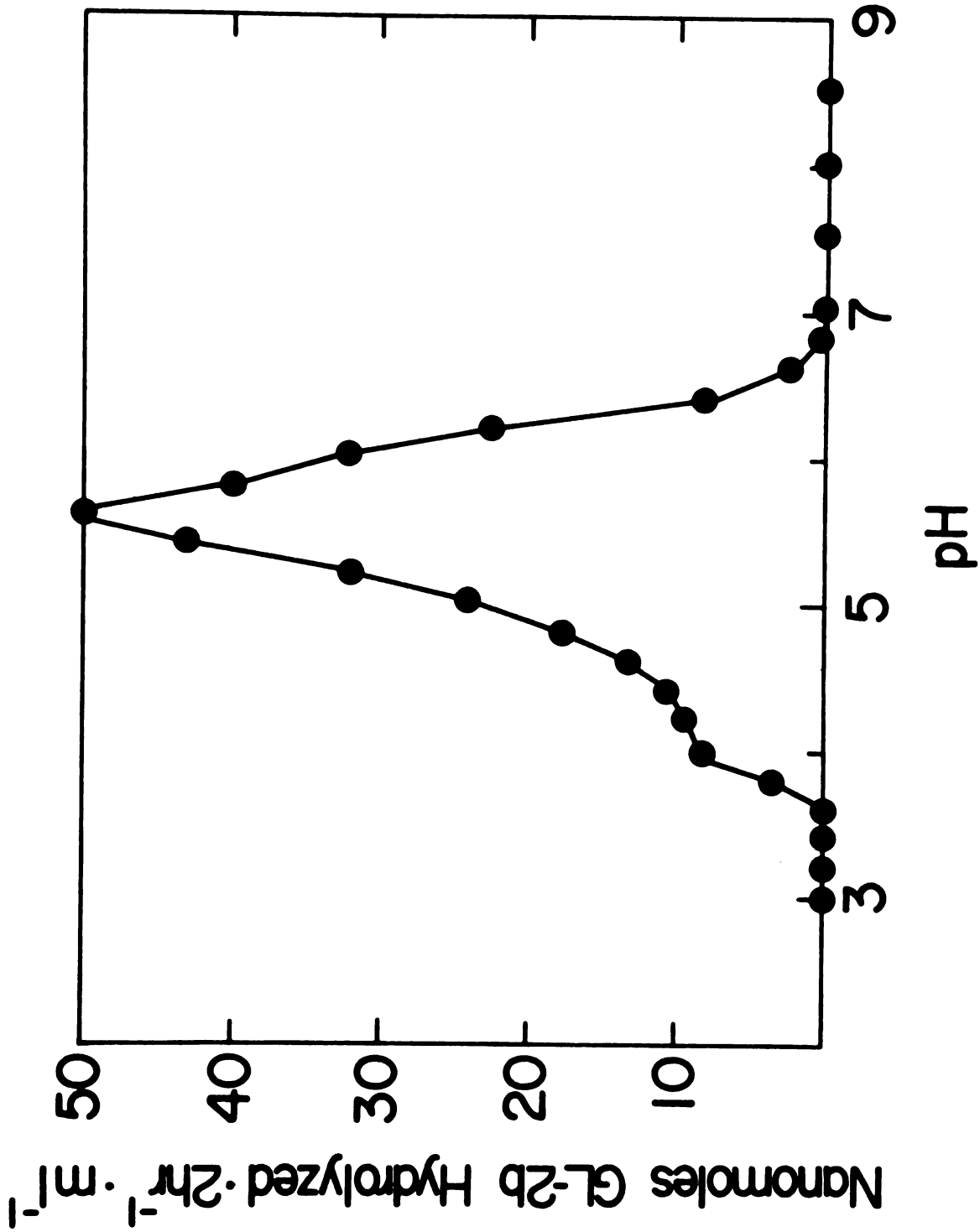

Nanomoles GL-2b Hydrolyzed·2hr⁻¹·ml⁻¹
50
40
30
20
10
3
5
7
9
pH

Figure 35. Affinity Chromatography of Digalactosylceramide : Galactosyl Hydrolase

Whole plasma was adjusted to pH 5.4 with 0.1 M citric acid and applied to the affinity column equilibrated to pH 5.4.

(A) Affinity chromatography of normal plasma

(B) Affinity chromatography of hemizygous Fabry plasma

The data were corrected to a constant volume of 100 ml of plasma.

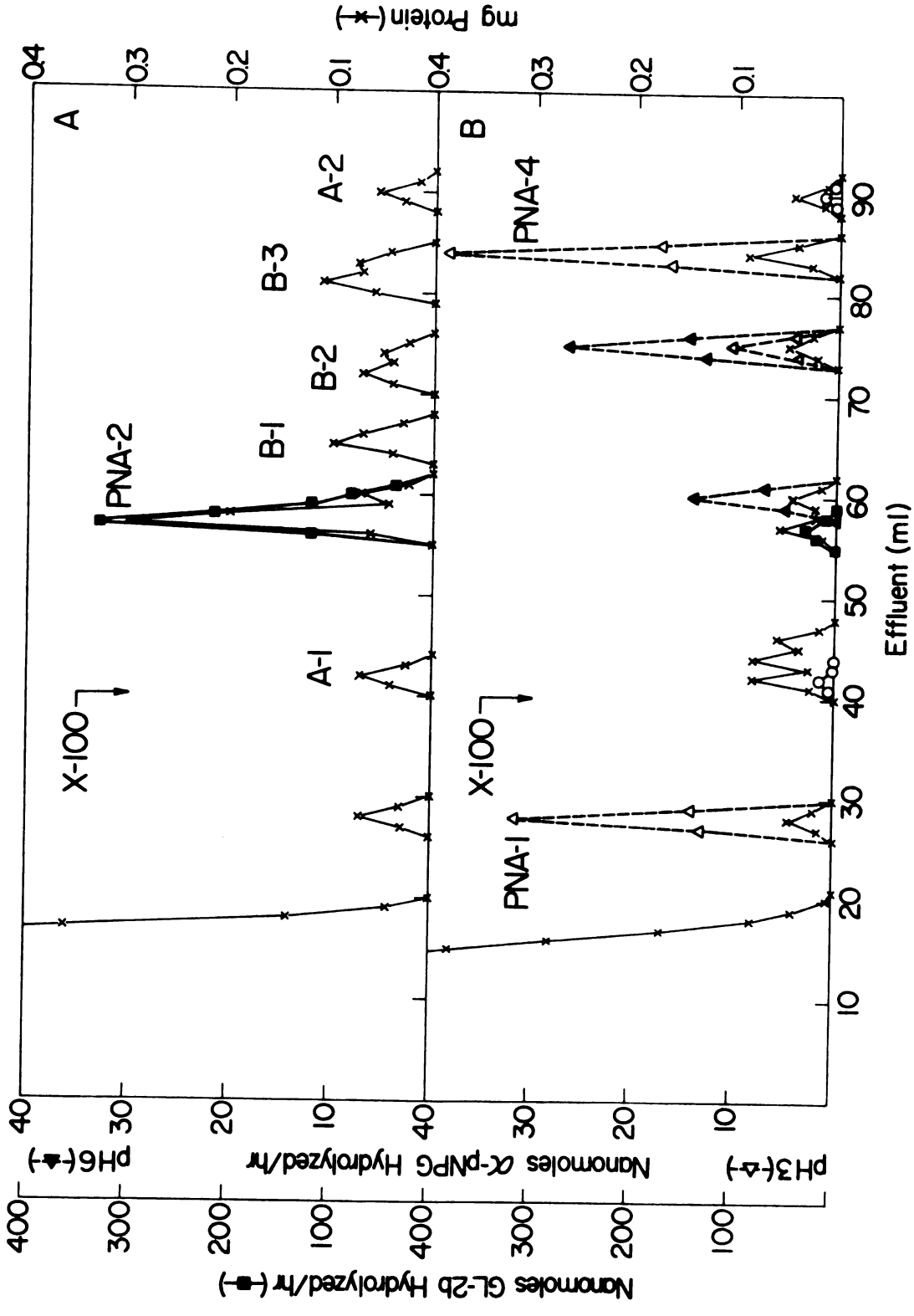
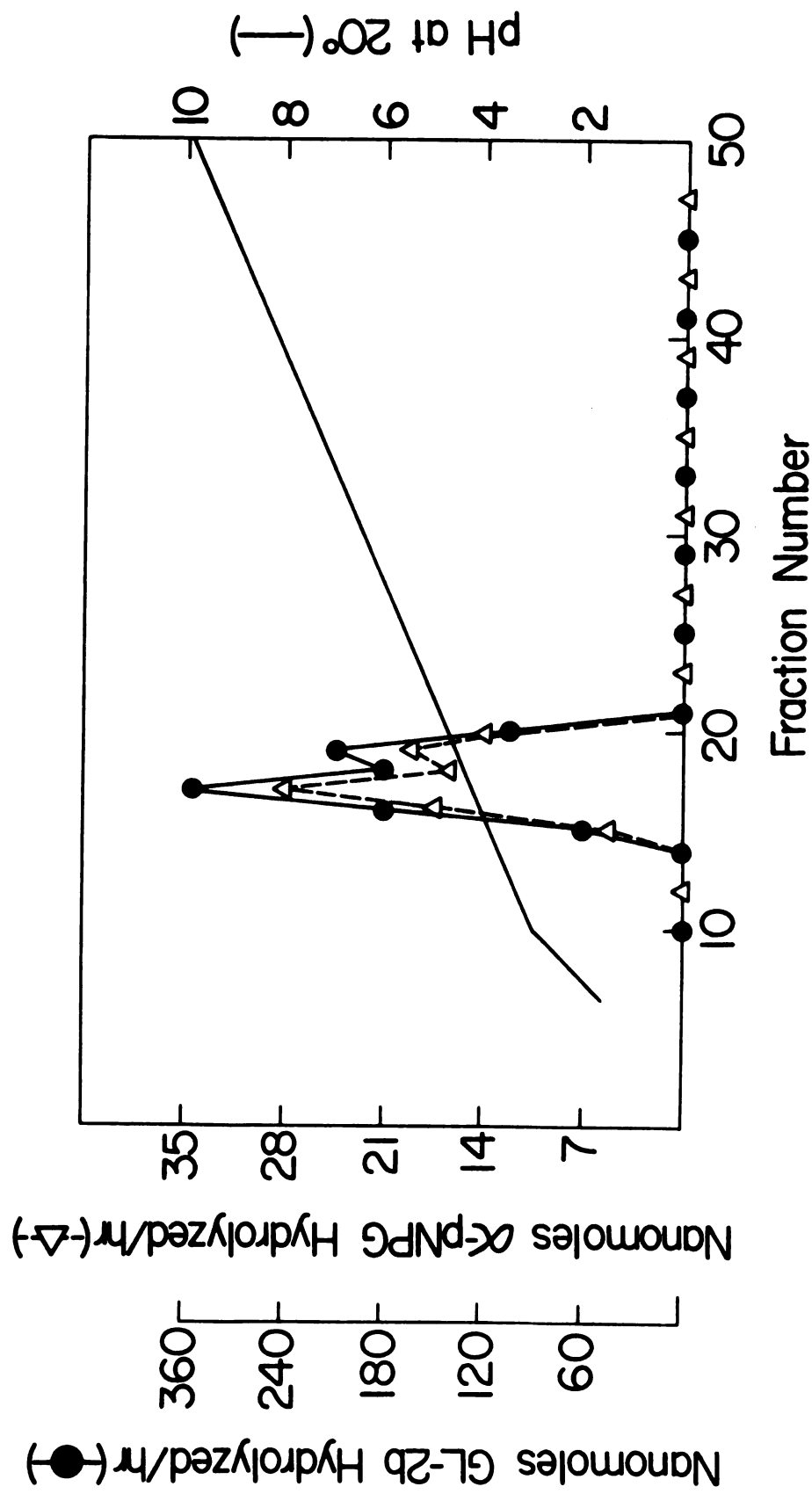

A
B
X-100
PNA-1
PNA-2
PNA-4
A-1
A-2
B-1
B-2
B-3
Nanomoles GL-2b Hydrolyzed/hr (■)
Nanomoles α-pNPG Hydrolyzed/hr
pH3(▲)
pH6(▲)
mg Protein (×)
Effluent (ml)

Figure 36. Isoelectric Focusing of Digalactosylceramide : Galactosyl Hydrolase

The proteins obtained from affinity chromatography were focused in a supportive sucrose gradient for 48 hr at 1°. The eluted fractions (2 ml) were assayed for enzymatic activity without removal of the carrier ampholytes.


