THE PURIFICATION AND PARTIAL CHARACTERIZATION OF α-GALACTOSIDASES A AND B FROM HUMAN LIVER

By

Kenneth James Dean

A DISSERTATION

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Department of Biochemistry

1978

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ABSTRACT

THE PURIFICATION AND PARTIAL CHARACTERIZATION OF α-GALACTOSIDASES A AND B FROM HUMAN LIVER

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α-Galactosidases A and B were purified 67,000-fold and 68,000-fold, respectively, from normal human liver. The purification procedure included ammonium sulfate precipitation, DEAE-cellulose chromatography, Sephadex G-150 chromatography, hydroxylapatite chromatography and ampholyte displacement chromatography on DEAE-cellulose.

α-Galactosidase A (EC 3.2.1.22) catalyzed the hydrolysis of 4-methylumbelliferyl-α-D-galactopyranoside, galabiaose and globotriaose at pH 4.5-4.6. The nonionic detergent Triton X-100 had no effect on the pH optimum for the hydrolysis of 4-methylumbelliferyl-α-D-galactopyranoside; however, the anionic detergent sodium taurocholate inhibited the hydrolysis of this substrate below pH 4.8. This enzyme also catalyzed the hydrolysis of galabiosylceramide and globotriglycosylceramide at pH 4.1 in the presence of 5.7 mM and 9.3 mM sodium taurocholate, respectively. These findings suggest that there is a strong electrostatic interaction between sodium taurocholate and α-galactosidase A below pH 4.8. α-Galactosidase A also catalyzed the hydrolysis of p-nitrophenyl-2-deoxy-α-D-galactopyranoside at pH 4.6, but did not hydrolyze o-nitrophenyl-α-N-acetylgalactosaminide, p-nitrophenyl-α-D-glucopyranoside or o-nitrophenyl-α-D-fucopyranoside. Furthermore, the hydrolysis of 4-methylumbelliferyl-α-D-galactopyranoside was not inhibited by 20 mM D-galactal or 20 mM D-(+)-fucose. These findings suggest that α-galactosidase A lacks absolute specificity for the hydroxyl group at C-2; however, replacement of this group with an acetamido group appears to prevent binding with the enzyme, possibly due to steric hindrance. α-Galactosidase A appears to have absolute specificity for the hydroxyl groups at C-4 and C-6.

α-Galactosidase B catalyzed the hydrolysis of 4-methylumbelliferyl-α-D-galactopyranoside, o-nitrophenyl-α-N-acetylgalactosaminide, globotriglycosylceramide, globopentaglycosylceramide, globotriaose and

globopentaose. Th
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globopentaose. The affinity of the enzyme toward substrates containing terminal α-N-acetylgalactosamine residues was approximately 5 times greater than its affinity toward substrates containing terminal α-galactose residues, based on the Michaelis constants with these substrates. Furthermore, the maximal velocities with substrates containing terminal α-N-acetylgalactosamine were 3 times greater than those with substrates containing terminal α-galactose residues. Therefore, it appears likely that this enzyme functions as an α-N-acetylgalactosaminidase (EC 3.2.1.49) rather than an α-galactosidase in vivo. α-Galactosidase B also catalyzed the hydrolysis of o-nitrophenyl-α-D-fucopyranoside and p-nitrophenyl-2-deoxy-α-D-galactopyranoside, but not p-nitrophenyl-α-D-glucopyranoside or 4-methumbelliferyl-α-N-acetylglucosaminide. One explanation for these results is that α-galactosidase B (α-N-acetylgalactosaminidase) lacks absolute specificity for the substituents at C-2 and C-6, but may have absolute specificity for the axial hydroxyl group at C-4.

To my dear

To my dear wife, Becky, who supported and encouraged me throughout my graduate studies.

I wish to exp
advisor and friend
development. I wo
Barker, Dr. Claren
discussions, and t
Sweeley's and Dr.
encouragement.

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I wish to express my gratitude to Dr. Charles C. Sweeley, my advisor and friend, for his guidance and concern for my professional development. I would also like to express my appreciation to Dr. Robert Barker, Dr. Clarence H. Suelter and Dr. John F. Holland for many helpful discussions, and to the graduate students and postdoctorates in Dr. Sweeley's and Dr. Barker's research groups for their friendship and encouragement.

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Uridine di
α-D-galactopyran
UDP-GlcNAc; uric
osine diphosphat
mannopyranoside,
CMP-NeuAc; gluco
glycosylceramide
pentaglycosylcer
nectetraglycosyl
Galabiosylceramic
4-MU-α-Gal; o-nit
phenyl-2-deoxy-α-
D-fucopyranoside,
p-NP-α-Glc; 4-met
4-methylumbellife
belliferyl-β-D-ga
xylopyranoside, 4
4-MU-α-L-Ara; gala

ABBREVIATIONS

Uridine diphosphate-α-D-glucopyranoside, UDP-Glc; uridine diphosphate-α-D-galactopyranoside, UDP-Gal; uridine diphosphate-α-N-acetylglucosaminide, UDP-GlcNAc; uridine diphosphate-α-N-acetylgalactosaminide, UDP-GalNAc; guanosine diphosphate-β-L-fucopyranoside, GDP-Fuc; guanosine diphosphate-α-D-mannopyranoside, GDP-Man; cytosine monophosphate-α-N-acetylneuraminic acid, CMP-NeuAc; glucosylceramide, GlcCer; lactosylceramide, LacCer; globotriglycosylceramide, $GbOse_3Cer$; globotetraglycosylceramide, $GbOse_4Cer$; globopentaglycosylceramide (Forssman antigen), $GbOse_5Cer$; IV^4-α-galactosyllactoneotetraglycosylceramide, IV-α-Gal-$LcnOse_4Cer$; galactosylceramide, GalCer; Galabiosylceramide, $GaOse_2Cer$; 4-methylumbelliferyl-α-D-galactopyranoside, 4-MU-α-Gal; o-nitrophenyl-α-N-acetylgalactosaminide, o-NP-α-GalNAc; p-nitrophenyl-2-deoxy-α-D-galactopyranoside, p-NP-2-deoxy-α-Gal; o-nitrophenyl-α-D-fucopyranoside, o-NP-α-D-Fuc; p-nitrophenyl-α-D-glucopyranoside, p-NP-α-Glc; 4-methylumbelliferyl-β-N-acetylgalactosaminide, 4-MU-β-GalNAc; 4-methylumbelliferyl-α-N-acetylglucosaminide, 4-MU-α-GlcNAc; 4-methylumbelliferyl-β-D-galactopyranoside, 4-MU-β-Gal; 4-methylumbelliferyl-β-D-xylopyranoside, 4-MU-β-Xyl; 4-methylumbelliferyl-α-L-arabinopyranoside, 4-MU-α-L-Ara; galactose, Gal; lactose, Lac; raffinose, Raf.

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INTRODUCTION

The enzymes involved in the metabolism of glycoconjugates have been an area of much interest due to their involvement in human glycolipid, glycoprotein and mucopolysaccharide storage diseases. These hereditary disorders of complex carbohydrate metabolism are characterized by the absence or deficiency of specific exoglycosidases or glycosyltransferases, which results in the accumulation of the glycolipid, glycoprotein or mucopolysaccharide substrate(s) of the missing enzyme(s). Several excellent reviews on the pathology and biochemistry of these disorders (1-3) and diagnostic procedures (4) have been published. Research on these enzymes has intensified in recent years with the realization that enzyme replacement therapy may be efficacious in treating some of these disorders. Many of the enzymes have now been purified and their physical and kinetic properties partially characterized.

This thesis describes the purification and partial characterization of α-galactosidases from normal human liver. One of these enzymes, α-galactosidase A (EC 3.2.1.22), is absent or deficient in Fabry's disease, an X-linked glycosphingolipidosis in which glycolipids containing terminal α-galactose residues (galabiosylceramide, globotriglycosylceramide and blood group B-active glycolipids) accumulate. The other α-galactosidase present in human liver (form B) is not absent in Fabry's disease, but in the course of these investigations was also found to be an α-N-acetylgalactosaminidase. This enzyme is probably more active as an α-N-acetylgalactosaminidase (EC 3.2.1.49) rather than an α-galactosidase in vivo, hydrolyzing globopentaglycosylceramide (Forssman antigen), and blood group A-active glycolipids and glycoproteins, all of which contain terminal α-N-acetylgalactosamine residues.

A complete review of the chemistry and metabolism of glycoconjugates is certainly beyond the scope of this thesis. A general discussion of current concepts of the biosynthesis and catabolism of glycoconjugates will be presented, followed by a discussion of the chemistry and metabolism of glycoconjugates containing terminal α-galactose and α-N-acetylgalactosamine residues found in man.

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REVIEW OF THE LITERATURE

The term "glycoconjugates" encompasses a large class of compounds, consisting of glycolipids, glycoproteins and proteoglycans, which have the common feature of bearing covalently attached carbohydrate substituents. Examples of oligosaccharide structures typical of each of these classes of glycoconjugates are shown in Figure 1. At first approach, the great diversity of oligosaccharide structures in glycoconjugates may discourage the reader from further study; however, close examination of these structures reveals that they are not entirely unique. Many of them have the same oligosaccharide backbone or core, and differ only in the nature and position of substituents on the core structure. Complex carbohydrates that contain the same oligosaccharide backbone or core structure may be classified as members of the same family. Recently, Sweeley and Siddiqui (5) described the classification of glycolipids into seven families with different tetrasaccharide core structures. The carbohydrate substituents on glycoproteins and proteoglycans can be classified into families on the basis of their amino acid linkage (to serine, threonine, hydroxylysine or asparagine) and the structures of their core oligosaccharides (6-9).

The compounds within each family of complex carbohydrates are related by more than their common structural features, since they also have common metabolic pathways and may also have similar functions. The reader is referred to several excellent reviews (5-15), which discuss the diverse structures, functions and metabolism of glycoconjugates in greater detail than will be presented here.

I. Current Concepts of Glycoconjugate Metabolism

The biosynthetic pathways for the carbohydrate portions of glycolipids and glycoproteins are thought to proceed by somewhat different mechanisms. The biosynthesis of glycolipids appears to occur primarily in the Golgi apparatus (11-16). Glycosphingolipid biosynthesis is initiated by the transfer of galactose or glucose from UDP-Gal or UDP-Glc directly to the hydroxyl group at C-1 of ceramide. A second, minor

Figure 1. Examples of Oligosaccharide Structures of Typical Glycoconjugates

The oligosaccharides of (I) a glycolipid [II^3-α-N-acetylneuraminosyl-gangliotriglycosylceramide (G_{M2} ganglioside that accumulates in Tay-Sachs and Sandhoff's diseases) (3,5)]; (II) a circulatory glycoprotein [this glycoprotein may also bear a Fuc(α1→6) substituent on the asparagine-linked N-acetylglucosamine residue, or a GlcNAc(β1→4) substituent on the mannose residue at the branching point (6)] and; (III) a mucopolysaccharide [chondroitin-4-sulfate that accumulates in Morquio's Syndrome. The oligosaccharide shown is the core structure for this mucopolysaccharide, which may contain repeating GlcUA(β1→3)GalNAc-4-SO_4 units (3)]. Mucopolysaccharides, or proteoglycans, contain much more carbohydrate than the glycoproteins do, and are sometimes referred to as glycopeptides.

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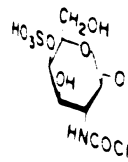
III



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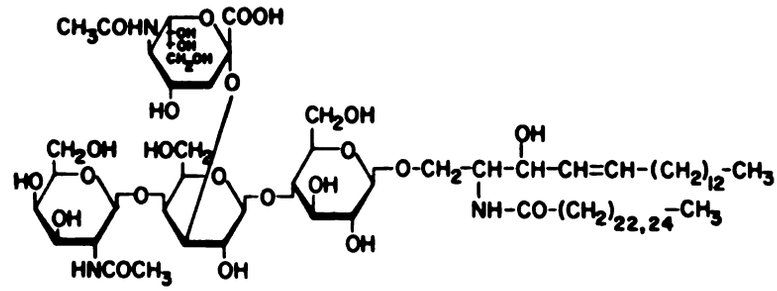

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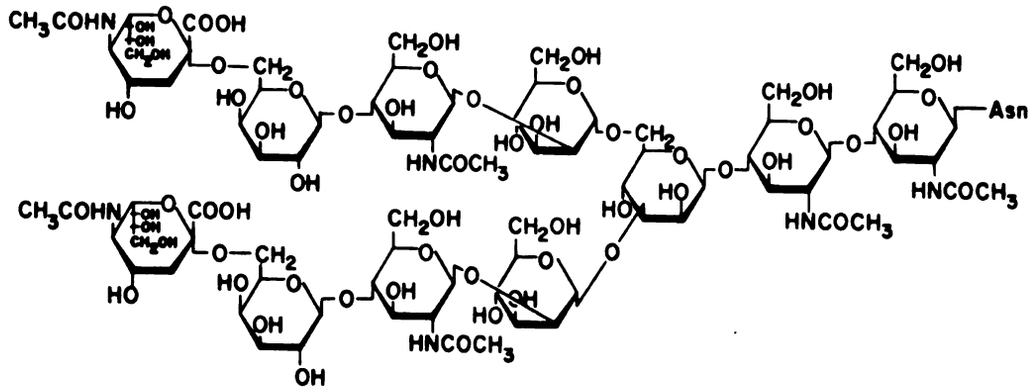

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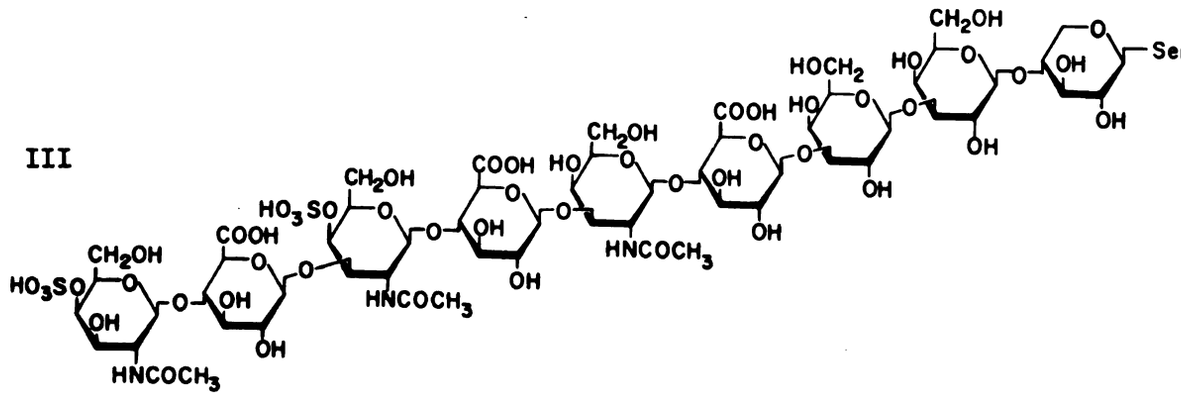
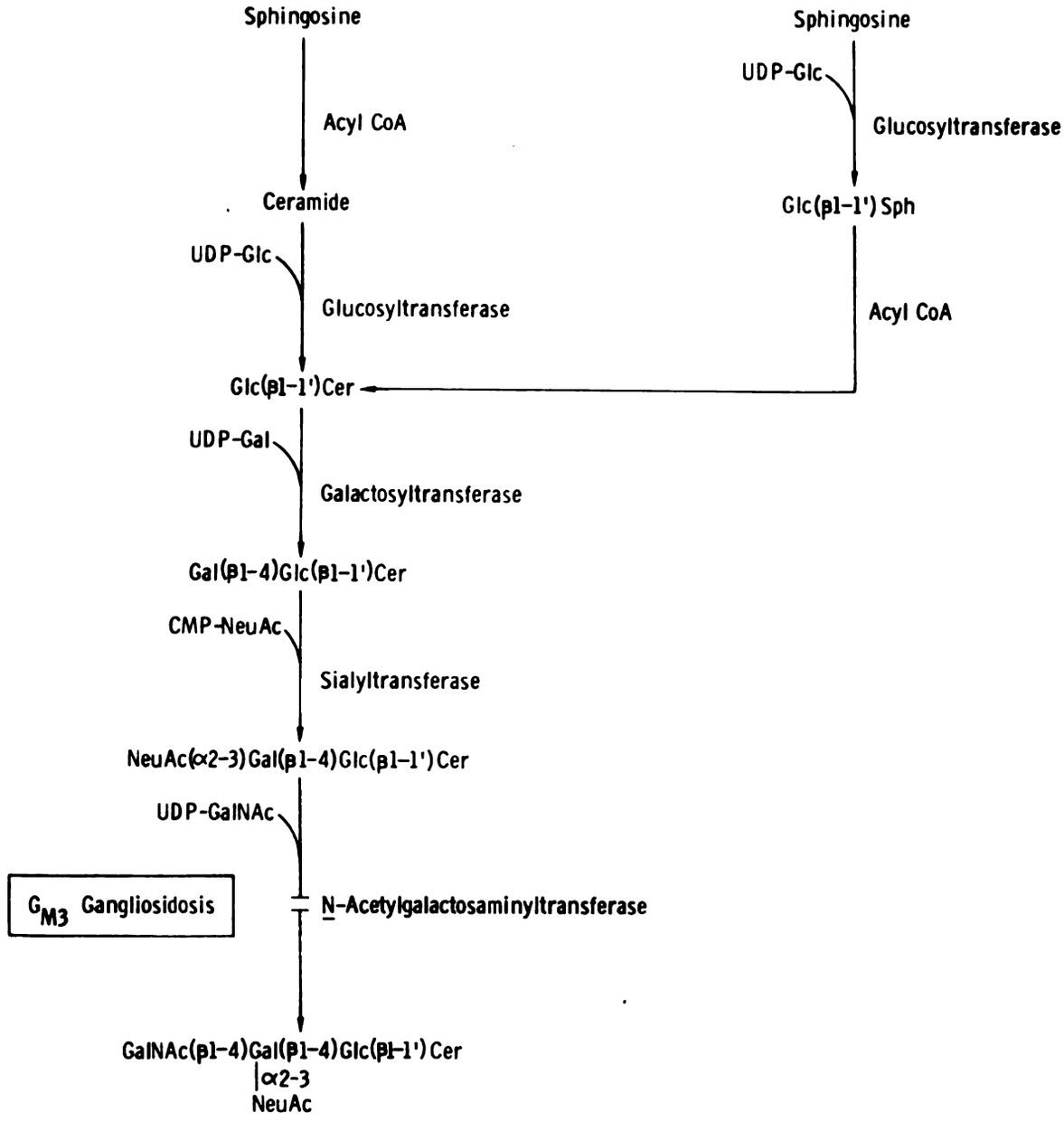


Figure 2. Typical Pathway of Glycosphingolipid Biosynthesis

The biosynthesis of II^3-α-N-acetylneuraminosyl-gangliotriglycosyl-ceramide (G_{M2} ganglioside) is shown.


Sphingosine
Acyl CoA
Ceramide
UDP-Glc
Glucosyltransferase
Glc(β1-1')Cer
UDP-Gal
Galactosyltransferase
Gal(β1-4)Glc(β1-1')Cer
CMP-NeuAc
Sialyltransferase
NeuAc(α2-3)Gal(β1-4)Glc(β1-1')Cer
UDP-GalNAc
G M3 Gangliosidosis
N-Acetylgalactosaminyltransferase
GalNAc(β1-4)Gal(β1-4)Glc(β1-1')Cer
α2-3
NeuAc
Sphingosine
UDP-Glc
Glucosyltransferase
Glc(β1-1')Sph
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pathway may exist for the biosynthesis of cerebrosides, involving the transfer of galactose or glucose to sphingosine to form psychosine (galactosylsphingosine) or glucosylsphingosine, which is then acylated by acyl CoA to form the corresponding cerebroside (17-19). The carbohydrate chain of the glycolipid may then be extended by the sequential transfer of monosaccharides from the appropriate sugar nucleotide (UDP-Gal, UDP-GalNAc, GDP-Fuc or CMP-NeuAc) to the nonreducing end of the oligosaccharide moiety of the acceptor glycolipid (10,11). An example of glycolipid biosynthesis is shown in Figure 2.

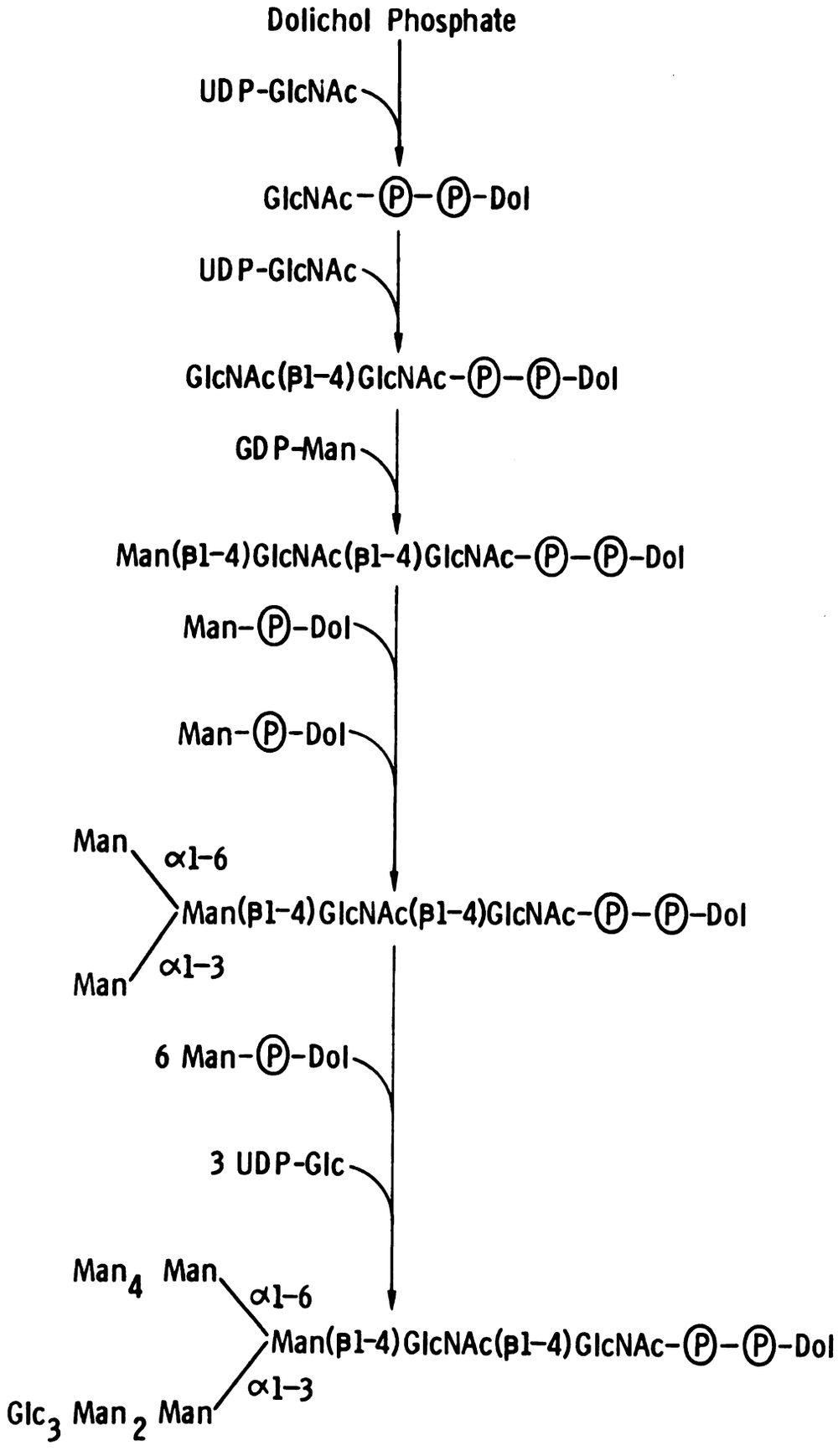
In contrast to the direct manner in which glycolipids are synthesized, with the sequential transfer of monosaccharides from sugar nucleotide donors directly to the nascent glycosphingolipid, an intermediate acceptor is involved in the biosynthesis of the core oligosaccharide of some glycoproteins, as shown in Figure 3. The biosynthesis of glycoproteins with asparagine-linked carbohydrate has been described by Schacter et al. (8, 9), and will be presented as a general model for glycoprotein biosynthesis. However, this is not intended to preclude the existence of other pathways for glycoprotein biosynthesis; the biosynthesis of glycoproteins with serine or threonine-linked carbohydrate is probably carried out by a different pathway (22).

The biosynthesis of the core oligosaccharide of asparagine-linked complex carbohydrates is thought to take place in the rough endoplasmic reticulum (8,9). The synthesis of the core oligosaccharide is initiated by the transfer of N-acetylglucosamine from UDP-GlcNAc to a polyisoprenoid phospholipid acceptor, dolichol phosphate (7), to form N-acetylglucosaminylpyrophosphoryldolichol. The carbohydrate chain is then extended by the sequential transfer of N-acetylglucosamine and mannose from UDP-GlcNAc and GDP-Man to form Man(β1→4)GlcNAc(β1→4)GlcNAc-P-P-Dol. The carbohydrate chain is further extended, with branching, by the sequential transfer of mannose residues from mannosylphosphoryldolichol (generated by the transfer of mannose from GDP-Man to dolichol phosphate) to the nonreducing end of the nascent glycopyrophosphoryldolichol (7,23). The transfer of mannose residues appears to be followed by the transfer of three glucose residues to the nascent core oligosaccharide (9,25,26). The entire oligosaccharide is then transferred from dolichol pyrophosphate to specific asparagine residues on the protein (24), a process which explicitly requires the terminal glucose residues for effective transfer (25,26). This is followed

Figure 3. The Biosynthesis of Glycopyrophosphoryldolichol

The biosynthesis of the pyrophosphoryldolichol intermediate involved in the biosynthesis of asparagine-linked complex carbohydrates is shown (7).

Glc


Dolichol Phosphate
UDP-GlcNAc
GlcNAc-Ⓟ-Ⓟ-Dol
UDP-GlcNAc
GlcNAc(β1-4)GlcNAc-Ⓟ-Ⓟ-Dol
GDP-Man
Man(β1-4)GlcNAc(β1-4)GlcNAc-Ⓟ-Ⓟ-Dol
Man-Ⓟ-Dol
Man-Ⓟ-Dol
Man
α1-6
Man(β1-4)GlcNAc(β1-4)GlcNAc-Ⓟ-Ⓟ-Dol
Man
α1-3
6 Man-Ⓟ-Dol
3 UDP-Glc
Man4 Man
α1-6
Man(β1-4)GlcNAc(β1-4)GlcNAc-Ⓟ-Ⓟ-Dol
α1-3
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by a series of processing reactions to remove excess glucose and mannose residues and reveal the core ologosaccharide (27,28). Intracellular vesicular glycoproteins, such as the lysosomal acid hydrolases, may be sequestered before processing to the core oligosaccharide is complete. Glycoproteins bearing only the core oligosaccharide subsequently appear in the Golgi apparatus, where the carbohydrate chains are extended by the sequential transfer of monosaccharides directly from sugar nucleotides (UDP-GlcNAc, UDP-Gal, UDP-GalNAc, GDP-Fuc, GDP-Man and CMP-NeuAc) to the oligosaccharide core, as shown in Figure 4.

The glycosyl transfer reactions involved in the biosynthesis of glycoconjugates are catalyzed by glycosyltransferases, which are specific for the sugar nucleotide donor, the oligosaccharide acceptor, and the linkage and anomerity of the glycosidic bond being formed (9-11). These enzymes are found in the cisternae of the rough endoplasmic reticulum and Golgi, as has been mentioned, and on the plasma membrane. Roseman (10), and others (29-31), have suggested that the glycosyltransferases may be associated in multienzyme complexes, which carry out the entire synthesis of specific oligosaccharides. This concept stems from the observation that the product of one glycosyl transfer reaction becomes the substrate for the next glycosyltransferase in the sequence. Roseman (10) has also suggested that the glycosyltransferases located on the plasma membrane could play a role in intercellular adhesion. This hypothesis has been embroiled in controversy (32,33) since its publication and has not been conclusively resolved.

The catabolism of glycoconjugates is carried out by exoglycosidases (glycosylhydrolases), as shown in Figure 5. These enzymes have been studied more extensively than the glycosyltransferases, partly because they are easily solubilized in aqueous solutions, and partly because of the involvement of many of these enzymes in human glycoconjugate storage diseases (1-4), whereas only one glycosyltransferase (G_{M3}: β-N-acetylgalactosaminyltransferase) has been shown to be associated with a disorder of glycoconjugate metabolism (20,21). The glycosidases were thought to be localized primarily in the lysosome (34-36), however sialylated forms of these enzymes are also present in plasma (37-39), where they may play a significant role in modulating the turnover of circulating glycoproteins, and neutral glycosidases are thought to be present in the endoplasmic reticulum, where glycoprotein processing reactions are carried out. Indeed,

Figure 4. The Biosynthesis of a Glycoprotein with Asparagine-Linked Carbohydrate.

The biosynthesis of the carbohydrate portion of a glycoprotein with asparagine-linked oligosaccharides is shown. This proceeds with transfer of the oligosaccharide from dolichol pyrophosphate to asparagine residues on the glycoprotein, followed by processing of the oligosaccharide to reveal the core structure. Carbohydrate residues are added by specific glycosyltransferases in the Golgi apparatus to complete the structure of the glycoprotein (8,9).

NeuAcα2

NeuAcα2-

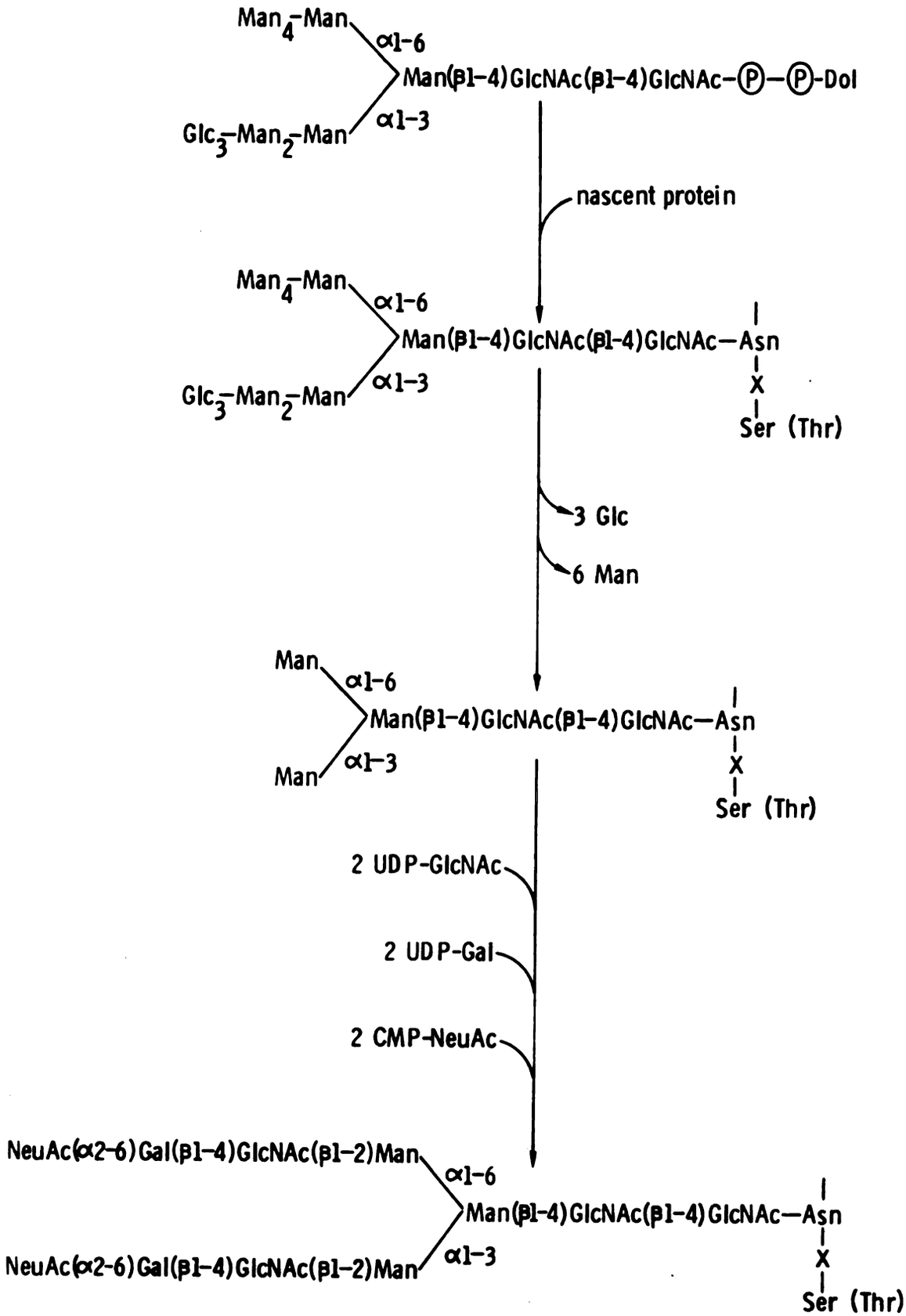
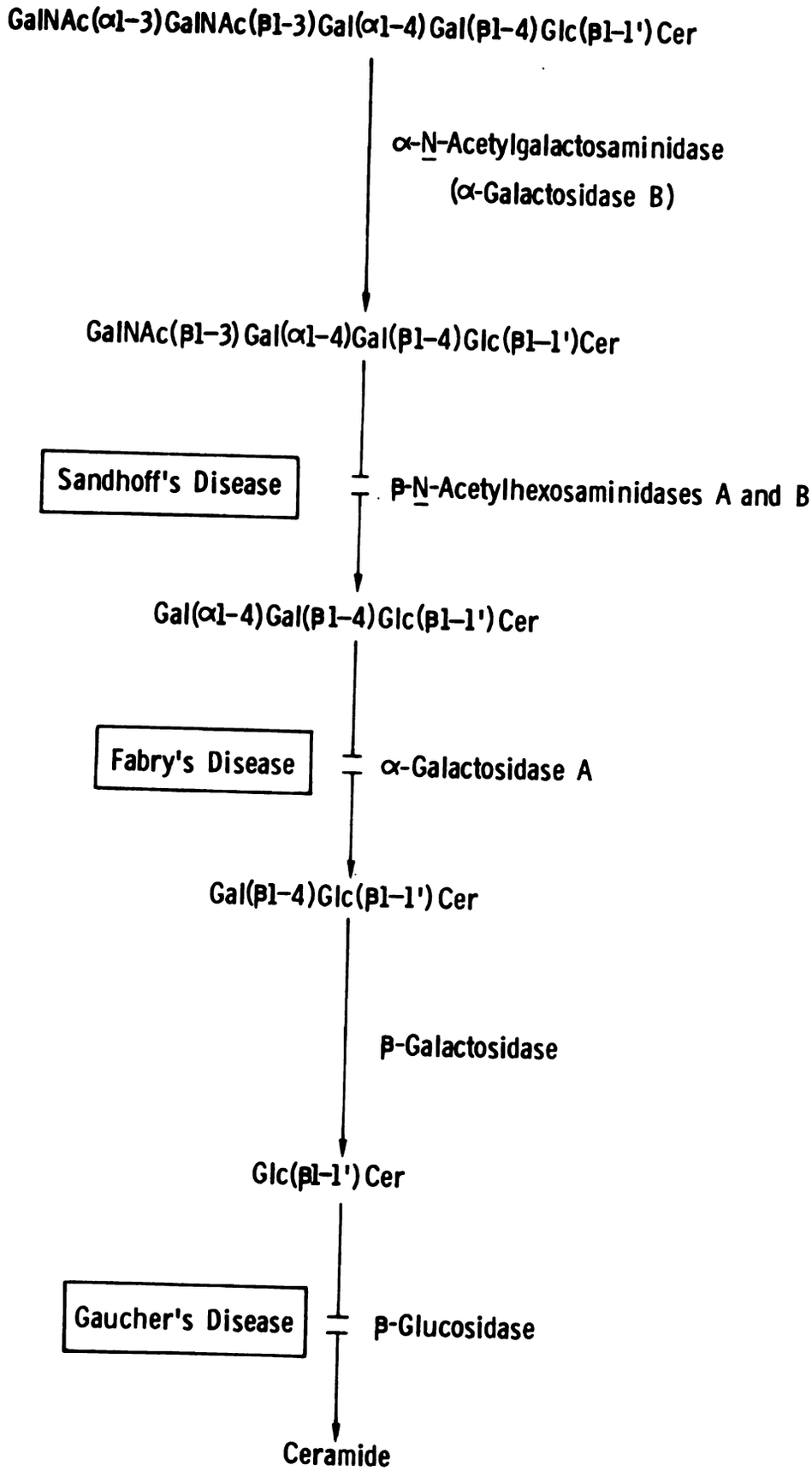

Man4–Man
α1-6
Man(β1-4)GlcNAc(β1-4)GlcNAc–Ⓟ–Ⓟ-Dol
α1-3
Glc3–Man2–Man
nascent protein
Man4–Man
α1-6
Man(β1-4)GlcNAc(β1-4)GlcNAc–Asn
X
Ser (Thr)
α1-3
Glc3–Man2–Man
3 Glc
6 Man
Man
α1-6
Man(β1-4)GlcNAc(β1-4)GlcNAc–Asn
X
Ser (Thr)
α1-3
Man
2 UDP-GlcNAc
2 UDP-Gal
2 CMP-NeuAc
NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man
α1-6
Man(β1-4)GlcNAc(β1-4)GlcNAc–Asn
X
Ser (Thr)
α1-3
NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man

Figure 5. An Example of Glycoconjugate Catabolism

The catabolism of the glycosphingolipids of the globo family is shown.


GalNAc(α1–3)GalNAc(β1-3)Gal(α1–4)Gal(β1–4)Glc(β1–1')Cer
α-N-Acetylgalactosaminidase
(α-Galactosidase B)
GalNAc(β1–3)Gal(α1–4)Gal(β1–4)Glc(β1–1')Cer
Sandhoff's Disease
β-N-Acetylhexosaminidases A and B
Gal(α1–4)Gal(β1–4)Glc(β1–1')Cer
Fabry's Disease
α-Galactosidase A
Gal(β1–4)Glc(β1–1')Cer
β-Galactosidase
Glc(β1–1')Cer
Gaucher's Disease
β-Glucosidase
Ceramide

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a glucosidase has been solubilized from a microsomal fraction of thyroid which has optimal activity toward dolichol pyrophasphate-linked oligosaccharides containing glucose at pH 6.5-7.5 (27).

Many circulating glycoproteins bear carbohydrate moieties with one or two units of the trisaccharide NeuAc(α2→6)Gal(β1→4)GlcNAc- linked to their oligosaccharide (6). These glycoproteins have extended lifetimes in circulation; however, removal of the terminal sialic acid residue results in rapid clearance from circulation, with uptake by parenchymal cells in the liver (40-43). Studies by Ashwell and coworkers (43-46) have revealed that uptake of asialoglycoproteins is mediated by a membrane-bound receptor on parenchymal cells, which binds glycoproteins containing terminal β-galactose residues. A receptor that binds glycoproteins containing terminal N-acetylgl cosamine residues has also been purified and partially characterized (47-49), and receptors with other carbohydrate-binding specificities will surely be found. These receptors are often referred to as lectins because of their similarity to the carbohydrate-binding proteins found in plants (50).

An interesting variation on this theme is the discovery that phosphorylation of mannose residues on β-glucuronidase is required for rapid uptake by skin fibroblasts (51,52). This marker for rapid uptake has also been identified on other glycoproteins (53-55).

Circulating glycosidases may regulate the turnover of circulating glycoconjugates by hydrolyzing their terminal carbohydrate substituent and exposing their internal carbohydrate residues to hepatic receptors. Glycoconjugates associated with the plasma membrane, either as components of the membrane or bound to receptors on the cell surface, presumable enter the cell by endocytosis and subsequently appear in the lysosomes. Monosaccharide residues are sequentially hydrolyzed from the nonreducing end of the oligosaccharides by the lysosomal glycosidases.

Unlike the glycosyltransferases, which appear to have absolute specificity for the sugar being transferred, the oligosaccharide acceptor, and the linkage and anomerity of the glycosidic bond being formed, several of the glycosidases lack absolute specificity for the monosaccharide being hydrolyzed (56-60), the glycoconjugate substrate (glycolipid, glycoprotein or proteoglycan) (61-63), and the linkage (but not the anomerity) of the glycosidic bond being hydrolyzed. Aside from these differences in the

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specificities of the glycosyltransferases and glycosidases, the biosynthesis and catabolism of glycoconjugates appear to be carried out in parallel, but converse, pathways.

II. The Chemistry and Metabolism of Glycoconjugates Containing Terminal α-Galactose Residues

A. Purification and Characterization of Glycoconjugates

Sources and methods for purifying glycolipids (5,12,64-66), glycoproteins (6,13-15,66,67) and proteoglycans (68,69), and methods for determining the composition (5,11,66-68) and structure (11,15,66) of the carbohydrate portions of glycoconjugates have been extensively reviewed, and only recent developments in glycoconjugate purification and characterization will be discussed.

Crude glycolipid preparations have been fractionated into their individual components by silicic acid column chromatography (65,66) and thin-layer chromatography on glass plates coated with silica gel G (70). Improved resolution of glycolipids has been obtained using a new porous silica bead, Iatrobeads (Iatron Laboratories, Inc., Tokyo, Japan) (71,72). Other improvements in silicic acid chromatography include the use of borate-coated silicic acid, which enabled Korniat and Hof (73) to resolve glucosylceramide and galactosylceramide, and the use of DEAE-silica gel to isolate neutral glycolipids and gangliosides (74). High resolution of glycolipids has been obtained by thin-layer chromatography using plates coated with silica gel of fixed pore size (Silica Gel 60, E. Merck, Darmstadt, West Germany) in solvents containing $CaCl_2$ (75,76).

Recent advances in glycoprotein purification include the use of Sepharose-linked lectins to isolate glycoproteins containing specific carbohydrates (77,78), affinity chromatography (79-81), ampholyte displacement chromatography (82), and hydrophobic chromatography on octoyl-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) (39).

Recently developed methods for structural analysis of complex carbohydrates include high pressure liquid chromatography (83-88), natural abundance Fourier-transform ^{13}C NMR spectroscopy (11,89), the use of purified bacterial endoglycosidases (90-94) to hydrolyze specific internal glycosidic bonds of oligosaccharides, and the use of anhydrous hydrogen fluoride to hydrolyze O-glycosidic bonds of oligosaccharides, but not the

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B. Structure and Function of Glycoconjugates Containing Terminal α-Galactose Residues

Glycoconjugates of human origin that contain terminal α-galactose residues are quite varied in structure, ranging from the relatively simple diglycosyl glycolipid, galabiosylceramide, to the complex blood group B-active megalosaccharides (Figure 6). The occurrence and function of each of these glycoconjugates will be discussed.

Galabiosylceramide. The biological function of $GaOse_2Cer$ is not known at the present time, although it may serve as a precursor for galabiosylceramide-II^3-sulfate, which probably occurs in kidney, and may play a role in sodium ion transport (95-99).

The occurrence of $GaOse_2Cer$ is relatively restricted in man: it is not found in normal or Fabry plasma or erythrocytes, but has been found in normal (100,101) and Fabry (102-106) kidney, Fabry urine sediment (100), heart (108), lung (108), pancreas (105) and Tay-Sachs brain (109). This lipid is also found in kidneys of Balb/c, C57/BL and A strain mice (103). The concentration of $GaOse_2Cer$ was higher in kidneys from male mice (111) and it was found that testosterone stimulated the appearance of this glycolipid in kidneys of female mice (112).

Globotriglycosylceramide. Globotriglycosylceramide has been identified by Naiki and Marcus (113) as the blood group P^k antigen. Individuals with this blood type are rare, as the P^k phenotype, unlike other blood group phenotypes, is inherited as a recessive trait (114-116). The P^k antigen can be detected immunologically on erythrocytes (116-120), fibroblasts and lymphocytes (121-123) from individuals with this phenotype, but not on normal erythrocytes, which also contain $GbOse_3Cer$ (but in much lower levels). Marcus et al. (124) found that type P^k erythrocytes contain almost 5 times more $GbOse_3Cer$ than normal erythrocytes, but are devoid of globoside (the blood group P antigen), which is the major glycosphingolipid present in normal erythrocytes (125). It was recently demonstrated that fibroblasts from individuals with this phenotype lack $GbOse_3Cer$: β-N-acetylgalactosaminyltransferase activity (126,127).

Globotriglycosylceramide was first isolated by Klenk and Lauenstein (128) in 1953, from human erythrocytes. Sweeley and Klionsky (102) reported abnormal quantities of this glycolipid accumulating in the kidney

Figure 6. Structures of Glycoconjugates Containing Terminal α-Galactose Residues.

The oligosaccharide portions of glycoconjugates containing terminal α-galactose residues are shown. They are: (I) galabiosylceramide; (II) globotriglycosylceramide[P^k glycolipid]; (III) IV^4-α-galactosyllactoneotetraglycosylceramide [P_1 glycolipid]; (IV) and (V) blood group B-active glycolipids [these oligosaccharides differ only in the linkage of the pentultimate galactose residue]; and (VI) megalosaccharide structure of blood group B glycoproteins.

I Gal(α1–4)Gal(β1–1')Cer

II Gal(α1–4)Gal(β1–4)Glc(β1–1')Cer

III Gal(α1–4)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc(β1–1')Cer

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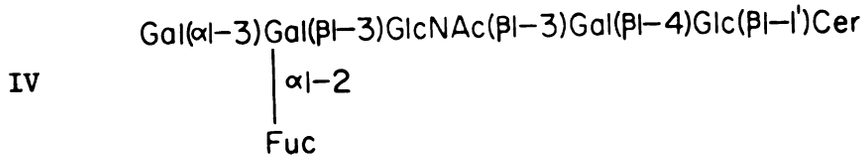

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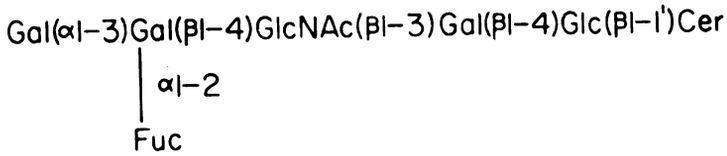

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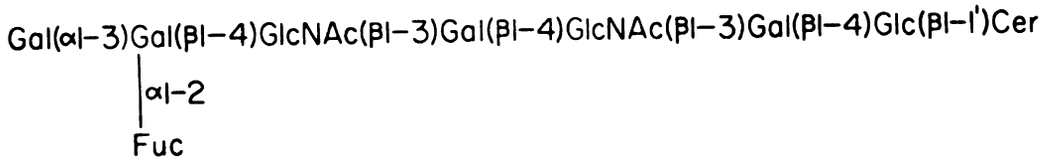

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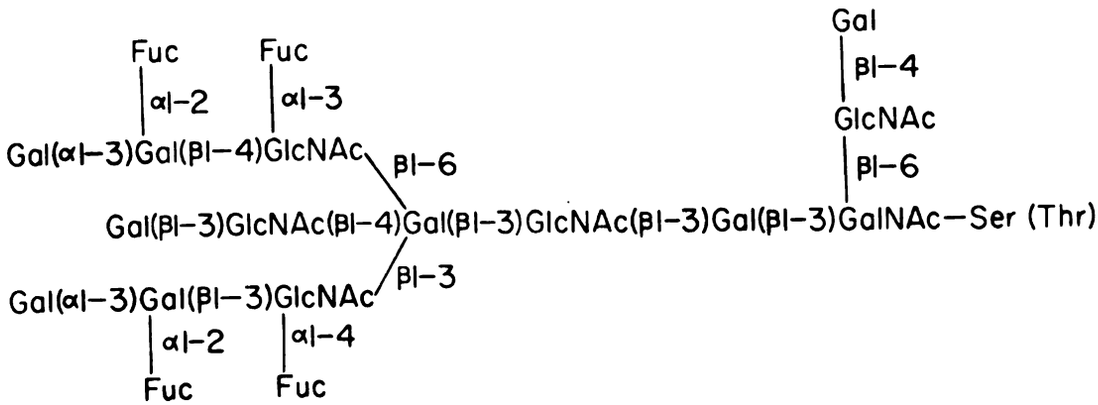

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from a patient with Fabry's disease in 1963, and it has now been found to occur in most extraneural tissues and fluids (5). The absence of $GbOse_3Cer$ in normal brain suggests that it is not synthesized by nervous tissue. This corresponds with the absence of α-galactosidase A from normal brain (129), which would not be required, as the substrate is not present, and the lack of central nervous system involvement in Fabry's disease (130-133).

The distribution of $GbOse_3Cer$ among lipoprotein fractions in normal (134-136) and Fabry (136) plasma has been examined; the majority of this glycolipid (>60%) was found to be associated with the low density lipoprotein fraction. The distribution of $GbOse_3Cer$ among lipoproteins in Fabry plasma was found to be in proportions similar to the normal distribution (although the concentration of $GbOse_3Cer$ in each fraction was 2-3 times higher than the control values).

IV^4-α-Galactosyllactoneotetraglycosylceramide. This glycolipid has been identified by Naiki et al. (137,138) as the human blood group P_1 antigen. Individuals with the P_1 phenotype are quite common (comprising approximately 75% of the population) (115,124), and this antigen has been detected immunologically on erythrocytes (117), fibroblasts and lymphocytes (122) from these individuals. Marcus (139) and Naiki et al. (138) have isolated IV^4-α-Gal-$LcnOse_4Cer$ from human erythrocytes, but this glycolipid has not been purified from other sources. Eto et al. (140) and Stellner et al. (141) isolated a glycolipid from rabbit erythrocytes with blood group B activity, differing from the human P_1 antigen only in the linkage of the terminal α-galactose residue. The rabbit erythrocyte glycolipid has a terminal Gal(α1→3) linkage, rather than a terminal Gal(α1→4) linkage found in the human P_1 antigen. It appears that the blood group activity of glycoconjugates is determined not only by the identity of the terminal carbohydrate residue and its anomerity, but also by its linkage to the pentultimate residue of the oligosaccharide. The accumulation of this glycolipid in patients with Fabry's disease has not been reported, although it would be expected to accumulate in patients with this phenotype.

Blood Group B-Active Glycoconjugates. The blood group B antigens are glycolipids and glycoproteins that have terminal Gal(α1→3)Gal(β1→3(4))GlcNAc trisaccharide groups in their carbohydrate moieties.

Three different blood group B-active glycolipids have been purified

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from human B erythrocytes (142-147) and Fabry pancreas (148), and their structures determined. Two of these glycolipids are hexaglycosylceramides differing only in the linkage of the pentultimate galactose residue, as shown in Figure 4. The third blood group B-active glycolipids found to date contain fucose; however, Eto et al. (140) and Stellner et al. (141) have purified a blood group B-active pentaglycosylceramide from rabbit erythrocytes that does not contain fucose, but does contain the terminal trisaccharide mentioned above. Gardas and Kościelak (149,150) recently reported the presence of a megaloglycolipid with blood group B activity in human erythrocyte membranes. This water-soluble substance may contain more than 20 carbohydrate residues linked to ceramide. Although the blood group B-active megaloglycolipid has not been purified or structurally characterized, a blood group H-active megaloglycolipid with 22 sugars has been purified from human erythrocyte membranes and the structure examined (151). This megaloglycolipid is presumably the precursor (the core structure) for blood group A- and B-active megaloglycolipids.

Blood group B-active glycoproteins have been isolated from human urine, saliva, meconium, gastric juice and ovarian cyst fluids (152-155). The structure of the carbohydrate portion of these glycoproteins is shown in Figure 6. This complex carbohydrate contains both the lacto and lactoneo structures found in blood group B-active glycosphingolipids (5,148), and the structure of the core oligosaccharide (with the terminal α-galactose residue removed) resembles the structure of the blood group H-active megaloglycolipid, suggesting similar synthetic and catabolic pathways.

Blood group B-active oligosaccharides have been isolated from urine of individuals with this blood type (155). The concentration of these oligosaccharides in urine varies with diet and the secretor status of the individual, with higher concentrations of oligosaccharides found in urine from unstarved secretors. These fucose-containing oligosaccharides vary in size from 3-7 sugars and are presumably derived from partially degraded blood group B-active glycoproteins.

The blood group B-active glycolipids have been reported to accumulate in Fabry patients with this blood type (148). Tondeur and Resibois (156) reported the accumulation of mucopolysaccharides in patients with Fabry's disease, but this finding has not been supported in studies by other investigators (157-160). Unfortunately, these studies were carried out prior to the discovery of blood group B-active glycolipid accumulation

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in Fabry pancreas (148), and the presence or absence of stored mucopolysaccharides was not correlated with blood type. Blood group B-active glycoproteins and oligosaccharides derived from these glycoproteins would be expected to accumulate in tissues of Fabry patients with this blood type, and to be present in high concentration in the urine of blood group B secretors with Fabry's disease.

C. The Biosynthesis of Glycoconjugates Containing Terminal α-Galactose Residues

None of the enzymes involved in the synthesis of glycoconjugates containing terminal α-galactose residues has been purified, except for the α-galactosyltransferase involved in the synthesis of blood group B-active glycoconjugates.

The synthesis of $GaOse_2Cer$, catalyzed by crude mouse kidney homogenates, has been examined by Gray and coworkers (161,162). These investigators found that testosterone stimulated the biosynthesis of $GaOse_2Cer$ in kidney of female mice (112), who had much lower levels of this glycolipid than male mice (111). However, this increase was not due to an alteration in α-galactosyltransferase activity, but rather appeared to be the result of an increase in galactosylceramide biosynthesis (112). From these studies, it appears that $GaOse_2Cer$ levels may be regulated by the availability of galactosylceramide.

The biosynthesis of $GbOse_3Cer$ has been examined by Stoffyn *et al.* (163) in rat kidney microsomes, and by Robbins *et al.* (164-169), Hakomori *et al.* (170-175), Macpherson *et al.* (176-178) and others (179,180) in normal and transformed hamster cells and newborn rat kidney cells in culture. These investigators found that when contact-inhibited cells became confluent, the levels of $GbOse_3Cer$ appeared to increase, but when transformed cells were used, the concentration of $GbOse_3Cer$ was not altered by cell population density. The biosynthesis of $GbOse_3Cer$ has been studied as a function of cell cycle in NIL cells (168) and human KB cells (181-184). Globotriglycosylceramide, and glycolipids in general, were synthesized during the G_1 phase of the cell cycle.

Vance *et al.* (185) examined the biosynthesis and catabolism of neutral glycolipids in plasma of normal humans and a patient with Fabry's disease by labelling the newly synthesized glycolipids with $[6,6\text{-}^2H_2]$glucose. These workers found that the incorportaion $[6,6\text{-}^2H_2]$glucose into plasma

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$GbOse_3Cer$ was reduced in the patient with Fabry's disease. It was suggested that the $[^2H]GbOse_3Cer$ may have been diluted by unlabeled $GbOse_3Cer$ in the patient's plasma, or that the rate of $GbOse_3Cer$ biosynthesis may be reduced in patients with Fabry's disease due to the high levels of this glycolipid that accumulate in tissues of these patients.

Recently, Kijimoto-Ochiai et al. (126) examined the biosynthesis of $GbOse_3Cer$ and $GbOse_4Cer$ in fibroblasts from individuals with blood group P^k and p phenotypes. They found that individuals with the P^k phenotype lack the β-N-acetylgalactosaminyltransferase for $GbOse_4Cer$ biosynthesis. Similarly, individuals with the p phenotype are missing the α-galactosyltransferase involved in the biosynthesis of $GbOse_3Cer$. These glycosyltransferase deficiencies do not result in pathological disorders of glycolipid metabolism; individuals with these phenotypes appear to be physically normal and to have normal life spans (126). This is in contrast to G_{M3} gangliosidosis (G_{M3}: β-N-acetylgalactosaminyltransferase deficiency), which is characterized by hepatosplenomegaly, poor physical and motor development and central nervous system dysfunction (20,21). Nevertheless, the blood group p, P^k (and H) phenotypes may be classified as hereditary glycosyltransferase deficiencies.

The biosynthesis of IV^4-α-Gal-$LcnOse_4Cer$ has not been investigated at the time of this writing.

The synthesis of $GaOse_2Cer$, $GbOse_3Cer$ and IV^4-α-Gal-$LcnOse_4Cer$ are carried out by α-galactosyltransferases which catalyze the formation of terminal Gal(α1→4)Gal glycosidic bonds. Kościelak et al. (164) have suggested that $GbOse_3Cer$ (P^k antigen) and IV^4-α-Gal-$LcnOse_4Cer$ (P_1 antigen) may be synthesized by the same α-galactosyltransferase. Galabiosylceramide may also be synthesized by this transferase. This hypothesis is supported by the absence of both $GbOse_3Cer$ and IV^4-α-$LcnOse_4Cer$ in erythrocytes of the p phenotype (115); however, this hypothesis has not been investigated in further detail.

The α-galactosyltransferase involved in the biosynthesis of blood group B-active glycoconjugates catalyzes the formation of terminal Gal(α1→3)Gal(2←1αFuc) glycosidic bonds. This glycosyltransferase has been purified from plasma of type B individuals by Carne et al. (187) and Nagai et al. (188). These investigators found that the enzyme was a dimer with subunits of 40,000 daltons. It required Mn^{+2} for maximal activity and the pH optimum was at pH 7.0-7.5 (188). The enzyme transferred

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galactose from UDP-Gal to H-active glycoproteins, H-active megaloglycolipid and also Fuc(α1→2)Gal, 2'-fucosyllactose and lacto-N-fucopentaose I (187).

Alterations in the biosynthesis of blood group B-active glycosphingolipids have been reported in human carcinomas. Blood group B antigens were missing from urinary epithelial, gastrointestinal, uterine, lung, pancreatic, and adenocarcinomas (68,188,189,257,258). Stellner and Hakomori (189) reported that the disappearance of blood group B-active glycolipids was due to the loss of the α-galactosyltransferase involved in the biosynthesis of these glycolipids.

The α-galactosyltransferase involved in the biosynthesis of blood group B-active glycoconjugates is clearly distinct from the enzyme(s) involved in the synthesis of $GaOse_2Cer$, $GbOse_3Cer$ and IV^4-α-Gal-$LcnOse_4Cer$. The blood group B-active glycocnjugates contain terminal Gal(α1→3)Gal linkages, while $GaOse_2Cer$, $GbOse_3Cer$ and IV^4-α-Gal-$LcnOse_4Cer$ contain terminal Gal(α1→4)Gal linkages. Replacement of a terminal Gal(α1→3) linkage with a terminal Gal(α1→4) linkage, as in IV^3-α-Gal-$LcnOse_4Cer$ from rabbit erythrocytes, compared with human erythrocyte IV^4-α-Gal-$LcnOse_4Cer$, results in a change in blood group specificity.

The similarities between the structures of the blood group H-active megaloglycolipid (151) and the blood group B megalosaccharide (6) suggest that these complex carbohydrates may be synthesized by a common pathway. The synthesis of these blood group-active glycoconjugates may proceed with the transfer of sugars from sugar nucleotides directly to the nascent glycolipid or glycoprotein.

D. The Catabolism of Glycoconjugates Containing Terminal α-Galactose Residues

The occurence of $GbOse_3Cer$ hydrolase was reported in 1967 by Brady *et al.* (190), who detected this hydrolase activity in particulate fractions of rat brain, liver, kidney, spleen and small intestine. These investigators subsequently found this enzyme activity in normal human intestine; it was absent in hemizygous male Fabry patients and reduced in heterozygous female patients (191). This finding suggested that the accumulation of glycolipids in Fabry's disease was due to the absence of $GbOse_3Cer$ hydrolase.

The identity of $GbOse_3Cer$ hydrolase as an α-galactosidase was suggested by Kint (192) in 1970. He reported that leukocytes from a patient

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with Fabry's disease were unable to hydrolyze the water-soluble artificial substrates, p-nitrophenyl-α-D-galactopyranoside (p-NP-α-Gal) or 4-methylunbelliferyl-α-D-galactopyranoside (4-MU-α-Gal), suggesting that Fabry's disease is due to an α-galactosidase deficiency. This finding was supported by similar studies using cultured skin fibroblasts and leukocytes (193-196). Structural studies on $GbOse_3Cer$ (194-201) and $GaOse_2Cer$ (202) confirmed that the terminal galactose residue was linked in an α-configuration, while the pentultimate galactose residue was linked in the β configuration.

In 1971, Beutler and Kuhl (203) and Kint (204) reported the presence of two forms of α-galactosidase activity in normal human tissues, designated α-galactosidase A and B. These forms could be distinguished by their different thermostabilities, Michaelis constants with artificial substrates, and electrophoretic mobilities at pH 7.0. Beutler and Kuhl found that only the A form was missing in patients with Fabry's disease, while α-galactosidase B persisted. Furthermore, the B form isolated from Fabry tissues, leukocytes and fibroblasts (203,206) was indistinguishable from normal α-galactosidase B activity. These α-galactosidase activities have now been examined in normal human intestinal mucosal scrapings (206), liver (206-215), heart (206), brain (209), spleen (206,216), kidney (206, 210,217,218), skeletal muscle (206), placenta (77,220-223), fibroblasts (206,214,224-228), leukocytes (206,227,228), endothelial cells (229), plasma (39,218,228,230-232), urine (218,228,233,234) and tears (235). α-Galactosidase A is the major form in these tissues and fluids, with the exception of brain, while the B form represents 20% or less of the total α-galactosidase activity. In contrast, α-galactosidase B is the major form in brain, with only traces of the A form detectable (209).

The presence of two forms of α-galactosidase activity posed a dilemma to investigators in this area. It was found that treatment with C. perfringens neuraminidase reduced the electrophoretic mobility of α-galactosidase A to approximately that of the B form (204,206,209,219, 220). This observation led Kint (204,209) to suggest that neuraminidase treatment converted the A form to the B form. The failure of neuraminidase treatment to alter the electrophoretic mobility of the B form (206) appeared to support this hypothesis. However, closer examination of the desialylated A form by Beutler and Kuhl (219,220) using antibodies prepared against purified α-galactosidases A and B, and by Romeo et al. (211)

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using isoelectric focusing, revealed that desialylated α-galactosidase A differs from the B form in antigenicity and isoelectric point. It is now apparent that neuraminidase treatment of α-galactosidase does not convert it to the B form, although it appears that sialic acid may be a component of the carbohydrate moiety of α-galactosidase A and may not be present in the B form. Subsequent studies by Dean et al. (60,236,237) on the substrate specificities of α-galactosidase A and B revealed that the B form is also an α-N-acetylgalactosaminidase, with much greater activity toward o-nitrophenyl-α-N-acetylgalactosaminide (o-NP-α-GalNAc) and $GbOse_5Cer$ (Forssman antigen) than towards substrates containing terminal α-galactose residues. It was concluded from these studies that α-galactosidase B probably functions as an α-N-acetylgalactosaminidase rather than an α-galactosidase in vivo. This finding was confirmed by Schram et al. (238) using antibodies to the purified enzymes.

It appears that a single enzyme, α-galactosidase A (EC 3.2.1.22), carries out the hydrolysis of terminal α-galactose residues, regardless of their linkage to the oligosaccharide. The absence of this enzyme in patients with Fabry's disease results in the accumulation of glycolipids, and probably glycoproteins, glycopeptides and oligosaccharides, containing terminal α-galactose residues.

III. The Chemistry and Metabolism of Glycoconjugates Containing Terminal α-N-Acetylgalactosamine Residues

The glycoconjugates of human origin that contain terminal α-N-acetylgalactosamine residues share the property of having blood group A activity or cross-reactivity. In contrast to the common occurrence in humans of many of the glycoconjugates containing terminal α-galactose residues, glycoconjugates containing terminal α-N-acetylgalactosamine residues are absent in a majority of the population. Furthermore, there are no known pathological disorders of metabolism involving glycoconjugates containing terminal α-N-acetylgalactosamine residues. Nevertheless, these glycoconjugates are of interest because of their blood group activity (115) and their involvement in human carcinomas (68,188,189,239,240,257-260).

A. Structure and Function of Glycoconjugates Containing Terminal α-N-Acetylgalactosamine Residues

The Structures of the carbohydrate portions of the glycoconjugates found in man containing terminal α-*N*-acetylgalactosamine residues are shown in Figure 7. The occurrence and function of each of these glycoconjugates will be discussed.

Globopentaglycosylceramide. In 1911, Forssman (241) reported that the injection of extracts from guinea pig kidney into rabbits resulted in the formation of hemolysins for sheep red blood cells. The antigen that evoked the formation of these hemolysins, the Forssman antigen, was subsequently found to be a heterogenetic antigen, occurring in many species of animals and bacteria (242). Brunius (242) found that the antigen was a glycolipid containing galactosamine, and Papirmesiter and Mallette (243) reported that it contained hexose, hexosamine, fatty acid and base. The structure of the Forssman antigen isolated from horse spleen was determined by Siddiqui and Hakomori (244) in 1971. The accuracy of the structure proposed by Siddiqui and Hakomori has been confirmed using Forssman antigen from dog intestine (245), goat erythrocytes (246) and guinea pig tissues (247). The Forssman antigen (GbOse$_5$Cer) has not been isolated from human erythrocytes, and was thought not to occur in humans until Hakomori *et al.* (239) reported its presence in gastrointestinal mucosa of some normal individuals. The presence of GbOse$_5$Cer in gastrointestinal mucosa was not dependent on the blood type of the individual. These investigators also suggested that GbOse$_5$Cer may be present in low levels in erythrocytes from some individuals. Globopentaglycosylceramide is also expressed in human hepatic biliary adenocarcinoma tissue (240) and in gastric and colonic tumors (239).

Globopentaglycosylceramide cross-reacts extensively with blood group A antigens (248,249). This specificity probably resides in the terminal GalNAc(α1→3) moiety, which is common to GbOse$_5$Cer and the blood group A antigens.