Nanomoles GL-2b Hydrolyzed/hr (-●-)
360
240
180
120
60
Nanomoles α-pNPG Hydrolyzed/hr (-△-)
35
28
21
14
7
10
20
30
40
50
Fraction Number
pH at 20° (——)
10
8
6
4
2

Comparison of normal and Fabry ceramide digalactosidase activity by cellulose acetate electrophoresis, shown in Figure 37, revealed that there are two enzymatically active components in normal plasma, whereas only the enzyme of slower electrophoretic mobility is present in Fabry plasma.

URINARY CERAMIDE TRIHEXOSIDASES

Crude urine was assayed for ceramide trihexosidase activity using galactose dehydrogenase (Method 1). It was found that the ceramide trihexosidases are excreted in the urine, with about three times as much activity associated with Form B as with Form A. As shown in Figure 38, these enzymes have the same binding capacity on the affinity column as the plasma enzymes and are eluted in nearly the same fractions. The concentration of the ceramide trihexosidases in urine was estimated to be 50 μg per liter for Form A and 170 μg per liter for Form B.

INTERCONVERSION OF THE CERAMIDE TRIHEXOSIDASES

Preliminary Neuraminidase Experiments

The effect of neuraminidase on the enzymatic activity of the ceramide trihexosidases was studied using the crude A forms obtained from Cohn fraction IV-1. Cohn fraction IV-1, having detectable enzymatic activity at only pH 5.4 (Form A) was incubated with commercially purified neuraminidase from *Clostridium perfringens*. The effect of neuraminidase on the pH optimum of ceramide trihexosidase,

Figure 37. Comparison of Normal and Fabry Digalactosyl-ceramide : Galactosyl Hydrolase Activity by Cellulose Acetate Electrophoresis

The proteins obtained from affinity chromatography were concentrated against polyethylene glycol 6000 and electrophoresed at room temperature. The strips are (1) normal enzyme stained for glycoprotein, (2) normal enzyme stained for activity, and (3) hemizygous Fabry enzyme stained for activity. The strips were redrawn for purposes of photography.

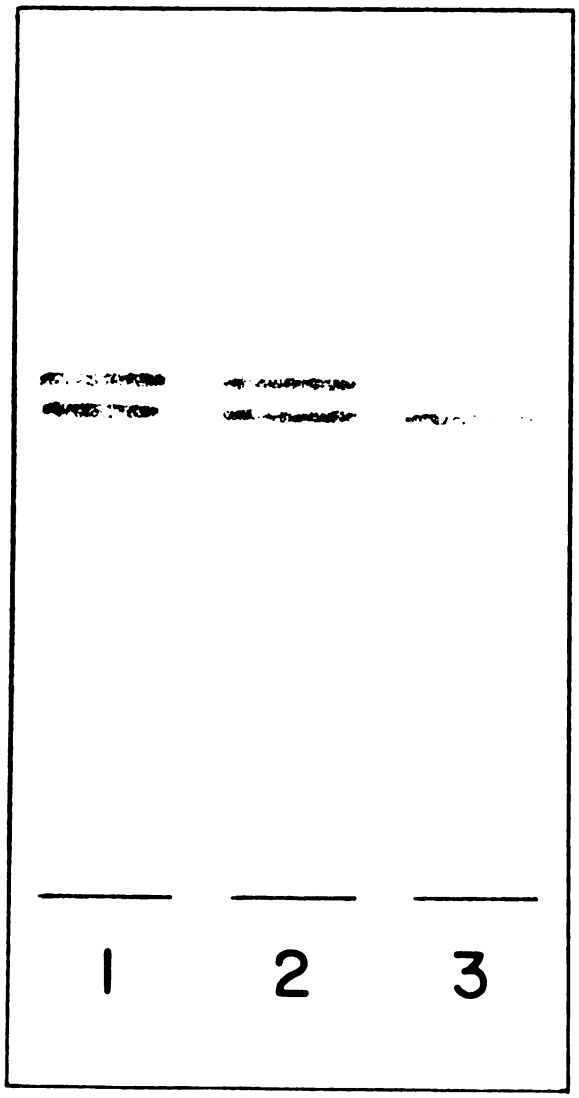

1
2
3

Figure 38. Affinity Chromatography of Concentrated Urine

Urine was concentrated 15-fold using an Amicon hollow fiber ultrafiltration system. An aliquot equivalent to 3 liters of urine was adjusted to pH 5.4 with 0.1 M citric acid and applied to the affinity column equilibrated to pH 5.4 with 0.001 M MES buffer.

pH5.4(○)
pH7.2(●)
Nanomoles GL-3 Hydrolyzed/hr
200
150
100
50
pH3(△)
pH6(▲)
Nanomoles α-pNG Hydrolyzed/hr
40
30
20
10
mg Protein(×)
0.60
0.40
0.20
0.16
0.12
0.08
0.04
X-100
PNA-1
A-1
B-2
PNB-2
B-3
PNA-4
A-2
10
20
30
40
50
60
70
80
90
100
Effluent (ml)

Form A, is shown in Figure 39. Following neuraminidase treatment there was substantial activity at pH 7.2 (Form B), but at pH 5.4 the activity had disappeared completely, within the limits of the method of assay. The control had no detectable activity at pH 7.2 although 30% of the ceramide trihexosidase activity at pH 5.4 was lost during the incubation. This loss of activity is explained by the instability of Form A, which had a half-life of 5 hours under the conditions of the experiment.

Incubation of whole plasma with neuraminidase enabled a study of the time course of the reaction, which shows a correlation of sialic acid release with the change in enzymatic activity at both pH optima, as shown in Figure 40. There was no detectable release of sialic acid in the controls, although there appeared to be a similar, though less marked, conversion of the ceramide trihexosidases, Form A to Form B during the first hour of the incubation. Thereafter both enzymes progressively lost activity in the control samples. A second series of controls contained 2-4 x 10^{-3} M N-acetylneuraminic acid, which neither inhibited enzymatic activity at pH 5.4 nor enhanced activity at pH 7.2.

Neuraminidase Treatment of Purified Ceramide Trihexosidase, Form A-1

Electrophoretically pure ceramide trihexosidase, Form A-1, was treated with neuraminidase purified by affinity chromatography. Ceramide trihexosidase (300 µg) was

Figure 39. Effect of Neuraminidase on the pH Optimum of the Ceramide Trihexosidases, Form A

Ceramide trihexosidases, Form A, derived from Cohn fraction IV-1 were treated with commercially prepared *Clostridium perfringens* neuraminidase. The comparison is between neuraminidase treated (-●-) and control (-o-) incubations carried out as described in Materials and Methods.

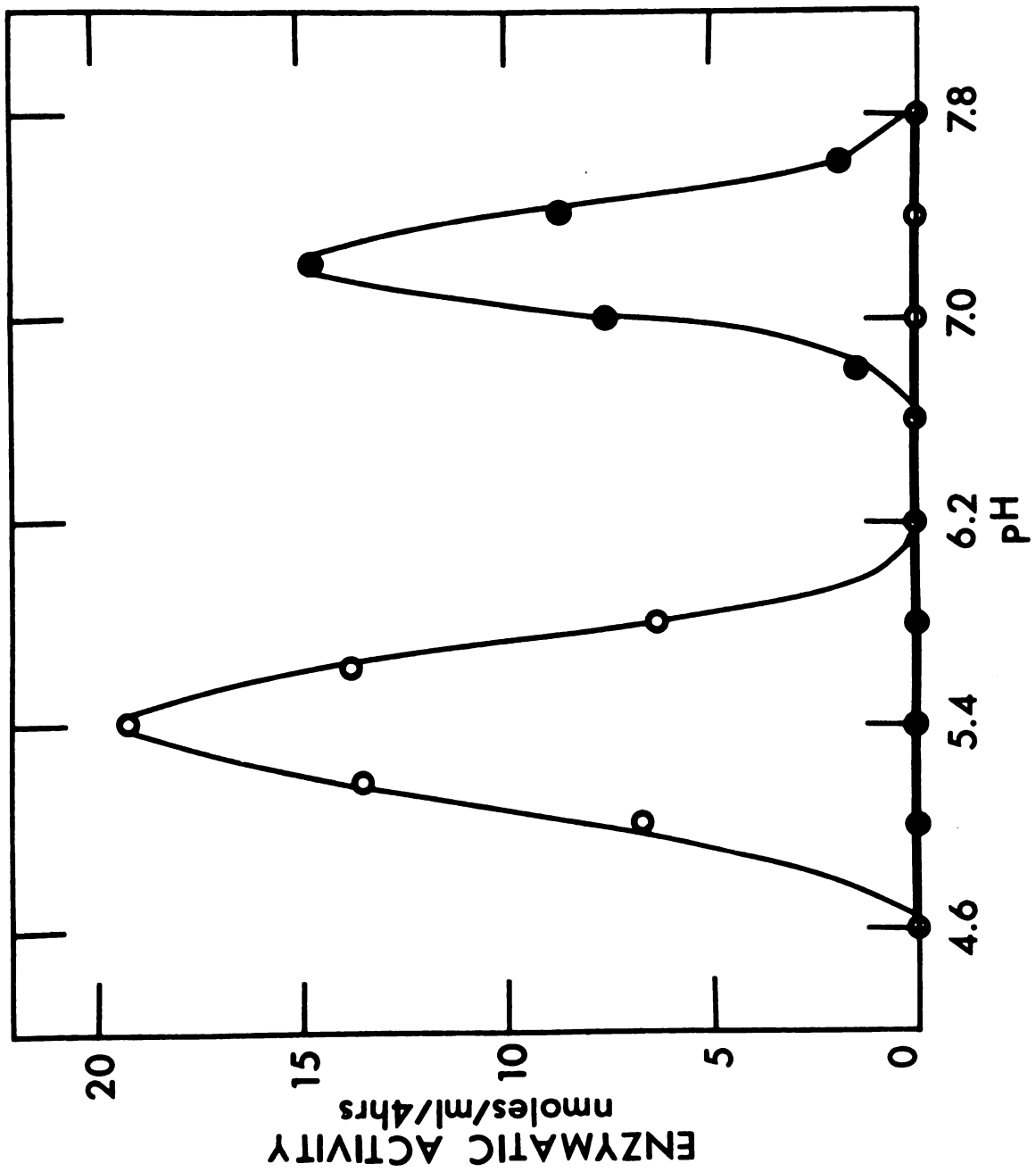

ENZYMATIC ACTIVITY
nmoles/ml/4hrs
0
5
10
15
20
4.6
5.4
6.2
7.0
7.8
pH

Figure 40 (A). Correlation of Sialic Acid Release with Changes in Enzymatic Activity

Time course showing the correlation of sialic acid released with decreased enzymatic activity at pH 5.4 when normal human plasma is treated with neuraminidase. Enzymatic activity at pH 5.4 in the presence of neuraminidase (-●-); the loss of enzymatic activity in the absence of neuraminidase (--●--); and the level of sialic acid in the presence (-■-) and absence (--■--) of neuraminidase.

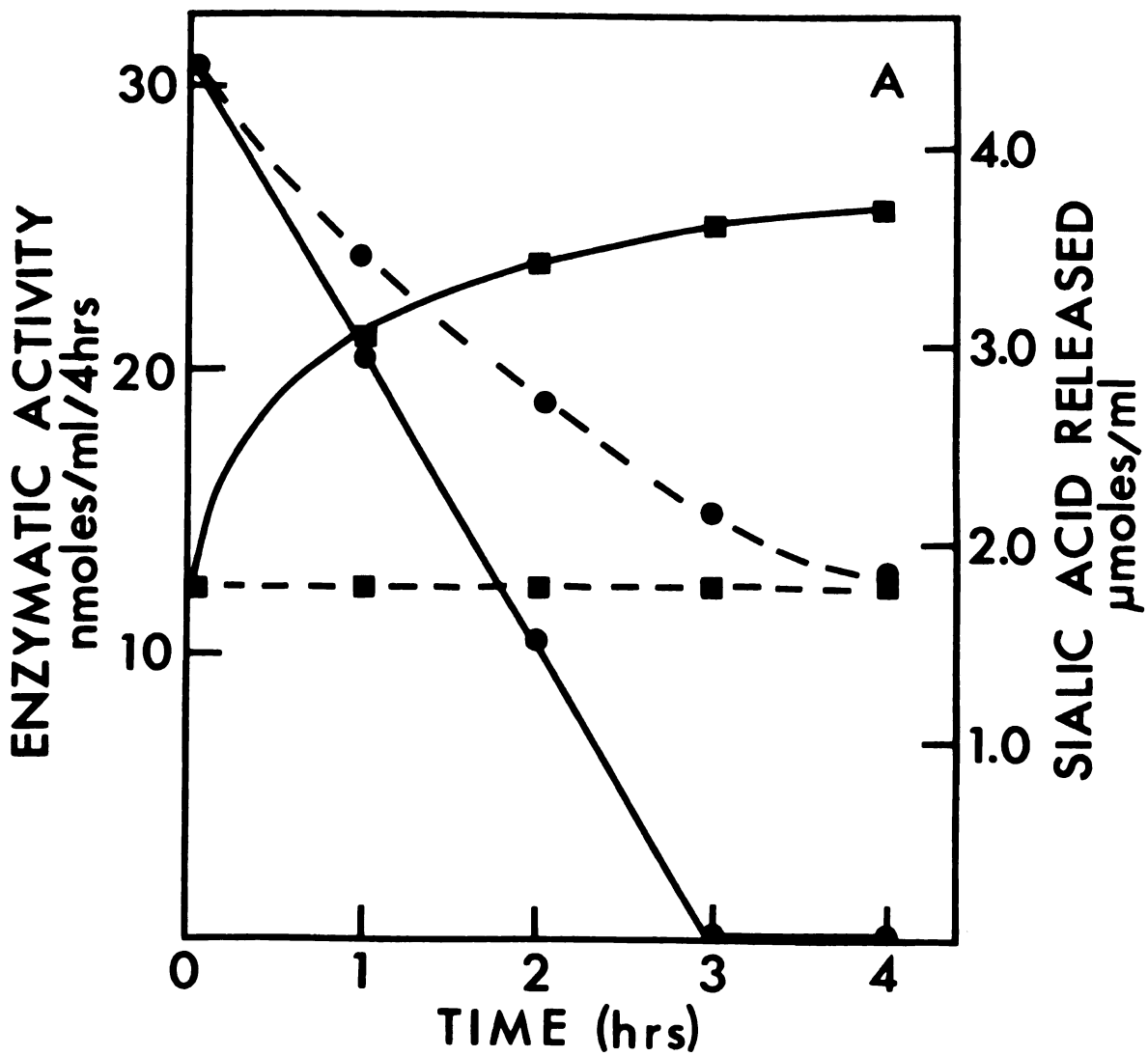
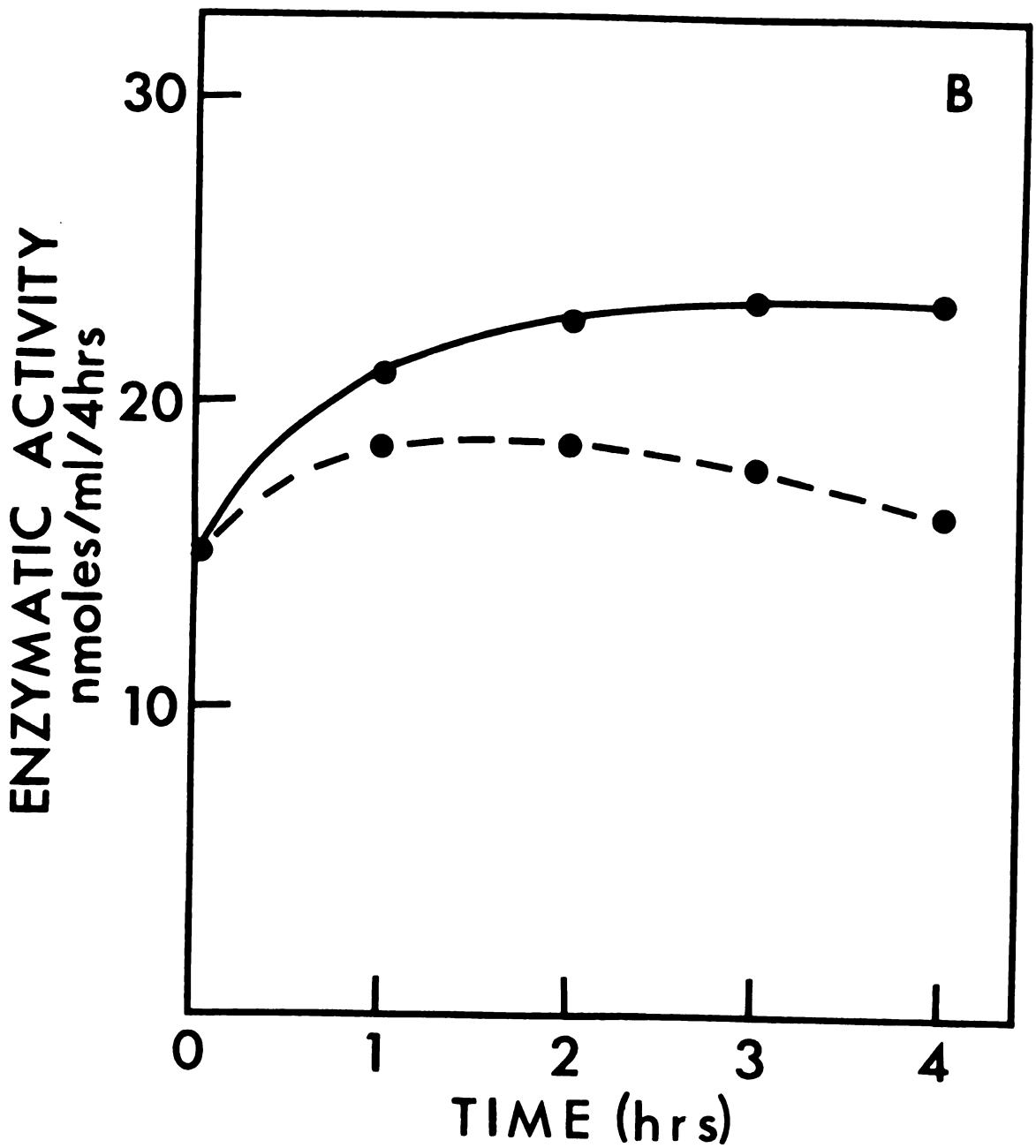

A
30
20
10
4.0
3.0
2.0
1.0
0
1
2
3
4
TIME (hrs)
ENZYMATIC ACTIVITY
nmoles/ml/4hrs
SIALIC ACID RELEASED
μmoles/ml

Figure 40 (B). Correlation of Sialic Acid Release with Changes in Enzymatic Activity

Enzymatic activity at pH 7.2 in the presence (-●-) and absence (--●--) of neuraminidase.


B
30
20
10
ENZYMATIC ACTIVITY
nmoles/ml/4hrs
0
1
2
3
4
TIME (hrs)

incubated with 50 units of neuraminidase for 4 hours. The time course of the reaction was followed by cellulose acetate electrophoresis and isoelectric focusing.

As shown in Figure 41, one hour of neuraminidase treatment altered the activity of ceramide trihexosidase, Form A-1, by forming 8 proteins of lower electrophoretic mobility. Isoelectric focusing of the ceramide trihexosidases after 2 hours incubation with neuraminidase, shown in Figure 42, gave a complex pattern of 14 enzymatically active proteins. Seven of these proteins were active at pH 5.4 (Form A) and the other proteins were active at pH 7.2 (Form B). One of the proteins active at pH 5.4 had a pI of 3.0 and probably represents unchanged ceramide trihexosidase, Form A-1. One of the proteins enzymatically active at pH 7.2 had a pI of 8.4 which is the same as the pI of ceramide trihexosidase, Form B-V. After a 4 hour incubation with neuraminidase (Figure 41) ceramide trihexosidase, Form A-1, was not detectable but an enzyme having the same electrophoretic mobility as ceramide trihexosidase, Form B-V, was present in addition to two proteins of intermediate electrophoretic mobility.

In the control, incubated 4 hours without neuraminidase, the majority of the protein had the same electrophoretic mobility as ceramide trihexosidase, Form A-1, although two proteins of slower electrophoretic mobility were found. These results suggest that ceramide trihexosidase, Form A-1,

Figure 41. Cellulose Acetate Electrophoresis of Ceramide Trihexosidase, Form, A-1 after Neuraminidase Treatment

Electrophoresis was performed at room temperature and the strips were stained for activity. The strips contain the following: (1) ceramide trihexosidase, Form A-1 obtained from affinity chromatography; (2) ceramide trihexosidases after a 1 hr incubation of Form A-1 with neuraminidase; (3) ceramide trihexosidases after 4 hr incubation with neuraminidase; (4) ceramide trihexosidase, Form A-1, incubated 4 hr without neuraminidase; and (5) ceramide trihexosidase, Form B-V, obtained from isoelectric focusing. The strips were redrawn for purposes of photography.