Blood Group A-Active Glycoconjugates. The blood group A antigens are glycolipids and glycoproteins that have a common terminal tetrasaccharide, shown below. Hakomori *et al.* (250) have isolated and partially

```
GalNAc(α1→3)Gal(β1→4)GlcNAc
             ↑
             |α1→2
             |
            Fuc
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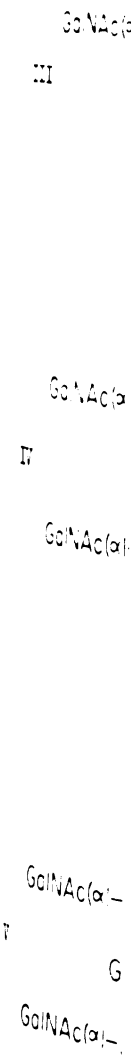
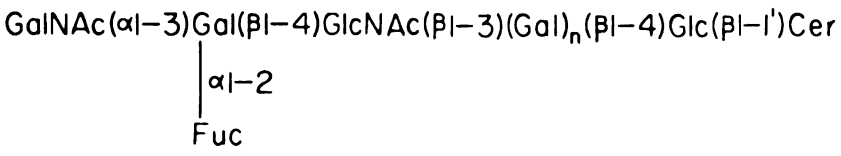
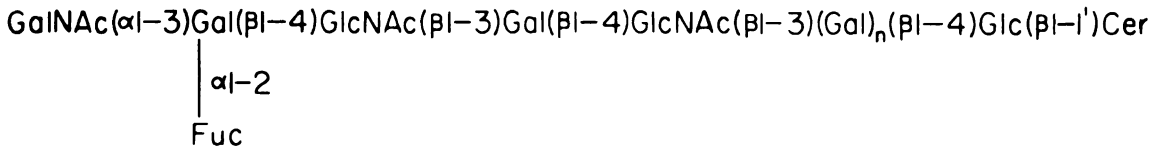
Figure 7. Structures of Glycoconjugates Containing Terminal α-N-Acetylgalactosamine Residues

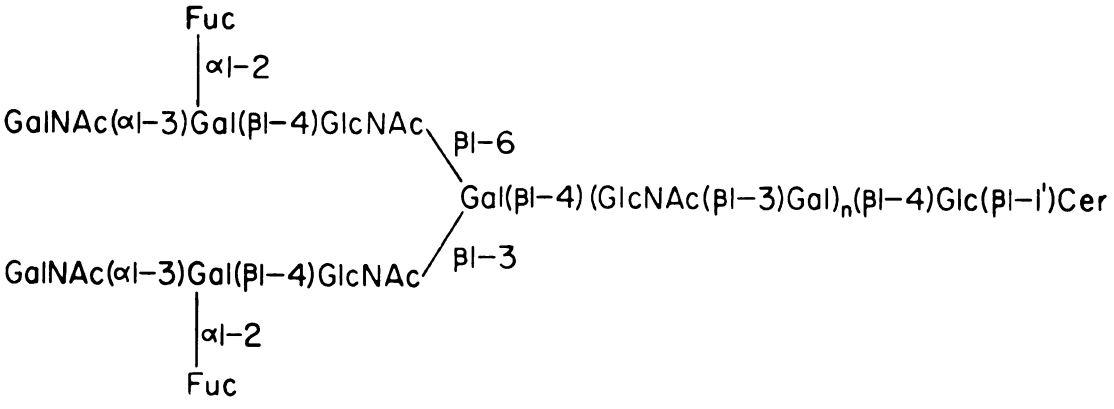


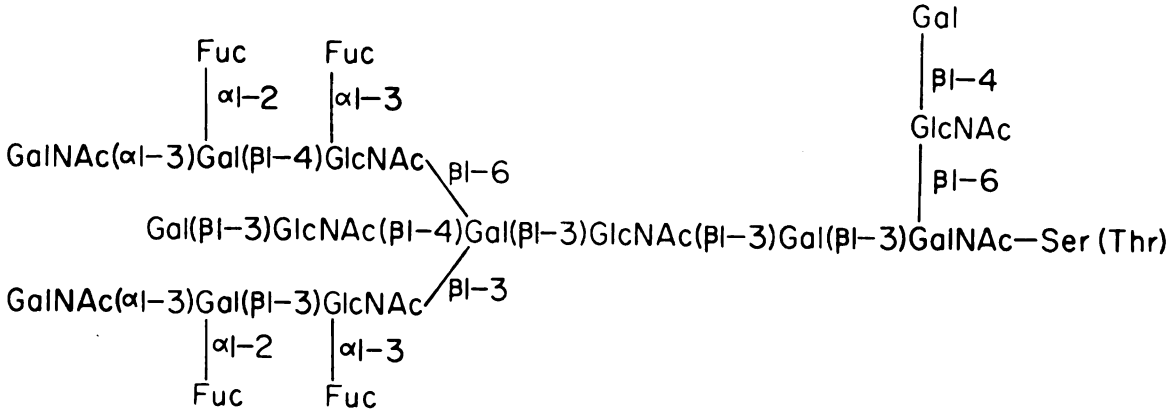
The oligosaccharide portions of glycoconjugates containing terminal α-N-acetylgalactosamine residues are shown. They are: (I) globopentaglycosylceramide (Forssman antigen); (II), (III) and (IV) blood group A-active glycolipids, a fourth A-active glycolipid has been described by Hakomori et al. (250) that resembles IV, but with excessive GlcNAc and an additional branching structure and; (V) the megalosaccharide structure of blood group A-active glycoproteins.

I GalNAc(α1–3)Gal(β1–3)Gal(α1–4)Gal(β1–4)Glc(β1–1')Cer

II


III


IV


V


characterized four variants of blood group A-active glycolipids. These glycolipids differ in the carbohydrate composition and length of their core oligosaccharides. Unlike the blood group B-active glycolipids, which may contain lacto or lactoneo core structures, the blood groyp A-active glycolipids appear to contain only the lactoneo structure. The blood group A^C glycolipid appears to have the same core structure as the blood group H-active megaloglycolipid described by Gardas (150). In addition to these blood group A-active glycolipids, Kościelak and coworkers (148,149,251) have reported the isolation of blood group A-active megaloglycolipids from human erythrocyte membranes that contain up to 60 carbohydrate residues.

Blood group A-active glycoproteins have been isolated from human urine, saliva, meconium, gastric juice and ovarian cyst fluids (152,153). The structure of the carbohydrate portion of these glycoproteins is shown in figure 7.

Blood group A-active oligosaccharides have been isolated from urine of individuals with this blood type (154). These oligosaccharides range in size from 3-7 sugars and are presumably derived from partial degradation of blood group A-active glycoproteins.

B. The Biosynthesis of Glycoconjugates Containing Terminal α-N-Acetylgalactosamine Residues

The α-N-acetylgalactosaminyltransferase involved in the synthesis of $GbOse_5Cer$ has not been purified at the time of this writing. This enzyme activity has been examined in crude preparations of guinea pig tissues by Kijimoto *et al.* (252). These investigators found that $GbOse_4Cer$: α-N-acetylgalactosaminyltransferase activity was highest in spleen and kidney, and lowest in liver. The enzyme required Mn^{+2} for maximal activity and the pH optimum was at pH 6.7.

The biosynthesis of $GbOse_5Cer$ has also been examined in normal and transformed cells in culture. The rate of biosynthesis of $GbOse_5Cer$ was found to be dependent on the cell cycle and the cell population density of hamster cells in culture. The biosynthesis of $GbOse_5Cer$ in contact-inhibited NIL cells was found to be maximal during G_1 phase (167,173). The biosynthesis of $GbOse_5Cer$ in contact-inhibited cells increased as the cells reached confluency (176,252); however, when these cells were transformed by hamster sarcoma or polyoma virus the density-dependent

increase in GbOse$_5$Cer biosynthesis was abolished (164-166,173,179,180).

Hakomori et al. (239) have reported the presence of GbOse$_5$Cer in gastric and colonic tunors of individuals who are normally lacking this antigen. Conversely, individuals with gastric and colonic tumors who normally possess GbOse$_5$Cer as a component of their gastrointestinal mucosa are lacking this antigen in their tumor tissue. These investigators speculated that the expression of the α-N-acetylgalactosaminyltransferase for GbOse$_5$Cer is repressed in normal individuals lacking this antigen, but becomes de-repressed in tumors.

The α-N-acetylgalactosaminyltransferase involved in the synthesis of GbOse$_5$Cer is clearly distinct from the α-N-acetylgalactosaminyltransferase for the biosynthesis of blood group A antigens; Hakomori et al. (239) detected GbOse$_5$Cer in the gastrointestinal mucosa of individuals of various blood group phenotypes.

The α-N-acetylgalactosaminyltransferase involved in the biosynthesis of blood group A-active glycoconjugates has been purified from human plasma (253-255) and porcine submaxillary glands (256,257). The enzyme was found to be a dimer with subunits of 46,000-57,000 daltons (255,256). It required Mn^{+2} for activity, which was optimal at pH 6.8 (253,255). The enzyme catalyzed the transfer of N-acetylgalactosamine from UDP-GalNAc to H-active glycolipids, glycoproteins and oligosaccharides (256).

The loss of blood group A antigens in urinary epithelial, gastrointestinal, uterine, lung, pancreatic, oral squamous cell and adenocarcinomas has been reported (68,189,190,240,258-261). Stellner and Hakomori (190) reported that the disappearance of blood group A- or B-active glycolipids in adenocarcinoma is due to the loss of the glycosyltransferase activities for the biosynthesis of these compounds.

C. The Catabolism of Glycoconjugates Containing Terminal α-N-Acetylgalactosamine Residues

Human α-N-acetylgalactosaminidase activity has not been the subject of such intensive study as human α-galactosidase, because it is not involved in any pathological disorders of glycoconjugate metabolism.

Human α-N-acetylgalactosaminidase was first isolated from liver by Yamamoto in 1968 (262). He found that α-N-acetylgalactosaminidase activity was present in normal liver regardless of the blood group phenotype of the donor. This enzyme catalyzed the conversion of blood group A antigens

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to H-active substance. In 1973, Callahan et al. (36) examined normal human brain, liver, spleen, kidney and fibroblasts for α-N-acetylgalactosaminidase activity. The enzyme activity was highest in liver extracts and lowest in brain. The enzyme was purified 124-fold from liver and the thermostability and enzymatic properties with p-NP-α-GalNAc were examined. The α-N-acetylgalactosaminidase activity was found to be thermostabile, retaining >80% of its original activity after 4 hours at 50°C, pH 4.3. The Michaelis constant with p-NP-α-GalNAc was 3.1 mM and the hydrolysis of this substance was competitively inhibited by N-acetylgalactosamine and galactose. Tallman et al. (263) observed that one form of placental α-galactosidase also exhibited α-hexosaminidase activity, however this activity was not described in further detail. In 1977, Dean et al. (60, 236,237) reported that α-galactosidase B from normal human liver was, in fact, an α-N-acetylgalactosaminidase, hydrolyzing $GbOse_5Cer$ and o-NP-α-GalNAc at much greater rates than substrates containing terminal α-galactose residues. This finding suggests that α-N-acetylgalactosaminidase lacks absolute specificity for the substituent at C-2 of galactose. Schram et al. (215,238) confirmed the identification of α-galactosidase B as an α-N-acetylgalactosaminidase using antibodies prepared against the purified enzyme.

From the studies of Yamamoto (262) and Dean et al. (60,236,237) it appears that a single α-N-acetylgalactosaminidase catalyzes the hydrolysis of globopentaglycosylceramide and blood group A antigens. This enzyme may be constitutive, since it was detected in tissues from persons with various blood types; however, the presence of Forssman antigen in the gastrointestinal mucosa or in erythrocyte membranes was not examined and the presence of this antigen may induce the appearance of α-N-acetylgalactosaminidase.

MATERIALS

SOLVENTS

Gene

Dry

Dry

Dry

CHEMICALS

D-Ga

D-(+)

Sodi

Sodi
[^{3}H]

Flura

DETERGENTS

Sodiu

Trito

SUBSTRATES

4-Me
a-D-G

P-Nit
Acety

P-Nit
Galact

MATERIALS AND METHODS

MATERIALS

SOLVENTS

General	Solvents were redistilled by constant flow-rotary evaporation.
Dry Methanol	Methanol (500 ml) was refluxed over magnesium turnings (2.5 g) and iodine (0.1 g) for 1 hour and then distilled and stored over 3A Davison molecular sieves.
Dry Pyridine	Pyridine was refluxed over barium oxide for 1 hour and then distilled and stored over potassium hydroxide pellets.
Dry Acetonitrile	Acetonitrile was redistilled and stored over type 3A Davison molecular sieves.

CHEMICALS

D-Galactal	Research Products International Corp. Elk Grove Village, IL
D-(+)-Fucose	Aldrich Chemical Company, Inc. Milwaukee, WI
Sodium Borohydride	Sigma Chemical Co. St. Louis, MO
Sodium Borohydride-[^{3}H]	New England Nuclear Boston, MA
Fluram	Pierce Chemical Co. Rockford, IL

DETERGENTS

Sodium Taurocholate	Calbiochem La Jolla, CA
Triton X-100	Research Products International Corp. Elk Grove Village, IL

SUBSTRATES

4-Methylumbelliferyl-α-D-Galactopyranoside	Research Products International Corp. Elk Grove Village, IL
p-Nitrophenyl-α-N-Acetylgalactosaminide	Research Products International Corp. Elk Grove Village, IL
p-Nitrophenyl-α-D-Galactopyranoside	Sigma Chemical Co. St. Louis, MO

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ENZYMES

Gal

Pro

Hor

4-Methylumbelliferyl-β-N-Acetylgalactosaminide	Pierce Chemical Co. St. Louis, MO

CHROMATOGRAPHY SUPPLIES

DEAE-Cellulose (DE-52)	Whatman, Inc. Clifton, NJ
Sephadex G-150	Pharmacia Fine Chemicals, Inc. Piscataway, NJ
Sephadex G-25 (Fine)	Sigma Chemical Co. St. Louis, NJ
Hypatite C (Hydroxylapatite)	Clarkson Chemical Company, Inc. Williamsport, PA
Unisil (100-200 mesh) (Silicic Acid)	Clarkson Chemical Company, Inc. Williamsport, PA
Iatrobeads	Iatron Laboratories, Inc. Tokyo, Japan
Silica Gel G Thin-Layer Chromatography Plates	Analtech, Inc. Newark, DE
Silica Gel 60 Thin-Layer Chromatography Plates	E. Merck Darmstadt, Germany
3% OV-225 on Supelcoport (80-100 mesh)	Supelco, Inc. Bellefonte, PA
5% SE-30 on Gas Chrom Q (80-100 mesh)	Applied Science Laboratories, Inc. State College, PA
3% SP-2340 on Supelcoport (100-120 mesh)	Supelco, Inc. Bellefonte, PA
Dowex 50W-X8 (50-100 mesh)	Sigma Chemical Co. St. Louis, MO
AG 501-X8 (20-50 mesh)	Bio-Rad Laboratories Richmond, CA
AG 1-X8 (20-50 mesh)	Bio-Rad Laboratories Richmond, CA
Dowex 1-X8 (50-100 mesh)	Sigma Chemical Co. St. Louis, MO

ENZYMES

Galactose Oxidase	Worthington Biochemical Corp. Freehold, NJ
Pronase	Calbiochem La Jolla, CA
Horse Radish Peroxidase	Worthington Biochemical Corp. Freehold, NJ

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MISCELLANEOUS REAGENTS

Bio-Solv BBS-3	Beckman Instruments, Inc. Fullerton, CA
PPO (2,5-Diphenyloxazole)	Research Products International Corp. Elk Grove Village, IL
Dimethyl-POPOP (1,4-bis-2-[4-methyl-5-phenyloxazolyl]-benzene	Research Products International Corp. Elk Grove Village, IL
Carrier Ampholytes (pH 3-5)	LKB Produkter AB Bromma, Sweden
Phenylmethylsulfonyl-fluoride	Sigma Chemical Co. St. Louis, MO
Protein Molecular Weight Standards	Boehringer Mannheim Corp. New York, NY

Porcine intestines were obtained from Peets Packing Company, Chessaning, MI. Normal human liver was kindly provided by Dr. Leo Walker, St. Lawrence Hospital, Lansing, MI; Dr. Wanderly De Mendonza, Ingham Medical Center, Lansing, Mi; Dr. Lawrence Simpson, E.W. Sparrow Hospital, Lansing, MI and Dr. Allen Yates, Dept. of Pathology, Ohio State University, Columbus, OH. Tissues were stored frozen at -80°C prior to use.

All other reagents and materials used in these studies were of reagent grade.

METHODS

I. Purification of α-Galactosidases A and B from Human Liver

Extraction. Approximately 1 kg of normal human liver was homogenized with two volumes (w/v) of 1 mM phenylmethylsulfonylfluoride (PMSF) in a Waring blender at 4°C. It was necessary to stir the PMSF in distilled water for several hours, with warming, to dissolve it. The PMSF solution was not buffered, as the pH of the liver homogenate in distilled water approximated the pH of maximum stability for hepatic α-galactosidases at pH 6.5 (212). The homogenate was centrifuged at 100,000 x g for 1 hour to obtain a clear supernatant.

Ammonium Sulfate Precipitation. The 100,000 x g supernatant was adjusted to 30% of saturation with ammonium sulfate, with gentle stirring, at 4°C. The solution was centrifuged to remove precipitated material. The supernatant was adjusted to 60% of saturation with ammonium sulfate, and the precipitate was collected by centrifugation. The pellet was suspended in approximately 200 ml of distilled water and dialyzed for 24 hours versus 10 liters of 10 mM sodium phosphate buffer, pH 6.5. The buffer was changed once during dialysis.

DEAE-Cellulose Chromatography. The dialyzed solution was centrifuged to remove precipitated material and was applied to a DEAE-cellulose column (4.3 x 25 cm) that had been prepared according to the manufacturer's instructions and equilibrated with 10 mM sodium phosphate buffer, pH 6.5, at 4°C. The enzymes were eluted with a linear gradient of NaCl (0-0.3 M NaCl) in sodium phosphate buffer. The gradient was monitored with a Radiometer-Copenhagen Model CDM3 conductivity meter. Fractions containing α-galactosidase activity were pooled and concentrated approximately 10-fold in an Amicon Model 52 ultrafiltration apparatus equipped with an 800 ml reservoir and a PM 10 membrane.

Sephadex G-150 Chromatography. The crude enzyme solution (<50 ml) was applied to a Sephadex G-150 column (3.0 x 110 cm) that had been prepared according to the manufacturer's instructions and equilibrated with 10 mM sodium phosphate buffer, pH 6.5, containing 0.02% sodium azide, at 4°C. The enzymes were eluted with the same buffer. The fractions containing α-galactosidase activity were pooled and concentrated approximately 10-fold in the Amicon Model 52 ultrafiltration cell equipped with a PM 10

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membrane. The concentrated solution was dialyzed versus two changes of 1 mM sodium phosphate buffer, pH 6.5, for 24 hours.

Hydroxylapatite Chromatography. The dialyzed enzyme solution was applied to a hydroxylapatite column (3.0 x 20 cm) that had been equilibrated with 1 mM sodium phosphate buffer, pH 6.5, at 4°C. The enzymes were eluted with a linear gradient of sodium phosphate buffer, pH 6.5 (1-50 mM), and then with 200 mM sodium phosphate buffer, pH 6.5. The gradient was monitored with a Radiometer-Copenhagen Model CDM3 conductivity meter. The fractions containing α-galactosidases A and B were pooled separately.

Ampholyte Displacement Chromatography. α-Galactosidases A and B, that had been separated by hydroxylapatite chromatography, were applied individually to DEAE-cellulose columns (1 x 5 cm) that had previously been equilibrated with 10 mM sodium phosphate buffer, pH 5.0. The column was then washed with 2 volumes of distilled water to remove the buffer. The enzymes were eluted with 15 ml of a carrier ampholyte solution consisting of 1.0 ml of carrier ampholytes (pH 3-5) diluted 1:15 with distilled water. Following application of the ampholyte solution, the column was washed with distilled water. The fractions (0.5 ml) containing α-galactosidase activity were pooled and the ampholytes were removed by Sephadex G-150 chromatography (2 x 90 cm column) as previously described.

II. Preparation of Substrates

A. Preparation of Glycolipid Substrates

Globotriglycosylceramide was purified from porcine small intestines as described below. Galabiosylceramide was purified from Fabry kidney (264) and globopentaglycosylceramide was purified from canine intestines (265).

Globotriglycosylceramide. The procedure of Suzuki et al. (266) was modified for the purification of $Gb0se_3Cer$. Approximately 23 kg of porcine small intestines were trimmed of mesenteric fat and connective tissue and washed with water. The intestines were cut into approximately 5 cm segments and homogenized in 5 volumes of acetone in a Waring blender. The homogenate was stored overnight at 4°C. The acetone was removed by vacuum filtration on a Buchner funnel with Whatman No. 4 (fast) filter paper. The residue was air-dried at room temperature. The acetone powder was divided into 1 kg lots and extracted with 3 volumes of

chloroform-methanol, 2:1 (v/v) in a Waring blender at high speed. The extracts were collected by vacuum filtration on a Buchner funnel with Whatman No. 4 (fast) filter paper. The filter residues were re-extracted with 3 volumes of chloroform-methanol, 1:1 (v/v) and then 3 volumes of chloroform-methanol, 1:1 (v/v). The extracts were collected, pooled with the previous extracts and dried by rotary evaporation. The dry residue was dissolved in approximately 1 liter of chloroform-methanol, 2:1 (v/v) and vacuum-filtered on a Buchner funnel to remove undissolved material. The solution, which had appeared pale yellow as a suspension, was deep red following filtration. The filter residue was resuspended in chloroform-methanol, 2:1 (v/v) and re-filtered. The filtrate was concentrated by rotary evaporation to a final volume of approximately 300 ml, and 3 liters of acetone were added slowly with stirring. The mixture was cooled to 4°C in the cold room. The suspension was then centrifuged at 7,000 x g for 15 minutes at 4°C in stainless steel centrifuge bottles to collect the precipitated glycolipids. The pellets were removed from the centrifuge bottles and the supernatant was discarded. Approximately 65 g of crude glycolipids were obtained. Saponification of contaminating neutral lipids and phospholipids was carried out by dissolving the crude glycolipids in 300 ml of chloroform and adding an equal volume of 1.0 N aqueous potassium hydroxide. The solution was incubated at 37°C for 12 hours, with stirring. After 12 hours the solution was neutralized to approximately pH 7 with concentrated HCl, transferred to dialysis tubing and dialyzed versus several changes of distilled water for 5 days at 4°C. The lipids retained by the dialysis tubing were concentrated by rotary evaporation using absolute ethanol to azeotrope the water. The glycolipids were then lyophilyzed to dryness. Approximately 60 g of glycolipids were recovered.

Approximately 50 g of the glycolipid preparation were dissolved in 150 ml of chloroform and applied to a Unisil column (500 g of Unisil; 4.4 x 63 cm) that had been washed with several volumes of chloroform. The column was eluted by a linear gradient of increasing methanol concentration in chloroform, prepared by mixing 2 liters of chloroform with 2 liters of methanol in a gradient maker. Fractions (10 ml) were collected and periodically examined by thin-layer chromatography on silica gel G thin-layer plates (250 μ). Thin-layer plates of neutral glycolipids were developed in chloroform-methanol-water, 65:25:4 (v/v/v), and visualized

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by iodine vapors or by spraying with 0.5% orcinol in 4 N sulfuric acid and heating the plate at 100°C until the dark purple color developed. Thin-layer chromatograms of gangliosides were developed in chloroform-methanol-7% ammonium hydroxide, 55:40:10 (v/v/v), and visualized by iodine vapors, orcinol spray, or by spraying with resorcinol reagent, covering the sprayed area with a clean glass cover plate (to prevent evaporation of the reagent) and heating at 100°C until the blue color developed. The resorcinol reagent was prepared by mixing 10 ml of 2% aqueous resorcinol with 80 ml of concentrated HCl, 0.25 ml of 0.1 M aqueous $CuSO_4$ and distilled water to a final volume of 100 ml. The resorcinol reagent should be protected from light and refrigerated when not in use (267).

Some of the glycolipids were still contaminated by phospholipids, which were removed by mild alkali-catalyzed methanolysis as described by Vance and Sweeley (125). The lipids were dissolved in 10 ml of 0.6 N methanolic NaOH and 10 ml of chloroform. The solution was allowed to stand at room temperature for 1 hour, after which 1.2 volumes (referring to the original volume of methanolic NaOH used) of 0.5 N methanolic HCl, 1.7 volumes of water and 3.4 volumes of chloroform were added and the biphasic system was mixed. The mixture was then centrifuged at low speed to recover the glycolipids in the lower, chloroform phase. Next, the chloroform phase was washed twice with theoretical upper phase (268) [chloroform-methanol-water, 3:48:47 (v/v/v)] and then dried under a stream of nitrogen at 50°C.

An alternative to gradient elution of the Unisil column is stepwise elution with solutions containing increasing concentrations of methanol in chloroform, as described by Suzuki et al. (266).

The final yield was approximately 600 mg of pure globotriglycosylceramide.

[^{3}H]Globotriglycosylceramide. Globotriglycosylceramide was labeled with tritium at C-6 of the terminal galactose residue by the method of Suzuki and Suzuki (269). A portion (50 mg) of the purified globotriglycosylceramide (above) was placed in a large screw-cap test tube with 4 ml of freshly distilled tetrahydrofuran and 4 ml of 0.1 M potassium phosphate buffer, pH 7.0. Galactose oxidase (427 units) was dissolved in 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.0, and was added to the reaction mixture. The solution was incubated at room temperature for 4 hours with gentle shaking. An additional 427 units of galactose oxidase, dissolved

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in 0.5 ml of potassium phosphate buffer was added, and the incubation was continued overnight. The tetrahydrofuran was then removed from the solution under a stream of nitrogen and 5 volumes of chloroform-methanol, 2:1 (v/v), were added. The upper phase was removed and the lower phase was washed once with theoretical upper phase (268) [chloroform-methanol-water, 3:48:47 (v/v/v)]. The lower phase was dried in vacuo and 5 ml of tetrahydrofuran plus 0.4 ml of [^{3}H]-sodium borohydride solution (10 mCi/ml in 0.1 N NaOH) were added. The sample was incubated, with shaking, at room temperature overnight. Excess sodium borotritiide was destroyed by the addition of 0.7 ml of 10 N acetic acid in the hood. The tetrahydrofuran was then removed under a stream of nitrogen and 5 volumes of chloroform-methanol, 2:1 (v/v), were added. The upper phase was removed and the lower phase was washed once with theoretical upper phase. The lower phase, containing [^{3}H]-labeled glycolipid, was then dried under a stream of nitrogen and 5 ml of tetrahydrofuran and 10 mg of solid, unlabeled sodium borohydride were added. The solution was incubated overnight at room temperature, with shaking, after which the excess borohydride was destroyed by the addition of 0.7 ml of 10 N acetic acid in the hood. Tetrahydrofuran was removed under a stream of nitrogen and 5 volumes of chloroform-methanol, 2:1 (v/v), were added. The upper phase was removed and the lower phase was washed 10-15 times with theoretical upper phase. This exhaustive washing reduced the background radioactivity of the assay to a practical level. The [^{3}H]-labeled $Gb0se_3Cer$ was purified by preparative thin-layer chromatography on 500 μ silica gel G-coated plates developed in chloroform-methanol-water, 65:25:4 (v/v/v). The [^{3}H]-labeled glycolipid was located by brief exposure to iodine vapors, or by scanning the thin-layer plate on a Varian Aerograph Berthold Radio Scanner.

The glycolipid was eluted form the silica gel scrappings by washing the gel thoroughly with chloroform-methanol-water, 100:50:10 (v/v/v).

Radioactivity was determined with a Beckman LS-150 liquid scintillation counter. Scintillation solvent was prepared according to the method of Suzuki and Suzuki (269) and contained 7.0 g PPO (2,5-diphenyloxazole) and 0.6 g of dimethyl POPOP (1,4-bis-2-[methyl-5-phenyloxazolyl]-benzene) and 100 ml of Bio-Solv BBS-3 in 1000 ml of scintillation toluene. Prior to the addition of scintillation solvent, the sample was dried under a stream of nitrogen and redissolved in 0.5 ml of water. The sample was counted in 10 ml of scintillation solvent.

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Galabiosylceramide. Galabiosylceramide, which had been purified from a formalin-fixed Fabry kidney (264), was [^{3}H]-labeled at C-6 of the terminal galactose residue by the galactose oxidase-[^{3}H]borohydride method described above.

Globopentaglycosylceramide. Globopentaglycosylceramide, which had been purified from canine intestines (265), was tritium labeled at C-6 of the terminal N-acetylgalactosamine residue as follows. Globopentaglycosylceramide (20 mg) was placed in a large screw-capped test tube with 4 ml of freshly distilled tetrahydrofuran and 4 ml of 0.1 M potassium phosphate buffer, pH 7.0. Galactose oxidase (427 units) and horse radish peroxidase (1000 units) were dissolved in 0.5 ml of potassium phosphate buffer and added to the reaction mixture. The solution was incubated for 4 hours at room temperature with gently shaking, and then an additional 427 units of galactose oxidase were added. The incubation was continued overnight and then a final 427 units of galactose oxidase were added. The incubation was terminated 24 hours after the final addition of galactose oxidase by the removal of the tetrahydrofuran under a stream of nitrogen and the addition of 5 volumes of chloroform-methanol, 2:1 (v/v). The upper phase was removed and the lower phase was washed once with theoretical upper phase (chloroform-methanol-water, 3:48:47 (v/v/v)) (261). The lower phase dried in vacuo and 5 ml of tetrahydrofuran and 0.4 ml of [^{3}H]-sodium borohydride solution (10 mCi/ml in 0.1 N NaOH) were added. The sample was incubated with shaking at room temperature overnight. Excess sodium borotritiide was destroyed by the addition of 0.7 ml of 10 N acetic acid in the hood. The tetrahydrofuran was then removed under a stream of nitrogen and 5 volumes of chloro orm-methanol, 2:1 (v/v), were added. The upper phase was removed and the lower phase was washed once with theoretical upper phase. The lower phase was washed once with theoretical upper phase. The lower phase, containing the [^{3}H]-labeled glyolipid, was then dried under a stream of nitrogen and 5 ml of tetrahydrofuran and 10 mg of solid, unlabeled sodium borohydride were added. The solution was incubated overnight at room temperature with shaking, after which the excess borohydride was destroyed by the addition of 0.7 ml of 10 N acetic acid in the hood. Tetrahydrofuran was removed under a stream of nitrogen and 5 volumes of chloroform-methanol, 2:1 (v/v), were added. The upper phase was removed and the lower phase was washed 10-15 times with theoretical upper phase. This exhaustive

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washing reduced the background radioactivity of the assay to a practical level. The [^{3}H]$GbOse_5Cer$ was purified by preparative thin-layer chromatography on a 500 μ silica gel G-coated plate developed in chloroform-methanol-water, 65:25:4 (v/v/v). The [^{3}H]-labeled glycolipid was located by brief exposure to iodine vapors, or by scanning the thin-layer plate on the Varian Aerograph Berthold Radio Scanner. The [^{3}H]$GbOse_5Cer$ had an R_f identical to that of authentic $GbOse_5Cer$. The glycolipid was eluted from the silica gel scrapings by washing the gel thoroughly with chloroform-methanol-water, 100:50:10 (v/v/v).

B. Oligosaccharide Substrates

Oligosaccharides were prepared from [^{3}H]-labeled and unlabeled $GaOse_2Cer$, $GbOse_3Cer$ and $GbOse_5Cer$ by ozonolysis followed by treatment with mild base (264,270, 271). The procedure described below was used without modification for the preparation of $GbOse_3$, $GaOse_2$ and $GbOse_5$. Ozone was generated by passing oxygen through a high voltage spark chamber equipped with a Supelco high voltage generator. The generation of ozone was confirmed by bubbling the gas through a 2% KI solution. The glycolipid was dissolved in dry methanol to make a 0.4% solution and ozone bubbled through the solution for 4-5 hours. Following ozonolysis, the solvent was removed and the residue was dissolved in 0.2 M sodium carbonate to make a 1% solution. The reaction mixture was incubated at room temperature overnight. The reaction was terminated by neutralizing the solution on a column (1 x 8 cm) of Dowex 50W-X8 (H^+) ion exchange resin (50-100 mesh). Following application of the reaction mixture to the column, it was eluted with water. The aqueous solution was extracted once with 5 volumes of chloroform-methanol, 2:1 (v/v), and 3 times with hexane to remove the degraded products of ceramide. The aqueous phase was lyophilyzed to obtain the oligosaccharide.