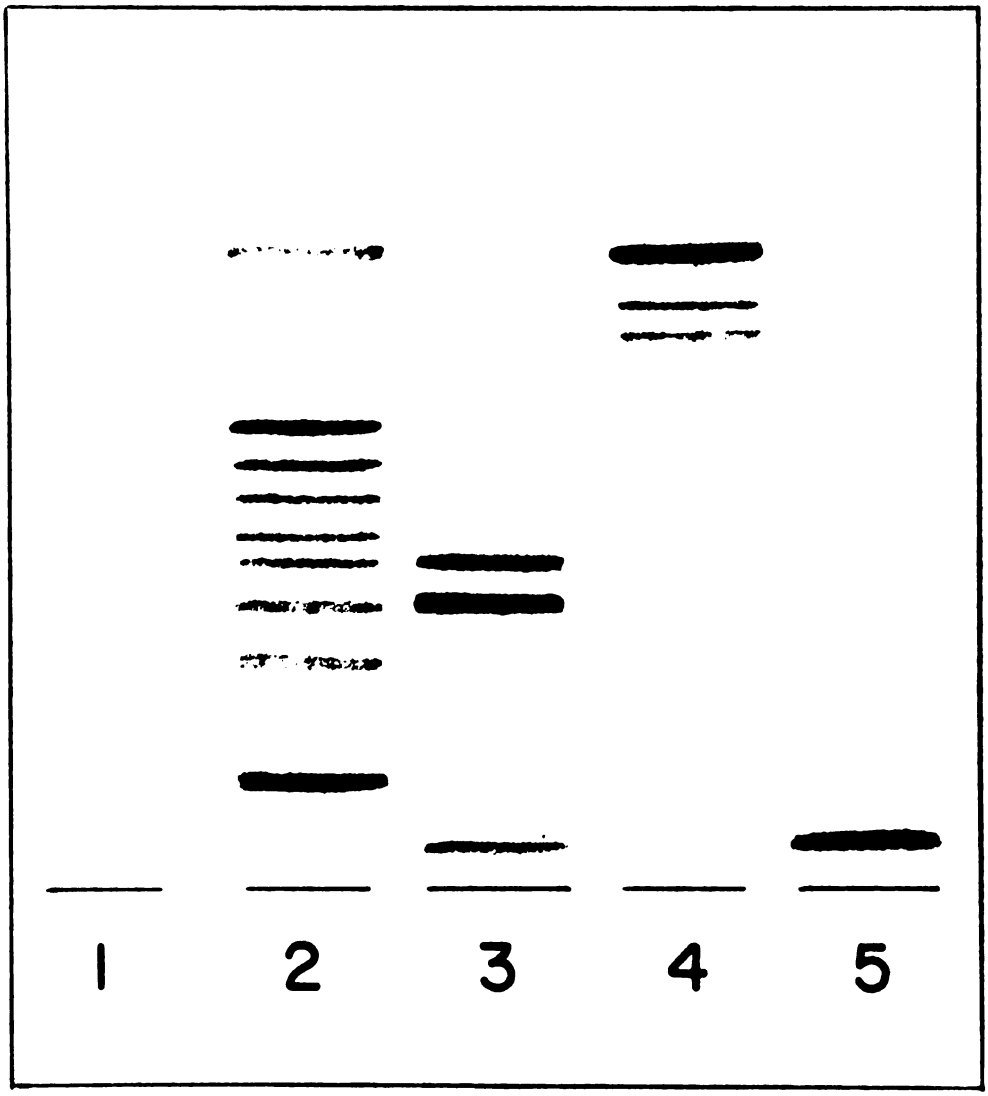
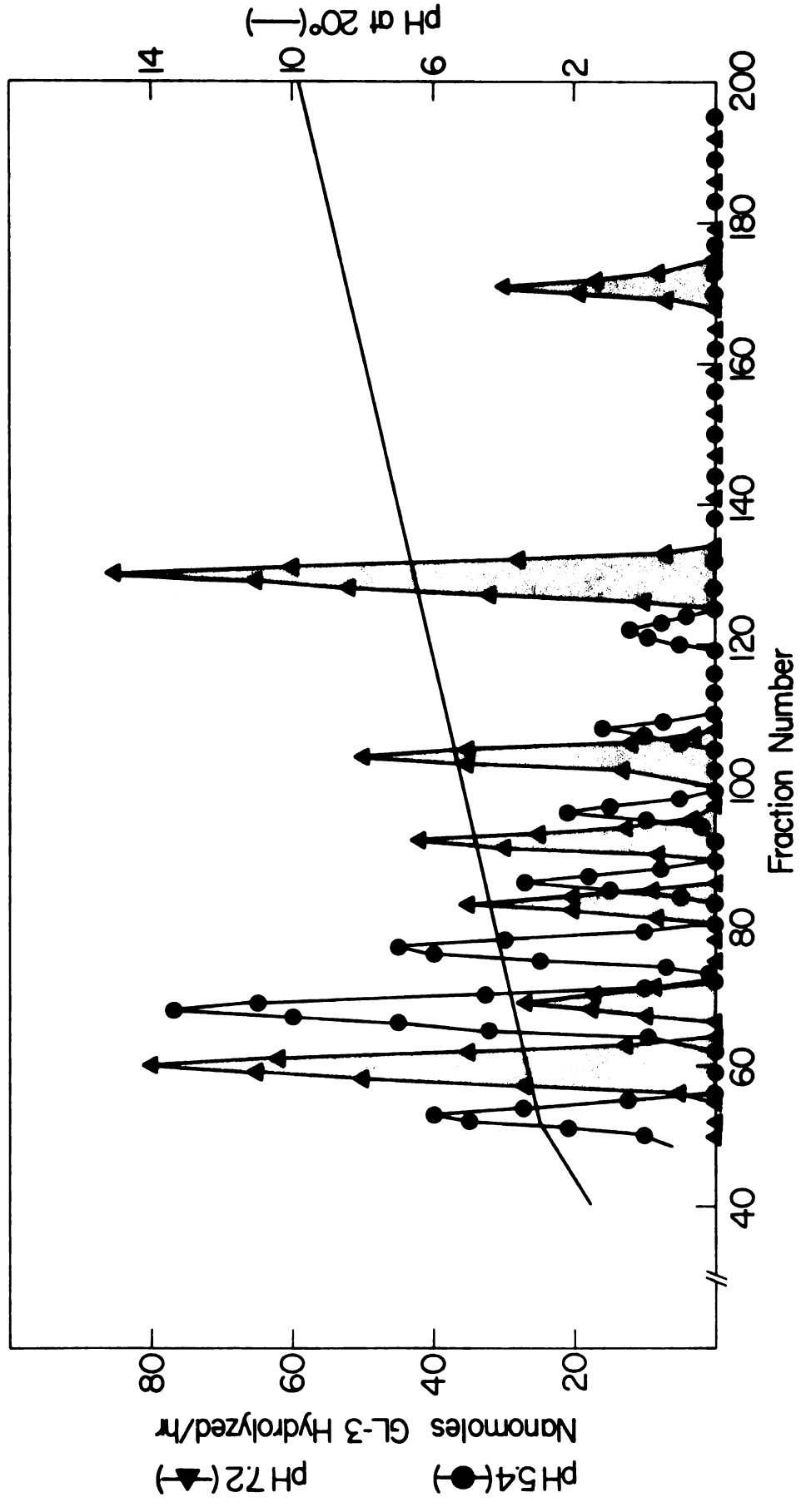

1
2
3
4
5

Figure 42. Isoelectric Focusing of Ceramide Trihexosidase, Form A-1, after Neuraminidase Treatment

After 2 hr of neuraminidase treatment ceramide trihexosidase, Form A-1, was focused in a supportive sucrose gradient for 48 hr at 1°. The fractions (0.5 ml) were assayed for enzymatic activity without removal of the carrier ampholytes.


pH 5.4 (●)
pH 7.2 (▲)
Nanomoles GL-3 Hydrolyzed/hr
80
60
40
20
40
60
80
100
120
140
160
180
200
Fraction Number
14
10
6
2
pH at 20° (—)

is a sialoglycoprotein which can be converted to the asialoglycoprotein, ceramide trihexosidase, Form B-V, by the action of neuraminidase.

Preliminary Studies on Incorporation of Sialic Acid into Ceramide Trihexosidase, Form B-V

A preliminary experiment was performed to determine whether there was any evidence for a sialyltransferase in porcine tissues which could convert human ceramide trihexosidase, Form B-V to Form A. Fresh porcine liver and kidney were homogenized in 0.25 M sucrose and incubated with the enzymes contained in whole plasma. The results of this experiment, indicated that both porcine liver and kidney homogenates could convert human plasma ceramide trihexosidase, Form B to Form A, although the kidney homogenate interconverted the proteins more rapidly.

Incorporation of [^{14}C]Sialic Acid and [^{14}C]N-Acetylglucosamine into Ceramide Trihexosidase, Form B

Ceramide trihexosidase, Form B-V, obtained by isoelectric focusing, was incubated with [^{14}C]CMP-sialic acid, using porcine kidney as a source of sialyltransferase. Three controls were run: one in which the basic protein was eliminated from the incubation to test for non-specific sialylation of other glycoproteins in the incubation mixture; one in which the [^{14}C]CMP-sialic acid was eliminated to test for spontaneous interconversion of the ceramide

trihexosidases; and one in which the [^{14}C]CMP-sialic acid and the enzyme were incubated together in the absence of kidney homogenate to test for possible adhesion of radioactivity to the protein.

Following 4 hours incubation with [^{14}C]CMP-sialic acid fourteen proteins were formed, as shown in Figure 43. One of these proteins had the same electrophoretic mobility as ceramide trihexosidase, Form B-V and one of them had the same electrophoretic mobility as ceramide trihexosidase, Form A. In the control, containing no [^{14}C]CMP-sialic acid, most of the protein was unchanged ceramide trihexosidase, Form B-V, although there were 4 other proteins of higher electrophoretic mobility.

As shown in Figure 44 sialic acid was incorporated into all of the proteins except the most basic ceramide trihexosidase, Form B-V. Assuming an average molecular weight of 95,000 for all of the proteins, it was calculated that the protein of lowest electrophoretic mobility, into which sialic acid was incorporated, contained 3 moles of sialic acid per mole of protein. Thereafter there appeared to be a consecutive addition of 1 mole of sialic acid per mole of protein until the protein having the fastest electrophoretic mobility contained 15 sialic acid residues. These results should be interpreted with care, however, since the lower limits of accuracy for quantitating protein were used in some cases. In addition, the individual

Figure 43. Cellulose Acetate Electrophoresis of Ceramide Trihexosidase, Form B-V, following Sialyltransferase Treatment

Electrophoresis was carried out at room temperature and the strips were stained for activity. The strips contain the following: (1) ceramide trihexosidase, Form B-V obtained by isoelectric focusing; (2) ceramide trihexosidase, Form A-1, obtained by affinity chromatography; (3) ceramide trihexosidase after 4 hr incubation with [^{14}C]CMP-sialic acid and kidney homogenate; and (4) ceramide trihexosidase, Form B-V, incubated 4 hr in the absence of [^{14}C]CMP-sialic acid. The strips were redrawn for purposes of photography.