The oligosaccharides were examined by thin-layer chromatography on silica gel G thin-layer plates developed in n-propanol-acetic acid-water, 85:12:3 (v/v/v), and by gas-liquid chromatography as trimethylsilyl methylglycosides (125).

C. Artificial Substrate Analogs

The synthetic substrate analogs, 4-MU-α-Gal and p-NP-α-Gal, were obtained from commercial sources. The artificial substrate, p-NP-α-GalNAc,

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became unavailable from commercial sources during the course of these investigations, and it was necessary to synthesize o-NP-α-GalNAc and p-NP-α-GalNAc, as previously described (133). The synthetic substrate analogs, p-nitrophenyl-2-deoxy-α-D-galactopyranoside and o-nitrophenyl-α-D-fucopyranoside (o-nitrophenyl-6-deoxy-α-D-galactopyranoside), were prepared by the following methods.

p-Nitrophenyl-2-Deoxy-α-D-Galactopyranoside. The methods of Dean and Sweeley (133) and Woods and Kramer (272) were modified for the preparation of p-nitrophenyl-2-deoxy-α-D-galactopyranoside. D-Galactal was acetylated by the method of Wallenfels and Lehman (273), as follows. Anhydrous sodium acetate (1.8 g) and acetic anhydride (60 ml) were heated in a 100 ml round-bottom flask at 100-120°C for 30 minutes. D-Galactal (3.0 g) was added to the acetylation mixture and the solution was heated at 100-120°C for 30 minutes. The acetylation mixture was allowed to cool to room temperature and was then poured slowly into 100 ml of cold 5% aqueous sodium carbonate, with stirring. The mixture was extracted twice with chloroform and the combined extracts were washed once with water. The chloroform was removed by rotary evaporation at ∿60°C to yield D-galactal-tri-O-acetate (5.5 g, 98% yield) as a yellow oil.

Phenyl-tri-O-acetyl-2-deoxy-α-D-galactopyranoside was prepared by shaking D-galactal-tri-O-acetate (5.5 g) and phenol (2.0 g) with 4 drops of concentrated HCl on a New Brunswick shaker for 4 hours at room temperature. The reaction was terminated by neutralizing the reaction mixture with 4 drops of 12.5 N NaOH. The reaction mixture was dissolved in ethylene dichloride and washed once with water, twice with 0.25 N NaOH and then one more time with water. The lower, ethylene dichloride phase, was dried with calcium chloride and the solvent removed by rotary evaporation at ∿60°C to yield an oil. Phenyl-tri-O-acetyl-2-deoxy-α-D-galactopyranoside was recrystallized from 95% ethanol as flat white plates (yield 1.9 g, m.p. 140-142°C).

Phenyl-tri-O-acetyl-2-deoxy-α-D-galactopyranoside (1.8 g), dissolved in 7.5 ml of acetic acid, was nitrated with 3 ml of a nitration mixture consisting of 3 volumes of 90% (fuming) nitric acid and 10 volumes of acetic anhydride. The solution was incubated at 37°C for 2 hours and then 18 ml of cold 2 M potassium acetate was added and the solution was allowed to stand at room temperature for 3 hours. The reaction mixture was then extracted 3 times with chloroform and the pooled chloroform extracts were

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washed once with cold 2 M aqueous sodium carbonate and once with cold water. The chloroform phase was dried with calcium chloride and the solvent removed by rotary evaporation. The product crystallized readily from 95% ethanol as long white needles (yield 0.7 g, m.p. 150-152°C).

The product was O-deacetylated by dissolving the crystals (0.7 g) in chloroform (10 ml) and heating the solution to boil until ∿20% of the solvent had escaped. Anhydrous methanol (8 ml) was added and the solution was heated to boiling again. Sodium methoxide (0.4 ml), prepared previously by mixing anhydrous methanol with sodium metal (3 g) in an ice bath in the hood until the evolution of hydrogen gas had ceased, was added to the boiling reaction mixture. The solution was removed from the heat, stoppered, and incubated at room temperature for 4 hours. At the end of the incubation, 1 drop of acetic acid was added to adjust the solution to ∿pH 6.5. The solvent was removed on the rotary evaporator and p-nitrophenyl-2-deoxy-α-D-galactopyranoside was recrystallized from 95% ethanol (yield 0.5 g, m.p. 178-181°C). Fourier-transform ^{13}C-NMR spectra were obtained on a 15.08 mHz Bruker WP-60 spectrometer.

o-Nitrophenyl-6-Deoxy-α-D-Galactopyranoside (o-Nitrophenyl-α-D-Fucopyranoside). D-(+)-Fucose (3.0 g) was acetylated by the method of Wallenfels and Lehman (273) as previously described for the synthesis of p-NP-2-deoxy-α-Gal. Tetra-O-acetyl-β-D-fucopyranoside was obtained as a yellow oil (6.0 g, 99% yield). Phenyl-tri-O-acetyl-α-D-fucopyranoside was prepared by the method of Conchie et al. (274), modified as described below. Tetra-O-acetyl-β-D-fucopyranoside was placed in a 100 ml round-bottom flask with 6.0 g of freshly distilled phenol. Anhydrous zinc chloride (2.0 g) (prepared by fusing zinc chloride in a crucible and allowing it to cool in a desiccator), dissolved in 5 ml of acetic acid-acetic anhydride, 95:5 (v/v), was added. The reaction mixture was immersed in an oil bath at 60-80°C and the pressure was reduced to 50-70 torr with a water aspirator. These reaction conditions were maintained for 2 hours with stirring, and then the heat was removed and the pressure returned to normal. The reaction mixture was allowed to cool overnight. The resulting red syrup was dissolved in ethylene dichloride and washed successively with water, 0.2 N NaOH and twice more with water, to remove zinc chloride and unreacted phenol. The lower ethylene dichloride phase, containing phenyl-tri-O-acetyl-α-D-fucopyranoside, was dried with calcium chloride and the solvent removed by rotary evaporation. The product was

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obtained as a yellow oil (6.3 g, 94% yield).

Phenyl-tri-O-acetyl-α-D-fucopyranoside was nitrated as previously described for the preparation of p-nitrophenyl-2-deoxy-α-D-galactopyranoside. The mixture of o- and p-nitrophenyl-tri-O-acetyl-α-D-fucopyranosides, obtained as a red oil (3.8 g, 51% yield), was de-O-acetylated as previously described. A mixture of o- and p-nitrophenyl-α-D-fucopyranosides was obtained as a red oil (1.9 g, 36% yield).

The o-nitrophenyl isomer was purified by Iatrobead chromatography. A column of Iatrobeads (1.5 x 30 cm) was prepared using Iatrobeads (not heat-activated) suspended in chloroform. The mixture of o- and p-nitrophenyl-α-D-fucopyranosides, dissolved in approximately 2 ml of chloroform containing several drops of methanol, was applied to the column and the column was washed with approximately 10 ml of chloroform. The o- and p-nitrophenyl-α-D-fucopyranosides were eluted with a methanol gradient in chloroform, prepared by mixing 100 ml of chloroform with 100 ml of chloroform-methanol, 7:3 (v/v), in a gradient maker. The ortho isomer eluted first, followed by the para isomer contaminated by the ortho isomer. Fractions containing pure o-nitrophenyl-α-D-fucopyranoside were pooled and recrystallized from absolute ethanol as white needles (0.8 g, 15% yield, m.p. 190-193°C). Fourier-transform ^{13}C-NMR spectra were obtained on a 15.08 mHz Bruker WP-60 spectrometer.

III. Assays

The ability of α-galactosidase A and α-galactosidase B (α-N-acetylgalactosaminidase) to hydrolyze glycosphingolipids, oligosaccharides and artificial substrates containing terminal α-galactose or α-N-acetylgalactosamine residues was investigated. The reaction conditions for assays with these substrates are described below.

A. Assays with Glycosphingolipid Substrates

The glycosphingolipids used in these studies are not soluble in water, and a detergent (sodium taurocholate) was therefore employed to solubilize them. The effect of sodium taurocholate on the hydrolysis of glycosphingolipids by α-galactosidases A and B will be discussed in a later section.

Assays with Globotriglycosylceramide. The reaction conditions for the hydrolysis of $Gb0se_3Cer$ were different for the reactions catalyzed by

α-galactosidase A and α-galactosidase B (α-N-acetylgalactosaminidase). The assay with α-galactosidase A was carried out as follows. An aliquot (100 µl) of 1 mM [^{3}H]GbOse$_3$Cer (1000 cpm/nmole) in chloroform-methanol, 2:1 (v/v), was mixed in a test tube with 100 µl of 0.5% sodium taurocholate (w/v) in theoretical upper phase (chloroform-methanol-water,3:48:47 (v/v/v). The solvent was removed under a stream of nitrogen and the residue was redissolved in 50 µl of Gomori citrate-phosphate buffer, pH 4.1 (275), with sonication. An aliquot (up to 50 µl) of the enzyme solution to be assayed was then added. The concentration of protein in the reaction mixture should not exceed 40 µg/100 µl, as it has been reported (207,217,218) that higher protein concentrations inhibit the hydrolysis of GbOse$_3$Cer, presumably by binding to the negatively charged detergent-glycolipid mixed micelles. Following the addition of the enzyme solution, the reaction mixture was taken up to a final volume of 100 µl with glass distilled water. The final concentrations of GbOse$_3$Cer and sodium taurocholate were 1.0 mM and 9.3 mM, respectively. The reaction mixture was incubated at 37°C for 1 hour and then the reaction was terminated by the addition of 100 µl of water, 400 µl of methanol and 200 µl of chloroform. The homogeneous solution was mixed on a Vortex mixer and then an additional 200 µl of chloroform was added, as described by Bligh and Dyer (276). The biphasic system was mixed and then centrifuged to resolve the phases. The upper phase was removed with a Pasteur pipet and the lower phase was washed once with 0.5 ml of theoretical upper phase (chloroform-methanol-water, 3:48:47 (v/v/v)). The pooled upper phases were washed once with 1 ml of theoretical lower phase (chloroform-methanol-water, 86:14:1 (v/v/v)). The upper phase was then transferred to a scintillation vial and dried under a stream of nitrogen. The residue was dissolved in 0.5 ml of water and 10 ml of scintillation solvent, consisting of 7.0 g PPO, 0.6 g of dimethyl-POPOP and 100 ml of Biosolv BBS-3 dissolved in 1,000 ml of toluene (269), was added. The solution was mixed and counted in a Beckman LS-150 liquid scintillation counter.

The assay for GbOse$_3$Cer hydrolysis by α-galactosidase B (α-N-acetylgalactosaminidase) differed slightly from the assay with α-galactosidase A. An aliquot (100 µl) of 1 mM [^{3}H] GbOse$_3$Cer (1000 cpm/nmole) in chloroform-methanol, 2:1 (v/v), was mixed in a test tube with 80 µl of 0.5% sodium taurocholate (w/v) in theoretical upper phase (chloroform-methanol-water, 3:48:47 (v/v/v)). The solvent was removed under a stream of

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nitrogen and the residue was redissolved, with sonication, in 50 µl of 0.1 M sodium citrate buffer, pH 4.3. An aliquot (up to 50 µl) of the enzyme solution to be assayed was then added, and the reaction mixture was taken up to a final volume of 100 µl with glass-distilled water. The final concentrations of $GbOse_3Cer$ and sodium taurocholate were 1.0 mM and 7.4 mM, respectively. The reaction mixture was incubated at 37°C for 6 hours, the reaction was terminated and the liberated [^{3}H]galactose was quantitated as previously described for the assay with α-galactosidase A.

Assays with Galabiosylceramide. The hydrolysis of $GaOse_2Cer$ was examined with α-galactosidase A only. An aliquot (100 µl) of 1 mM [^{3}H]$GaOse_2Cer$ (1000 cpm/nmole) in chloroform-methanol, 2:1 (v/v), was mixed in a test tube with 60 µl of 0.5% sodium taurocholate (w/v) in theoretical upper phase (chloroform-methanol-water, 3:48:47 (v/v/v)). The solvent was removed under a stream of nitrogen and the residue was redissolved, with sonication, in 50 µl of Gomori citrate-phosphate buffer, pH 4.1 (275). An aliquot (up to 50 µl) of the enzyme solution to be assayed was then added and the reaction mixture was taken up to a final volume of 100 µl with glass-distilled water. The final concentrations of $GaOse_2Cer$ and sodium taurocholate were 1.0 mM and 5.6 mM, respectively. The reaction mixture was incubated for 1 hour at 37°C, the reaction was terminated and the liberated [^{3}H]galactose was quantitated as previously described for the assay with $GbOse_3Cer$.

Assays with Globopentaglycosylceramide. An aliquot (100 µl) of 1 mM [^{3}H]$GbOse_5Cer$ (500 cpm/nmole) in chloroform-methanol, 2:1 (v/v), was mixed in a test tube with 80 µl of 0.5% sodium taurocholate (w/v) in theoretical upper phase (chloroform-methanol-water, 3:48:47 (v/v/v)). The solvent was removed under a stream of nitrogen and the residue was redissolved, with sonication, in 50 µl of 0.1 M sodium citrate buffer, pH 3.9. An aliquot (up to 50 µl) of the enzyme solution to be assayed was then added and the reaction mixture was taken up to a final volume of 100 µl with glass-distilled water. The final concentrations of $GbOse_5Cer$ and sodium taurocholate wer 1.0 mM and 7.4 mM, respectively. The reaction mixture was incubated at 37°C for 30 minutes, the reaction was terminated by the addition of 4 ml of chloroform-methanol, 2:1 (v/v), water (0.85 ml) was added, and the biphasic system was thoroughly mixed on a Vortex mixer and then centrifuged to resolve the phases. The upper phase was washed once with 2.5 ml

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B. Assays with Oligosaccharide Substrates

The oligosaccharides used in these studies were derived from glycosphingolipids by ozonolysis and treatment with mild base, as previously described. These complex carbohydrates were soluble in aqueous solutions, and sodium taurocholate was therefore omitted from the reaction mixtures. Reaction conditions for the hydrolysis of $GaOse_2$, $GbOse_3$ and $GbOse_5$ by α-galactosidase A or α-galactosidase B (α-N-acetylgalactosaminidase) are described below.

Assays with Galabiaose. The hydrolysis of $GaOse_2$ was examined with α-galactosidase A only. An aliquot (250 µl) of aqueous [^{3}H]$GaOse_2$ (500 cpm/nmole) was placed in a test tube and the solvent was removed under a stream of nitrogen. The residue was redissolved in 25 µl of Gomori citrate-phosphate buffer, pH 4.5 (275), and an aliquot (up to 25 µl) of the enzyme solution to be assayed was added. The reaction mixture was adjusted to a final volume of 50 µl with glass-distilled water. The final concentration of $GaOse_2$ was 5.0 mM. The reaction mixture was incubated at 37°C for 1 hour and then the reaction was terminated by the addition of 100 µl of methanol. The total reaction mixture was applied to a 500 µ silica gel G thin-layer plate, along with galactose and lactose standards, which were spotted on either side of the reaction mixture. The chromatograpm was developed in n-propanol-acetic acid-water, 85:12:3 (v/v/v), in a paper-lined tank that had been allowed to equilibrate for several hours prior to use. The galactose and lactose standards were visualized by covering the lanes of the reaction mixtures with a glass plate and spraying the exposed lanes with 0.5% orcinol in 4 N sulfuric acid. The plate was then placed in an oven at ∿100°C until a purple color developed. Regions of the unsprayed lanes that corresponded in R_f to the galactose standard were scrapped and the [^{3}H]galactose was eluted in a small column with 30 ml of 50% ethanol. The solvent was removed under a stream of nitrogen and the [^{3}H]galactose was quantitated as previously described.

Assays with Globotriaose. The hydrolysis of $GbOse_3$, catalyzed by both α-galactosidase A and α-galactosidase B (α-N-acetylgalactosaminidase), was examined. The assay for $GbOse_3$ hydrolysis by α-galactosidase A was carried out as follows. An aliquot (250 µl) of aqueous 1 mM [^{3}H]$GbOse_3$ (500

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cpm/nmole) was placed in a test tube and the solvent was removed under a stream of nitrogen. The residue was redissolved in 25 µl of Gomori citrate-phosphate buffer, pH 4.5 (275) and an aliquot (up to 25 µl) of the enzyme solution to be assayed was added. The reaction mixture was adjusted to a final volume of 50 µl with glass-distilled water. The final concentration of $GbOse_3$ was 5.0 mM. The reaction mixture was incubated at 37°C for 1 hour and then the reaction was terminated. The [^{3}H]galactose was isolated and quantitated as previously described for the assay with $GaOse_2$.

Assays for $GbOse_3$ hydrolysis by α-galactosidase B were carried out as follows. An aliquot (250 µl) of aqueous 1 mM [^{3}H]$GbOse_3$ was placed in a test tube and the solvent was removed under a stream of nitrogen. The residue was dissolved in 25 µl of Gomori citrate-phosphate buffer, pH 4.8 (275) and an aliquot (up to 25 µl) of the enzyme solution to be assayed was added. The reaction mixture was adjusted to a final volume of 50 µl with glass-distilled water. The final concentration of $GbOse_3$ was 5.0 mM. The reaction mixture was incubated at 37°C for 6 hours and then the reaction was terminated. The [^{3}H]galactose was isolated and quantitated as previously described for the assay with $GaOse_2$.

Assays with Globopentaose. The hydrolysis of $GbOse_5$ was examined with α-galactosidase B (α-N-acetylgalactosaminidase) only. An aliquot (250 µl) of aqueous 1 mM [^{3}H]$GbOse_5$ (500 cpm/nmole) was placed in a test tube and the solvent was removed under a stream of nitrogen. The residue was redissolved in 25 µl of Gomori citrate-phosphate buffer, pH 4.4, and an aliquot of the enzyme solution to be assayed (10 µl) was added. The reaction mixture was adjusted to a final volume of 50 µl with glass-distilled water. The final concentration of $GbOse_5$ was 5.0 mM. The reaction mixture was incubated at 37°C for 45 minutes and then the reaction was terminated and the liberated [^{3}H]N-acetylgalactosamine was isolated and quantitated as previously described for the assay with $GaOse_2$.

C. Assays with Artificial Substrates

Artificial substrates were employed to monitor the purification of α-galactosidases A and B because of their availability and the ease of the assay compared with assays employing glycolipid or oligosaccharide substrates. In addition, artificial substrates were employed to probe the substrate specificities of these enzymes.

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Assays with 4-Methylumbelliferyl-α-D-Galactopyranoside. The procedure described below is a modification of the method of Desnick et al. (228). A stock solution of 5.0 mM 4-MU-α-Gal was prepared in Gomori citrate-phosphate buffer, pH 4.6 (275). An aliquot (300 µl) of this solution was placed in a test tube and an aliquot (up to 50 µl) of the enzyme solution to be assayed was added and the reaction mixture was adjusted to a final volume of 350 µl with glass-distilled water. The final concentration of 4-MU-α-Gal in the reaction mixture was 4.3 mM. The reaction mixture was incubated at 37°C for 10-30 minutes, after which the reaction was terminated by the addition of 4.65 ml of 0.1 M ethylenediamine, pH 11. Fluorescence was read on an Aminco J4-7439 Fluoro-Colorimeter, equipped with a Turner 110-811 (Corning 7-60) excitation filter and Turner 110-831 and 110-816 (Wratten 48 and 2A) emission filters. The fluorimeter was first calibrated with standard solutions of 4-methyumbelliferone that were prepared and stored in water, and diluted up to volume with 0.1 M ethylenediamine just before use.

Assays with o- or p-Nitrophenyl-α-N-Acetylgalactosaminide. The procedure described below is a modification of the method of Sung and Sweeley (265). An aliquot (50 µl) of 10 mM o- or p-NP-α-GalNAc in 0.1 M sodium citrate buffer, pH 4.3, was placed in a test tube. An aliquot (up to 50 µl) of the enzyme solution to be assayed was added and the reaction mixture was adjusted to a final volume of 100 µl. The final concentration of o-NP-α-GalNAc was 5.0 mM. The reaction mixture was incubated at 37°C for 15-30 minutes, after which the reaction was terminated by the addition of 3.0 ml of saturated sodium borate, pH 9.7, and the optical density of the solution was read at 410 nm in a Gilford 2400 spectrophotometer.

Assays with Other Artificial Substrates. Assays with 4-methylumbelliferyl-β-N-acetylgalactosaminide, to check for contamination by β-N-acetylhexosaminidase, were carried out as follows. An aliquot (50 µl) of 2.0 mM 4-MU-β-GalNAc in Gomori citrate phosphate buffer, pH 4.4 (275), was placed in a test tube. An aliquot (up to 50 µl) of the enzyme solution to be assayed was added and the reaction mixture was adjusted to a final volume of 100 µl. The final concentration of 4-MU-β-GalNAc was 1.0 mM. The reaction mixture was incubated at 37°C for 10 minutes, after which the reaction was terminated by the addition of 4.9 ml of 0.1 M ethylenediamine and fluorescence was read on an Aminco J4-7439 Fluoro-Colorimeter, as previously described for assays with 4-MU-α-Gal.

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Assays with p-nitrophenyl-2-deoxy-α-D-galactopyranoside and o-nitrophenyl-α-D-fucopyranoside were carried out as previously described for assays with o- or p-NP-α-GalNAc. Michaelis constants (K_m) and maximal velocities (V_{max}) were calculated by weighted double reciprocal plots, as described by Wilkinson (278), on a CDC 6500 computer.

Protein Assays. Protein concentrations were determined by the method of Lowry et al. (277) using bovine serum albumin (0-200 µg/ml).

IV. Physical Characterization of α-Galactosidases A and B

Isoelectric focusing of α-galactosidases A and B was carried out in an LKB 8101 isoelectric focusing column in pH 3-5 carrier ampholytes, in a sucrose density gradient, as described by Vesterberg (279). Native polyacrylamide disc gel electrophoresis was carried out as described by Wrigley (280). The gels were stained for enzyme activity as described by Gabriel (281), or for protein as described by Malik and Berrie (282).

The molecular weights of native α-galactosidases A and B were determined by gel filtration on Sephadex G-150 (3.0 x 110 cm column) with 0.1 M sodium phosphate buffer, pH 6.5, using horse heart cytochrome c, bovine pancreas chymotrypsinogen A, Bovine serum albumin, rabbit muscle aldolase and beef liver catalase as molceular weight standards.

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RESULTS

I. Purification of α-Galactosidases A and B

The purification of α-galactosidases A and B from normal human liver is summarized in Table 1. α-Galactosidase A was purified about 67,000-fold, with approximately 16% recovery, and α-galactosidase B was purified 68,000-fold, with approximately 9% recovery.

Following homogenization, ammonium sulfate precipitation and dialysis, the crude enzyme preparation was chromatographed on DEAE-cellulose, as shown in Figure 8. β-N-Acetylhexosaminidase B eluted at a very low salt concentration, well ahead of the α-galactosidases. However, β-N-acetylhexosaminidase A eluted between α-galactosidases A and B, and was contained in the fractions pooled for further purification. β-N-Acetylhexosaminidase A also chromatographed with the α-galactosidase activity on Sephadex G-150, as shown in Figure 9. The most effective steps in the purification scheme were hydroxylapatite chromatography and ampholyte displacement chromatography on DEAE-cellulose. Hydroxylapatite chromatography gave approximately 400-fold purification of the α-galactosidases and completely resolved the A and B forms, with the B form eluting at 10 mM phosphate and the A form eluting at 30 mM phosphate, as shown in Figure 10. β-N-Acetylhexosaminidase A (not shown) eluted at 40 mM phosphate, contaminating α-galactosidase A. Ampholyte displacement chromatography on DEAE-cellulose is shown in Figures 11 and 12. β-N-Acetylhexosaminidase A eluted at pH 5.0, ahead of α-galactosidase A at pH 4.7, and α-galactosidase B, at pH 4.5.

The purified α-galactosidases were examined by native polyacrylamide disc gel electrophoresis, as shown in Figure 13. α-Galactosidase A migrated ahead of the B form, and appeared to have some minor contaminants, when relatively large amounts of protein were used. α-Galactosidase B was apparently homogeneous, even when relatively large amounts of protein were used.

Table 1. Purification of α-Galactosidases A and B from Human Liver

4-MU-α-Gal

Gb3Se2Cer

Table 1. Purification of α-Galactosidases A and B from Human Liver

step	4-MU-α-Gal total units (μmoles/min)	4-MU-α-Gal specific activity (nmoles/min·mg)	protein	-fold	$GbOse_3Cer$ total units (nmoles/min)	$GbOse_3Cer$ specific activity (nmoles/min·mg)
1. crude 100,000 x g supernatant	122.0	.36	336 g	1.0	60,480	0.18
2. 30-60% ammonium sulfate	105.4	2.2	47 g	6.1	19,943	0.42
3. NaCl-eluted DEAE-cellulose chromatography	59.4	6.0	9.9 g	16.7	2,695	0.27
4. Sephadex G-150 chromatography	102.1	15.7	6.5 g	43.6	9,126	1.40
5. hydroxylapatite chromatography						
A	49.9	899	55.5 mg	3,418	8,052	145.1
B	50.5	1,530	33.0 mg	15,773	50	1.52
6. ampholyte displacement chromatography						
A	19.4	17,636	1.1 mg	67,057	3,170	2,882
B	10.5	6,563	1.6 mg	67,660	12	7.30

Figure 8. DEAE-Cellulose Chromatography of α-Galactosidases A and B

The crude α-galactosidase preparation obtained by 30-60% ammonium sulfate precipitation was applied to a 4.3 x 25 cm DEAE-cellulose column and eluted with a linear gradient of NaCl (0-0.5 M). Fractions of approx-10 ml were collected.

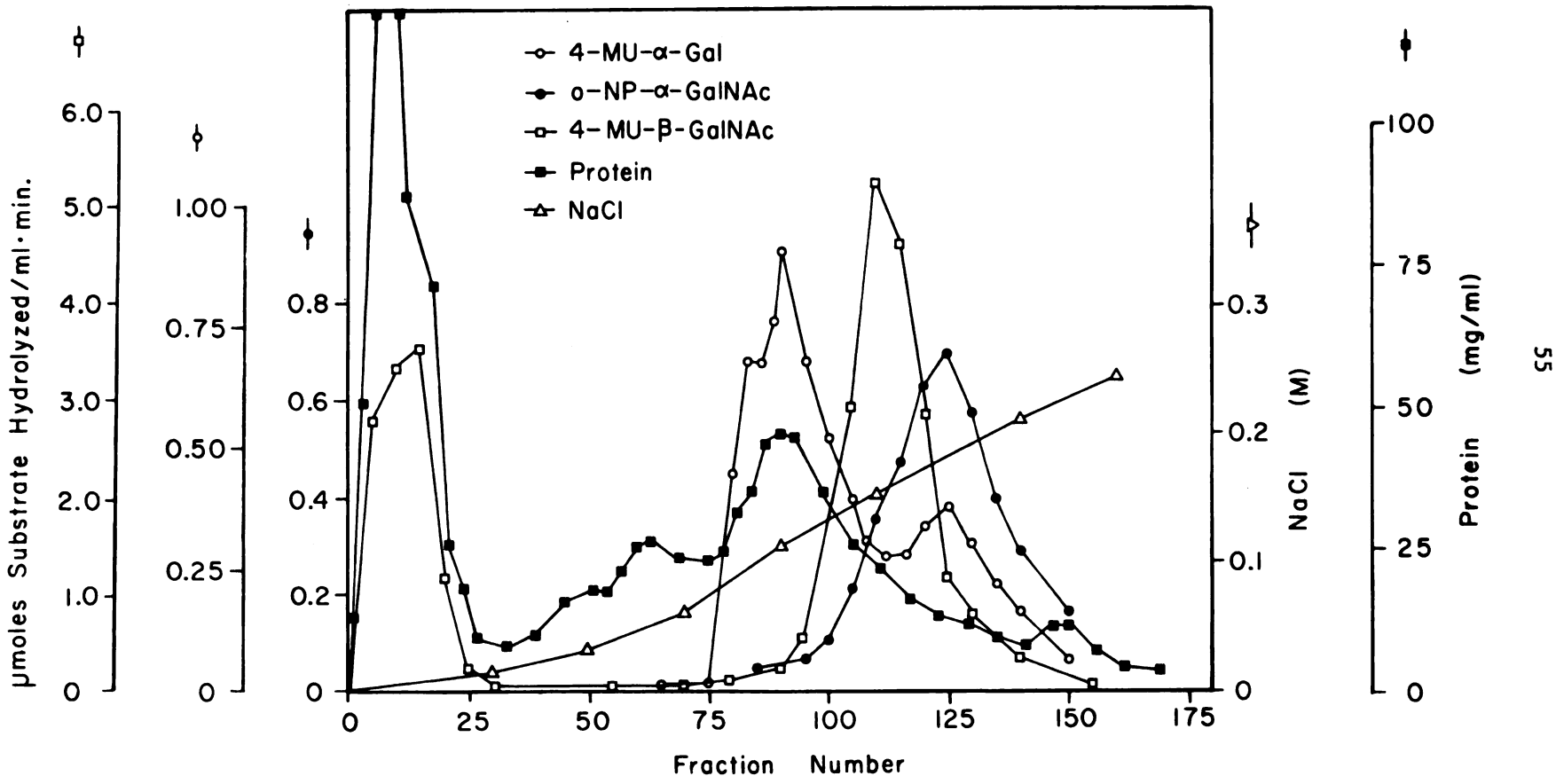

4-MU-α-Gal
o-NP-α-GalNAc
4-MU-β-GalNAc
Protein
NaCl
μmoles Substrate Hydrolyzed/ml·min.
NaCl (M)
Protein (mg/ml)
Fraction Number
0
25
50
75
100
125
150
175
6.0
5.0
4.0
3.0
2.0
1.0
0
1.00
0.75
0.50
0.25
0
0.8
0.6
0.4
0.2
0
0.3
0.2
0.1
0
100
75
50
25
0

Figure 9. Sephadex G-150 Chromatography of α-Galactosidases A and B

The partially purified α-galactosidases isolated by DEAE-cellulose chromatography were concentrated on an Amicon Model 52 ultrafiltration apparatus and applied to a 3.0 x 110 cm Sephadex G-150 column.

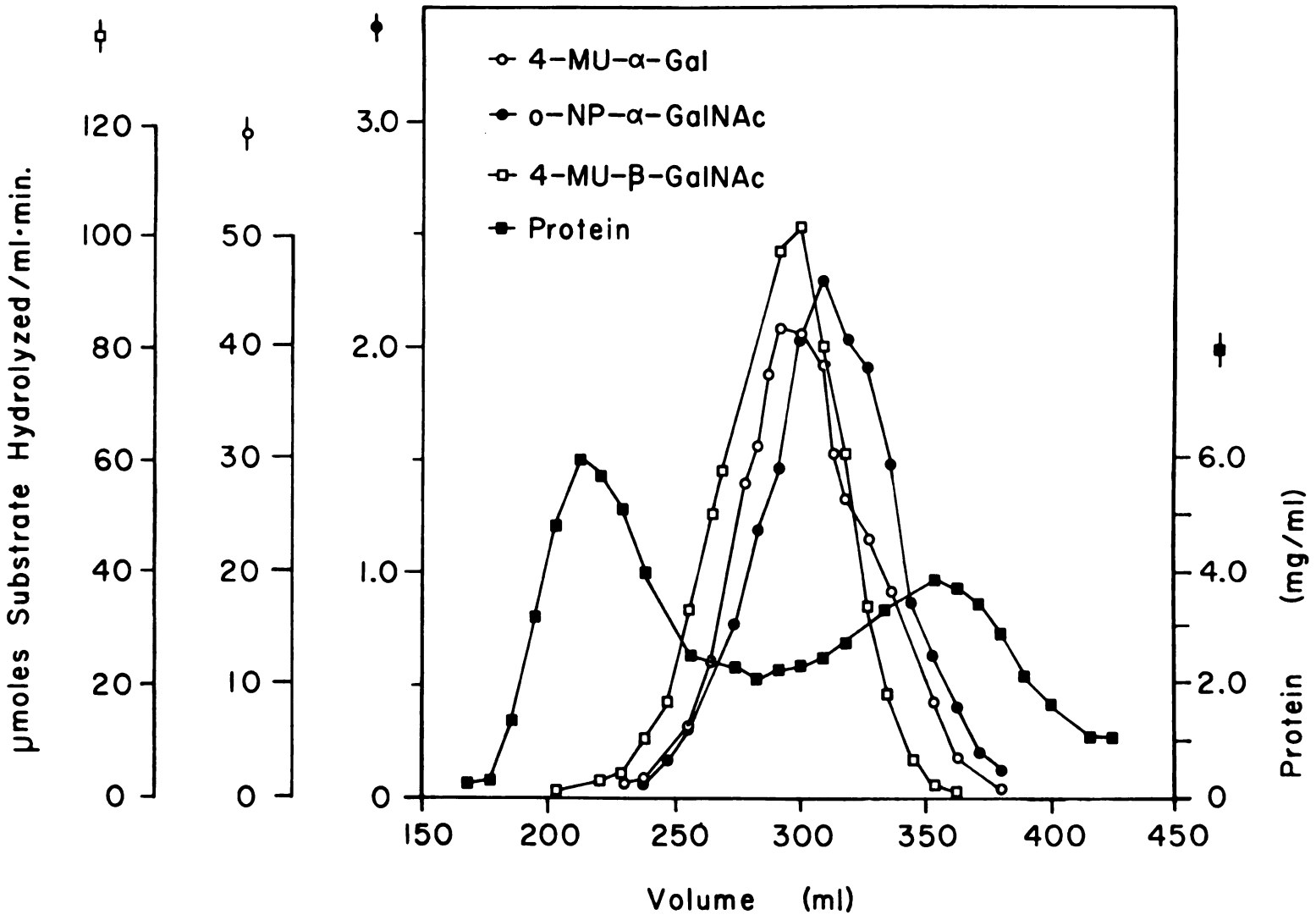

4-MU-α-Gal
o-NP-α-GalNAc
4-MU-β-GalNAc
Protein
μmoles Substrate Hydrolyzed/ml·min.
Protein (mg/ml)
Volume (ml)
120
100
80
60
40
20
0
50
40
30
20
10
0
3.0
2.0
1.0
0
6.0
4.0
2.0
0
150
200
250
300
350
400
450

Figure 10. Hydroxylapatite Chromatography of α-Galactosidases A and B

The partially purified α-galactosidases obtained by Sephadex G-150 column chromatography were applied to a 3.0 x 20 cm hydroxylapatite column. The enzymes were separated by eluting the column with a phosphate gradient (0-50 mM). Fractions of approximately 7 ml were collected.

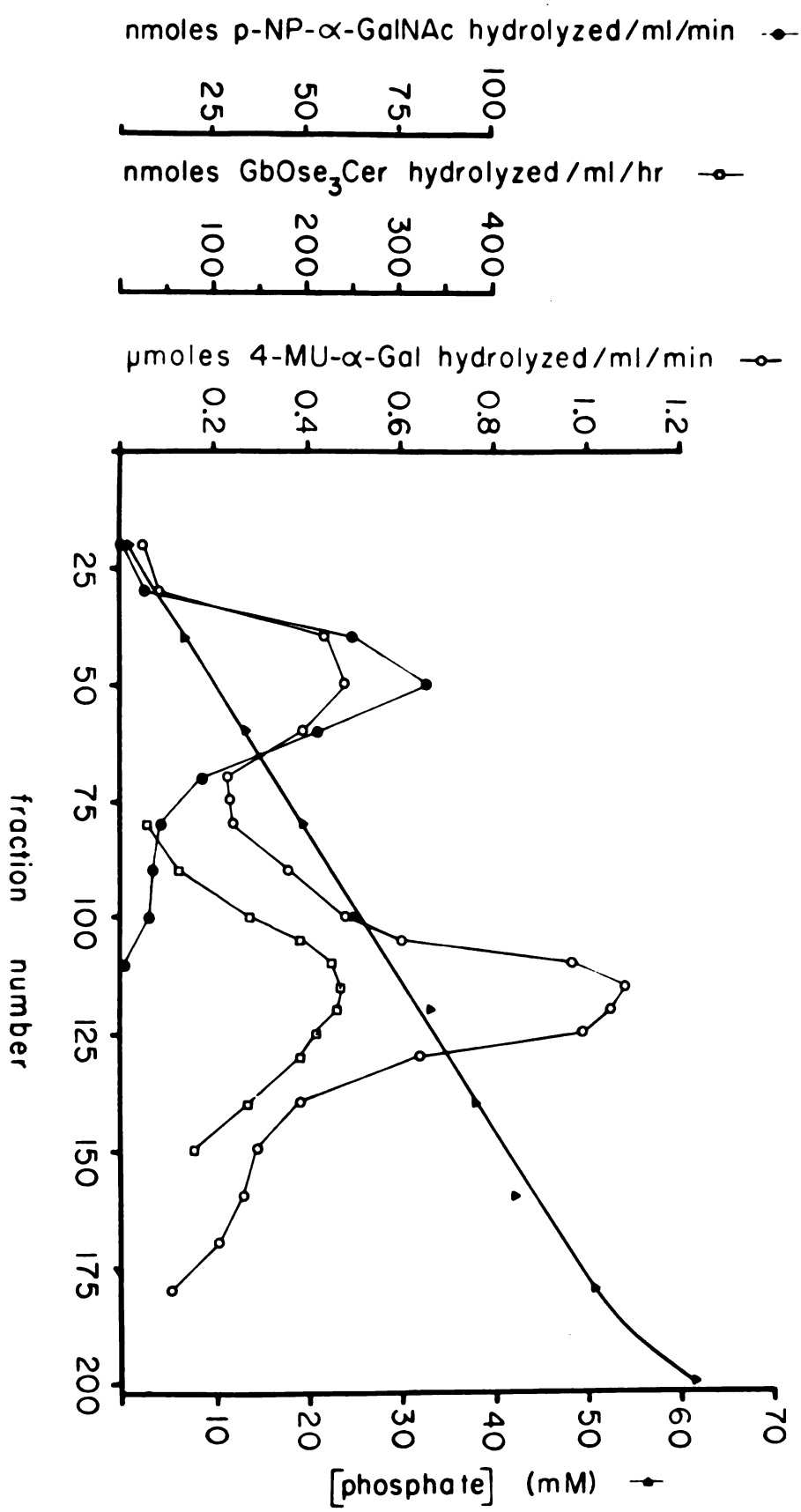
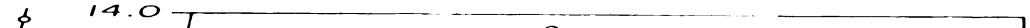

nmoles p-NP-α-GalNAc hydrolyzed/ml/min
25
50
75
100
nmoles GbOse3Cer hydrolyzed/ml/hr
100
200
300
400
μmoles 4-MU-α-Gal hydrolyzed/ml/min
0.2
0.4
0.6
0.8
1.0
1.2
fraction number
25
50
75
100
125
150
175
200
[phosphate] (mM)
10
20
30
40
50
60
70

Figure 11. Ampholyte Displacement Chromatography of α-Galactosidase A on DEAE-Cellulose

α-Galactosidase A was separated from the B form by hydroxylapatite chromatography and was purified by ampholyte displacement chromatography on DEAE-cellulose. The enzyme was applied to a 1 x 5 cm column of DEAE-cellulose and eluted with a solution of carrier ampholytes, pH 3-5. α-Galactosidase A eluted at pH 4.7. Fractions of approximately 1 ml were collected.



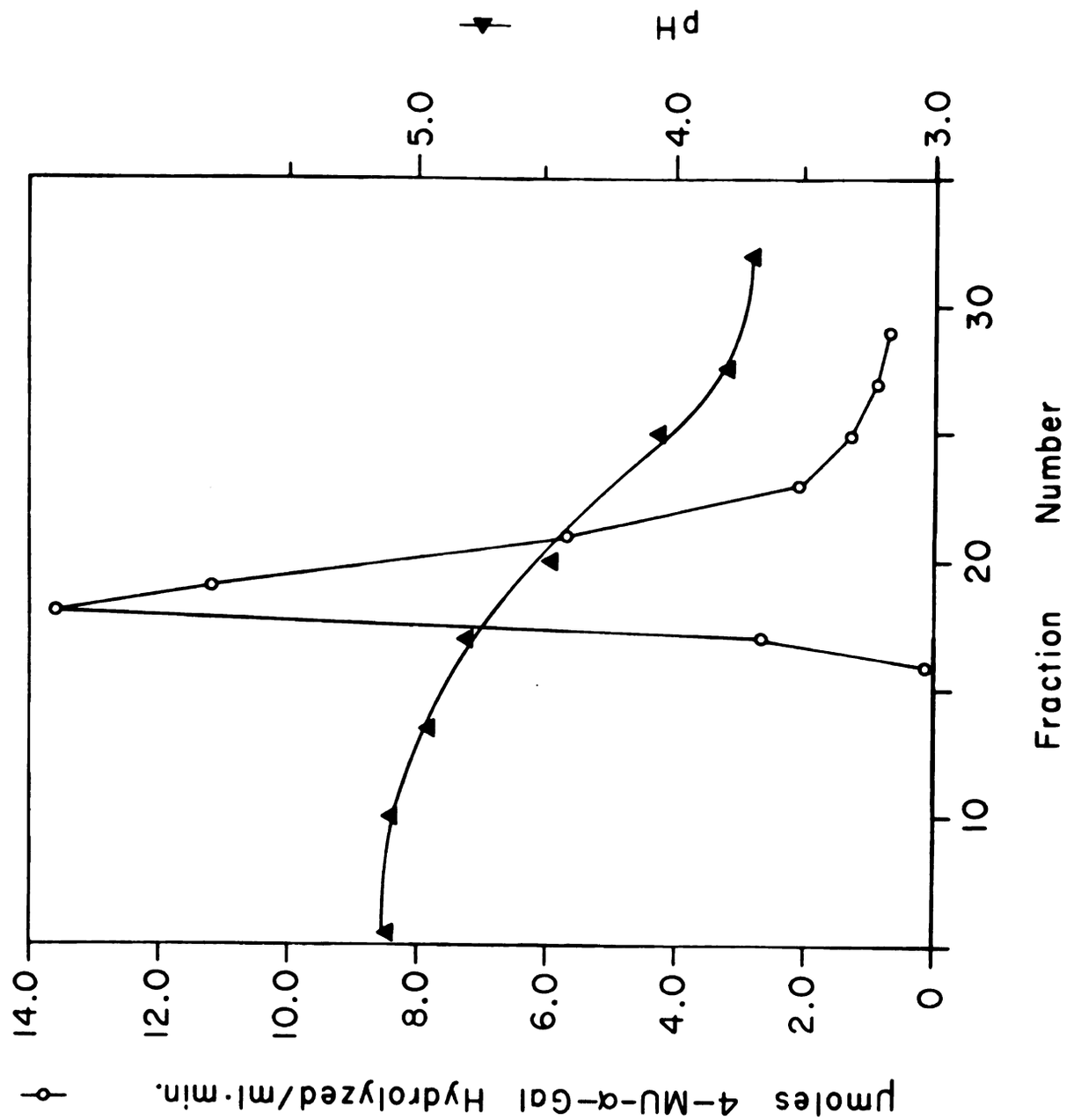

μmoles 4-MU-α-Gal Hydrolyzed/ml·min.
pH
Fraction Number
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
3.0
4.0
5.0
10
20
30

Figure 12. Ampholyte Displacement Chromatography of α-Galactosidase B

α-Galactosidase B was separated from the A form by hydroxylapatite chromatography and applied to a 1 x 5 cm DEAE-cellulose column. The enzyme was eluted with a solution of carrier ampholytes, pH 3-5. α-Galactose B eluted at pH 4.5. Fractions of approximately 1 ml were collected.