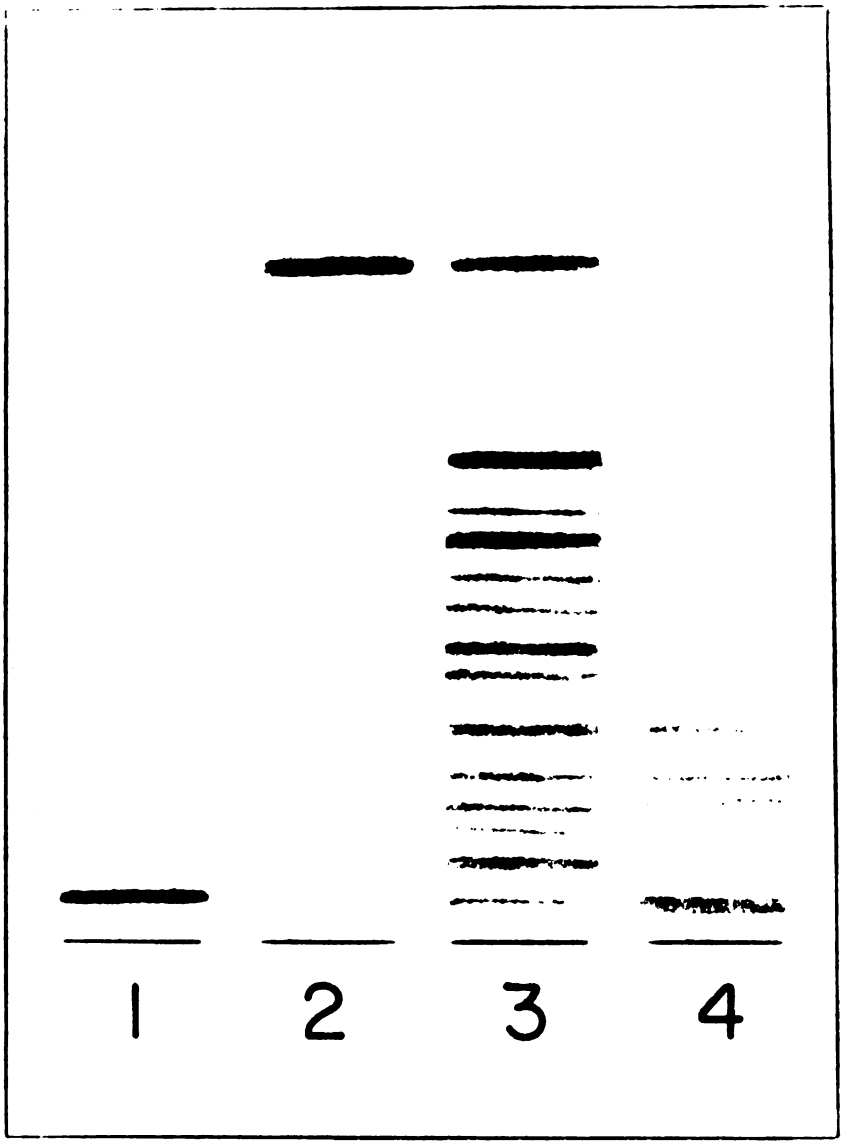
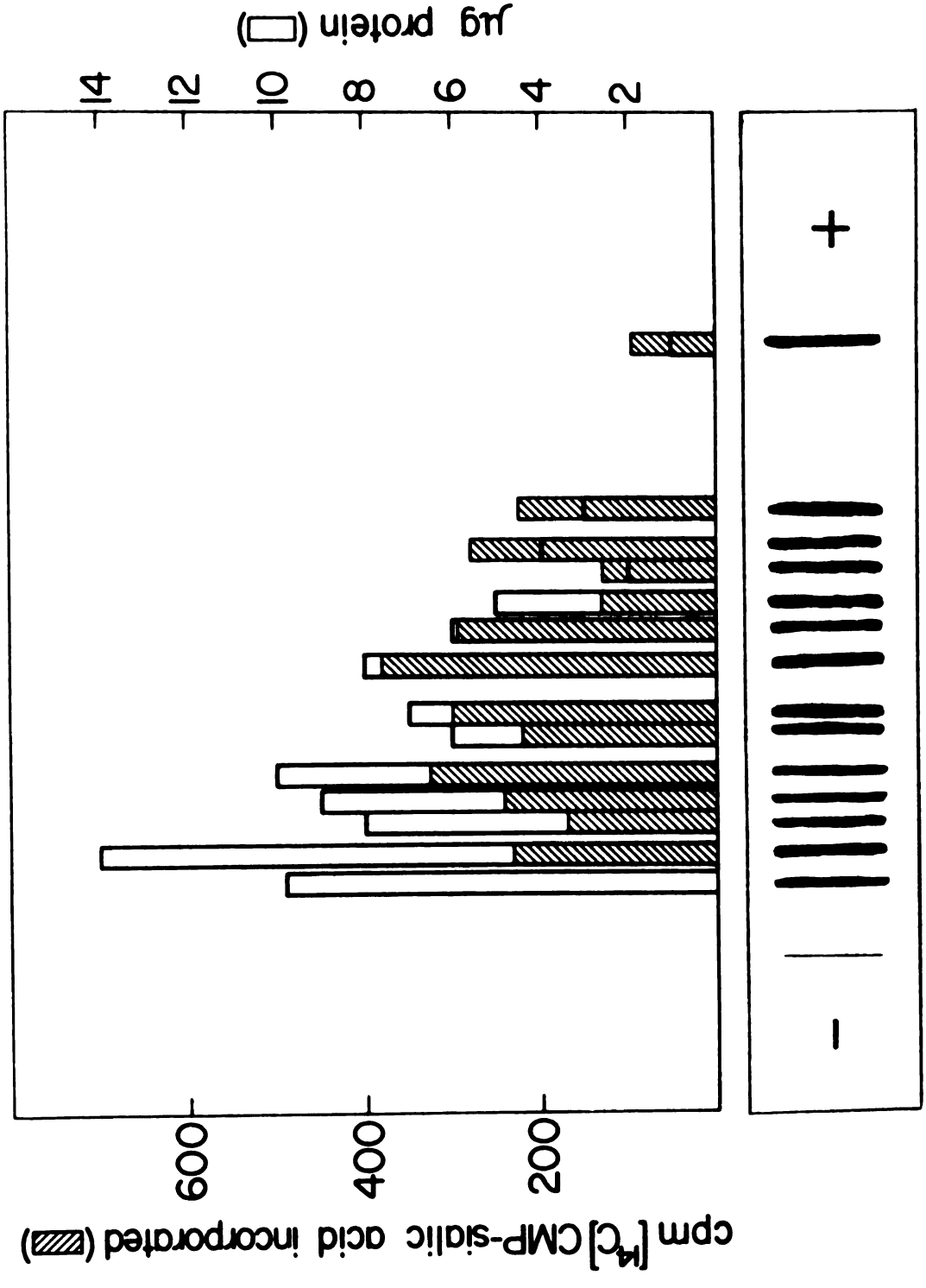

1
2
3
4

Figure 44. Incorporation of [^{14}C]Sialic Acid into Ceramide Trihexosidase, Form B-V

The cpm and μg protein were determined as described in Materials and Methods. All values were corrected using the data obtained from the controls.


cpm [14C] CMP-sialic acid incorporated (▨)
600
400
200
μg protein (□)
14
12
10
8
6
4
2
+
—

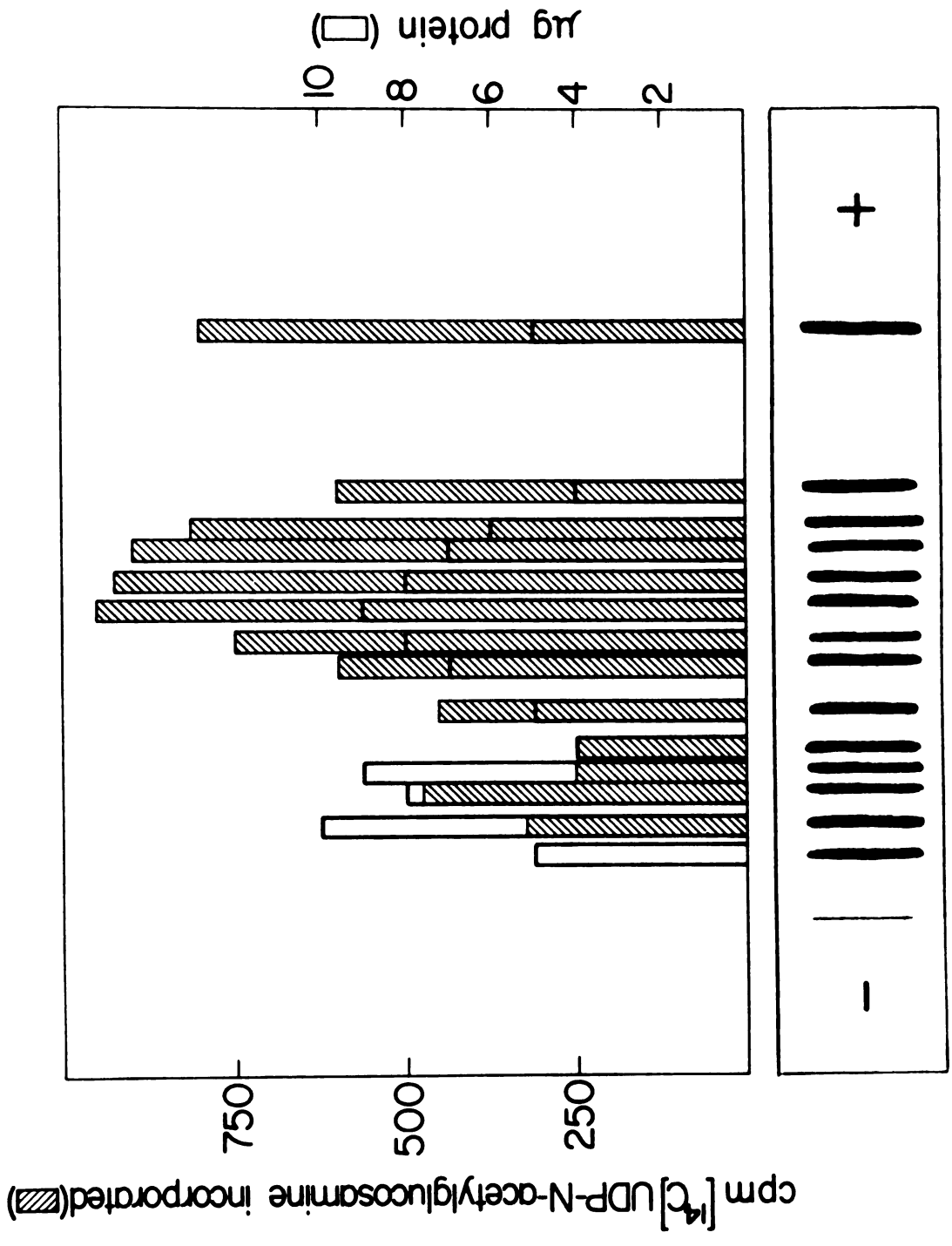
proteins would not have identical molecular weights. However, it is safe to assume that the most acidic enzyme contains 15 ± 4 residues of sialic acid.

Since the [^{14}C]CMP-sialic acid was known to be unstable below pH 9.0, this experiment was repeated using [^{14}C]UDP-N-acetylglucosamine, on the assumption that sialic acid incorporation into the basis protein would follow the pathway delineated for glycoprotein biosynthesis in liver (186). The pattern of radioactivity incorporated, shown in Figure 45, was basically the same as for incorporation of [^{14}C]CMP-sialic acid although the quantity of the more acidic proteins was greater. The possibility that N-acetylglucosamine was incorporated as such into the proteins cannot be ruled out. This is not likely, however, since the electrophoretic mobilities of the proteins formed by incorporation of [^{14}C]UDP-N-acetylglucosamine were identical with those of the proteins formed from [^{14}C]CMP-sialic acid.

In both experiments the total amount of radioactivity incorporated in the controls accounted for less than 5% of the total radioactivity incorporated into the proteins.

Figure 45. Incorporation of [^{14}C]N-Acetylglucosamine into Ceramide Trihexosidase, Form B-V

The cpm and μg protein were determined as described in Materials and Methods. All values were corrected using the data obtained from the controls.


cpm [14C] UDP-N-acetylglucosamine incorporated(▨)
750
500
250
μg protein (□)
10
8
6
4
2
+
—

DISCUSSION

When ceramide trihexosidase activity was discovered in human plasma it was observed that there were two pH optima which suggested that two forms of ceramide trihexosidase might be present in plasma. This possibility was confirmed when it was found that the ceramide trihexosidase activity at pH 5.4, called the A form, could be separated from the activity at pH 7.2 (B form) by the low temperature ethanol fractionation procedure described by Cohn *et al.* (164). A combination of affinity chromatography and isoelectric focusing separated the A form of ceramide trihexosidase activity into two glycoproteins which differed primarily in their kinetic properties, whereas the B form of the enzyme was separated into five proteins having distinct pI's.

The occurrence of multiple molecular forms of plasma proteins is not uncommon. This phenomenon has been detected in many instances by electrophoretic and immunochemical studies, and the multiple forms of some of these proteins have already been isolated. For example, at least eight forms of plasminogen having different isoelectric points have been obtained by isoelectric focusing (187) and affinity chromatography (188), six forms of hypoxanthine-guanine

phosphoribosyl transferase have been obtained by a combination of chromatography and isoelectric focusing (189), and a group of seven liver 4-methylumbelliferyl-α-galactosidases related to each other by their sialic acid content have been reported (190). This multiplicity of proteins often arises from variations in the combination of several polypeptide chains, chemical modifications of the completed protein, differences in the carbohydrate content of glycoproteins, or genetic heterogeneity resulting from multiple loci or multiple alleles at the same locus coding structurally distinct polypeptide chains of the same protein (133).

An understanding of the significance of the multiple forms of ceramide trihexosidase is particularly important since this enzyme might have therapeutic value in the treatment of Fabry's disease. This requires isolation of the individual molecular forms of the enzyme and characterization of their physical and biochemical properties.

CHARACTERISTICS OF THE CERAMIDE TRIHEXOSIDASES, FORM A

Purity of the Enzymes

The plasma ceramide trihexosidases obtained from Cohn fraction IV-1 appeared to be homogeneous as determined by a constant specific activity following affinity chromatography and isoelectric focusing, by a single band on polyacrylamide gel electrophoresis, and by the presence of a single protein,

coincident with enzymatic activity, on isoelectric focusing and sucrose density gradient centrifugation. Although both proteins appeared to be homogeneous by all of the criteria employed, most glycoproteins show microheterogeneity. Alterations in the carbohydrate moiety which affect neither the enzymatic activity nor the charge on the protein probably would not have been detected by these techniques. More stringent criteria, employing larger quantities of protein, will have to be employed before the ceramide trihexosidases can be regarded as completely homogeneous proteins. At the present time these enzymes can be assumed to be 90-95% pure.

Classification of the Ceramide Trihexosidases

The results of neuraminidase treatment and sialic acid-incorporation studies strongly indicate that the A and B forms of ceramide trihexosidase are glycoproteins which differ in sialic acid content. Neuraminidase treatment of Form A-1 ceramide trihexosidase converted it to a protein indistinguishable from B-V ceramide trihexosidase. [^{14}C]Sialic acid and [^{14}C]N-acetylglucosamine were incorporated into the B-V form of the enzyme forming a protein indistinguishable by electrophoretic mobility from the A forms of the enzyme. In addition all of the ceramide trihexosidases are stained by Schiff's-periodate on cellulose acetate strips. Direct proof of their classification can be obtained by sequencing the purified proteins.

If the completely sialylated form of ceramide trihexosidase contains 7-15 sialic acid residues attached to alternating carbohydrate moieties, it can be assumed that the ceramide trihexosidases are 5-10% carbohydrate.

On the basis of the sialic acid incorporation studies it seems that the B-V form of ceramide trihexosidase is an asialoglycoprotein which can be converted into the A form of the enzyme by the action of a porcine kidney sialyltransferase. Judging from the number of proteins which were produced by this method it is possible that plasma ceramide trihexosidase, form A, contains 10-15 sialic acid residues. This data supports the stepwise addition of sialyl residues to the B-V form of the enzyme. Studies on the properties of porcine serum and liver CMP-N-acetylneuraminic acid:glycoprotein sialyltransferases were reported by Hudgin and Schachter (191). The most effective acceptors for these enzymes were neuraminidase - treated α_1-glycoproteins and sialic acid incorporation appeared to occur whenever a terminal galactose was linked ($\beta 1 \rightarrow 4$) to a penultimate N-acetylglucosaminyl residue. These results should not be construed as an indication that the carbohydrate moiety of ceramide trihexosidase consists of repeating galactosyl-($\beta 1 \rightarrow 4$)N-acetylglucosaminyl residues, since most organ-specific sialyltransferases are a family of enzymes having different acceptor specificities (192, 193).

The neuraminidase studies might also support the stepwise removal of sialyl residues resulting in 15 enzymes differing in their net negative charge. The number of intermediate forms of ceramide trihexosidase also can be explained on the basis of seven sialic acid residues by assuming that one of these residues determines the pH optimum at which the enzyme has catalytic activity. This speculation implies that the removal of a specific sialyl moiety causes the formation of a group B protein which is consecutively desialylated to form B-V ceramide trihexosidase.

Although there is no evidence in the literature which indicates that sialic acid plays a role in the catalytic site of an enzyme, it is feasible that the removal of a charged residue might cause a conformational change in the protein enabling it to be catalytically active at a different pH optimum. For instance, it is known that arylsulfatase A and β-N-acetylhexosaminidase A are converted to their respective B forms by neuraminidase treatment (95) and that a group of seven 4-methyl-umbelliferyl-α-galactosidases are converted to one electrophoretically slow component by neuraminidase (190). In addition, several biologically active hormones containing sialic acid are either partially or completely inactivated by neuraminidase treatment. These hormones include follicle-stimulating hormone, human chorionic gonadotropin and erythropoietin (194). On the other hand, the catalytic properties of several glycoprotein

enzymes are not effected by neuraminidase. These enzymes include serum cholinesterase, γ-glutamyl transpeptidase, enterokinase and serum atropinesterase (194).

General Properties of the Ceramide Trihexosidases

The two plasma ceramide trihexosidases are remarkably similar in their molecular weights, electrophoretic characteristics, response to the lipid substrate under optimal conditions, and substrate specificity; they differ in their heat stability, solubility in butanol, and response to various inhibitors. The findings suggest that the two enzymes serve catalytic functions at different *in vivo* sites where their metabolic regulation may be partially controlled by the physiological environment. However it is difficult to attribute any physiological significance to the kinetic characteristics of these enzymes since little is known about either the substrate or protein concentration at a particular *in vivo* site.

Several sphingolipid hydrolases, including lactosylceramidase (195), ceramidase (195) and the β-N-acetylhexosaminidases (34, 144) are activated by detergents. This finding is usually explained as a requirement for an anionic detergent to form a molecular aggregate with a physical form suitable for hydrolysis of the lipophilic substrate (195). Likewise, the enhancement of enzymatic activity in the presence of specific salts (114) is normally attributed to an effect on the substrate environment. In the case of the

ceramide trihexosidases, these effects are more complex in the sense that detergents and salts may alter both the enzyme and the substrate.

Effect of Sodium Taurocholate and Sodium Chloride on GL-3: The action of sodium taurocholate and sodium chloride on GL-3 is most easily explained in terms of their effect on the detergent micelles which solubilize this lipophilic substrate. In micelles composed of ionic monomers, the charged groups are arranged near the surface of the aggregate. This results in a high charge density and produces an electrical field which polarizes the surrounding solvent molecules (196). This type of molecular aggregate may have the ability to fix an enzyme on its surface. The addition of sodium chloride to this micellar system may effect the substrate in two ways. 1) It lowers the critical micellar concentration (CMC) of the bile salt (185). This enables the formation of additional micelles which increases the micellar surface area. 2) The addition of salts can alter the conformation of the oligosaccharide which remains on the surface of the micelle (197). Both of these effects could make the substrate more accessible to the enzyme.

Effect of Salt and Detergent on the Enzyme: As shown in Figure 26 A and B both forms of ceramide trihexosidase have sigmoid substrate saturation curves. Under the conditions of this experiment the substrate should exist in a mixed

micellar form with the bile acid at low substrate concentrations. Thus the sigmoid saturation would presumably not be due to formation of micelles but rather would result from an alteration of the mixed micelle or of the enzyme itself as the substrate concentration is increased. If the enzyme conformation is affected, it is not likely that the sigmoidal substrate saturation curve represents typical allosteric kinetics since the specific activity of the enzyme is unusually dependent on enzyme concentration (Figure 27).

The following scheme is suggested as a working model to explain this dependency on enzyme concentration:

$$\text{Enzyme} + \text{micelle} \leftrightarrows \text{(enzyme-micelle complex)} \downarrow \text{product}$$

$$\text{(Enzyme-micelle) complex} + \text{enzyme} \leftrightarrows \text{(enzyme-micelle aggregate)}$$

This model assumes that the enzyme expressed its optimum activity when complexed with the mixed substrate-cholate micelle and that excess enzyme causes formation of an inactive enzyme-micelle aggregate.

Since the formation of the postulated inactive enzyme aggregate does not appear to have precedence in the literature, some remarks regarding this process are useful. The active enzyme complex would presumably be dynamic in the sense that the enzyme, once complexed to a specific micelle,

either remains complexed to that micelle and interacts with several substrate molecules on its surface or is released free in solution after completion of one catalytic event prior to formation of the next enzyme substrate complex.

The inactive enzyme aggregate could then reflect an interference with this dynamic process. Additional information regarding the formation of mixed micelles and enzyme-micelle complexes are required to describe this behavior in detail. Regardless of the lack of precedent for such a model, it is necessary to note that the description of these or similar micellar systems must include rigid control of enzyme concentration, ratio of enzyme to micelle concentration, and the type of salt employed.

Analysis of this model reveals the following characteristics. 1) Any perturbation which affects the surface of the micelle or the number of micelles may alter the association of the enzyme with the micelle or the availability of substrate on the surface of the micelle, thereby affecting the specific activity of the enzyme. For example, the addition of potassium or ammonium salts which reduce the observed specific activity might be expected to alter the electrostatic surface potential of the micelle and interfere with association of the enzyme. Likewise, the addition of the neutral detergent Triton X-100 might be expected to reduce the surface potential and also affect the specific activity of the enzyme. 2) Addition of excess enzyme should lead to rapid inactivation or a sharp decrease

in specific activity as was observed (Figure 27).