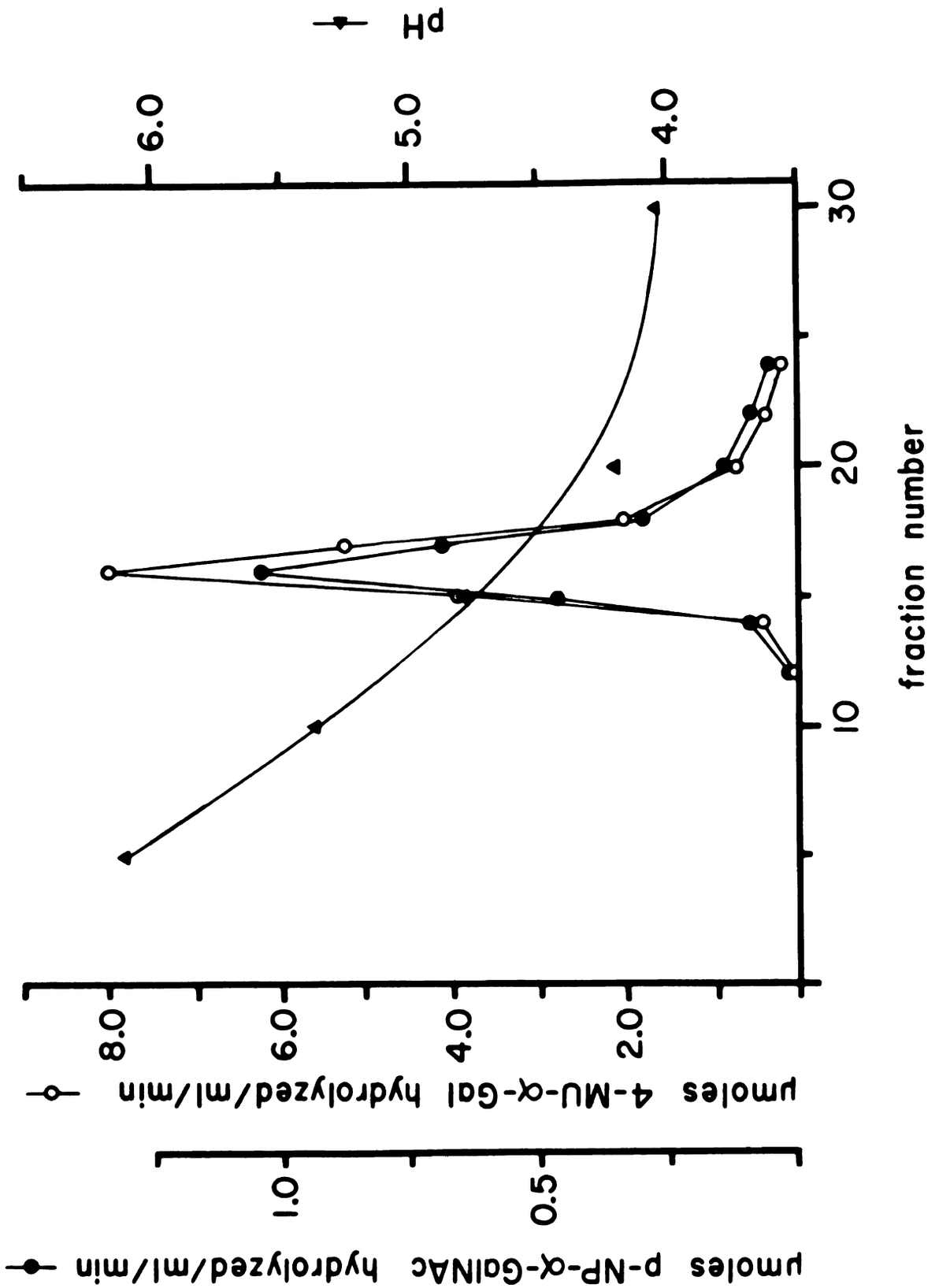

pH
6.0
5.0
4.0
fraction number
10
20
30
μmoles 4-MU-α-Gal hydrolyzed/ml/min
8.0
6.0
4.0
2.0
μmoles p-NP-α-GalNAc hydrolyzed/ml/min
1.0
0.5

Figure 13. Native Polyacrylamide Disc Gel Electrophoresis of α-Galactosidases A and B

Native polyacrylamide disc gel electrophoresis of α-galactosidases A and B at different stages in the purification procedure are shown: (A), DEAE-cellulose eluate (NaCl elution); (B), Sephadex G-150 eluate; (C), α-galactosidase B (α-N-acetylgalactosaminidase) following hydroxylapatite chromatography; (D), α-galactosidase B (α-N-acetylgalactosaminidase) following ampholyte displacement chromatography; (E) α-galactosidase A following hydroxylapatite chromatography; and (F) α-galactosidase A following ampholyte displacement chromatography. Electrophoresis was carried out in 7% gels at pH 8.3, as described by Wrigley (280).


A B C D

II. Enzymatic Properties of α-Galactosidases A and B

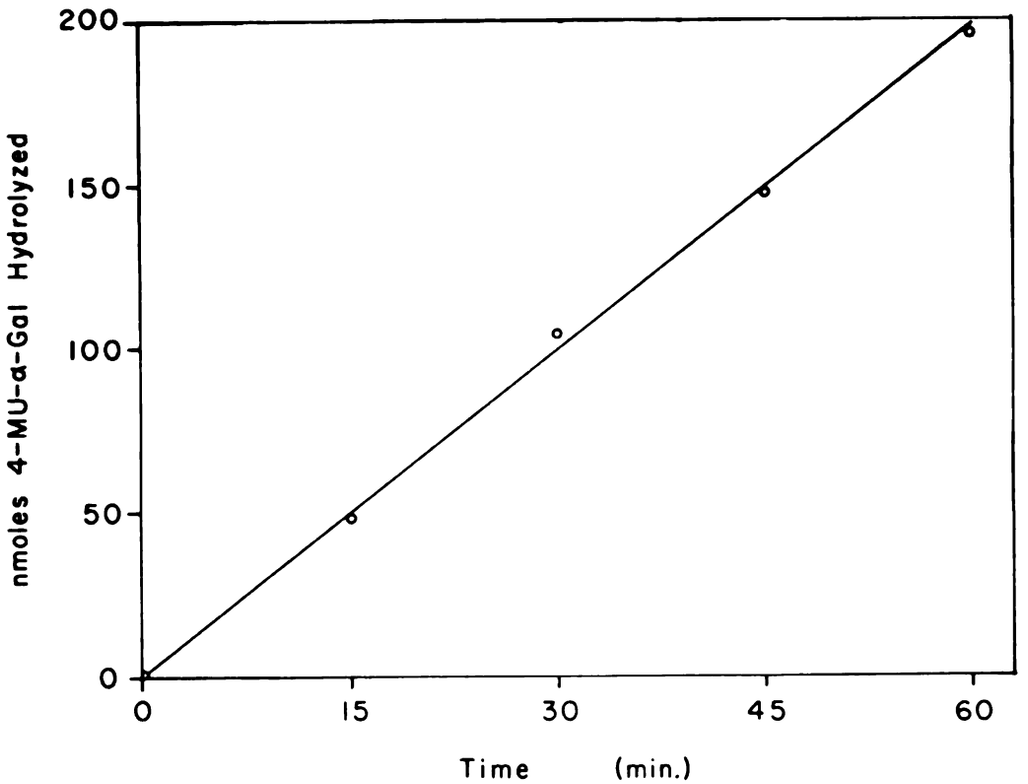
A. α-Galactosidase A

Artificial Substrates. An artificial substrate (4-MU-α-Gal) was used to monitor the purification of α-galactosidase A. The linearity of this assay with respect to time and enzyme concentration is shown in Figure 14. The pH optimum for 4-MU-α-Gal hydrolysis in the absence of detergents was at pH 4.6, as shown in Figure 15. The presence of the nonionic detergent Triton X-100 had no effect on the pH optimum of 4-MU-α-Gal hydrolysis, however the anionic detergent, sodium taurocholate, used to solubilize glycosphingolipids in aqueous reaction mixtures, inhibited the hydrolysis of 4-MU-α-Gal below pH 4.8. The Michaelis constant (K_m) for 4-MU-α-Gal hydrolysis was 2.9 mM at pH 4.6, with a Hill slope of 1.0, as shown in Figures 16 and 17. The maximal velocity was 28.7 μmoles/min·mg. The K_m was not dependent on pH, as shown in Figure 18A, and Lineweaver-Burk plots of 4-MU-α-Gal hydrolysis at pH 3.7,4.0,4.5 and 4.8 were linear, as shown in Figure 19. The V_{max} was dependent on pH, as shown in Figure 18B. The presence of 9.3 mM sodium taurocholate in the reaction mixture had a profound effect on the kinetics of 4-MU-α-Gal hydrolysis at pH 3.7,4.0,4.5, and 4.8, as shown in Figure 20. The Lineweaver-Burk plot of 4-MU-α-Gal hydrolysis at pH 3.7 was hyperbolic, however the hyperbolic character of the kinetics diminished as the pH was raised.

The artificial substrates p-nitrophenyl-2-deoxy-α-D-galactopyranoside and o-nitrophenyl-6-deoxy-α-D-galactopyranoside (o-nitrophenyl-α-D-fucopyranoside) were synthesized to examine the substrate specficity of α-galactosidase A. Fourier-transform ^{13}C-NMR spectra of these artificial substrates are shown in Figure 21. Figure 21A is the Fourier-transform ^{13}C-NMR spectrum of p-NP-α-Gal, which was used as a standard. The anomeric carbon of p-NP-β-Gal (spectrum not shown) appeared at 101.3 ppm. The ^{13}C-NMR spectrum of p-nitrophenyl-2-deoxy-tri-O-acetyl-α-D-galactopyranoside is shown in Figure 21B. Authentic p-NP-2-deoxy-α-Gal was not sufficiently soluble in water to obtain a spectrum, so the ^{13}C-NMR spectrum of acetylated p-NP-2-deoxy-α-Gal is shown. The anomeric carbon appeared at 96.4 ppm and the C-2 methylene appeared at 30.0 ppm. Signals at 170 ppm and 21 ppm are from the acetyl groups. The ^{13}C-NMR spectrum of o-NP-α-D-Fuc is shown in Figure 21C. The anomeric carbon appeared at 99.1 ppm and the signal at 16.6 ppm was due to the C-6 methyl group.

Figure 14. The Linearity of 4-MU-α-Gal Hydrolysis by α-Galactosidase A with Respect to Time and Enzyme Concentration

The hydrolysis of 4-MU-α-Gal by α-galactosidase A was linear with respect to time for up to 1 hour (top) and with respect to enzyme concentration up to 12 μg/350 μl (bottom).


200
150
100
50
0
nmoles 4-MU-α-Gal Hydrolyzed
0
15
30
45
60
Time (min.)

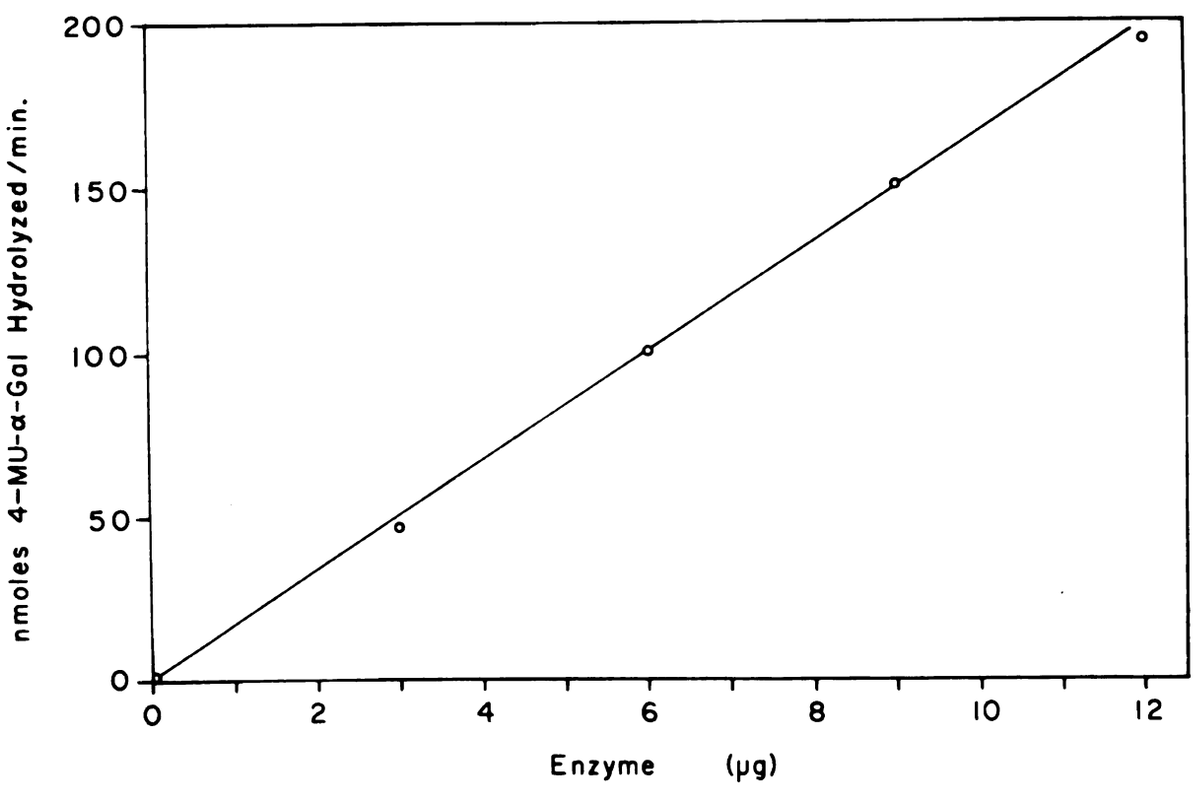

200
150
100
50
0
nmoles 4-MU-α-Gal Hydrolyzed/min.
0
2
4
6
8
10
12
Enzyme (μg)

Figure 15. The pH Optimum of 4-MU-α-Gal Hydrolysis with α-Galactosidase A.

The pH optimum for the hydrolysis of 4-MU-α-Gal with α-galactosidase A was at pH 4.6 with no detergent added, or with 0.3 mM Triton X-100, but 9.3 mM sodium taurocholate inhibited the hydrolysis of 4-MU-α-Gal below pH 4.8. The assays were carried out in Gomori citrate-phosphate buffer (275).

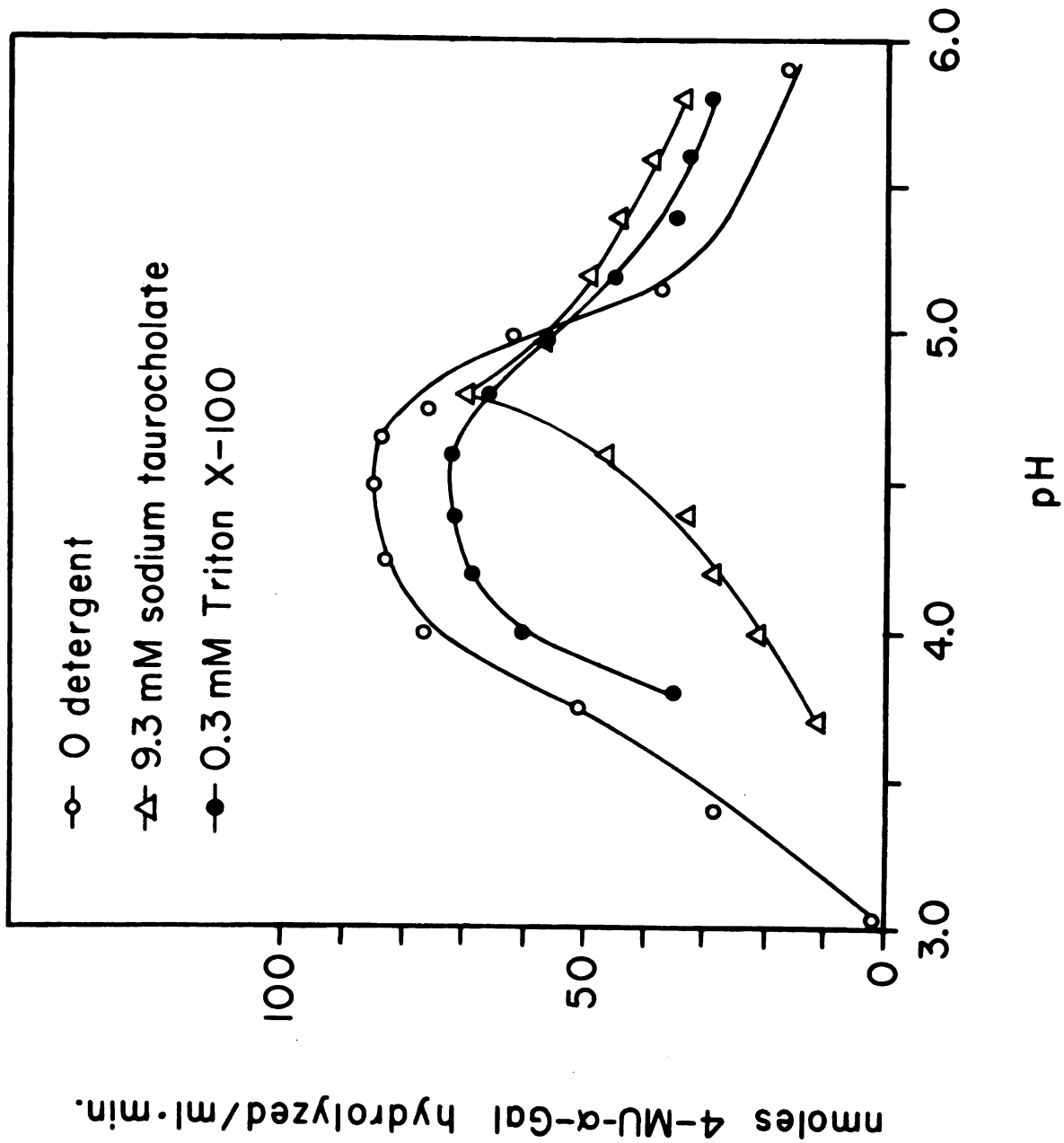

O detergent
9.3 mM sodium taurocholate
0.3 mM Triton X-100
nmoles 4-MU-α-Gal hydrolyzed/ml·min.
0
50
100
3.0
4.0
5.0
6.0
pH

Figure 16. The Lineweaver-Burk Plot of 4-MU-α-Gal Hydrolysis by α-Galactosidase A

The K_m with 4-MU-α-Gal was 2.9 mM and the V_{max} was 28.7 μmoles/min·mg. The error in the velocity measurements was approximately ±2.0 nmoles/min.

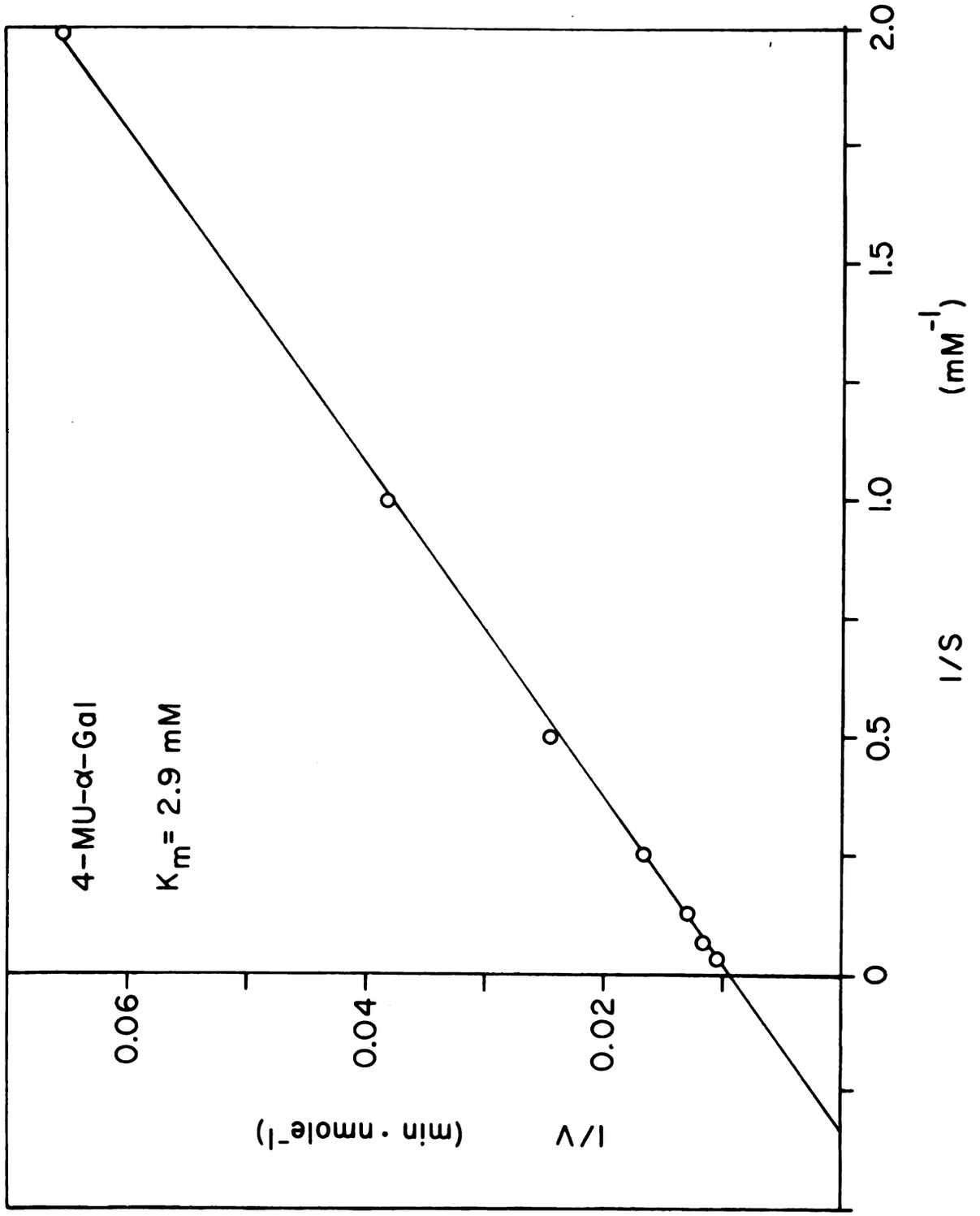

4-MU-α-Gal
K_m= 2.9 mM
1/V
(min · nmole⁻¹)
0.06
0.04
0.02
0
0.5
1.0
1.5
2.0
1/S
(mM⁻¹)

Figure 17. The Hill Plot of 4-MU-α-Gal Hydrolysis by α-Galactosidase A

The Hill plot of 4-MU-α-Gal hydrolysis was linear, with a slope of 1.0.

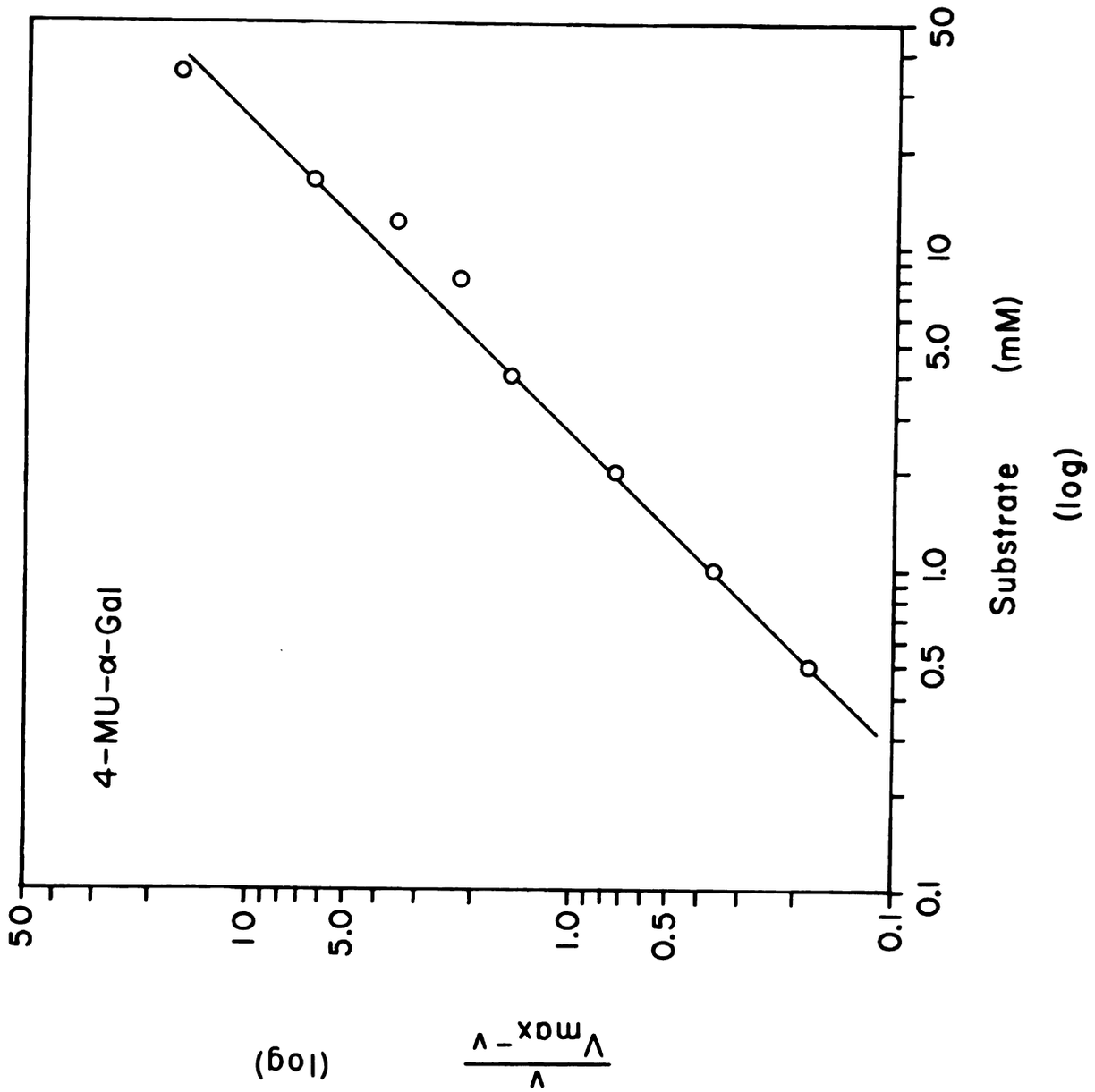

4-MU-α-Gal
50
10
5.0
1.0
0.5
0.1
0.1
0.5
1.0
5.0
10
50
Substrate (mM)
(log)
$\frac{v}{v_{max}-v}$
(log)

Figure 18. The Effect of pH on the K_M and V_{max} of 4-MU-α-Gal Hydrolysis by α-Galactosidase A.

The effect of pH on the Michaelis constant (A), and V_{max} (B) is shown. The standard deviation of the Michaelis constants was ±0.1 mM and for the maximal velocities was ±0.8 μmoles/min·mg.

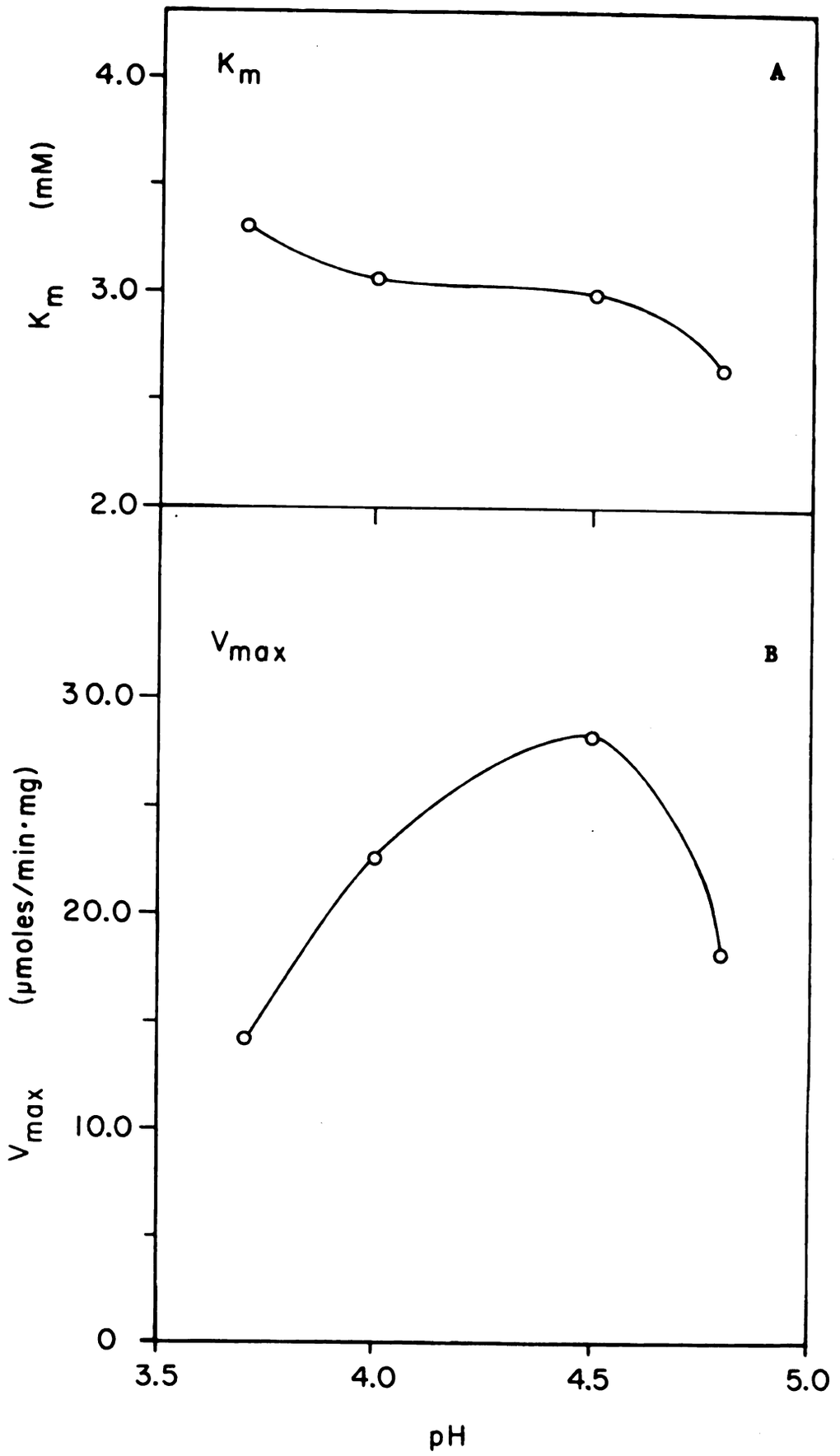

K_m
A
4.0
3.0
2.0
K_m (mM)
V_{max}
B
30.0
20.0
10.0
0
V_{max} (μmoles/min·mg)
3.5
4.0
4.5
5.0
pH

Figure 19. The Effect of pH on Lineweaver-Burk Plots of 4-MU-α-Gal Hydrolysis by α-Galactosidase A

The kinetics of 4-MU-α-Gal hydrolysis by α-galactosidase A were examined at pH 3.7, 4.0, 4.5 and 4.8. The error in the velocity measurements was approximately ±2.0 nmoles/min.

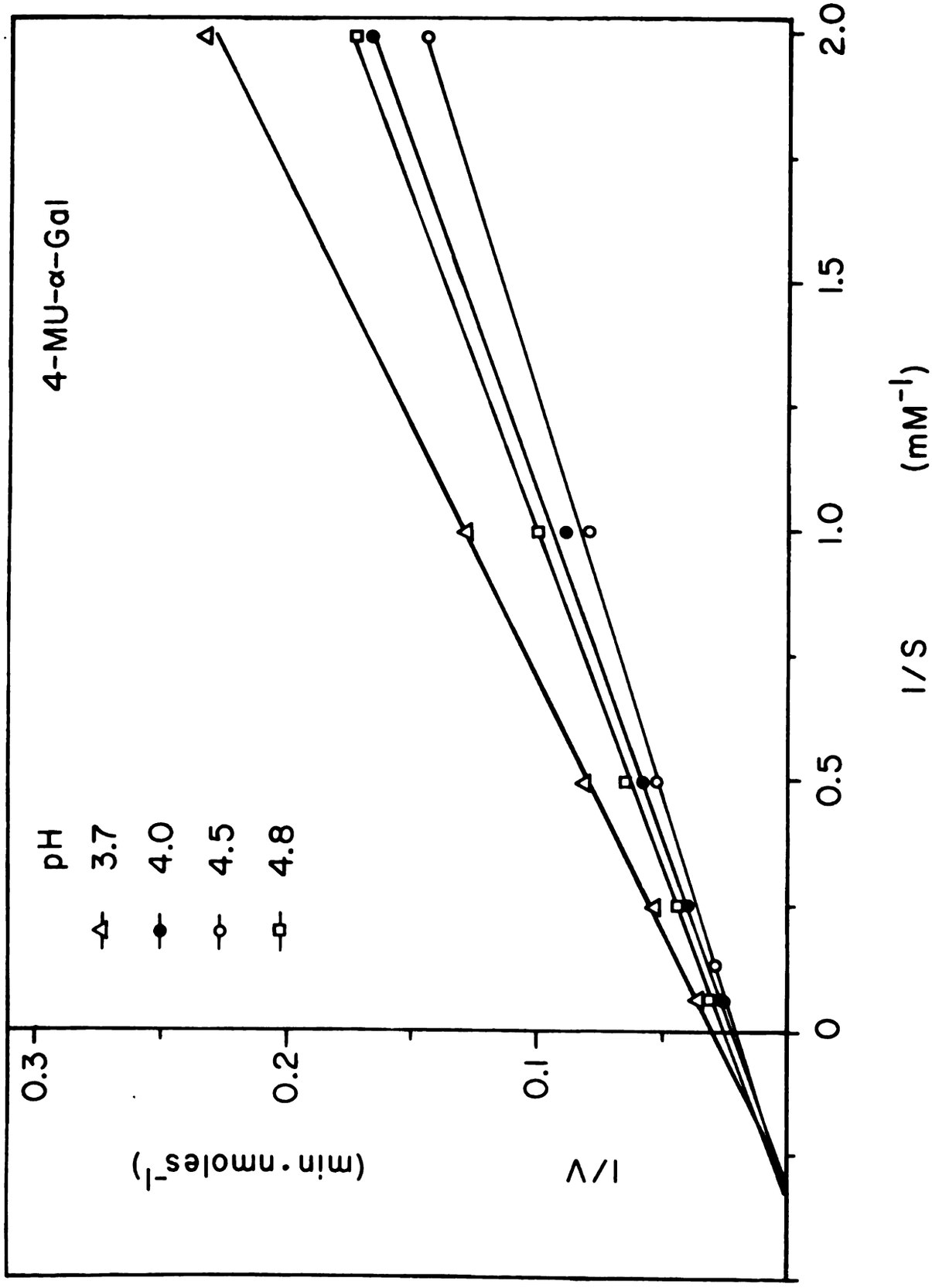

4-MU-α-Gal
pH
3.7
4.0
4.5
4.8
1/V
(min·nmoles⁻¹)
0.3
0.2
0.1
0
0.5
1.0
1.5
2.0
1/S
(mM⁻¹)

Figure 20. The Effect of pH on Lineweaver-Burk Plots of 4-MU-α-Gal Hydrolysis in the Presence of Sodium Taurocholate

The kinetics of 4-MU-α-Gal hydrolysis by α-galactosidase A were examined in the presence of 9.3 mM sodium taurocholate at pH 3.7, 4.0, 4.5 and 4.8. The error in the velocity measurements was approximately ±2.0 nmoles/min.

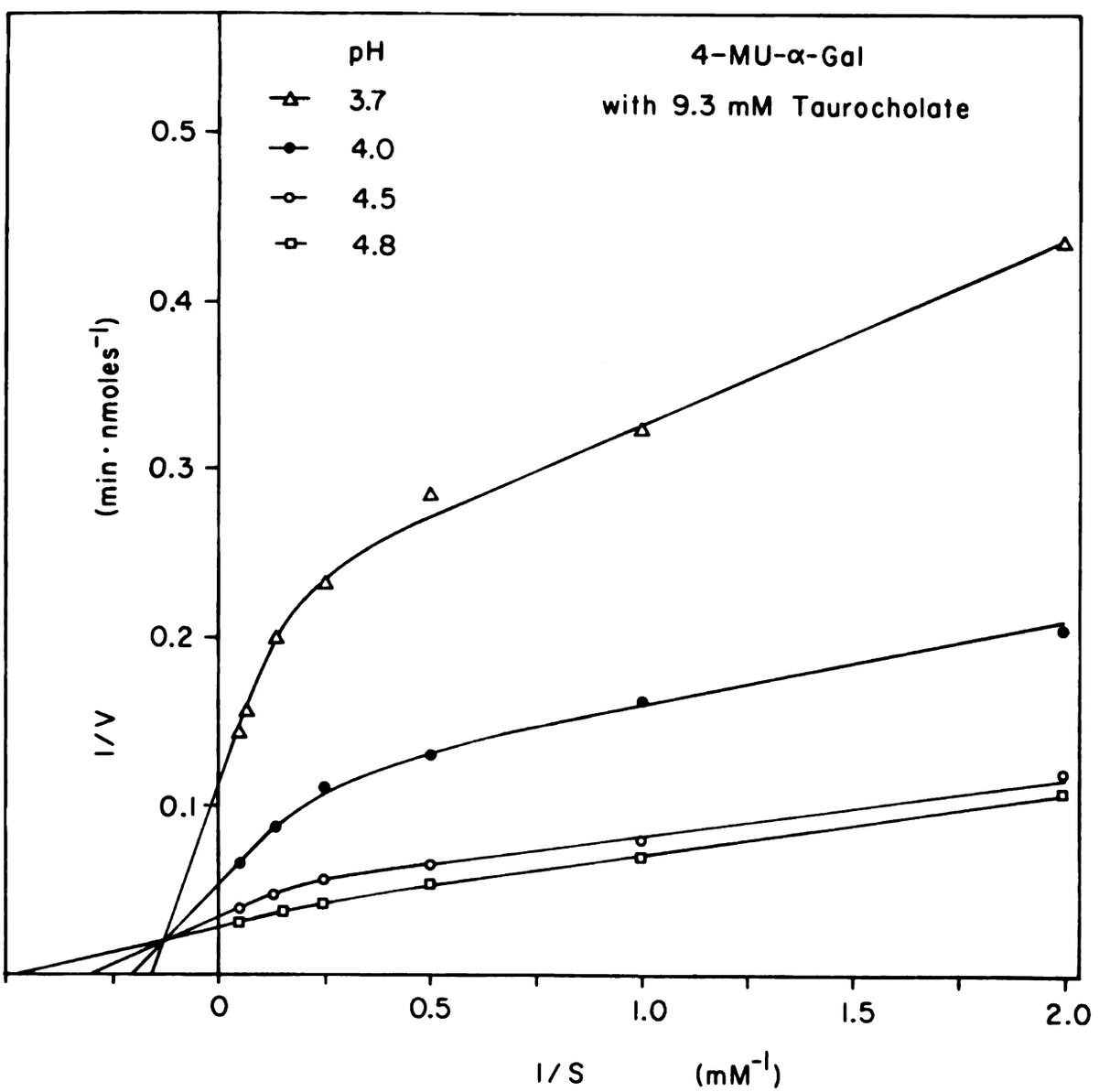
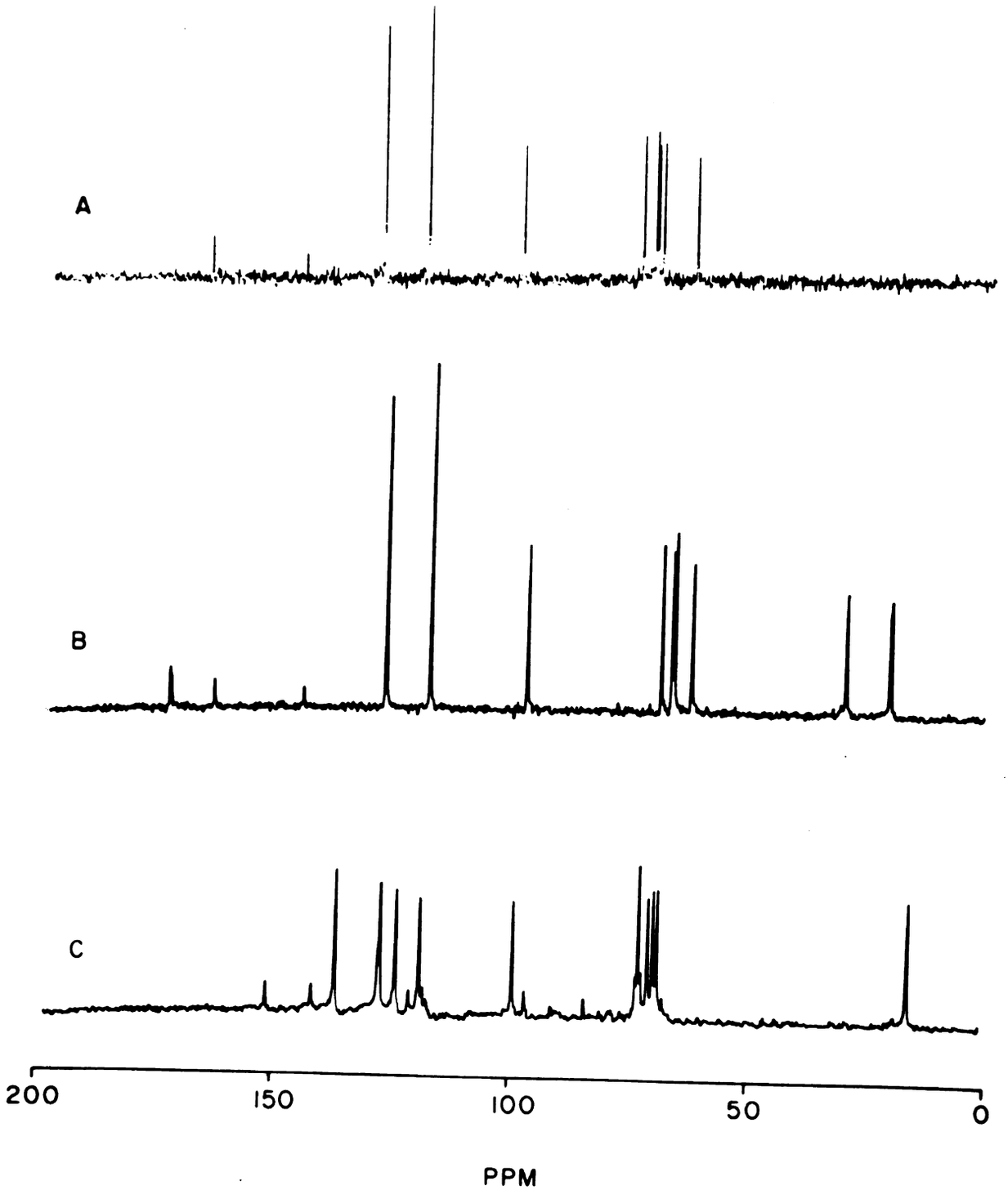

pH
3.7
4.0
4.5
4.8
4-MU-α-Gal
with 9.3 mM Taurocholate
0.5
0.4
0.3
0.2
0.1
1/V
(min · nmoles⁻¹)
0
0.5
1.0
1.5
2.0
1/S (mM⁻¹)

Figure 21. Fourier-Transform ^{13}C-NMR Spectra of Artificial Substrate Analogs.

Fourier-transform proton-decoupled ^{13}C-NMR spectra of p-nitrophenyl-α-D-galactopyranoside, (A); p-nitrophenyl-tri-O-acetyl-2-deoxy-α-D-galactopyranoside, (B); and o-nitrophenyl-α-D-fucopyranoside are shown. Spectra were obtained on a 15.08 mHz Bruker WP-60 NMR spectrometer.


A
B
C
200
150
100
50
0
PPM

α-Galactosidase A catalyzed the hydrolysis of p-nitrophenyl-2-deoxy-α-D-galactopyranoside (p-NP-2-deoxy-α-Gal). The hydrolysis of this substrate was optimal at pH 4.6. The K_m for the reaction was 8.2 mM, as shown in Figure 22, and the V_{max} was 37.4 μmoles/min·mg. The Hill slope was 1.0, as shown in Figure 23. α-Galactosidase A did not catalyze the hydrolysis of o-NP-α-D-Fuc, o-NP-α-GalNAc or p-NP-α-Glc. In addition, the hydrolysis of 4-MU-α-Gal was not inhibited by 10 mM o-NP-α-GalNAc, 20 mM D-galactal or 20 mM D-(+)-fucose.

Oligosaccharide Substrates. Thin-layer chromatograms of the oligosaccharides derived from [^{3}H]$GaOse_2Cer$ and [^{3}H]$GbOse_3Cer$ are shown in Figures 24 and 25, respectively. Examination of these oligosaccharides by gas-liquid chromatography, as described by Vance and Sweeley (125), showed that [^{3}H]$GaOse_2$ contained only galactose and [^{3}H]$GbOse_3$ contained galactose and glucose in the ratio of 2:1. The specific activities of [^{3}H]$GaOse_2$ and [^{3}H]$GbOse_3$ were 25,733 cpm/nmole and 86,169 cpm/nmole, respectively. The assays with these oligosaccharides were linear with respect to time (up to 3 hours) and the concentration of enzyme (up to 130 μg). The hydrolysis of $GaOse_2$ and $GbOse_3$ was optimal at pH 4.5 (Figure 26). The K_m with $GaOse_2$ was 5.5 mM, as shown in Figure 27, and the V_{max} was 15.8 μmoles/min·mg. The Hill slope was 1.0, as shown in Figure 28. The K_m with $GbOse_3$ was 4.1 mM (Figure 29) and the V_{max} was 13.8 μmoles/min·mg. The Hill slope for the hydrolysis of $GbOse_3$ was 1.1 (Figure 30).

Glycosphingolipid Substrates. Thin-layer chromatograms of purified [^{3}H]$GaOse_2Cer$ and [^{3}H]$GbOse_3Cer$ are shown in Figures 31 and 32, respectively. Examination of these glycolipids by gas-liquid chromatography showed that $GaOse_2Cer$ contained only galactose and $GbOse_3Cer$ contained galactose and glucose in the ratio of 2:1. The specific activities of [^{3}H]$GaOse_2Cer$ and [^{3}H]$GbOse_3Cer$ were 25,858 cpm/nmole and 26,768 cpm/nmole, respectively. Assays with $GaOse_2Cer$ and $GbOse_3Cer$ were linear with respect to time and enzyme concentration, as shown in Figure 33. These glycolipids are not soluble in water, and the anionic detergent sodium taurocholate was used to solubilize these substrates in the aqueous reaction mixture. Optimal hydrolysis of $GaOse_2Cer$ and $GbOse_3Cer$ was observed with 5.7 mM and 9.3 mM sodium taurocholate, respectively (Figure 34). No activity could be detected when the nonionic detergent Triton X-100 was substituted for sodium taurocholate (concentrations of 0.3-1.2 mM Triton X-100 and 1.0-8.0 mM $GbOse_3Cer$ were examined). The pH optimum for the hydrolysis of $GaOse_2Cer$

Figure 22. Lineweaver-Burk Plot of p-NP-2-deoxy-α-Gal Hydrolysis by α-Galactosidase A

The K_m with p-NP-2-deoxy-α-Gal was 8.2 mM and the V_{max} was 37.4 μmoles/min·mg. The error in the velocity measurements was approximately ±10.0 nmoles/min.

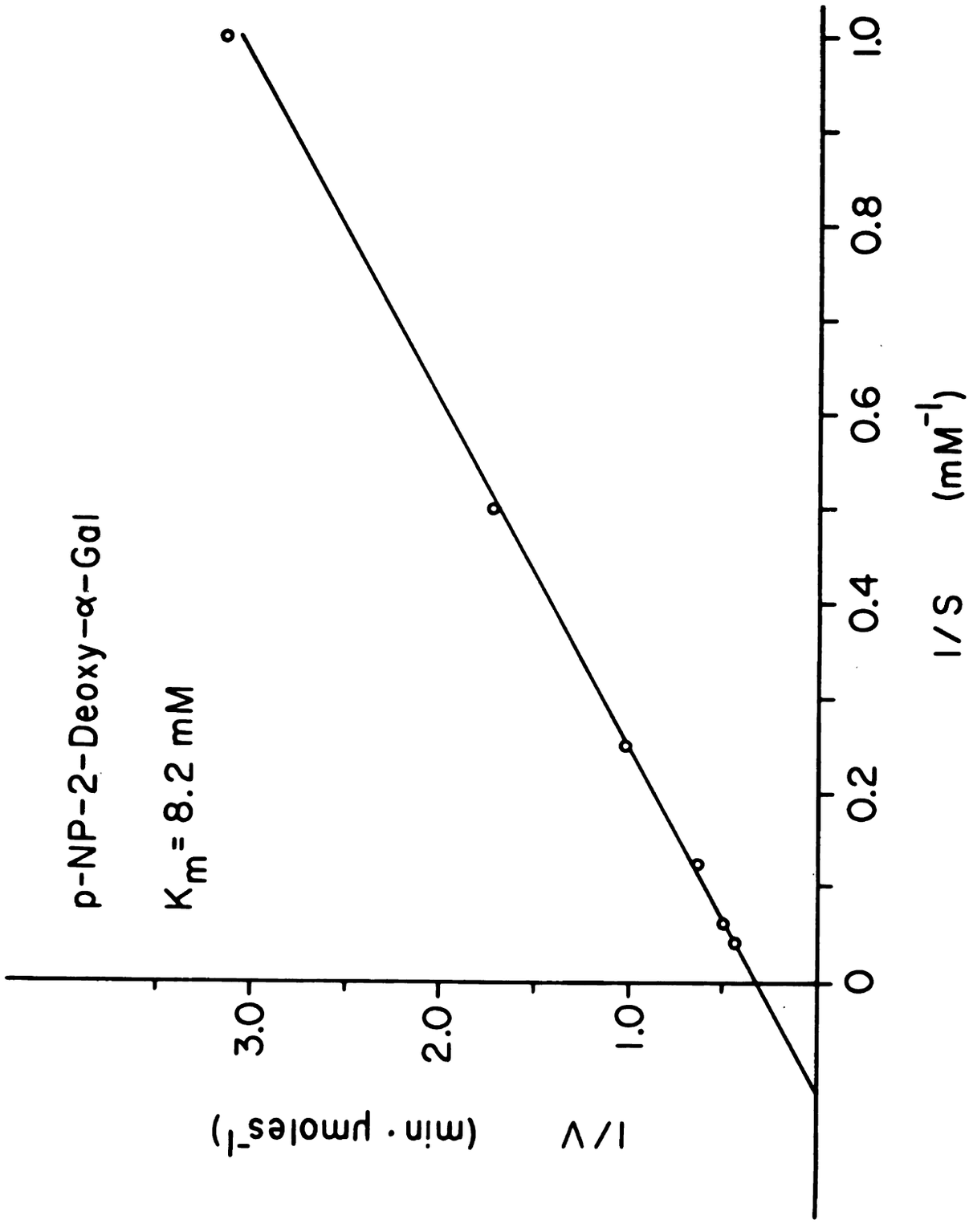

p-NP-2-Deoxy-α-Gal
K_m= 8.2 mM
1/V (min · μmoles^{-1})
3.0
2.0
1.0
0
0.2
0.4
0.6
0.8
1.0
1/S (mM^{-1})

Figure 23. The Hill Plot of p-NP-2-deoxy-α-Gal Hydrolysis by α-Galactosidase A

The Hill plot of p-NP-2-deoxy-α-Gal hydrolysis by α-galactosidase A was linear, with a slope of 1.0.

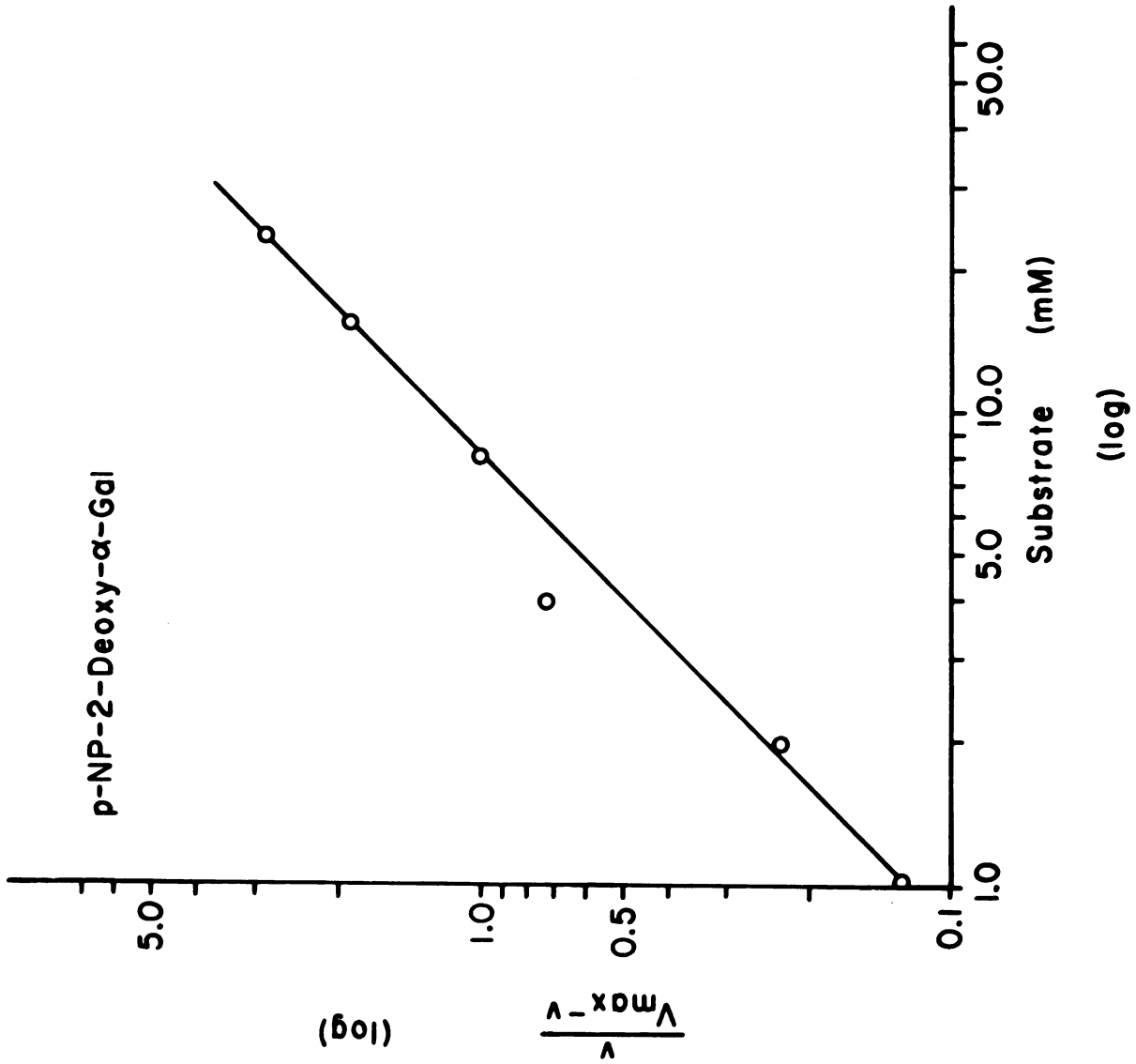

p-NP-2-Deoxy-α-Gal
5.0
1.0
0.5
0.1
1.0
5.0
10.0
50.0
Substrate (mM)
(log)
$\frac{v}{V_{max}-v}$
(log)

Figure 24. Thin-Layer Chromatogram of [^{3}H]Galabiaose

The thin-layer chromatogram of [^{3}H]galabiose was obtained on a silica gel G thin-layer plate. Galactose, lactose and raffinose were used as a standard. The radioactive oligosaccharide was scanned on a Varian Aerograph Berthold Radio Scanner, and had a specific activity of 25,733 cpm/nmole. The carbohydrates were visualized with orcinol.

n-Propanol-Acetic Acid-Water

(85:12:3)

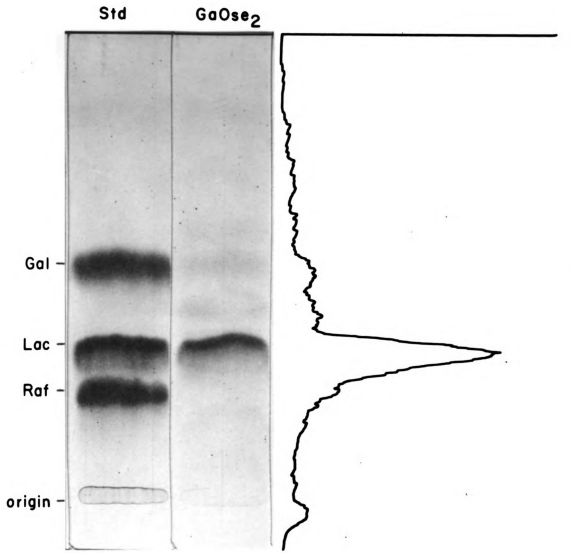


Figure 25. Thin-Layer Chromatogram of [^{3}H]Globotriaose

The thin-layer chromatogram of [^{3}H]globotriaose was obtained on a silica gel G thin-layer plate. Galactose, lactose and raffinose were used as standards. The radioactive oligosaccharide was scanned on a Varian Aerograph Berthold Radio Scanner and had a specific activity of 86,169 cpm/nmole. The carbohydrates were visualized with orcinol.

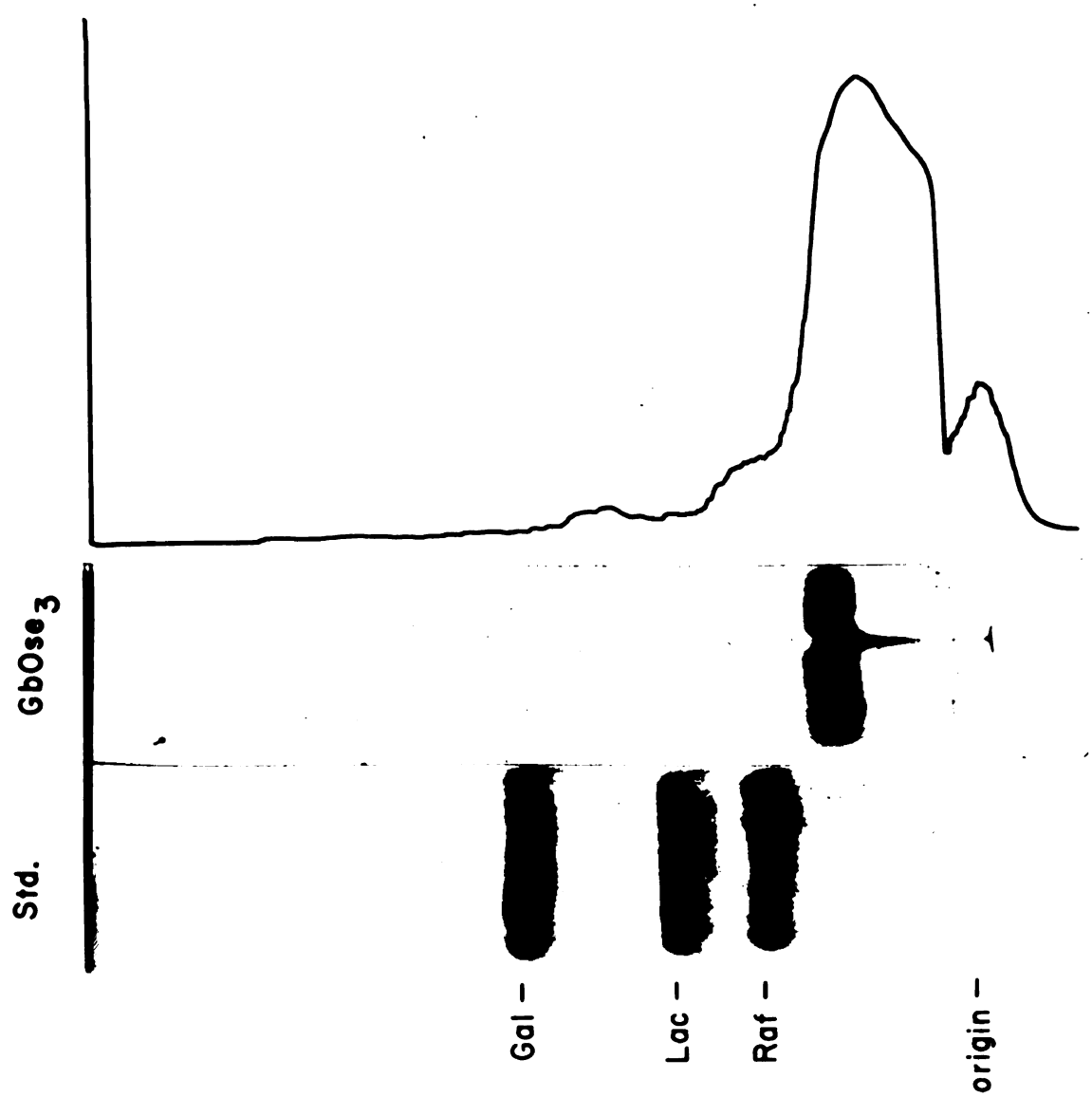

Std.
GbOse3
Gal –
Lac –
Raf –
origin –
n-Propanol-Acetic Acid-Water
(85:12:3)

Figure 26. The pH Optimum for the Hydrolysis of Galabiaose and Globotriaose by α-Galactosidase A

The hydrolysis of $GaOse_2$ and $GbOse_3$ was optimal at pH 4.5 with Gomori citrate-phosphate buffers (275).

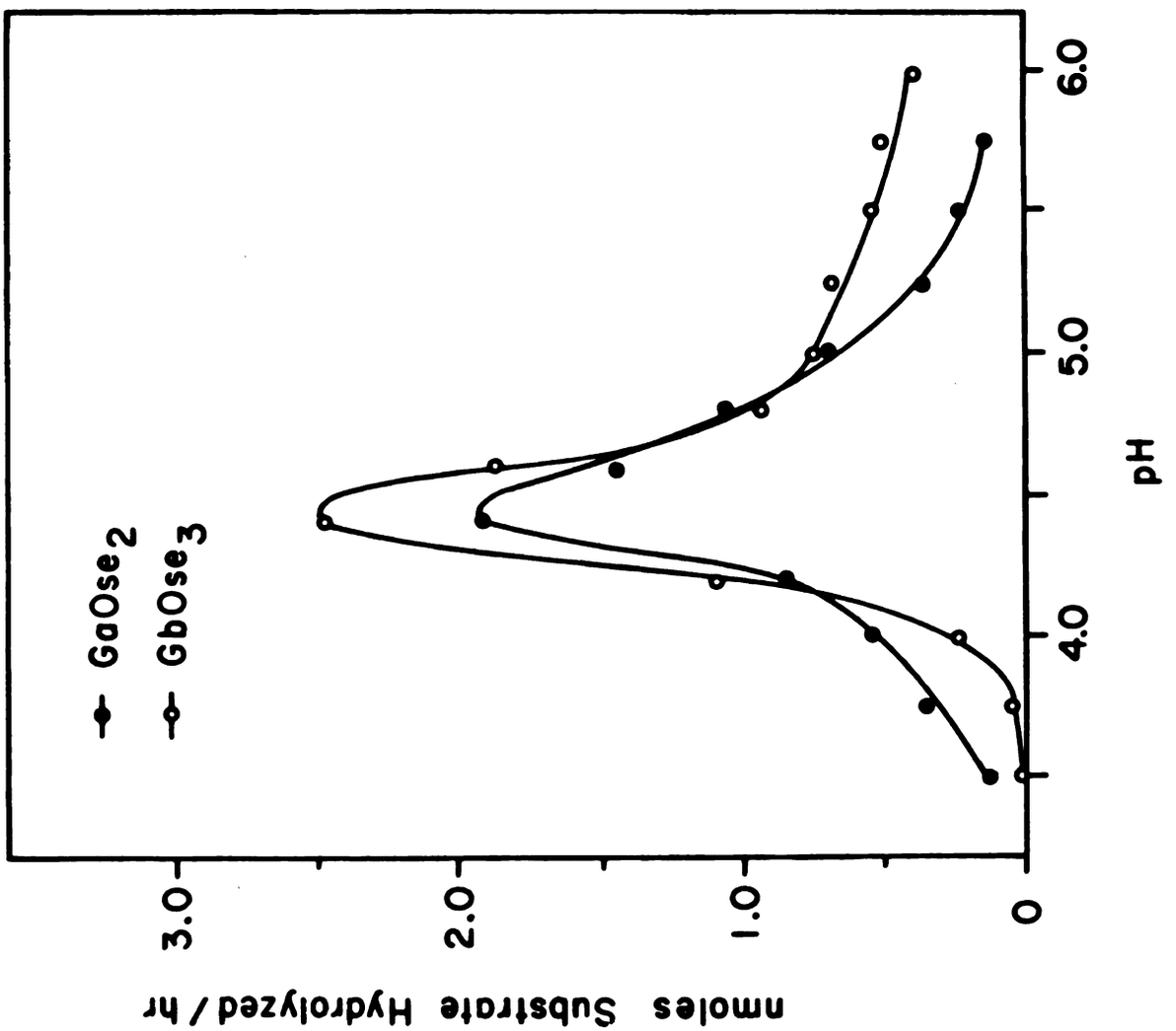

GaOse2
GbOse3
3.0
2.0
1.0
0
4.0
5.0
6.0
pH
nmoles Substrate Hydrolyzed / hr

Figure 27. Lineweaver-Burk Plot of $GaOse_2$ Hydrolysis by α-Galactosidase A

The K_m for the hydrolysis of $GaOse_2$ by α-galactosidase A was 5.5 mM and the V_{max} was 15.8 μmoles/min·mg. The error in the velocity measurements was approximately ±0.5 nmoles/min.

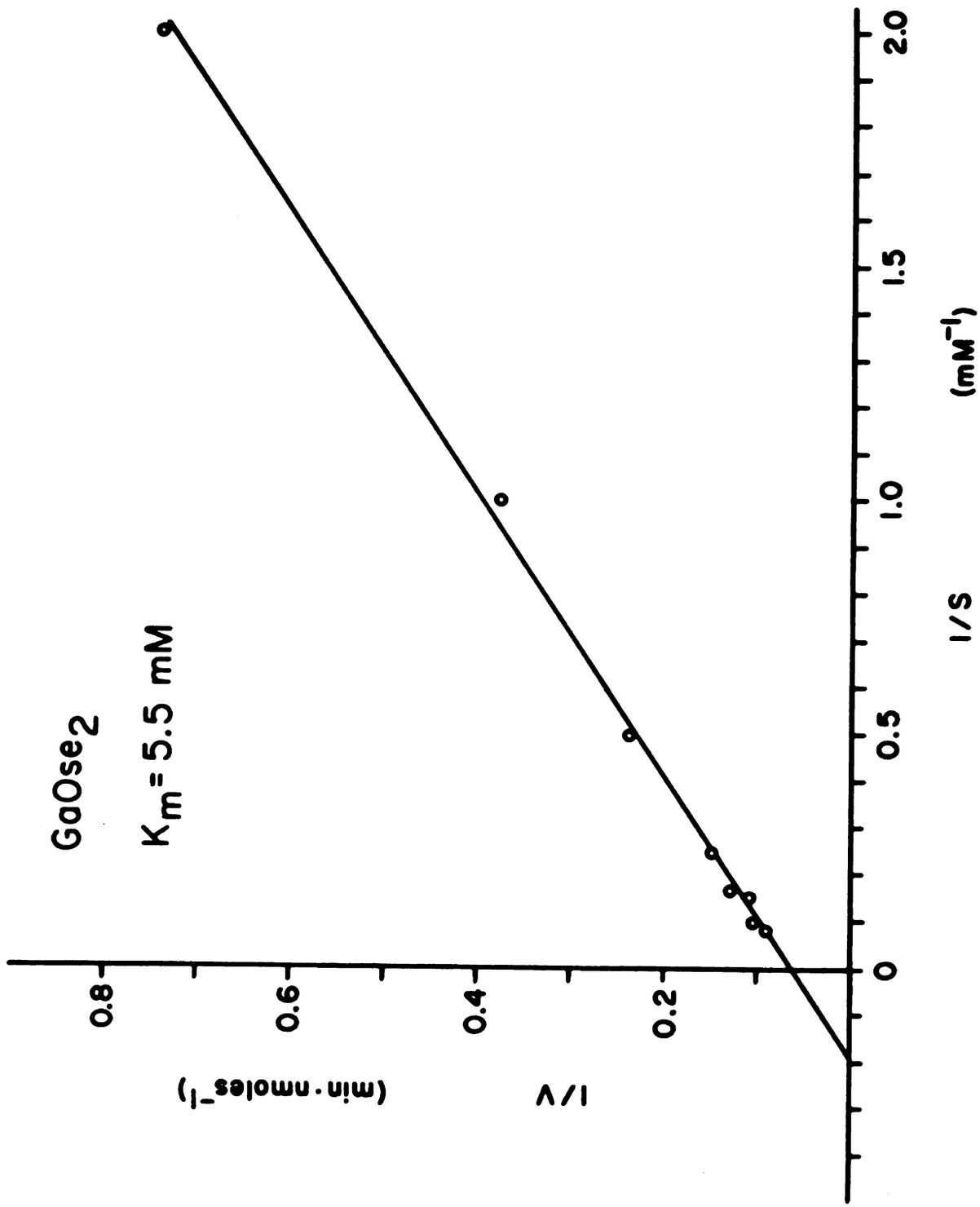

GaOse2
Km=5.5 mM
1/V
(min·nmoles⁻¹)
0.8
0.6
0.4
0.2
0
0.5
1.0
1.5
2.0
1/S
(mM⁻¹)

Figure 28. The Hill Plot of $GaOse_2$ Hydrolysis by α-Galactosidase A

The Hill plot of $GaOse_2$ hydrolysis by α-galactosidase A was linear, with a slope of 1.0.

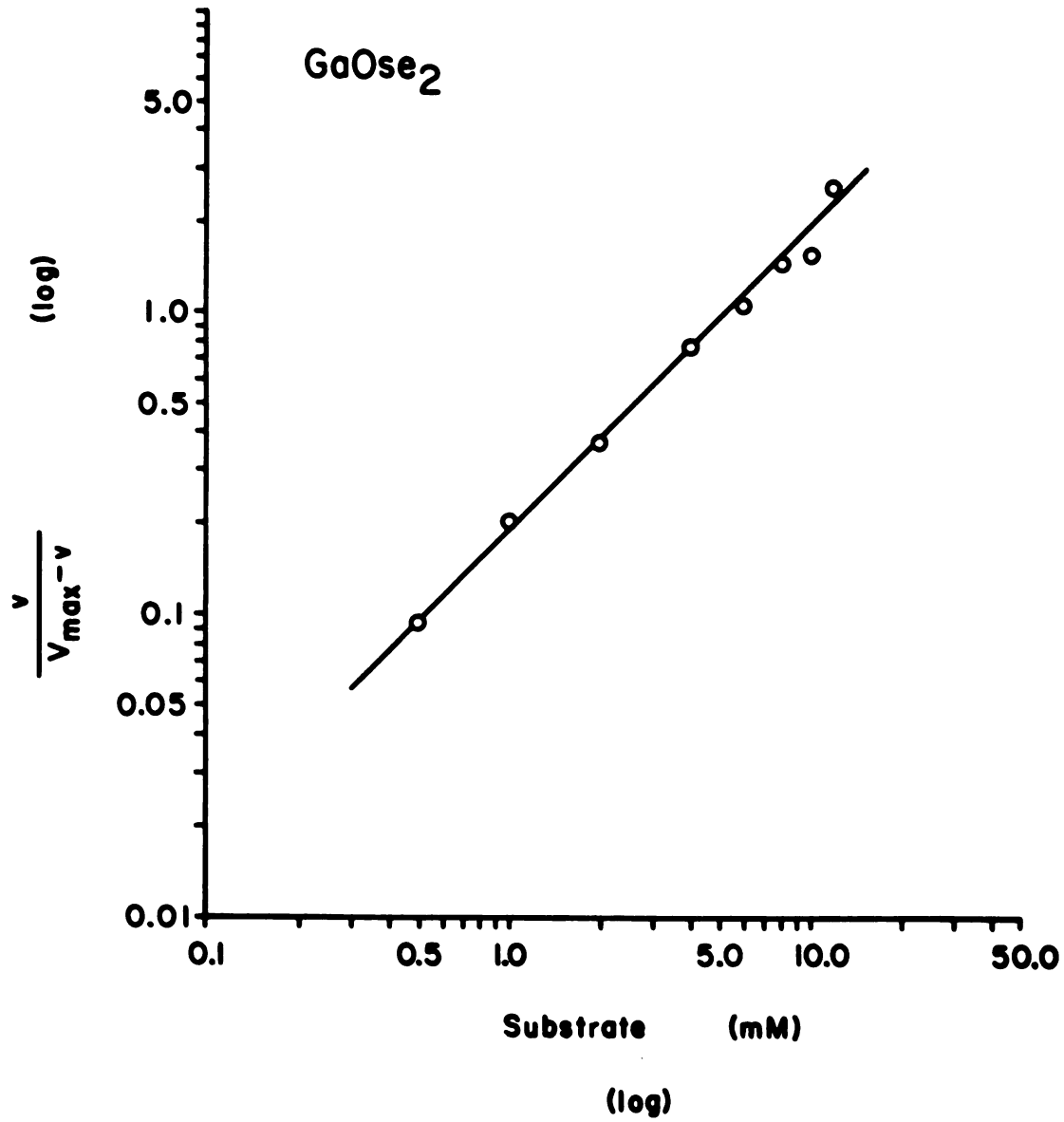

GaOse2
5.0
1.0
0.5
0.1
0.05
0.01
(log)
v / Vmax - v
0.1
0.5
1.0
5.0
10.0
50.0
Substrate (mM)
(log)

Figure 29. Lineweaver-Burk Plot of $GbOse_3$ Hydrolysis by α-Galactosidase A

The K_m for the hydrolysis of $GbOse_3$ by α-galactosidase A was 4.1 mM and the V_{max} was 13.8 μmoles/min·mg. The error in the velocity measurements was approximately 0.5 nmoles/min.

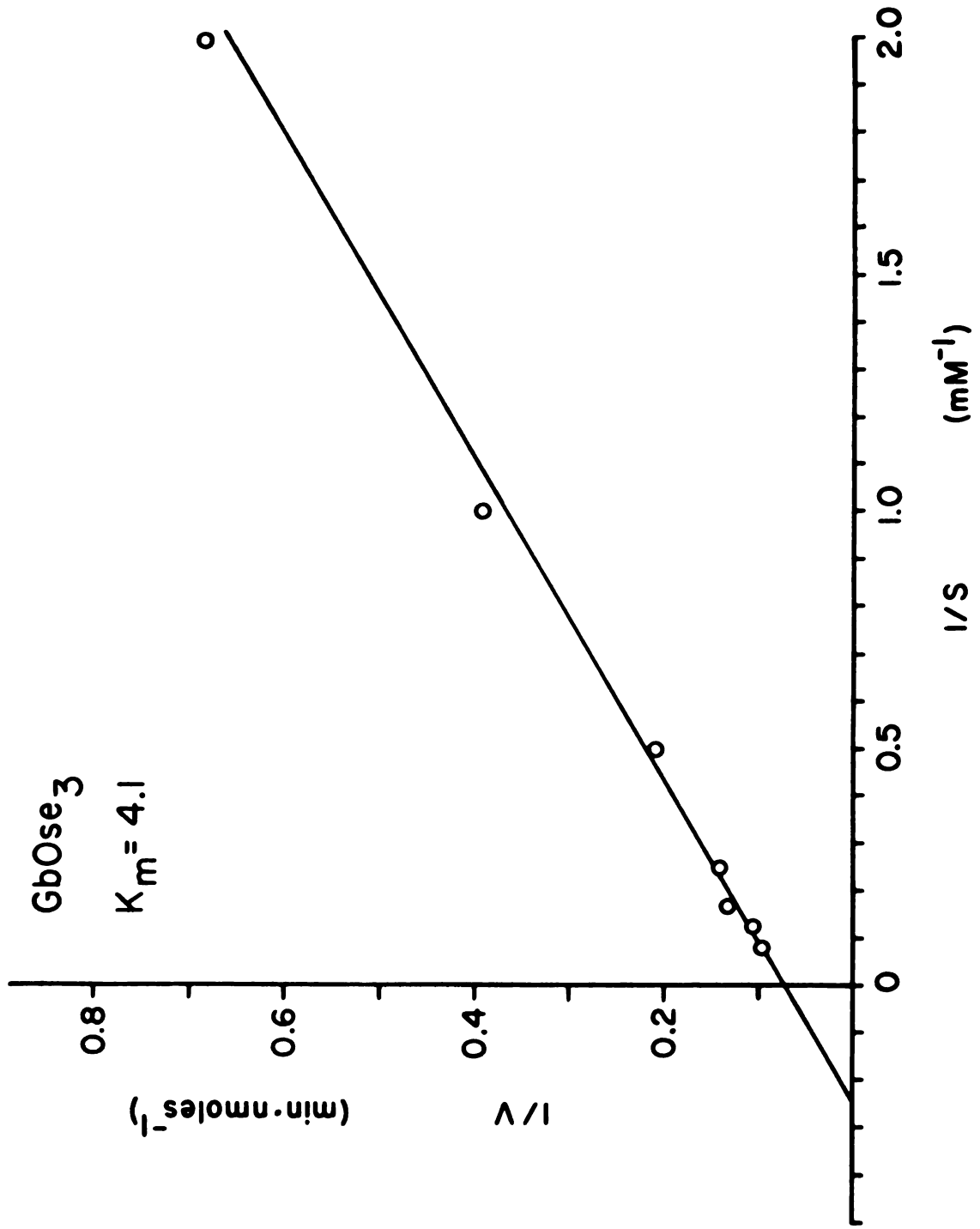

GbOse3
Km= 4.1
1/V
(min·nmoles-1)
0.8
0.6
0.4
0.2
0
0.5
1.0
1.5
2.0
1/S
(mM-1)

Figure 30. The Hill Plot of the Hydrolysis of $GbOse_3$ by α-Galactosidase A

The Hill plot of the hydrolysis of $GbOse_3$ by α-galactosidase A was linear with a slope of 1.1.

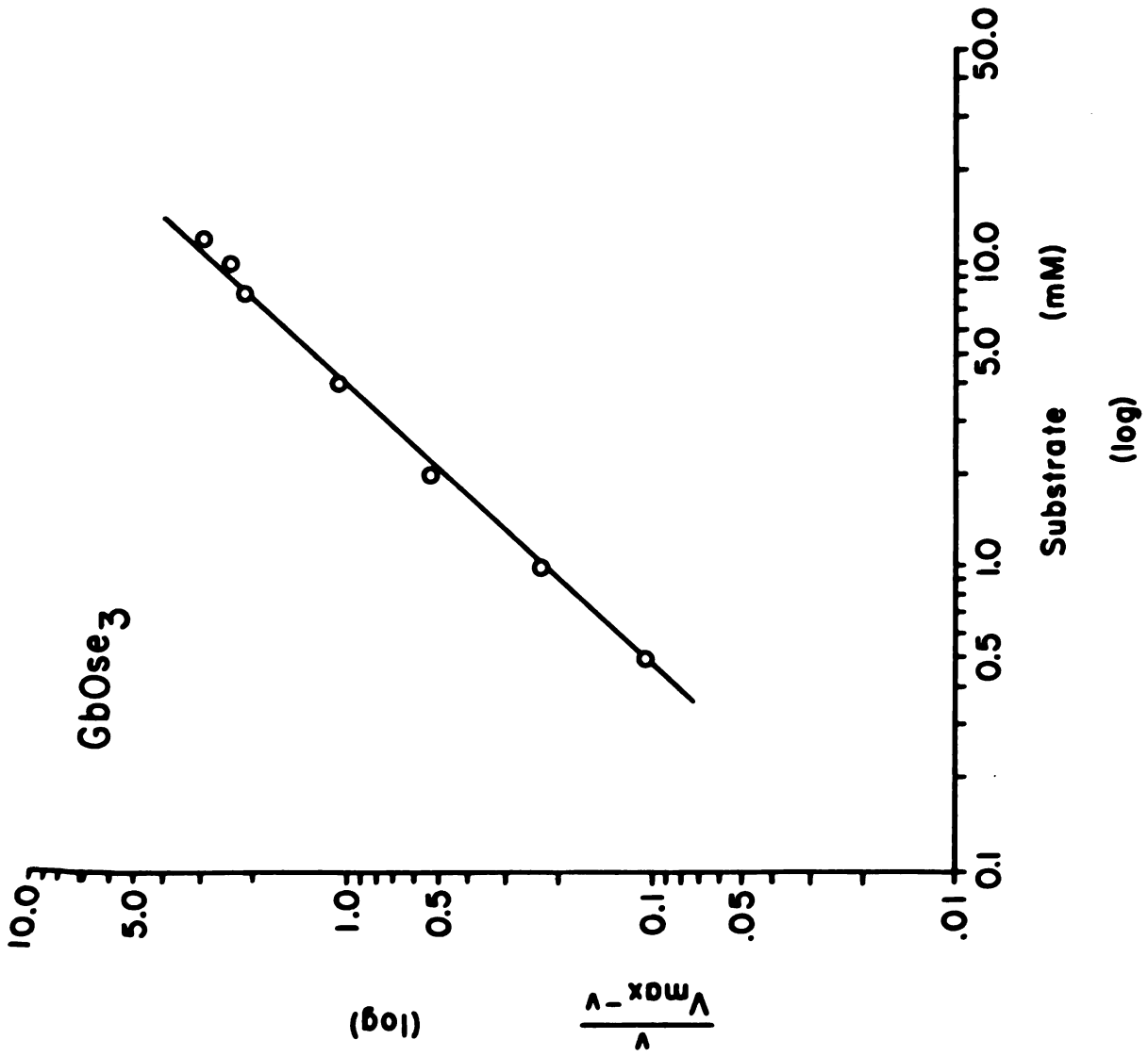

GbOse3
10.0
5.0
1.0
0.5
0.1
.05
.01
0.1
0.5
1.0
5.0
10.0
50.0
Substrate (mM)
(log)
v / Vmax - v
(log)

Figure 31. Thin-Layer Chromatogram of [^{3}H]Galabiosylceramide

The thin-layer chromatogram of [^{3}H]galabiosylceramide was obtained on a silica gel G thin-layer plate. Canine intestinal neutral glycolipids were used as a standard. The radioactive glycolipid was located by scanning on a Varian Aerograph Berthold Radio Scanner, and had a specific activity of 25,858 cpm/nmole. The glycolipids were visualized with orcinol.

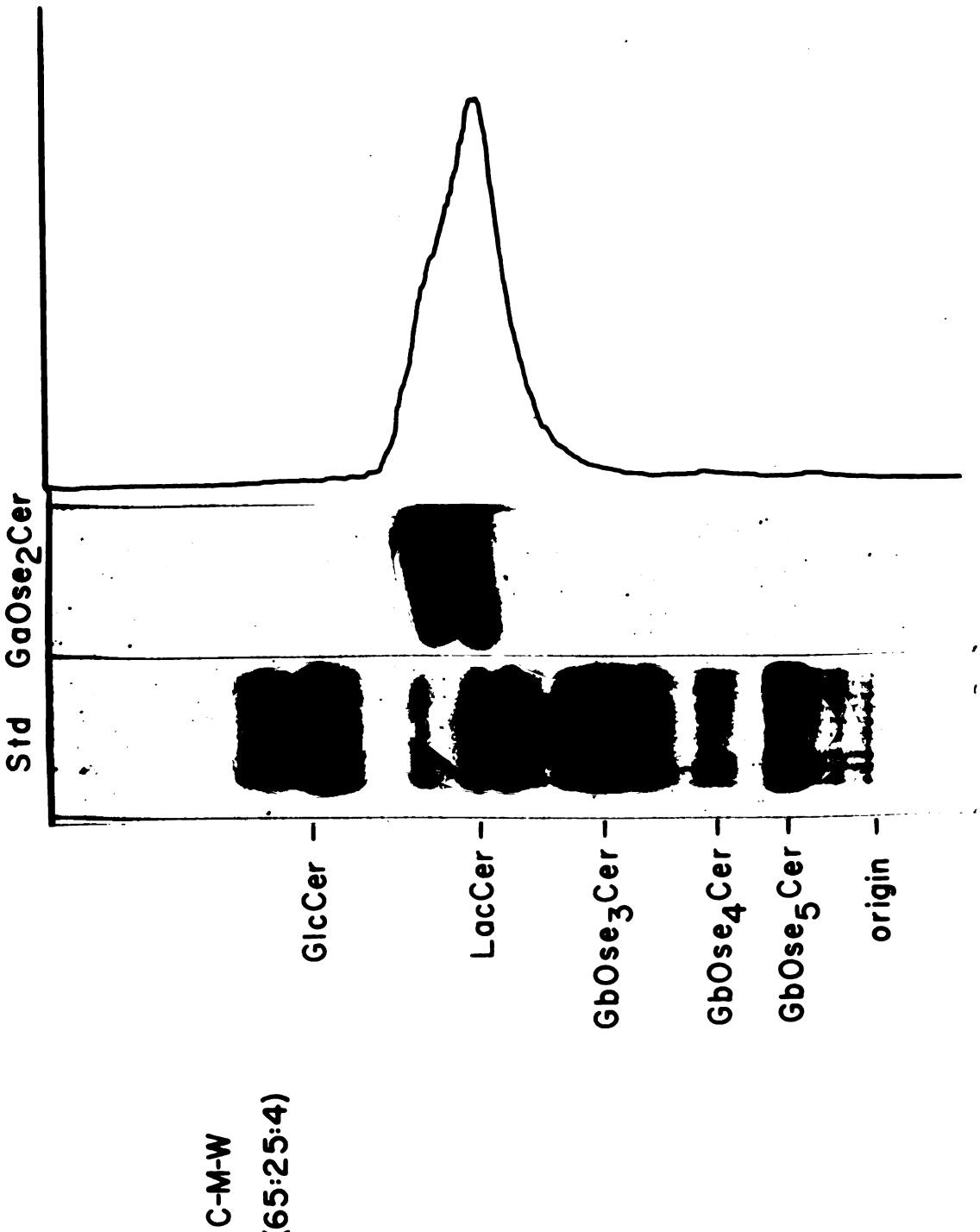

Std
GaOse2Cer
GlcCer
LacCer
GbOse3Cer
GbOse4Cer
GbOse5Cer
origin
C-M-W
(65:25:4)

Figure 32. Thin-Layer Chromatogram of [^{3}H]Globotriglycosylceramide

The thin-layer chromatogram of [^{3}H]globotriglycosylceramide was obtained on a silica gel G thin-layer plate. Canine intestinal neutral glycolipids were used as a standard. The radioactive glycolipid was located by scanning on a Varian Aerograph Berthold Radio Scanner, and had a specific activity of 26,768 cpm/nmole. The glycolipids were visualized with orcinol.

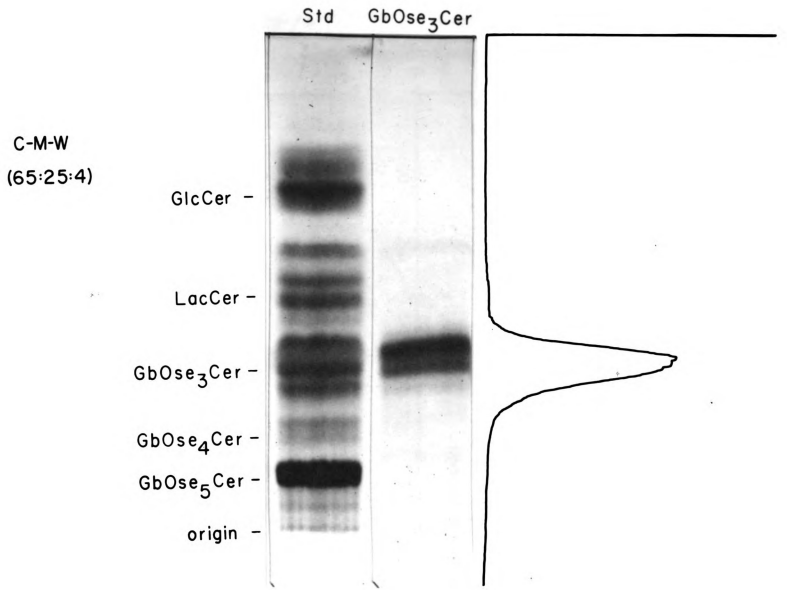
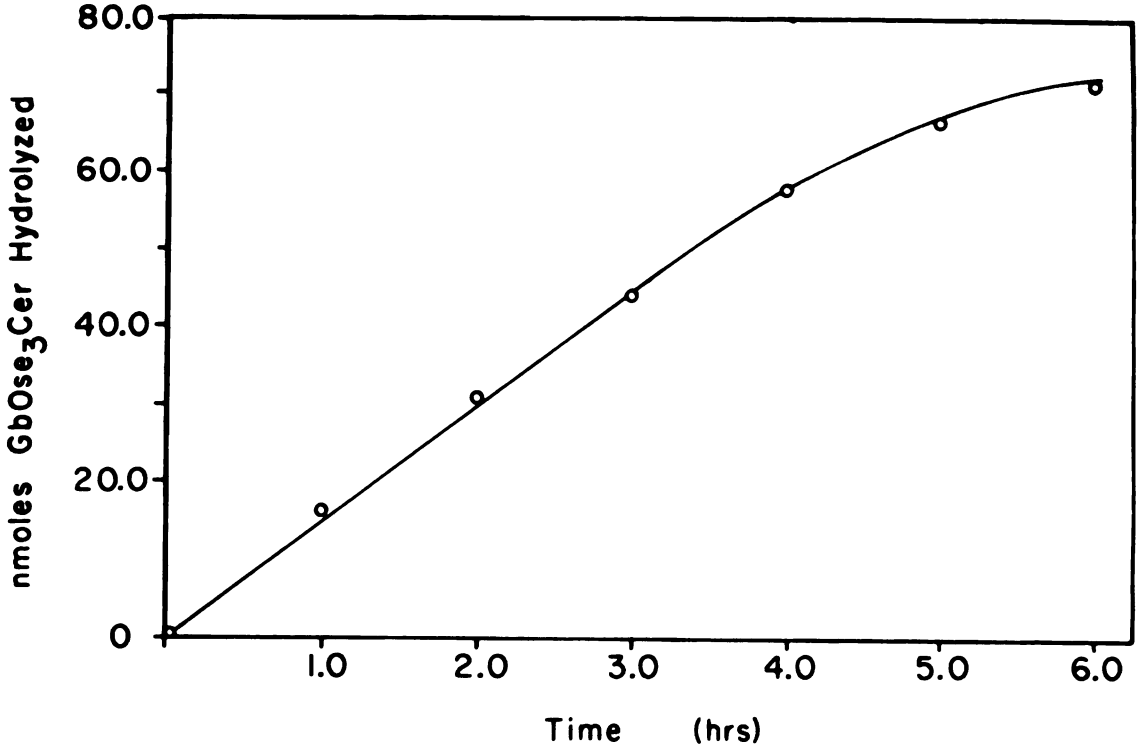