At low substrate concentrations the specific activity remains constant up to 1.4×10^{-7} M enzyme at which point a rapid decrease in activity is observed. Assuming that a decrease in activity is due to the formation of an inactive enzyme aggregate, addition of sodium taurocholate and sodium chloride should increase the concentration of micelles and thus increase the range of protein concentration over which constant specific activity is observed. Addition of inhibitor at this substrate concentration also extends the range of constant specific activity either by increasing the number of micelles or by allowing formation of a higher concentration of enzyme in the complexed form by allowing more active enzyme molecules per micelle.

At the intermediate concentration of substrate, the range of constant specific activity is greater than that observed at low substrate concentrations, but contrary to prediction the range of constant specific activity at this substrate concentration is not affected by sodium taurocholate or inhibitor. The significant increase in specific activity in the presence of inhibitor is not understood although this observation was made in one other experiment (Figure 28).

At the high substrate concentration, addition of taurocholate extends the range of protein over which a constant specific activity is observed presumably by providing an

increased concentration of micelles. The addition of inhibitor at this substrate concentration decreases the range of constant enzyme specific activity. Perhaps the combination of increased substrate and inhibitor concentration alters the cholate micelle surface allowing inactive enzyme aggregation at a lower concentration.

The range of constant specific activity with substrate alone at the high concentration is not increased over that observed at the intermediate concentration presumably because the substrate does not increase the number of micelles and the surface of the cholate micelle is already saturated. The addition of inhibitor at this substrate concentration now decreases the range of constant enzyme specific activity. Perhaps the inhibitor and increased substrate concentration alters the micelle surface allowing aggregation of enzyme at a lower concentration.

The unusual effects of inhibition by digalactosyl-ceramide, that is, the stimulatory effects at low substrate concentrations and inhibitory effects at high substrate concentrations confirms the observation made by Ho with partially purified placenta ceramide trihexosidase (123). Contrary to her interpretation of the results in terms of an enzyme with an effector site, examination of Figure 27 shows that the sigmoidal substrate saturation curve should not be observed at low enzyme concentration. In other words, if the enzyme concentration is sufficiently low, the specific

activity in the absence of taurocholate is identical to that observed in the presence of taurocholate resulting in hyperbolic kinetics.

In view of the concentration dependency of the enzyme and the effect of various substances on this phenomenon, it should be pointed out that the inhibition studies conducted under a carefully standardized set of conditions may reflect alterations in the micellar system rather than classical inhibition kinetics.

Classification by Gatt's Kinetic System: Since the hydrolysis of GL-3 is unusually dependent on the enzyme concentration it is difficult to attach significance to the "pseudo-micellar" system of Gatt. It is possible that the ceramide trihexosidases will hydrolyze some oligosaccharides in the presence of lecithin. This suggests that the enzymes may be somewhat nonspecific exogalactosidases under the proper conditions. These experiments also indicate that the A-2 form of ceramide trihexosidase has a different mechanism for hydrolyzing oligosaccharides than the natural glycolipid substrate. According to Gatt's classification system (124) the A-2 form of the enzyme hydrolyzes trisaccharide equally well in the presence and absence of substrate micelles. However it should be noted that the concentration of lecithin is high enough to provide a mixed lipid-substrate micelle at all substrate concentrations.

Butanol Solubility

The butanol method is normally applied with animal tissues to either solubilize the protein or to release the protein from the membrane. A list of several proteins obtained in solution or purified by butanol can be made. These proteins include actin, cholinesterase, lactic dehydrogenase and xanthine oxidase (165). Many of these proteins are lipoprotein or glycoprotein in nature and sequencing of a few of them has shown that they contain a large number of basic amino acid residues. It appears that butanol provides a hydrophobic environment for the water-insoluble portions of these molecules.

Most of these proteins are not completely butanol soluble and only xanthine oxidase was activated by butanol (198). However the C_{55}-isoprenoid alcohol phosphokinase isolated from the bacterial membrane of *Staphylococcus aureus* is soluble and stable in water-saturated butanol (199, 200). This enzyme is a lipoprotein which has an absolute dependency for a phospholipid cofactor. However the phospholipid can be replaced by detergent *in vitro* and the enzyme is optimally active in the presence of 0.5 M sodium chloride. Unlike the ceramide trihexosidases, the reaction is linear with increasing concentrations of enzyme.

AFFINITY CHROMATOGRAPHY

Protein purification by affinity chromatography utilizes the specific and reversible interaction of an enzyme with its substrate or a specific inhibitor. Purification is achieved by chromatographing a mixture of crude proteins on a column containing an enzyme-specific substrate or inhibitor attached by a covalent linkage to agarose beads. Proteins exhibiting affinity for the ligand will be retarded on the column to an extent related to their binding capacity for the ligand. The bound protein is eluted from the column by changing the elution medium so as to favor dissociation of the enzyme-ligand complex.

The general principles and specificity of this method were demonstrated by the use of specific adsorbents in the purification of staphylococcal nuclease directly from crude extracts, α-chymotrypsin, carboxypeptidase A, avidin, neuraminidase, and β-galactosidase (159, 162, 201).

In most instances it is desirable to attach an enzyme-specific ligand to the matrix backbone to minimize the adsorption of non-specific proteins. This was the approach taken by Breslow and Sloan for purification of glucocerebrosidase, sphingomyelinase and arylsulfatase (94, 179). In the case of the ceramide trihexosidases, it was fortuitous that the specific ligand could not be attached to the matrix and that the available carbohydrate substrate had an (α1→6) linkage. This probably aided in the adsorption of the

specific α-galactosidases since steric hinderance of the binding site was minimized.

In all probability the ceramide trihexosidases would not have been separated by an affinity column adsorbent containing a trihexosylsphingosine ligand. There is no evidence to indicate that the ceramide trihexosidases have different affinities for the lipid substrate, whereas there are several indications that the enzymes have different affinities for the carbohydrate ligand. 1) Five of the seven ceramide trihexosidases bind to the affinity column with varying degrees of strength and are eluted in different fractions. 2) The A-1 form of ceramide trihexosidase binds strongly to the column only in the presence of lecithin, but is only retarded in its absence. 3) Kinetic investigations employing the system proposed by Gatt (124) indicate that the two A forms of ceramide trihexosidase recognize different forms of carbohydrate substrates.

α-Galactosidases of Whole Plasma

Investigation of normal plasma revealed that the A forms of ceramide trihexosidase were present in nearly equal quantities. This indicates that some of the enzymatic activity was lost during the enzyme purification. It is likely that the ceramide trihexosidases are lost during Cohn fractionation since the specific activity of these fractions is 0.07 as compared to 0.2 in whole plasma. In addition,

100 ml of plasma contains as many μg of ceramide trihexosidase as are obtained from the amount of Cohn fraction representing 2-3 liters of plasma. Although there is no evidence, it is possible that most of the ceramide trihexosidase activity occurs as an inactive form in Cohn fraction V, since this is the fraction from which glycoproteins are classically isolated (183). The reason for their inactivation is unknown but other experiments have indicated that an absence of enzymatic activity cannot be equated with an absence of the enzyme.

Digalactosylceramide:Galactosyl Hydrolase

Normal plasma digalactosylceramide:galactosyl hydrolase activity is coincident with the activity of the non-specific α-galactosidase PN-A-2. This enzyme is separated into two active components by isoelectric focusing and cellulose acetate electrophoresis. In Fabry plasma only the protein of slower electrophoretic mobility is detectable.

Beutler and Kuhl recently reported that the normal plasma "ceramide trihexosidase" which hydrolyzes 4-methylumbelliferyl-α-galactoside consists of two isozymes distinguishable by electrophoretic mobility (121). They further reported that only the enzyme of slower electrophoretic mobility was detectable in Fabry plasma and that it was indistinguishable from the normal plasma enzyme on electrophoresis.

The correlation between digalactosylceramide:galactosyl hydrolase and Beutler's 4-methylumbelliferylgalactoside may be coincidental. However it is possible that other investigators are measuring GL-2b hydrolase rather than ceramide trihexosidase. The studies of Crawhall and Banfalvi (122) correlate well with Beutler's studies, but the lack of detailed information in these reports forbids any definite conclusions.

A deficiency of GL-2b hydrolase explains the accumulation of this glycosphingolipid in Fabry kidney (49) and urinary sediment (182). It was previously assumed that ceramide trihexosidase would hydrolyze GL-2b since the lipid has a terminal residue identical to that of GL-3. However, purified ceramide trihexosidase does not hydrolyze GL-2b to an appreciable extent, although the lipid competitively inhibits both of the A forms of the enzyme.

Non-Specific α-Galactosidases of Whole Plasma

The question of why the artificial substrates can be used successfully to diagnose Fabry's disease when ceramide trihexosidase does not hydrolyze p-nitrophenyl-α-galactoside or 4-methylumbelliferyl-α-galactoside cannot be answered on the basis of present knowledge. However, it cannot be disputed that a depressed plasma α-galactosidase level for these artificial substrates is one of the biochemical characteristic of Fabry's disease.

It is known that the artificial substrates are measuring the combined activities of a group of α-galactosidases whose specific functions are unknown. There are six detectable peaks of non-specific enzymatic activity obtained by affinity chromatography of whole plasma. The unexpected discrepancy of Fabry α-galactosidase activity before and after affinity chromatography is not easily explained. However it is known that enzymatic or electrophoretic quantitation of total α-galactosidase levels often leads to erroneous assumptions concerning the level of a particular glycoprotein. For instance, the total glycoprotein level is depressed in renal diseases (202), cancer (203) and wounding (204), but the isolation and partial purification of specific proteins showed them to be 2- or 3-fold elevated while other proteins in the preparation showed normal or slightly depressed activity (204).

It might be postulated that an inhibitor of the non-specific galactosidases accumulates in Fabry plasma and is removed by affinity chromatography. It is attractive to hypothesize that the accumulated proteins, presumably inactive forms of ceramide trihexosidase, are inhibitory to these galactosidases. However, the catalytically inactive proteins are not present in sufficient quantity to depress the total α-galactosidase activity. In addition, the Fabry heterozygote has nearly the same concentration of catalytically inactive proteins as the hemizygote but has a higher α-galactosidase activity.

A second alternative is that there is a depressed level of acidic α-galactosidases resulting from the renal complications of the disease. This is not a feasible suggestion because each Fabry differs in renal deficiency and the heterozygotes are more severely affected than the hemizygotes in some cases (205).

The most practical suggestion is that an inhibitor of unknown composition is present in Fabry plasma and causes partial inactivation of the non-specific α-galactosidases. Furthermore this inhibitor is removed by affinity chromatography, suggesting that it is not α-galactosidase in nature.

This hypothesis derives some support from the fact that several ions are known to reversibly inactivate the ceramide trihexosidases. In addition, the plasma enzymes become rapidly inactive in whole plasma but the catalytic activity is regained by affinity chromatography. There is an unknown component eluted two to three fractions ahead of the first ceramide trihexosidase fraction in plasma containing no enzymatic activity. This component is yellow, non-protein in nature, and its color is destroyed by the addition of base. This may be only coincidental but these fractions are not obtained with fresh plasma which has catalytic activity at the time of chromatography. The addition of this component to active α-galactosidase fractions might determine whether it inhibits these enzymes.

GENETIC IMPLICATIONS OF MULTIPLE CERAMIDE TRIHEXOSIDASE DEFICIENCIES

Fabry's disease has been shown to be an X-linked disorder by pedigree studies (59) and by the demonstration that clones of fibroblasts from heterozygotes display a bimodal population with respect to α-galactosidase activity (53). The nature of the enzyme deficiency could be explained on the basis of either an X-linked regulator gene or X-linkage of the structural locus for the enzyme. A structural gene mutation could be proved by demonstrating an abnormal residual α-galactosidase activity in the cells of patients with Fabry's disease. If no abnormal proteins were found, a regulatory mutation would be possible. The question of whether Fabry's disease involves a structural or a regulatory mutation has been considered in four recent papers (56, 190, 206-7).

Beutler and Kuhl found that the 4-methylumbelliferyl-α-galactosidase activity of leukocytes and fibroblasts consisted of two α-galactosidases differing in electrophoretic mobility (206). These proteins were designated as isozymes A and B, A being the more electrophoretically rapid. The residual enzyme activity in leukocytes and fibroblasts of Fabry patients appeared to represent an increased quantity of the normal heat stable B form rather than an abnormal A form. Since these authors were unable to find an abnormal enzyme they entertained the possibility that the mutation was regulatory.

Nadler and Wood confirmed these findings but suggested that the enzymatic defect could be attributed to the absence of a specific sialyltransferase (56). This conclusion was made by analogy with Tay-Sach's disease and metachromatic leukodystropy in which the acidic isozyme is absent. This suggests that the basic form of the enzyme is the precursor of the acidic form to which it is converted by the addition of sialic acid residues.

Ho *et al.* demonstrated similar findings in liver (190). However the liver α-galactosidases were separated into seven proteins by isoelectric focusing. On starch gel electrophoresis three of these proteins migrated with the A isozyme, two with the B isozyme and two remained at the origin. Fabry liver contained only one B form of activity.

Neuraminidase treatment of control liver supernates resulted in rapid conversion of the A forms to B forms of the α-galactosidase. Examination of the thermolability of the B enzyme following neuraminidase treatment revealed that control B activity was thermolabile while that from Fabry liver was thermostable. These authors suggested that Fabry's disease might be the result of a structural gene mutation or a specific sialyltransferase deficiency.

Sutton and Omenn reviewed these papers and dismissed the possibility of a sialyltransferase deficiency on the basis that neuraminidase treatment of the α-galactosidases did not alter their catalytic properties (207).

In contrast to these findings it was found that the B forms of ceramide trihexosidase were absent in Fabry plasma while the A forms were present. In addition catalytically inactive proteins were found in hemizygous and heterozygous Fabry plasma. There is not enough information concerning the Fabry ceramide trihexosidases, human genetics, or the intermediary metabolism of glycoproteins to enable a reasonable hypothesis as to whether these enzyme alterations are due to a regulatory or a structural mutation. However, the following suggestions are offered as an explanation for the ceramide trihexosidase pattern in Fabry plasma.

It is possible that the A-1 form of ceramide trihexosidase is the metabolically most significant form of the enzyme *in vivo* and that the B forms are rapidly sialylated when synthesized in an attempt to regain a catalytically active form of this enzyme. It must be further assumed that interconversion of B to A in normal persons is controlled to maintain a dynamic equilibrium between the enzymes since normals have a constant ratio of A to B activity.

Since the catalytically inactive proteins have a slightly greater binding capacity to the affinity column than the A-1 form of ceramide trihexosidase, it is possible that they are partially sialylated B forms. However attempts to incorporate sialic acid into these proteins were unsuccessful. This suggests that these proteins are either fully sialylated or not recognized by the sialyltransferases.

If the second alternative is correct, there would be a defective glycosyltransferase resulting in the formation of a carbohydrate sequence not recognized by the available sialyltransferases. Alternatively, there could be an alteration at the catalytic site of the B form. In either case, an incomplete mutation must be assumed to explain the fact that the Fabry hemizygote has some protein which appears to be the normal A-1 form in binding capacity to the affinity column and electrophoretic mobility.

It is suggested that the A-2 form of ceramide trihexosidase arises from the A-1 form. When the A-1 form is desialylated, it is rapidly removed from the circulation, resialylated and sent back into the circulation as A-2. This is suggested because the A-2 form of ceramide trihexosidase has the same electrophoretic properties as the A-1 form, but is more susceptible to inhibition and may hydrolyze oligosaccharide substrates in a non-micellar system. These factors make it appear to be an altered form of a true glycosphingolipid hydrolase. In addition, whole kidney homogenate has A and B activity but the B activity rapidly disappears accompanied by an increase in A activity. Affinity chromatography showed this activity to be of the A-2 form. This suggests that there are two pools of B enzymes; one is newly synthesized and converted to the A-1 form of the enzyme and the other is desialylated ceramide trihexosidase, Form A-1.

This might be disproved by studying the kinetics of the enzyme formed by incorporation of [^{14}C]sialic acid into the B form of the enzyme. Tissue culture studies might also be used to determine whether the B-V form of ceramide trihexosidase can be taken up by the organ, resialylated and sent back into the culture medium as a catalytically active protein having the kinetic properties of either of the A forms of ceramide trihexosidase.

Although this scheme may be biochemically feasible it explains neither the genetics of the ceramide trihexosidases nor their relationship to the 4-methylumbelliferyl-α-galactosides.

FEASIBILITY OF ENZYME REPLACEMENT THERAPY

The results of plasma infusion experiments indicate that the ceramide trihexosidases have the capability of hydrolyzing GL-3 *in vivo* and can be expected to decrease the level of accumulated plasma substrate. The enhancement of enzymatic activity in these experiments might have resulted from activation of the Fabry enzyme, but was more likely the result of several factors acting together to cause activation of the normal enzyme which was infused. When heparin is infused intravenously, there is a release of lipoprotein lipase from the vascular bed (208). This might result in the same type of activation observed after addition of butanol to crude enzyme preparations. Butanol causes a 100-150% activation in the activity of the ceramide trihexosidases.

The amount of enzyme infused in these pilot studies was not sufficient to indicate whether enzyme replacement would effect any clinical improvement in the disease. However, enzyme replacement by renal transplantation has indicated that the presence of catalytically active enzymes in Fabry patients will lead to the eventual control of the disease (209-10).

The significance of the two primary forms of ceramide trihexosidase in plasma remains obscure. Attempts to determine the physiological role of these forms will be complicated since the B form is composed of several enzymatically active components. Certainly the question of their chemical and physiological relationship will have to be examined more critically when individual components are available in sufficient quantities for analyses of their carbohydrate composition and for studies of several kinetic parameters.

The turnover rates in plasma of injected lysosmal hydrolases will be of considerable importance in the treatment of storage diseases by enzyme replacement therapy. It is not known whether there is a difference in the A and B forms of ceramide trihexosidase in this respect. However, recent information suggests that the presence of sialic acid residues on glycoproteins prolongs their lifetime in circulation. It has been found that desialylated forms of some plasma glycoproteins can be removed from the circulation

by liver parenchymal cells (211). The binding of these proteins to the hepatic membrane was shown to involve the obligatory presence of sialic acid on what was presumed to be a glyoprotein acceptor site (212). In addition, it was suggested that this binding could be reversed by cytoplasmic neuraminidase or changes in calcium ion concentration and pH (212). Perhaps the same mechanism will be involved in the removal of the B form of ceramide trihexosidase at a rapid rate if it is injected into patients or control subjects.

It has not been determined whether binding of desialylated glycoproteins serves exclusively as a mechanism for cellular absorption and catabolism of circulating glycoproteins, or whether these receptor sites might also be utilized for resialylation and subsequent release of reformed sialoglycoprotein into the circulation.

Although studies on the turnover rates of the A and B forms of ceramide trihexosidase in plasma will be important in the choice of an appropriate form for therapeutic trials, another question is of equal importance to the ultimate therapeutic usefulness of lysosomal hydrolases in genetic storage diseases. Whether the catabolism of accumulated substances in abnormal cells can be achieved by injection of these enzymes depends on their accessibility to target organs. It is presumed that such cells will be able to form pinocytotic vesicles containing the hydrolase and that there will be transport in the cytoplasm to the secondary lysosomes

where stored material is located. It has been shown with cultured fibroblasts from Fabry patients that a plant α-galactosidase can decrease the amount of cellular ceramide trihexoside when added to the medium (213). It is difficult to predict which of the ceramide trihexosidases will be best able to penetrate abnormal cells *in vivo* and which will have the longest intercellular lifetime.

Another question to consider is whether the full compliment of α-galactosidases might have utility in the treatment of Fabry's disease. The Fabry patient has a depressed level of some non-specific α-galactosidases, GL-2b hydrolase and the ceramide trihexosidases. The *in vivo* role of these individual enzymes has not been determined and it may be that some of the clinical symptoms of the disease arise from the absence of non-specific hydrolases.

SUMMARY

Plasma ceramide trihexosidase activity was separated into seven proteins. These glycoproteins consisted of two groups of enzymes related by their sialic acid content. The A form of ceramide trihexosidase was purified from Cohn fraction IV-1 by a series of steps including ammonium sulfate precipitation, butanol treatment, acetone precipitation and affinity chromatography.

The biochemical characteristics of the two A forms of ceramide trihexosidase were investigated. It was found that the two enzymes were remarkably similar in their molecular

weights, electrophoretic characteristics, response to the lipid substrate under optimal conditions, and substrate specificity; they differed in heat stability, solubility in butanol and response to various inhibitors.

The ceramide trihexosidases were activated by sodium taurocholate and sodium chloride and their hydrolysis of GL-3 was non-linear with increasing enzyme concentration.

An α-galactosidase affinity column adsorbent was prepared and used to study the enzymes in normal and Fabry plasma. It was found that normal plasma contained six non-specific α-galactosidases in addition to the ceramide trihexosidases. Affinity chromatography of Fabry plasma revealed that the A forms of ceramide trihexosidase were partially inactive, whereas the B forms were completely absent. There was also an accumulation of catalytically inactive proteins and an alteration of the specific activity of several of the non-specific α-galactosidases in Fabry plasma.

Through the use of affinity chromatography a specific enzyme for the hydrolysis of GL-2b was discovered in normal plasma. This enzyme was separated into two enzymatically active components by isoelectric focusing and cellulose acetate electrophoresis. Only the enzyme of slower electrophoretic mobility was present in Fabry plasma.

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APPENDIX

List of Publications

Mapes, C.A., Anderson, R.L., and Sweeley, C.C. Trihexosylceramide:Galactosyl Hydrolase in Normal Human Serum and Plasma and its Absence in Patients with Fabry's Disease. Fed. Proc., 29, 409 (1970).

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Mapes, C.A., and Sweeley, C.C. Properties of Ceramide Trihexosidase. In: Proceedings of the Symposium on Enzyme Replacement in Genetic Diseases. R.J. Desnick, W. Krivit and R. Bernlohr (Editors) The National Foundation, New York.

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In Review

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