Std
GbOse3Cer
C-M-W
(65:25:4)
GlcCer
LacCer
GbOse3Cer
GbOse4Cer
GbOse5Cer
origin

Figure 33. The Linearity of $GbOse_3Cer$ Hydrolysis by α-Galactosidase A with Respect to Time and Enzyme Concentration

The hydrolysis of $GbOse_3Cer$ was linear with respect to time for up to 4 hours (top) and with respect to enzyme concentration, up to 40 µg/100 µl (bottom).


80.0
60.0
40.0
20.0
0
nmoles GbOse3Cer Hydrolyzed
1.0
2.0
3.0
4.0
5.0
6.0
Time (hrs)

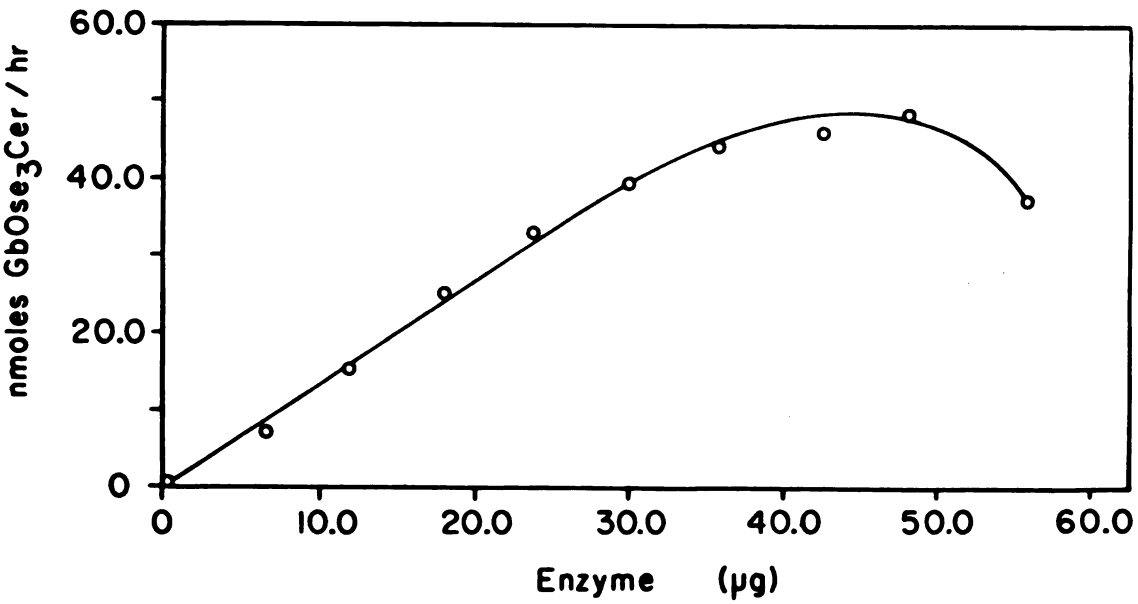
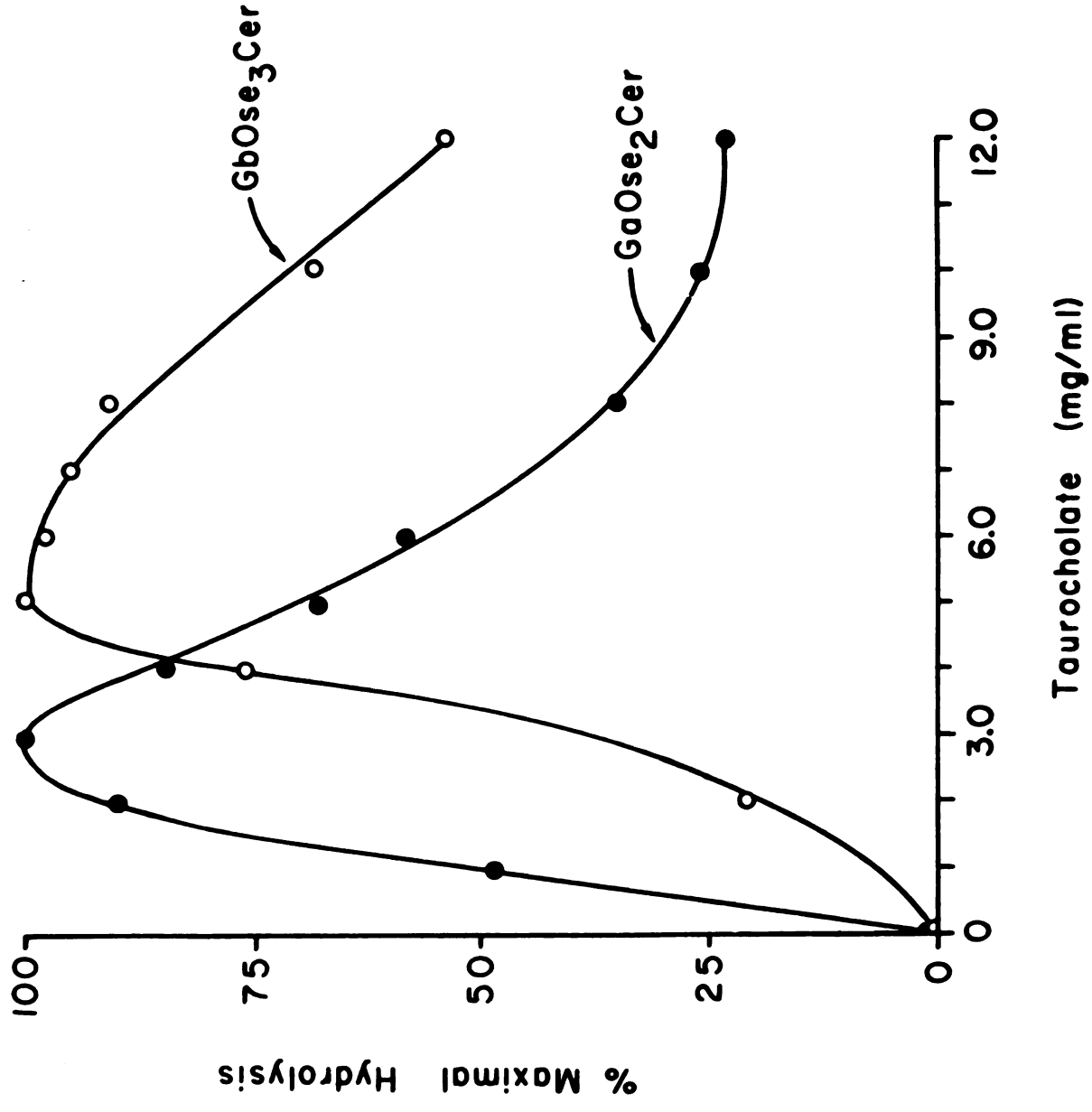

60.0
40.0
20.0
0
nmoles GbOse3Cer / hr
0
10.0
20.0
30.0
40.0
50.0
60.0
Enzyme (µg)

Figure 34. The Optimum Taurocholate Concentration for the Hydrolysis of Galabiosylceramide and Globotriglycosylceramide

The hydrolysis of $GaOse_2Cer$ and $GbOse_3Cer$ by α-Galactosidase A was optimal with 5.7 mM and 9.3 mM sodium taurocholate, respectively. Assays were carried out in Gomori citrate-phosphate buffer, pH 4.1 (275).


GbOse3Cer
GaOse2Cer
% Maximal Hydrolysis
100
75
50
25
0
0
3.0
6.0
9.0
12.0
Taurocholate (mg/ml)

and $GbOse_3Cer$ was at pH 4.1 (Figure 35), in contrast to the pH optimum for the hydrolysis of 4-MU-α-Gal in the presence of 9.3 mM sodium taurocholate, at pH 4.8, and for 4-MU-α-Gal, p-NP-2-deoxy-α-Gal, $GaOse_2$ and $GbOse_3$ in the absence of sodium taurocholate, at pH 4.5-4.6.

The Lineweaver-Burk plot of $GaOse_2Cer$ hydrolysis by α-Galactosidase A was sigmoidal, with inhibition at high substrate concentrations (Figure 36). The apparent K_m with $GaOse_2Cer$ was 0.28 mM and the apparent V_{max} was 1.09 μmoles/min·mg. The Hill plot of $GaOse_2Cer$ hydrolysis was not linear, but approximated a line with a slope of 1.1, as shown in Figure 37.

The kinetics of $GbOse_3Cer$ hydrolysis catalyzed by α-galactosidase A resembled those with $GaOse_2Cer$. The Lineweaver-Burk plot of $GbOse_3Cer$ was sigmoidal, with inhibition at high substrate concentrations. The apparent K_m with $GbOse_3Cer$ was 0.18 mM and the apparent V_{max} was 1.16 μmoles/min·mg (Figure 38). The Hill plot of $GbOse_3Cer$ hydrolysis was not linear, but approximated a line with a slope of 1.0, as shown in Figure 39.

B. α-Galactosidase B

Artificial Substrates. The artificial substrate 4-MU-α-Gal was used to monitor the purification of α-galactosidase B. The hydrolysis of this substrate was linear with respect to time and enzyme concentration (Figure 40). The pH optimum for the hydrolysis of 4-MU-α-Gal was at pH 4.7, as shown in Figure 41. The K_m was 6.8 mM and the V_{max} was 18.73 μmoles/min·mg, as shown in Figure 42. The Hill slope was 1.1, as shown in Figure 43. α-*N*-Acetylgalactosaminidase activity co-chromatographed with α-galactosidase B throughout the purification procedure (Figures 8-10 and 12). To determine whether a single enzyme catalyzed the hydrolysis of both α-galactose and α-*N*-acetylgalactosamine residues, or whether these activities only coincided during purification, the physical and kinetic properties of these activities were investigated. Both α-galactosidase B and α-*N*-acetylgalactosaminidase activities were thermostable compared with α-galactosidase A, retaining approximately 60% of their original activity after 30 minutes at 55°C (Figure 44). Furthermore, the thermal inactivation curves for α-galactosidase B and α-*N*-acetylgalactosaminidase activities coincided. Isoelectric focusing of a partially purified preparation of α-galactosidase B revealed that both α-galactosidase B and α-*N*-acetylgalactosaminidase had isoelectric points at pI = 4.5 (Figure 45). β-*N*-Acetylhexosaminidase A, contaminating this preparation, had pI = 5.0. To determine whether a single enzyme

Figure 35. The pH Optimum for the Hydrolysis of $GaOse_2Cer$ and $GbOse_3Cer$

The hydrolysis of $GaOse_2Cer$ and $GbOse_3Cer$ was optimal at pH 4.1 with 5.7 mM and 9.3 mM sodium taurocholate, respectively. The assays were carried out in Gomori citrate-phosphate buffers (275).

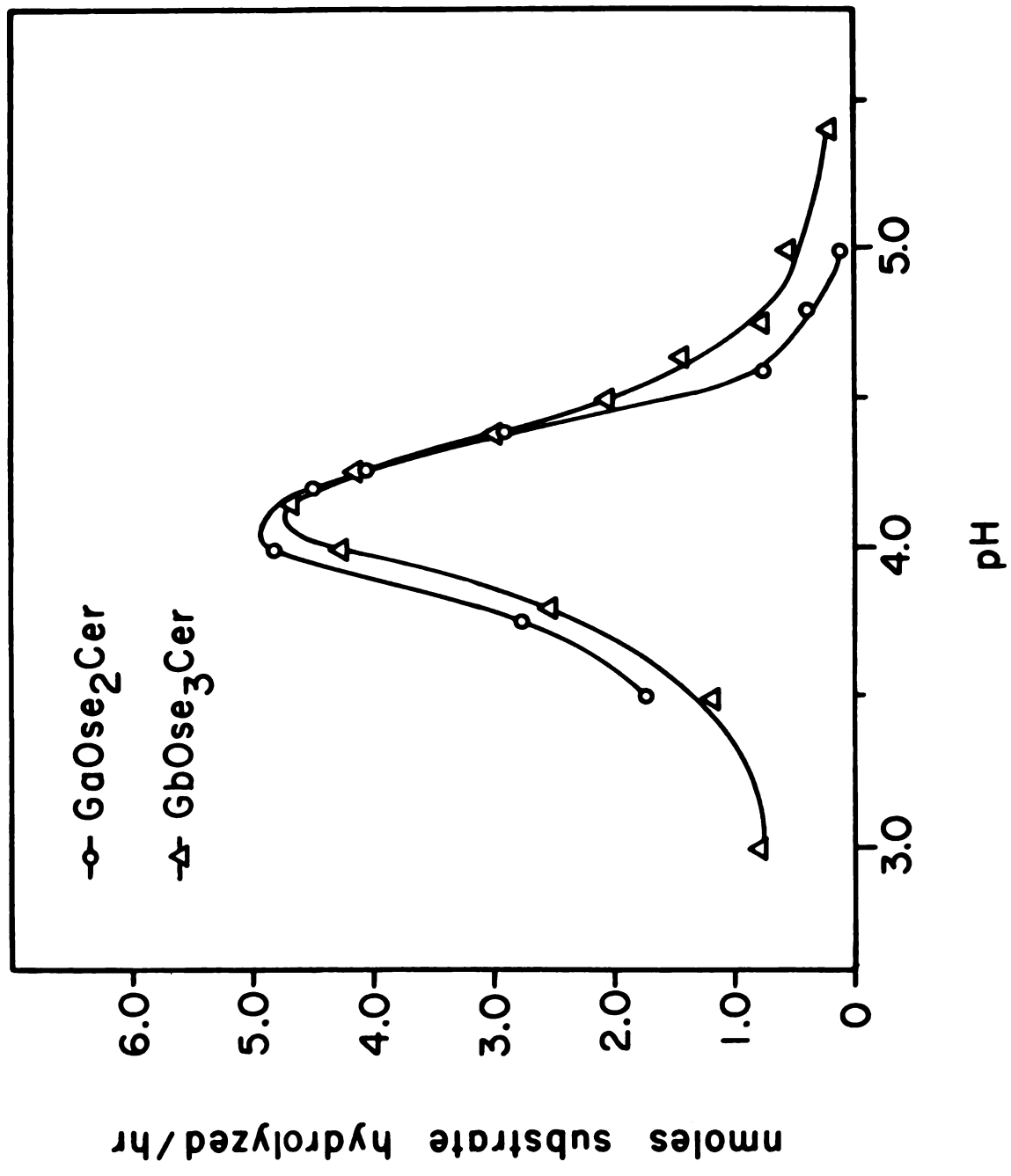

nmoles substrate hydrolyzed/hr
6.0
5.0
4.0
3.0
2.0
1.0
0
3.0
4.0
5.0
pH
GaOse2Cer
GbOse3Cer

Figure 36. Lineweaver-Burk Plot of $GaOse_2Cer$ Hydrolysis by α-Galactosidase A

The Lineweaver-Burk plot for the hydrolysis of $GaOse_2Cer$ was sigmoidal, with an apparent K_m of 0.32 mM and an apparent V_{max} of 1.09 μmoles/min•mg. The error in the velocity measurements was approximately ±0.5 nmoles/hr.

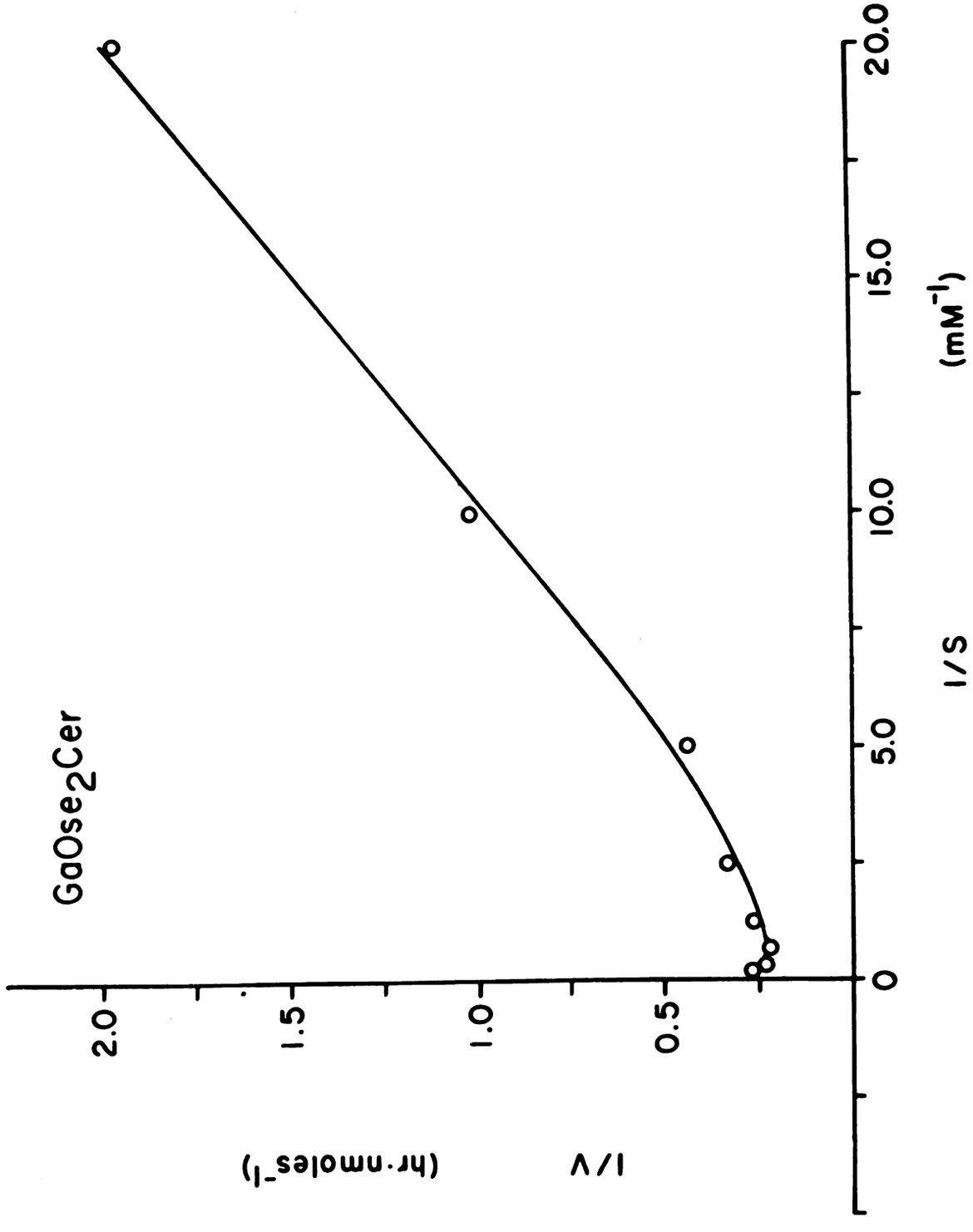

GaOse2Cer
1/V
(hr.nmoles-1)
2.0
1.5
1.0
0.5
0
5.0
10.0
15.0
20.0
1/S
(mM-1)

Figure 37. The Hill Plot of $GaOse_2Cer$ Hydrolysis by α-Galactosidase A.

The Hill plot of $GaOse_2Cer$ hydrolysis was not linear, but a line with a slope of 1.0.

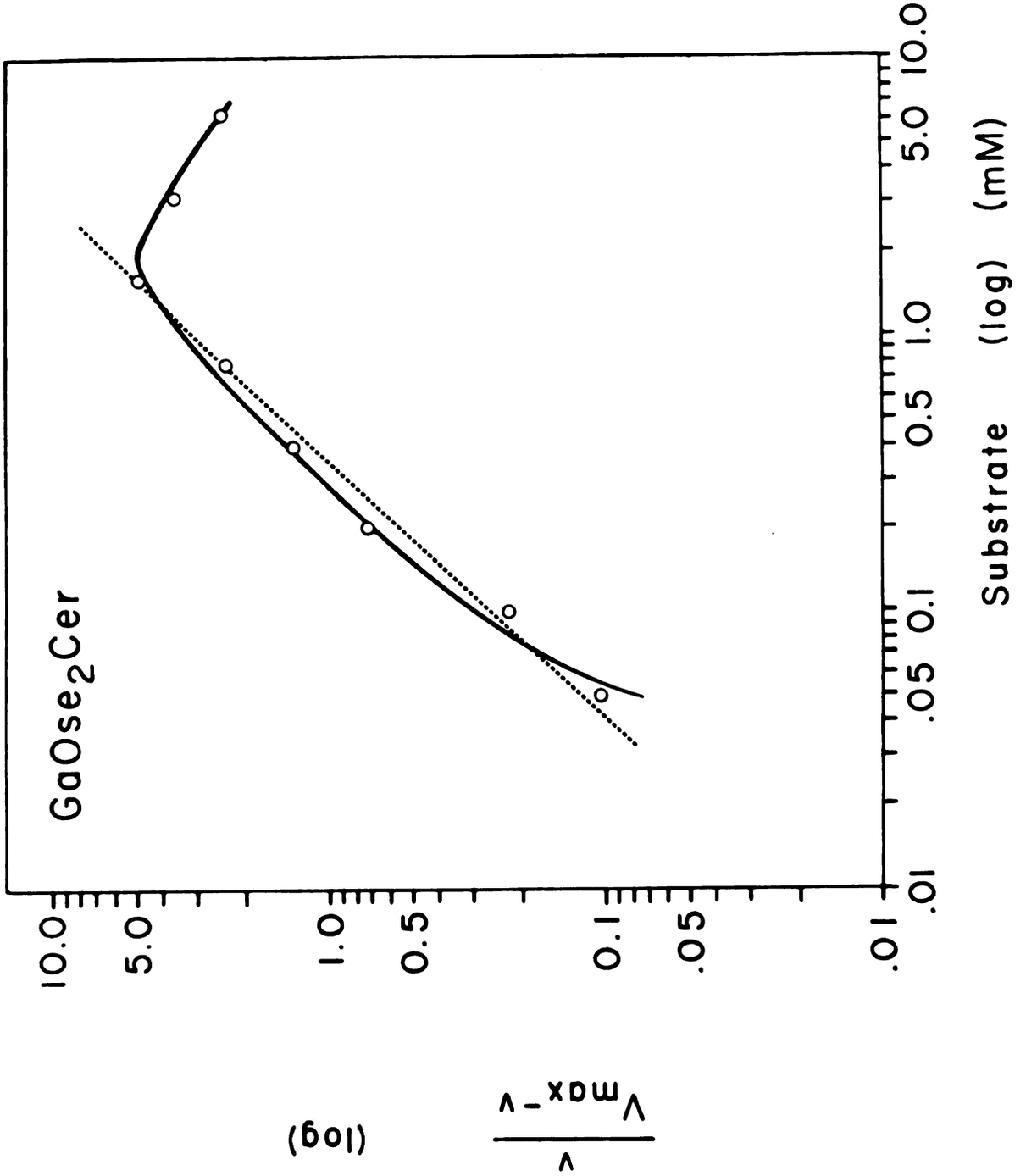

GaOse2Cer
Substrate (log) (mM)
.01
.05
0.1
0.5
1.0
5.0
10.0
v / vmax - v (log)

Figure 38. Lineweaver-Burk Plot of $GbOse_3Cer$ Hydrolysis by α-Galactosidase A

The Lineweaver-Burk plot of $GbOse_3Cer$ hydrolysis was sigmoidal, with an apparent K_m of 0.39 mM and an apparent V_{max} of 1.16 μmoles/min·mg. The error in the velocity measurements was approximately ±4.0 nmoles/hr·ml.

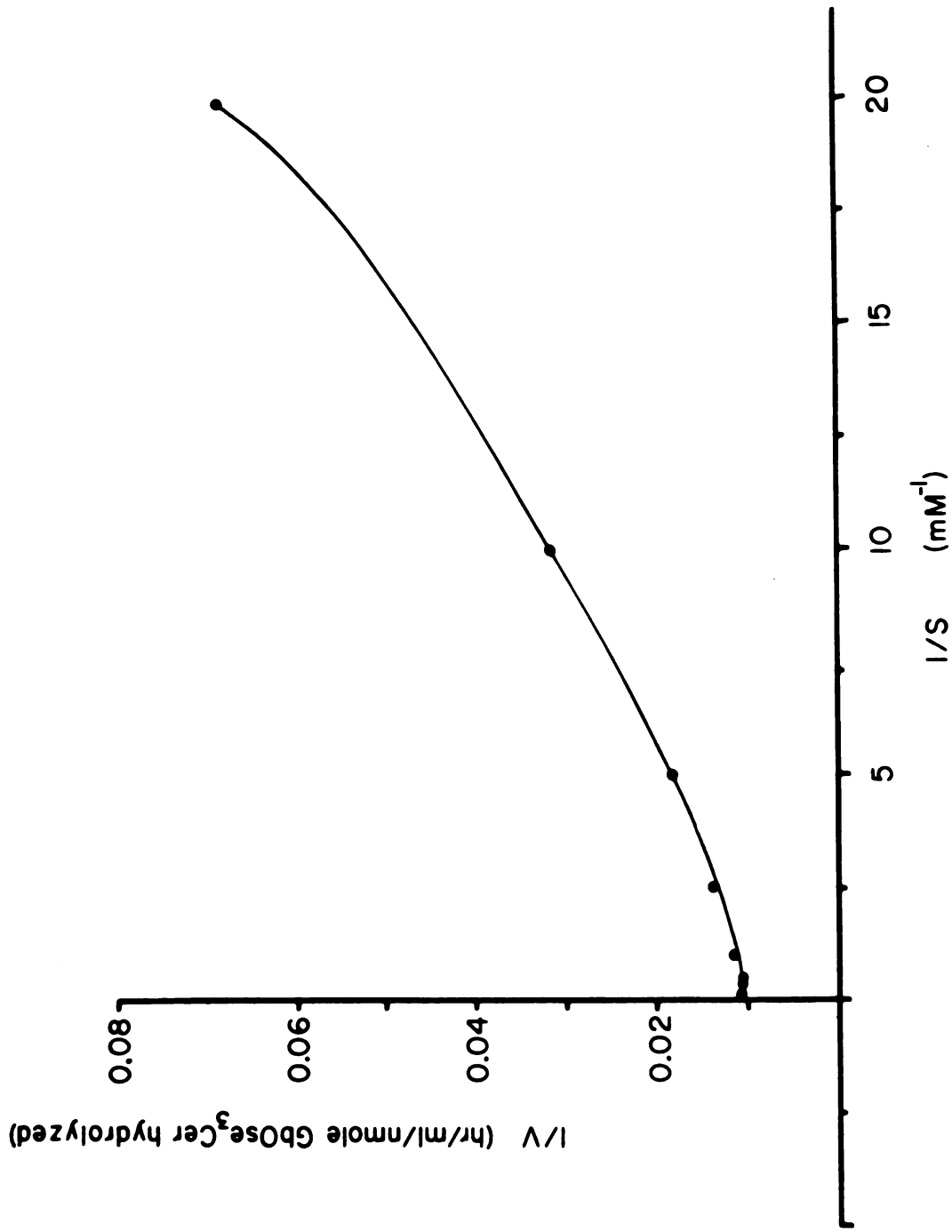

0.08
0.06
0.04
0.02
1/V (hr/ml/nmole GbOse3Cer hydrolyzed)
5
10
15
20
1/S (mM-1)

Figure 39. The Hill Plot of $GbOse_3Cer$ Hydrolysis by α-Galactosidase A

The Hill plot of $GbOse_3Cer$ hydrolysis was not linear, but approximated a line with a slope of 1.0.

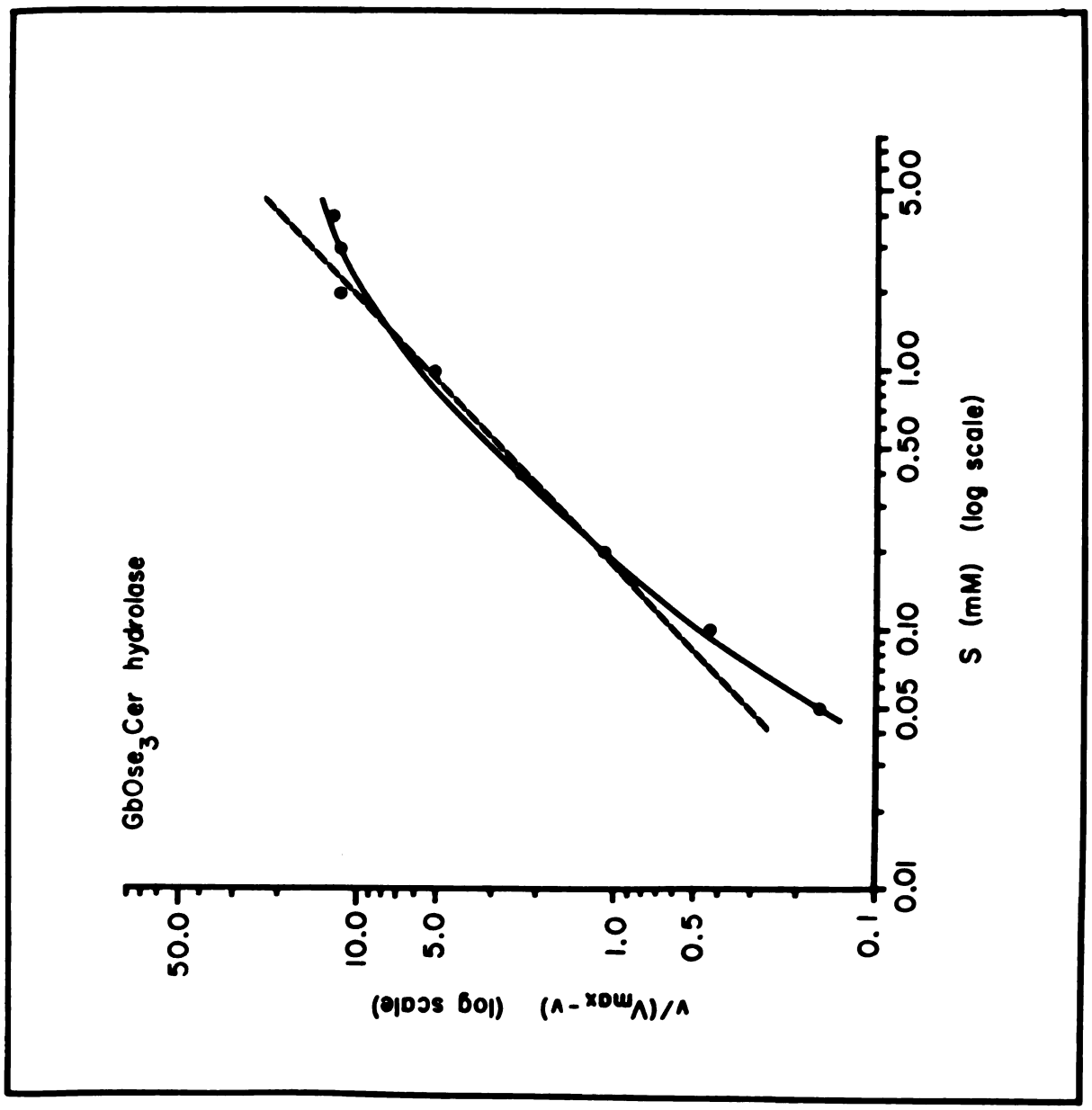
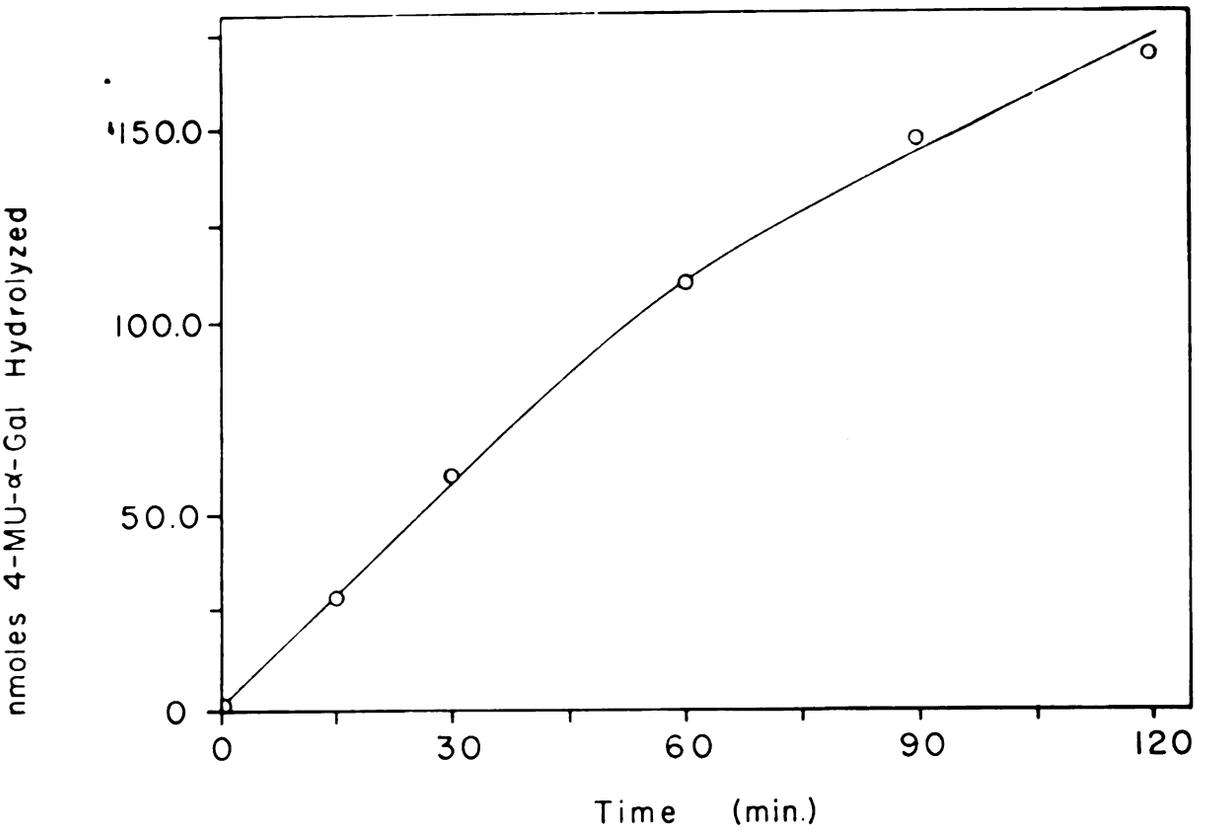

GbOse3Cer hydrolase
v/(Vmax - v) (log scale)
50.0
10.0
5.0
1.0
0.5
0.1
0.01
0.05
0.10
0.50
1.00
5.00
S (mM) (log scale)

Figure 40. The Linearity of 4-MU-α-Gal Hydrolysis by α-Galactosidase B with Respect to Time and Enzyme Concentration

The Hydrolysis of 4-MU-α-Gal by α-galactosidase B was linear with respect to time for up to 1 hour (top), and with respect to enzyme concentration, up to 100 µg/100 µl (bottom).


nmoles 4-MU-α-Gal Hydrolyzed
150.0
100.0
50.0
0
0
30
60
90
120
Time (min.)

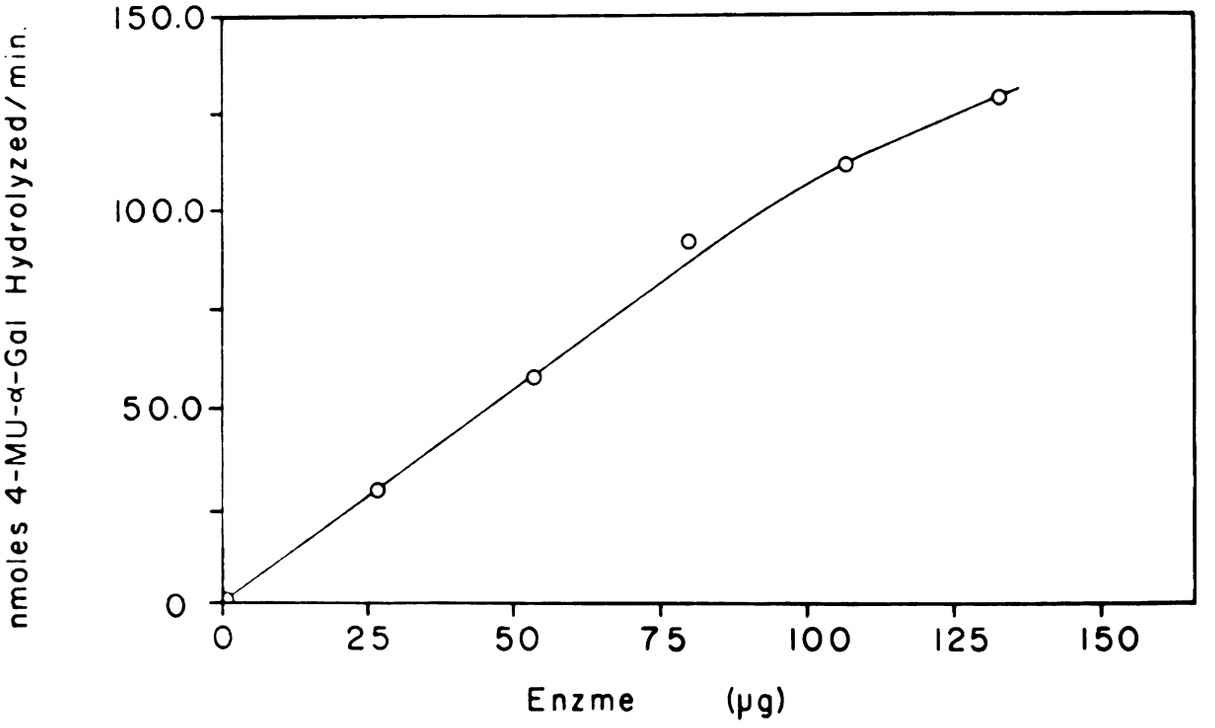

nmoles 4-MU-α-Gal Hydrolyzed/min.
150.0
100.0
50.0
0
0
25
50
75
100
125
150
Enzme (μg)

Figure 41. The pH Optima for the Hydrolysis of 4-MU-α-Gal and o-NP-α-GalNAc by α-Galactosidase B

The hydrolysis of 4-MU-α-Gal by α-galactosidase B was optimal at pH 4.7. The hydrolysis of o-NP-α-GalNAc by this enzyme was optimal at pH 4.3.

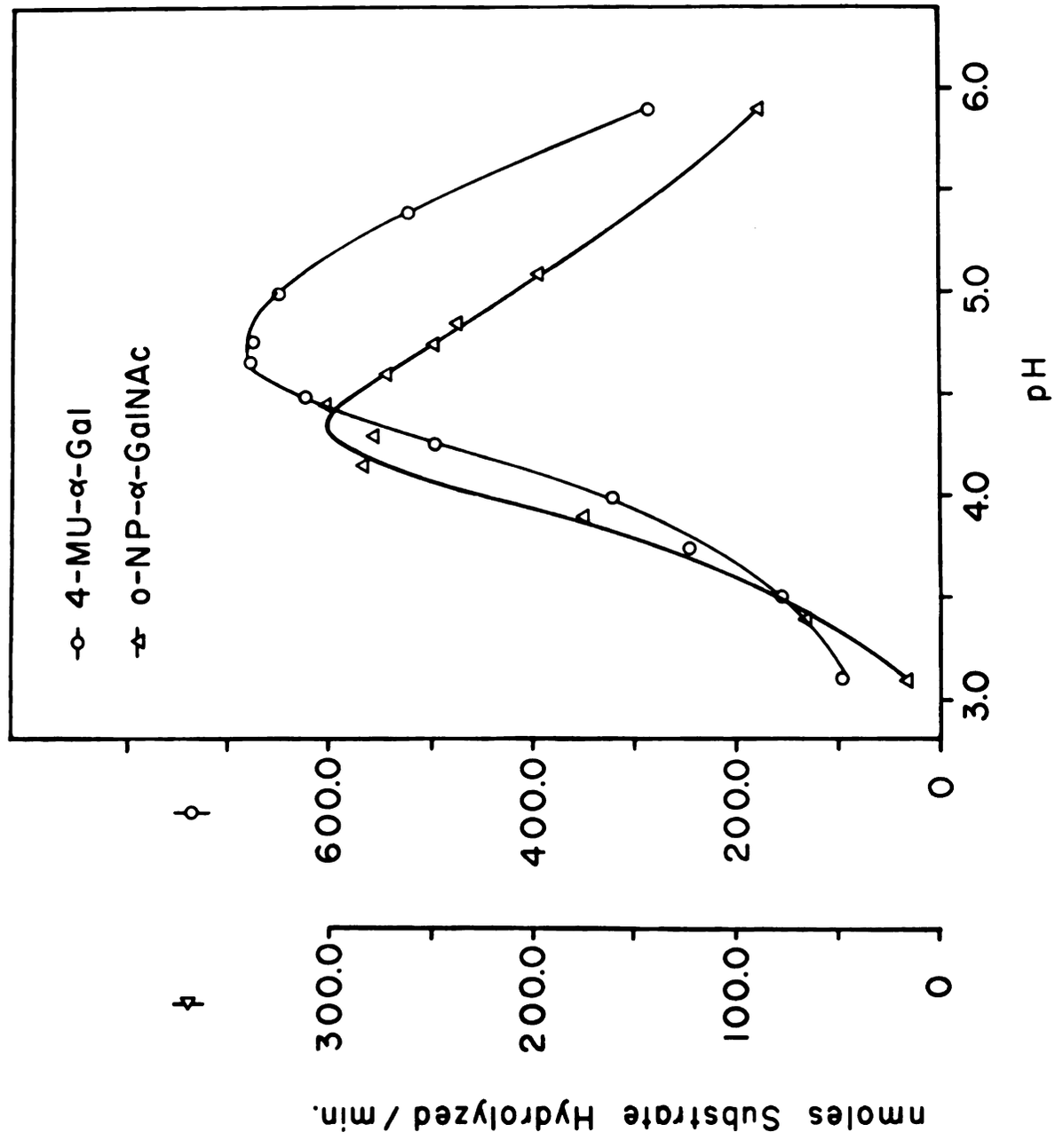

nmoles Substrate Hydrolyzed / min.
300.0
200.0
100.0
0
600.0
400.0
200.0
0
3.0
4.0
5.0
6.0
pH
4-MU-α-Gal
o-NP-α-GalNAc

Figure 42. Lineweaver-Burk Plot of 4-MU-α-Gal Hydrolysis by α-Galactosidase B

The K_m for the hydrolysis of 4-MU-α-Gal by α-galactosidase B was 6.8 mM and the V_{max} was 18.73 μmoles/min·mg. The error in the velocity measurements was approximately ±2.0 nmoles/min.

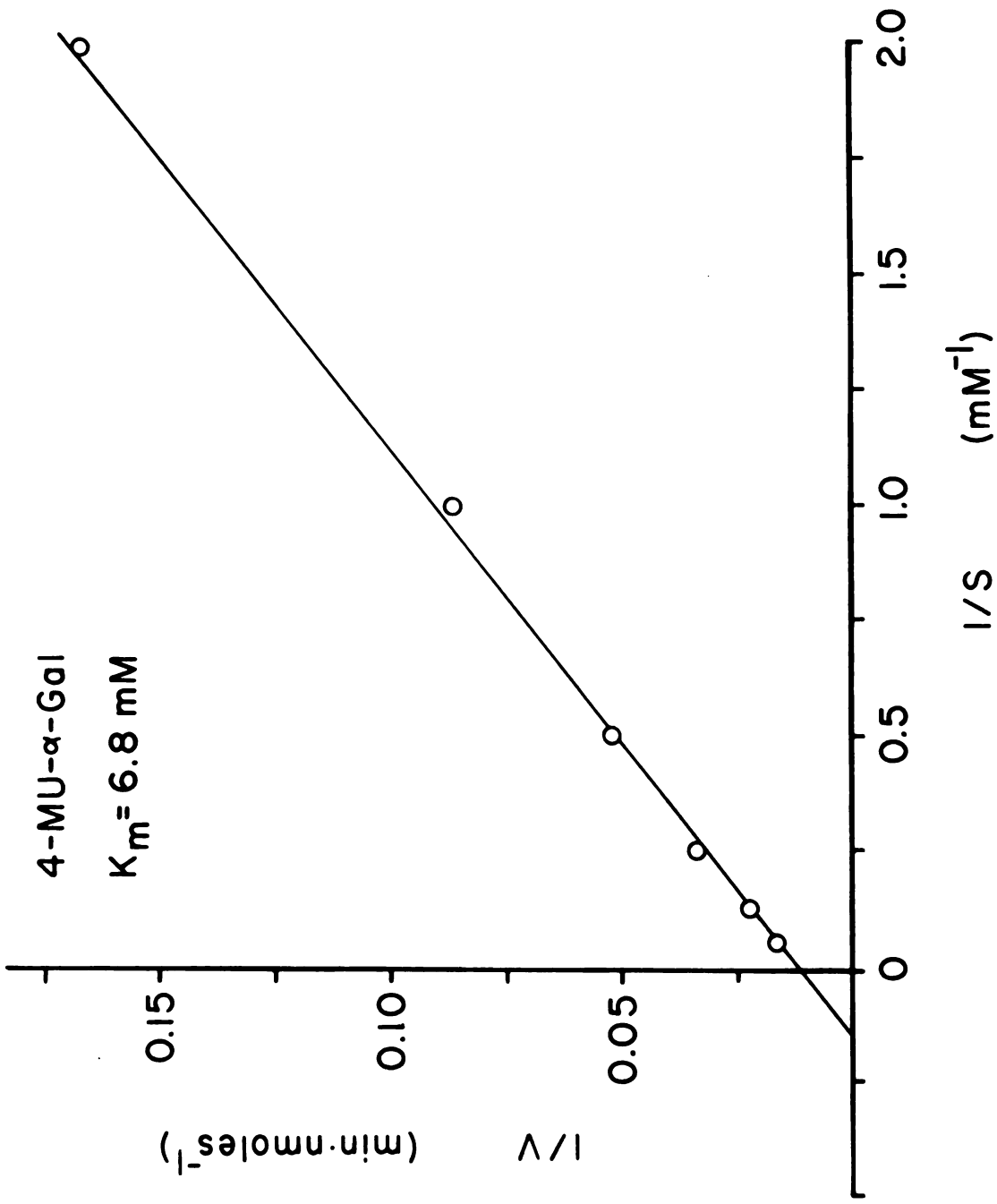

4-MU-α-Gal
K_m= 6.8 mM
1/V (min·nmoles^{-1})
0.15
0.10
0.05
0
0.5
1.0
1.5
2.0
1/S (mM^{-1})

Figure 43. The Hill Plot of 4-MU-α-Gal Hydrolysis by α-Galactosidase B

The Hill plot of 4-MU-α-Gal hydrolysis by α-galactosidase B was linear, with a slope of 1.1.

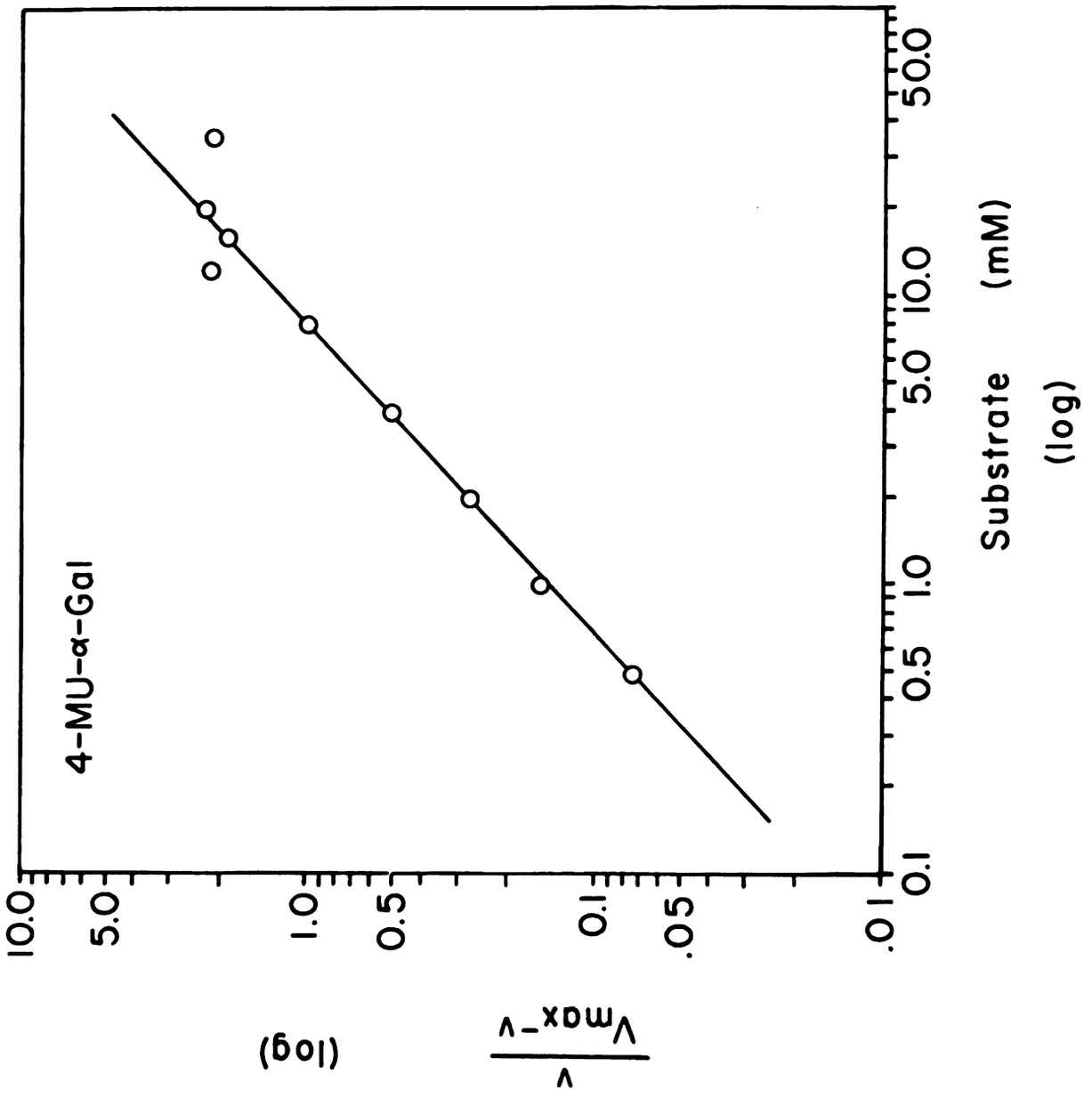

4-MU-α-Gal
10.0
5.0
1.0
0.5
0.1
.05
.01
0.1
0.5
1.0
5.0
10.0
50.0
Substrate (mM)
(log)
v / Vmax-v
(log)

Figure 44. Thermal Inactivation Curves for α-Galactosidase A and B, and α-N-Acetylgalactosaminidase Activities

Thermal inactivation of α-galactosidases A (●) and B (○) were monitored with 4-MU-α-Gal, and α-N-acetylgalactosaminidase (▲) was monitored with o-NP-α-GalNAc. Thermal inactivation was carried out at 55°C at pH 6.5.

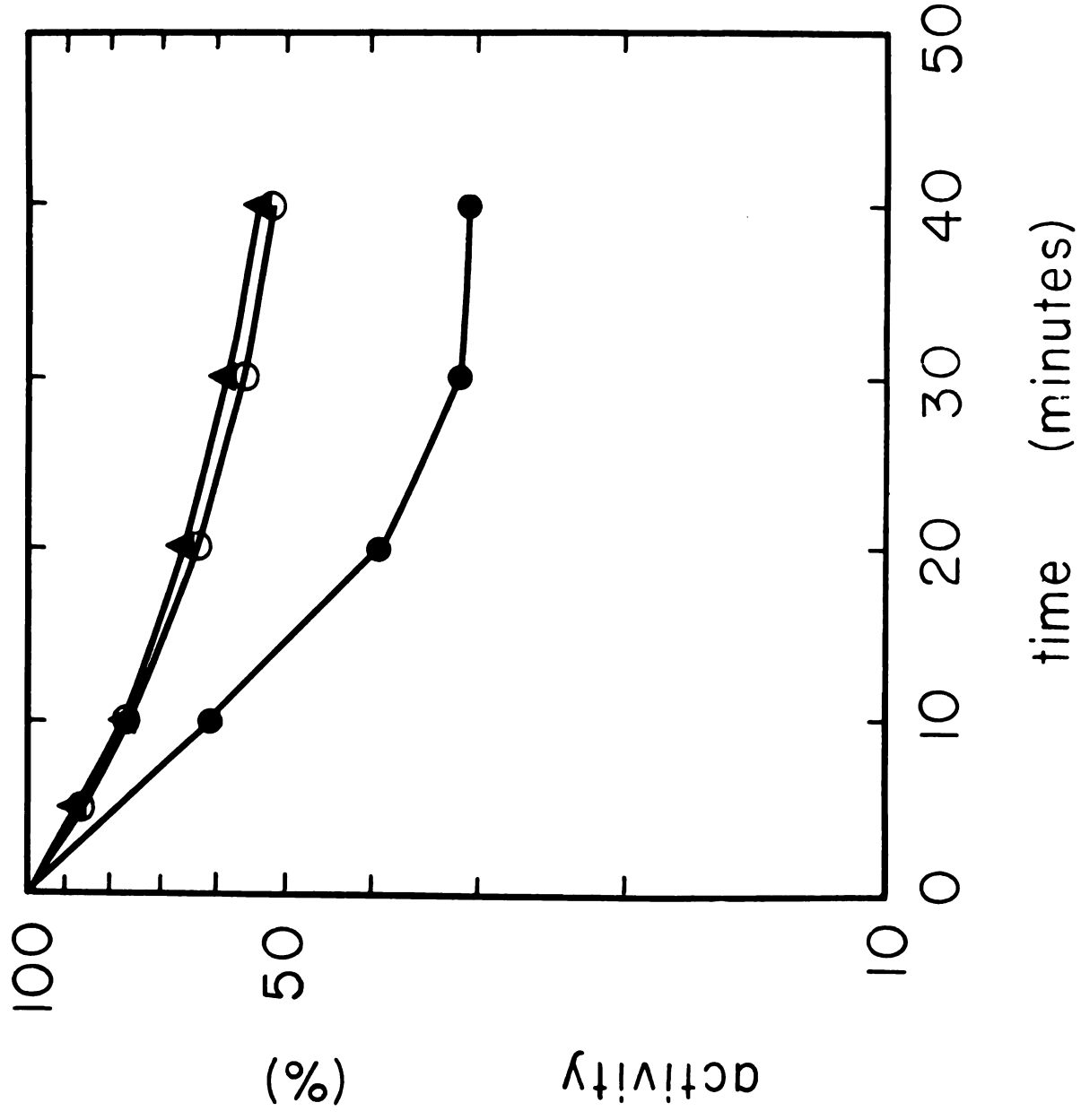

activity
(%)
100
50
10
0
10
20
30
40
50
time (minutes)

Figure 45. Isoelectric Focusing of a Partially Purified Preparation of α-Galactosidase B

Isoelectric focusing was performed in an LKB 8101 isoelectric focusing column with LKB carrier ampholytes, pH 3-5, in a sucrose density gradient, as described by Vesterberg (279). α-Galactosidase B had pI = 4.5.

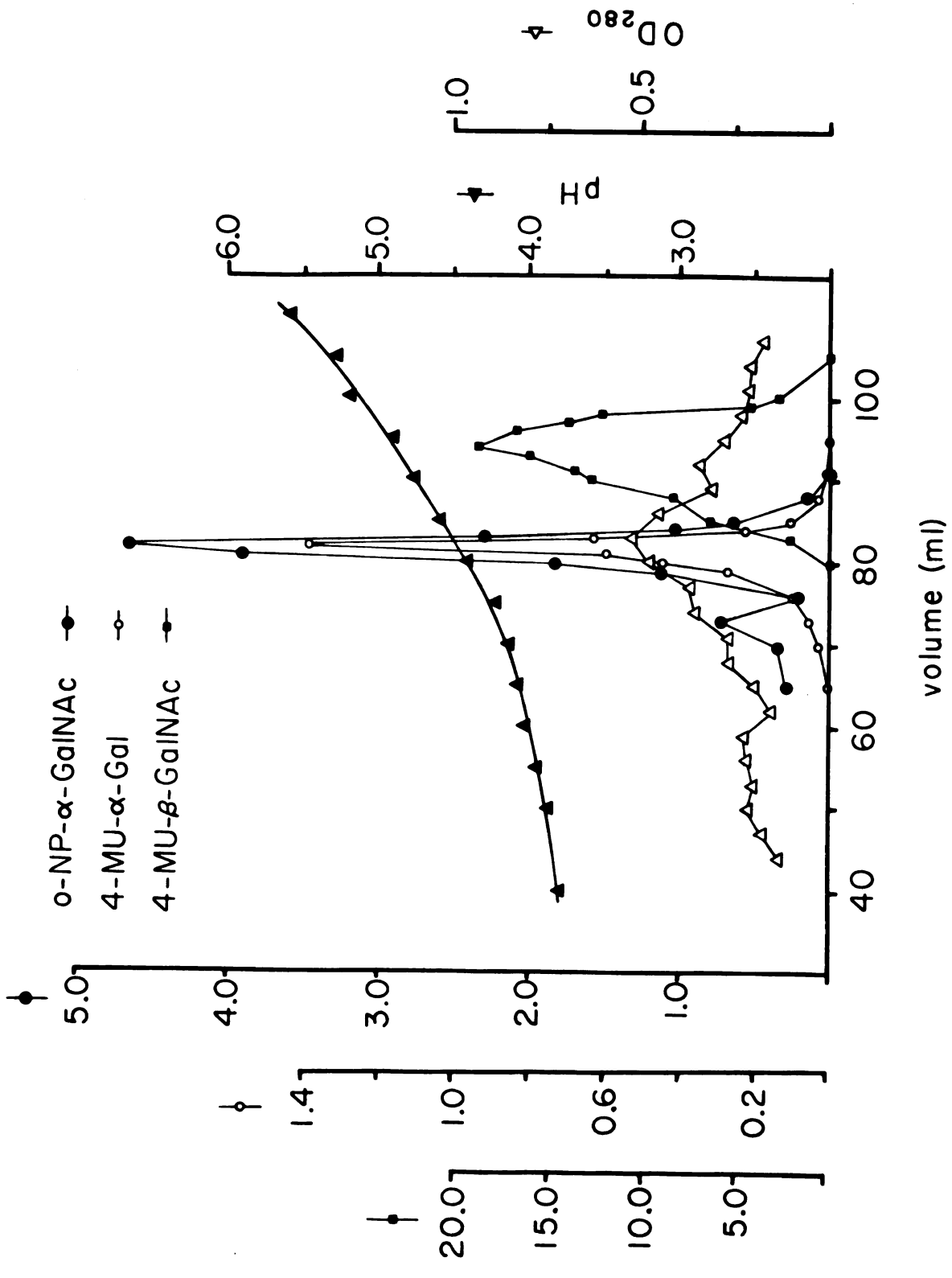
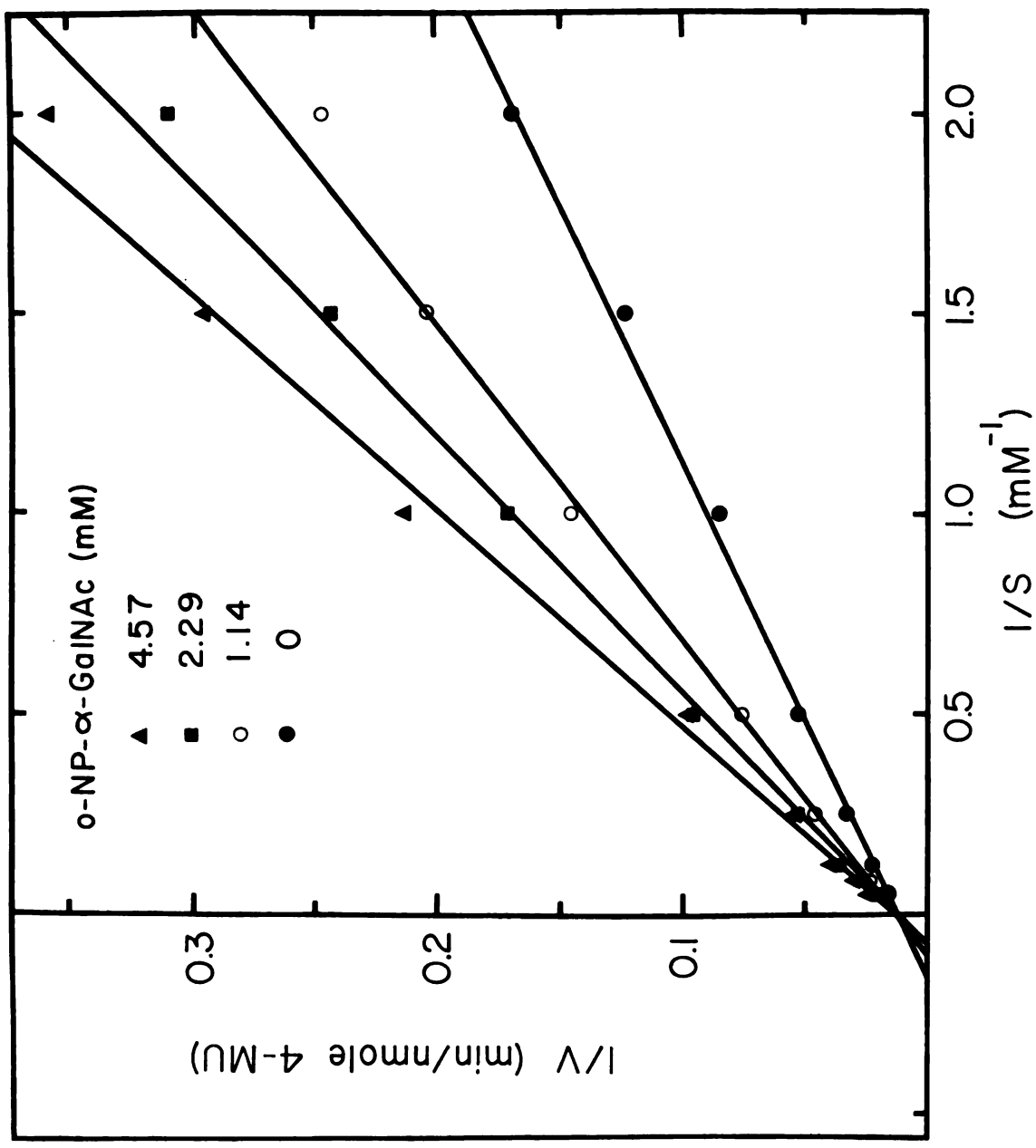

μmoles sub. hydrolyzed / min.
o-NP-α-GalNAc
4-MU-α-Gal
4-MU-β-GalNAc
pH
OD280
volume (ml)

Figure 46. The Competitive Inhibition of 4-MU-α-Gal Hydrolysis by o-NP-α-GalNAc with α-Galactosidase B

The hydrolysis of 4-MU-α-Gal by α-galactosidase B at pH 4.7 was examined in the presence of o-NP-α-GalNAc. The hydrolysis of 4-MU-α-Gal was competitively inhibited by o-NP-α-GalNAc, with K_i = 2.7 mM. The error in the velocity measurements was approximately ±1.0 nmoles/min.


o-NP-α-GalNAc (mM)
4.57
2.29
1.14
0
1/V (min/nmole 4-MU)
0.3
0.2
0.1
0.5
1.0
1.5
2.0
1/S (mM⁻¹)

catalyzed the hydrolysis of both α-galactose and α-N-acetylgalactosamine residues, the hydrolysis of 4-MU-α-Gal in the presence of o-NP-α-GalNAc was examined. The measurement of 4-MU-α-Gal hydrolysis in the presence of o-NP-α-GalNAc was possible because 4-methylumbelliferone is measured at 365 nm excitation and 450 emission and o-NP-α-GalNAc absorbs at different wavelengths (265 nm and 322 nm (133)). The hydrolysis of 4-MU-α-Gal was competitively inhibited by o-NP-α-GalNAc with a K_I of 2.7 mM, as shown in Figure 46. This was lower than the K_m with 4-MU-α-Gal at 6.8 mM.

The hydrolysis of o-NP-α-GalNAc catalyzed by α-galactosidase B was linear with respect to time (up to 1 hour) and enzyme concentration (up to 70 µg). The hydrolysis of this substrate was optimal at pH 4.3, as shown in Figure 41. Th K_m with o-NP-α-GalNAc was 1.3 mM (Figure 47) and the V_{max} was 59.95 µmoles/min·mg. The Hill plot of o-NP-α-GalNAc hydrolysis was linear, with a slope of 1.1, as shown in Figure 48.

α-Galactosidase B also catalyzed the hydrolysis of p-NP-2-deoxy-α-Gal and o-NP-α-D-Fuc. The hydrolysis of these substrates was linear with respect to time (up to 1 hour with p-NP-2-deoxy-α-Gal and up to 3 hours with o-NP-α-D-Fuc) and enzyme concentration (up to 80 µg with the 2-deoxy substrate and up to 320 µg with the 6-deoxy substrate). The hydrolysis of these substrates was optimal at pH 4.7. The K_m with p-NP-2-deoxy-α-Gal was 14.7 mM (Figure 49) and the V_{max} was 215.7 µmoles/min·mg. The Hill plot of p-NP-2-deoxy-α-Gal hydrolysis was linear, with a slope of 1.0 (Figure 50). The K_m with o-NP-α-D-Fuc was 8.8 mM (Figure 51) and the V_{max} was 0.16 µmoles/min·mg. The Hill plot of o-NP-α-D-Fuc hydrolysis was linear with a slope of 1.0 (Figure 52).

Oligosaccharide Substrates. The thin-layer chromatogram of [^{3}H]$GbOse_5$ is shown in Figure 53. The specific activity of the [^{3}H]-labeled oligosaccharide was 8,071 cpm/nmole. The hydrolysis of this pentasaccharide was linear with respect to time (up to 3 hours) and with respect to enzyme concentration (up to 170 µg of enzyme with 30 minute incubations). The hydrolysis of $GbOse_5$ was optimal at pH 4.4 (Figure 54). The K_m with this substrate was 3.7 mM (Figure 55) and the V_{max} was 9.24 µmoles/min·mg. The Hill plot of $GbOse_5$ hydrolysis was linear, with a slope of 1.0 (Figure 56).

α-Galactosidase B also catalyzed the hydrolysis of $GbOse_3$, however prolonged incubation periods were required to detect the hydrolysis of this substrate. The hydrolysis of $GbOse_3$ was optimal at pH 4.8, as shown in Figure 53. The K_m with this oligosaccharide was 9.1 mM (Figure 57) and the

Figure 47. Lineweaver-Burk Plot of o-NP-α-GalNAc Hydrolysis by α-Galactosidase B

The K_m for the hydrolysis of o-NP-α-GalNAc by α-galactosidase B was 1.3 mM and the V_{max} was 59.95 μmoles/min·mg. The error in the velocity measurements was approximately ±2.0 nmoles/min.

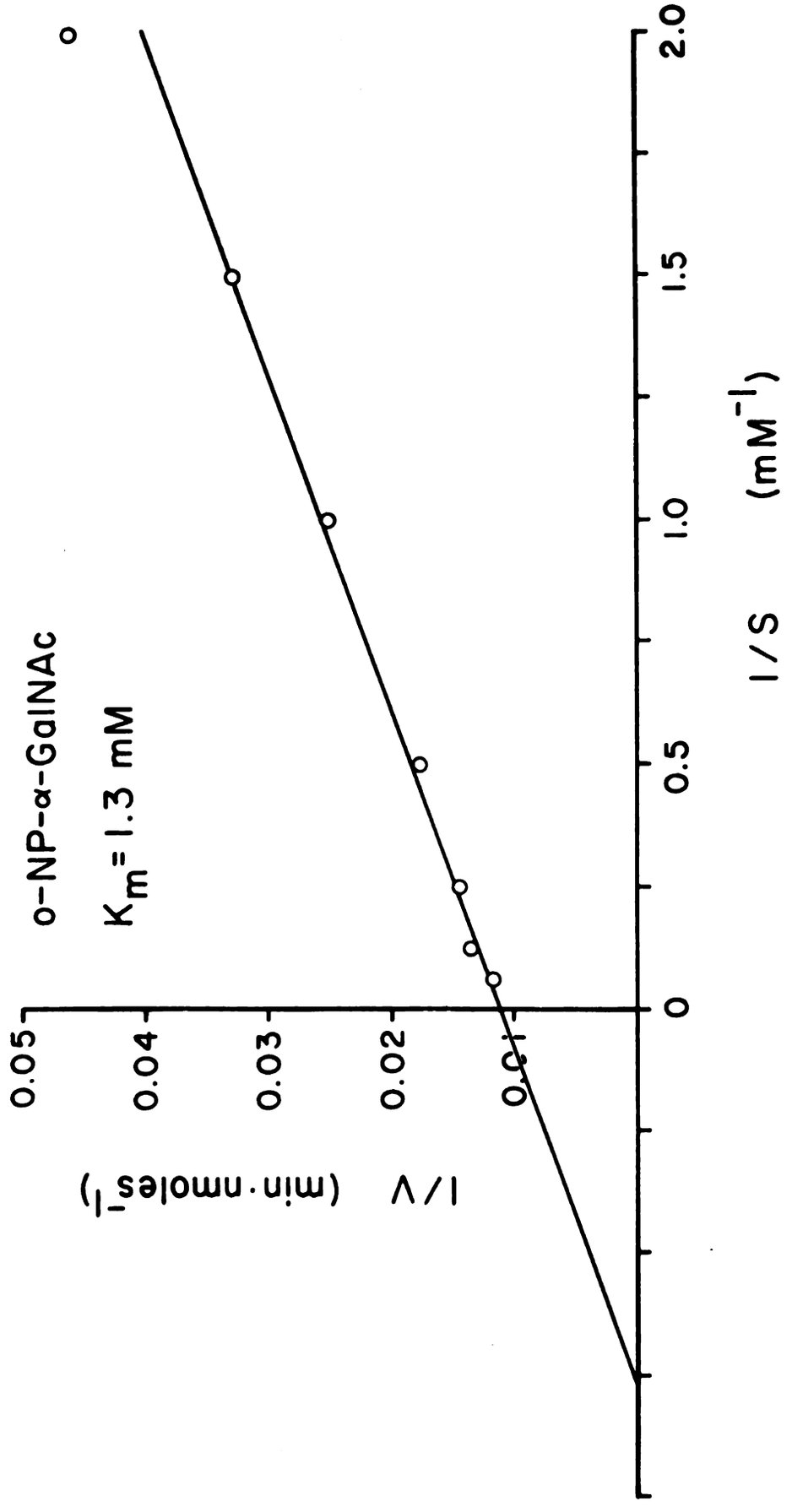

o-NP-α-GalNAc
K_m= 1.3 mM
1/V (min·nmoles^{-1})
0.05
0.04
0.03
0.02
0.01
0
0.5
1.0
1.5
2.0
1/S (mM^{-1})

Figure 48. The Hill Plot of o-NP-α-GalNAc Hydrolysis by α-Galactosidase B

The Hill plot of o-NP-α-GalNAc hydrolysis by α-galactosidase B was linear, with a slope of 1.1.

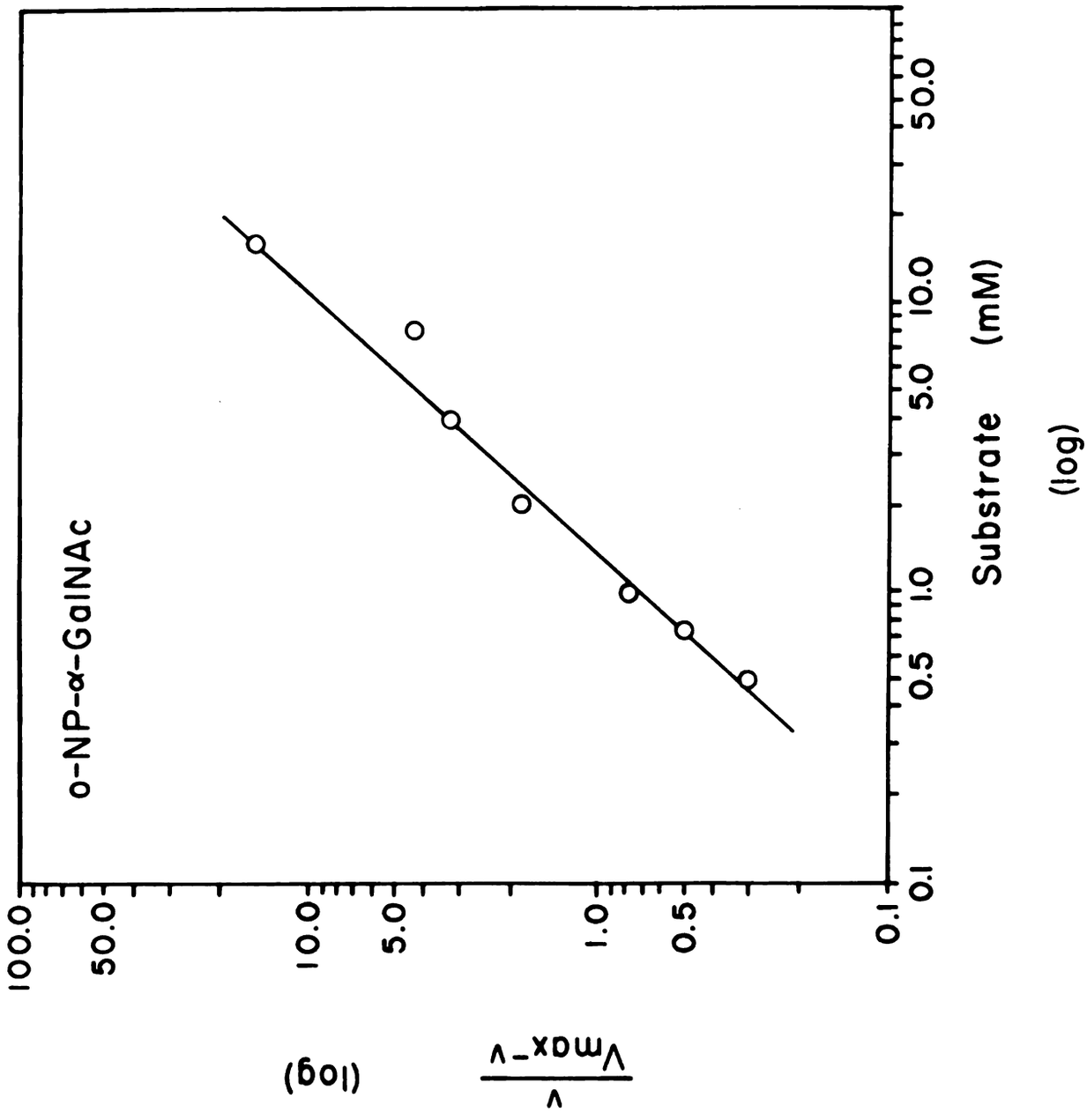

o-NP-α-GalNAc
Substrate (mM)
(log)
0.1
0.5
1.0
5.0
10.0
50.0
100.0
v / Vmax-v
(log)

Figure 49. Lineweaver-Burk Plot of the Hydrolysis of p-NP-2-Deoxy-α-Gal by α-Galactosidase B

The K_m for the hydrolysis of p-NP-2-deoxy-α-Gal was 14.7 mM and the V_{max} was 215.7 μmoles/min·mg. The error in the velocity measurements was approximately ±20.0 nmoles/min.

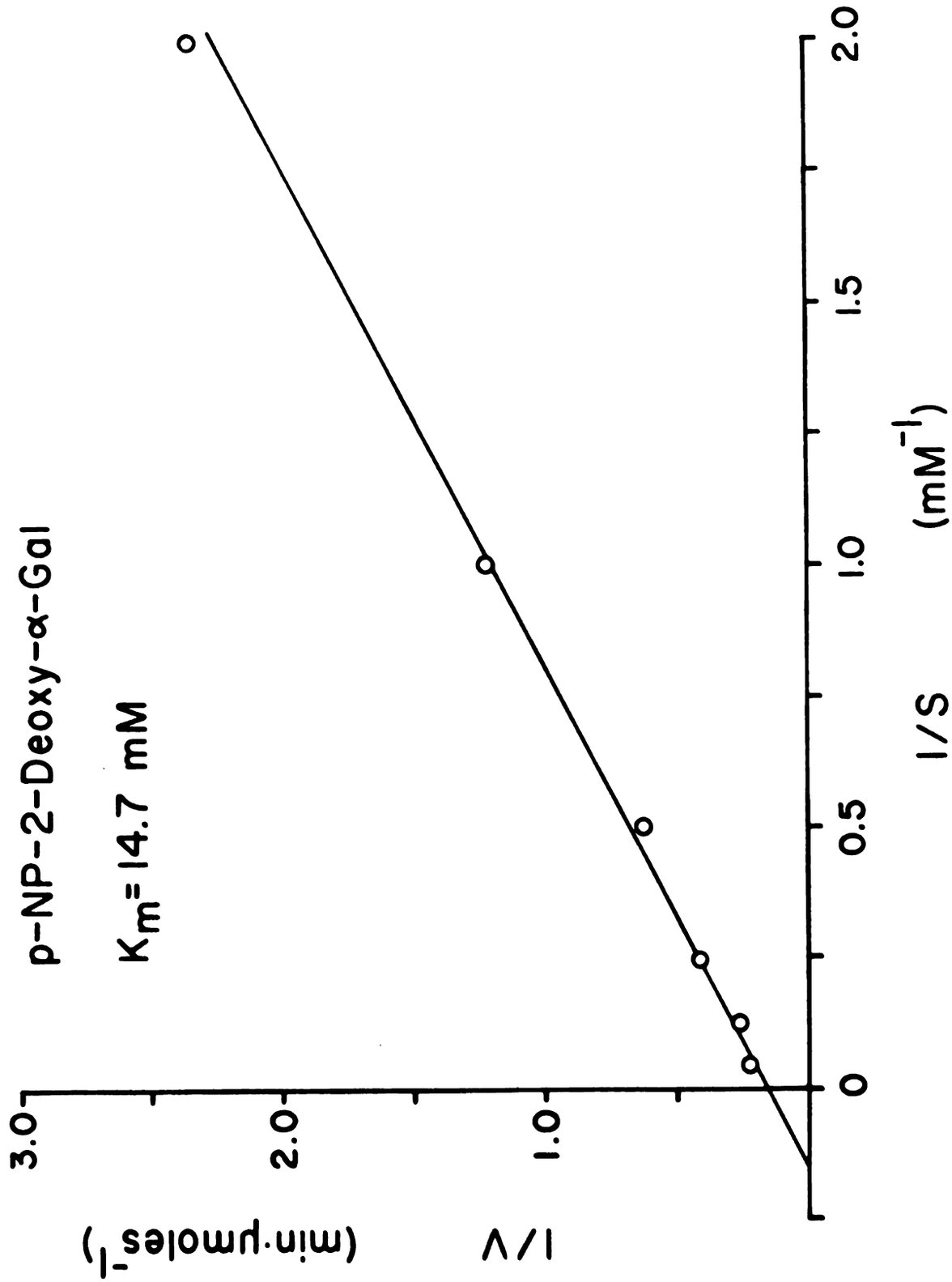

p-NP-2-Deoxy-α-Gal
K_m= 14.7 mM
1/V
(min·μmoles^{-1})
3.0
2.0
1.0
0
0.5
1.0
1.5
2.0
1/S
(mM^{-1})

Figure 50. The Hill Plot of p-NP-2-Deoxy-α-Gal Hydrolysis by α-Galactosidase B

The Hill plot of p-NP-2-deoxy-α-Gal hydrolysis was linear, with a slope of 1.0.

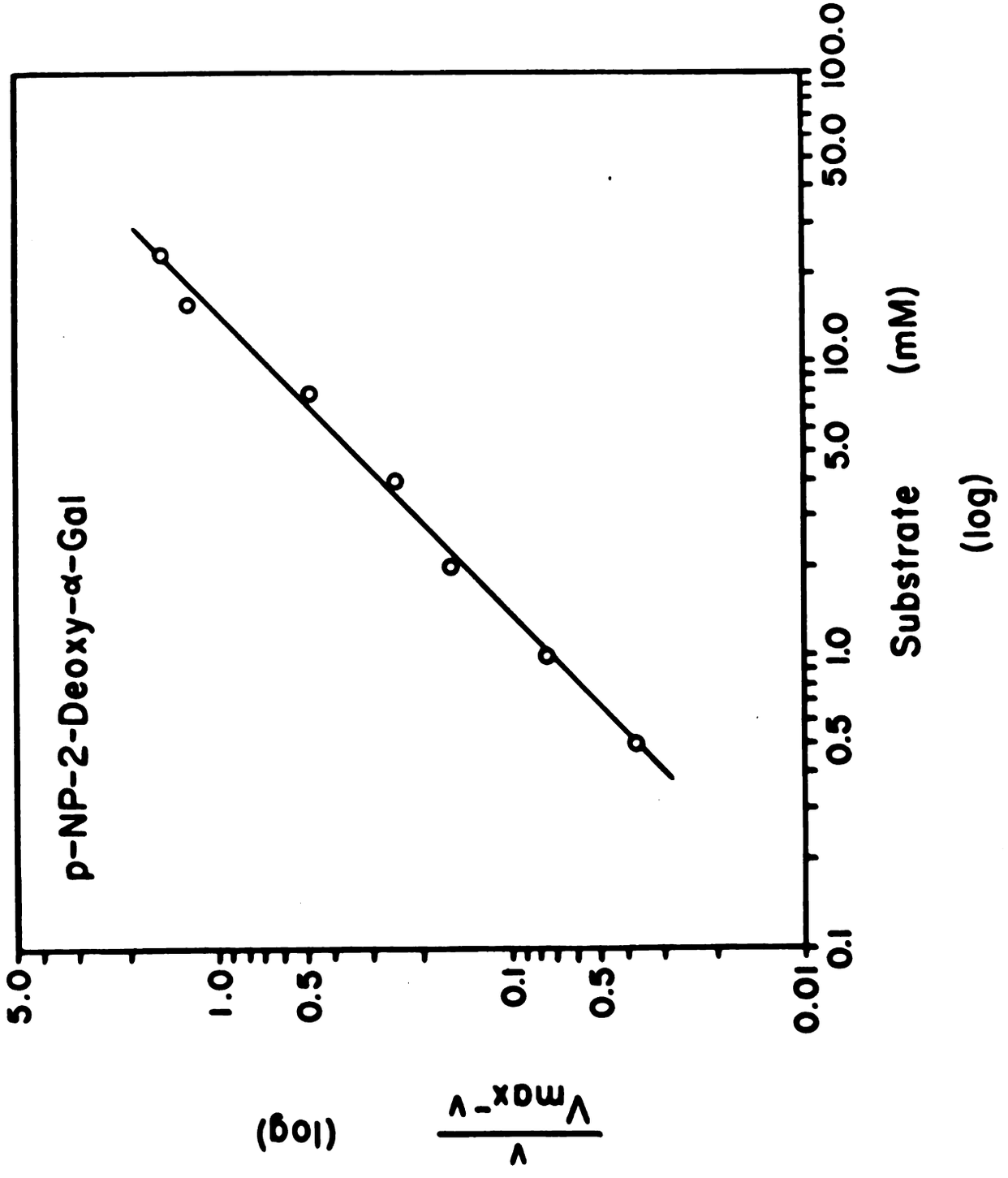

p-NP-2-Deoxy-α-Gal
5.0
1.0
0.5
0.1
0.5
0.01
0.1
0.5
1.0
5.0
10.0
50.0
100.0
Substrate (mM)
(log)
v / Vmax-v
(log)

Figure 51. Lineweaver-Burk Plot for the Hydrolysis of o-NP-α-D-Fuc by α-Galactosidase B

The K_m for the hydrolysis of o-NP-α-D-Fuc was 8.8 mM and the V_{max} was 0.16 μmoles/min·mg. The error in the velocity measurements was approximately ±1.0 nmoles/min.

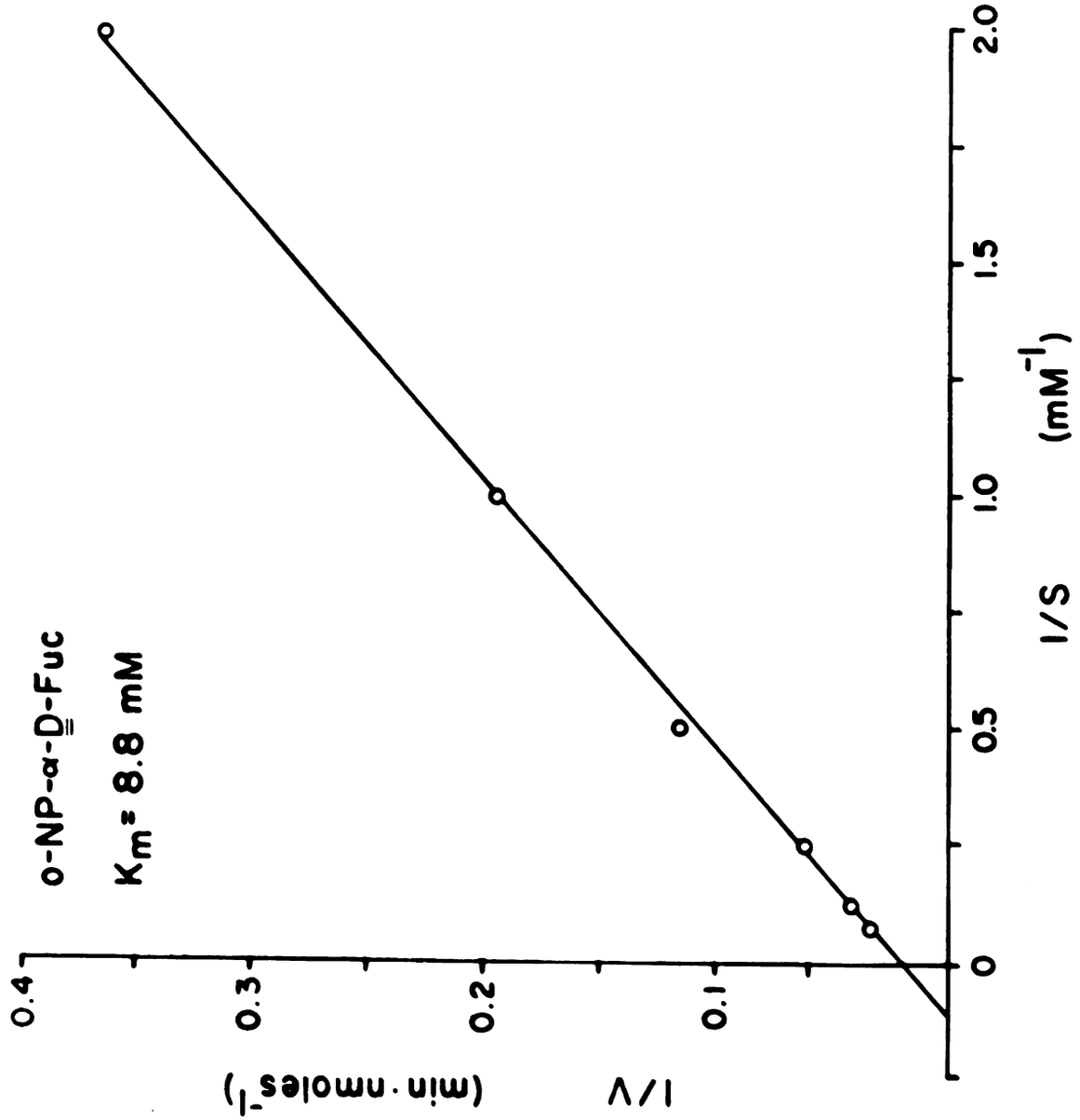

o-NP-α-D-Fuc
K_m = 8.8 mM
1/V
(min · nmoles^{-1})
0.4
0.3
0.2
0.1
0
0.5
1.0
1.5
2.0
1/S
(mM^{-1})

Figure 52. The Hill Plot of o-NP-α-D-Fuc Hydrolysis by α-Galactosidase B

The Hill plot of o-NP-α-D-Fuc hydrolysis was linear, with a slope of 1.0.

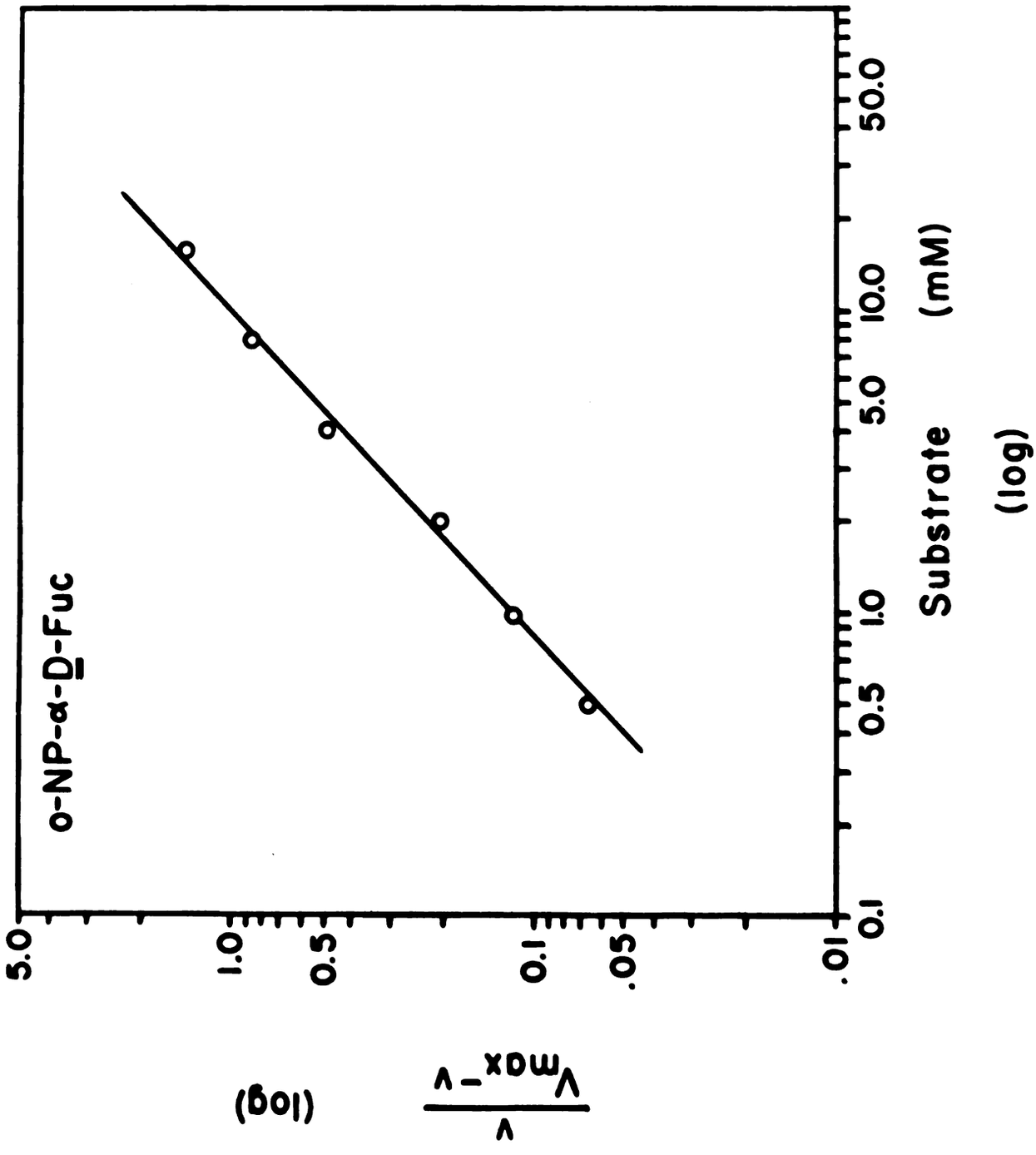

o-NP-α-D-Fuc
5.0
1.0
0.5
0.1
.05
.01
0.1
0.5
1.0
5.0
10.0
50.0
Substrate (mM)
(log)
$\frac{v}{V_{max}-v}$
(log)

Figure 53. Thin-Layer Chromatogram of [^{3}H]Globopentaose

The thin-layer chromatogram of [^{3}H]globopentaose was obtained on a silica gel G thin-layer plate. The radioactive oligosaccharide was scanned on a Varian Aerograph Berthold Radio Scanner, and had a specific activity of 8,071 cpm/nmole. The carbohydrates were visualized with orcinol.

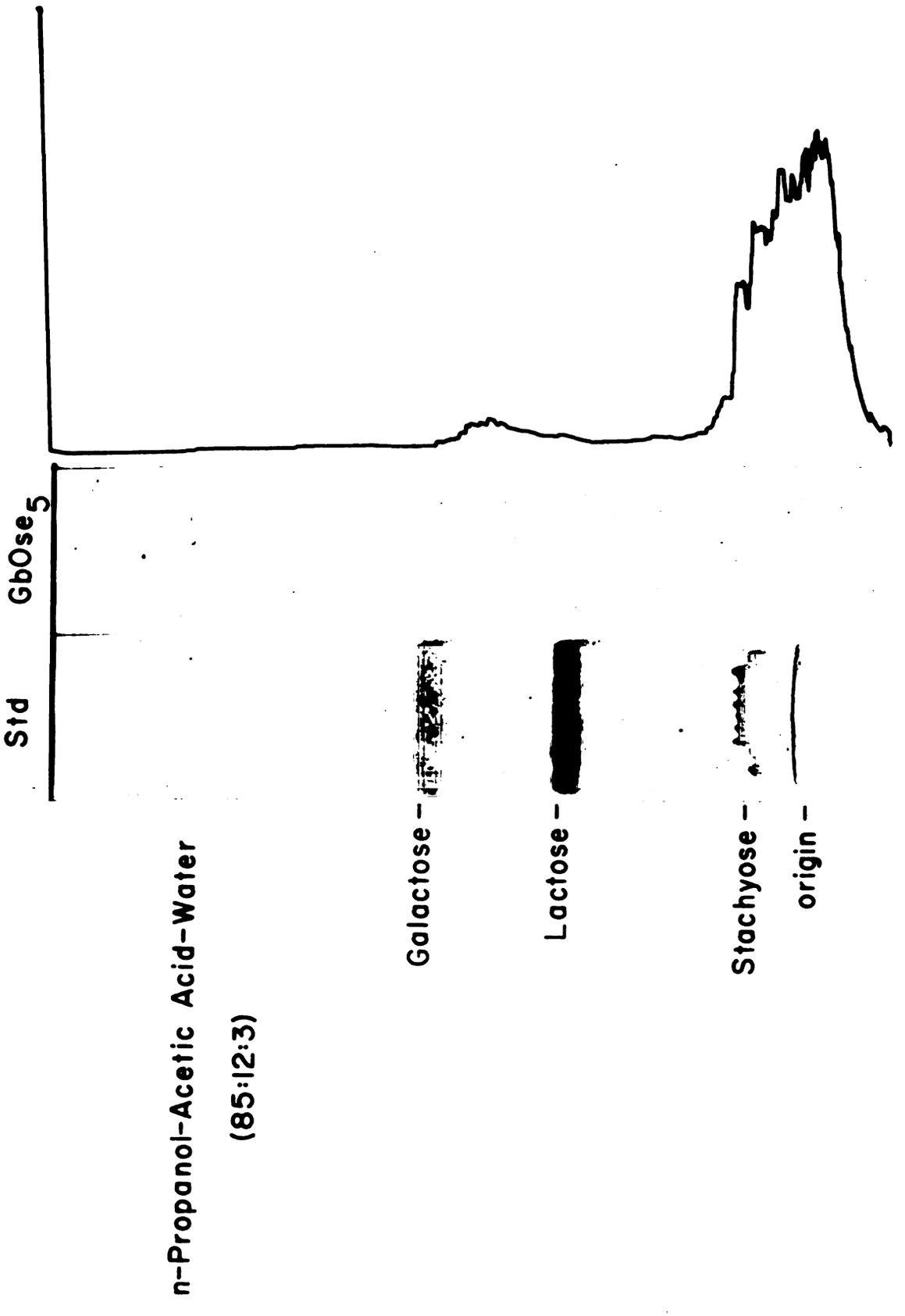

Std
GbOse5
n-Propanol-Acetic Acid-Water
(85:12:3)
Galactose –
Lactose –
Stachyose –
origin –

Figure 54. The pH Optima for the Hydrolysis of Globotriaose and Globopentaose by α-Galactosidase B

The hydrolysis of $GbOse_3$ and $GbOse_5$ by α-galactosidase B was optimal at pH 4.8 and pH 4.4, respectively. Gomori citrate-phosphate buffers (275) were used.

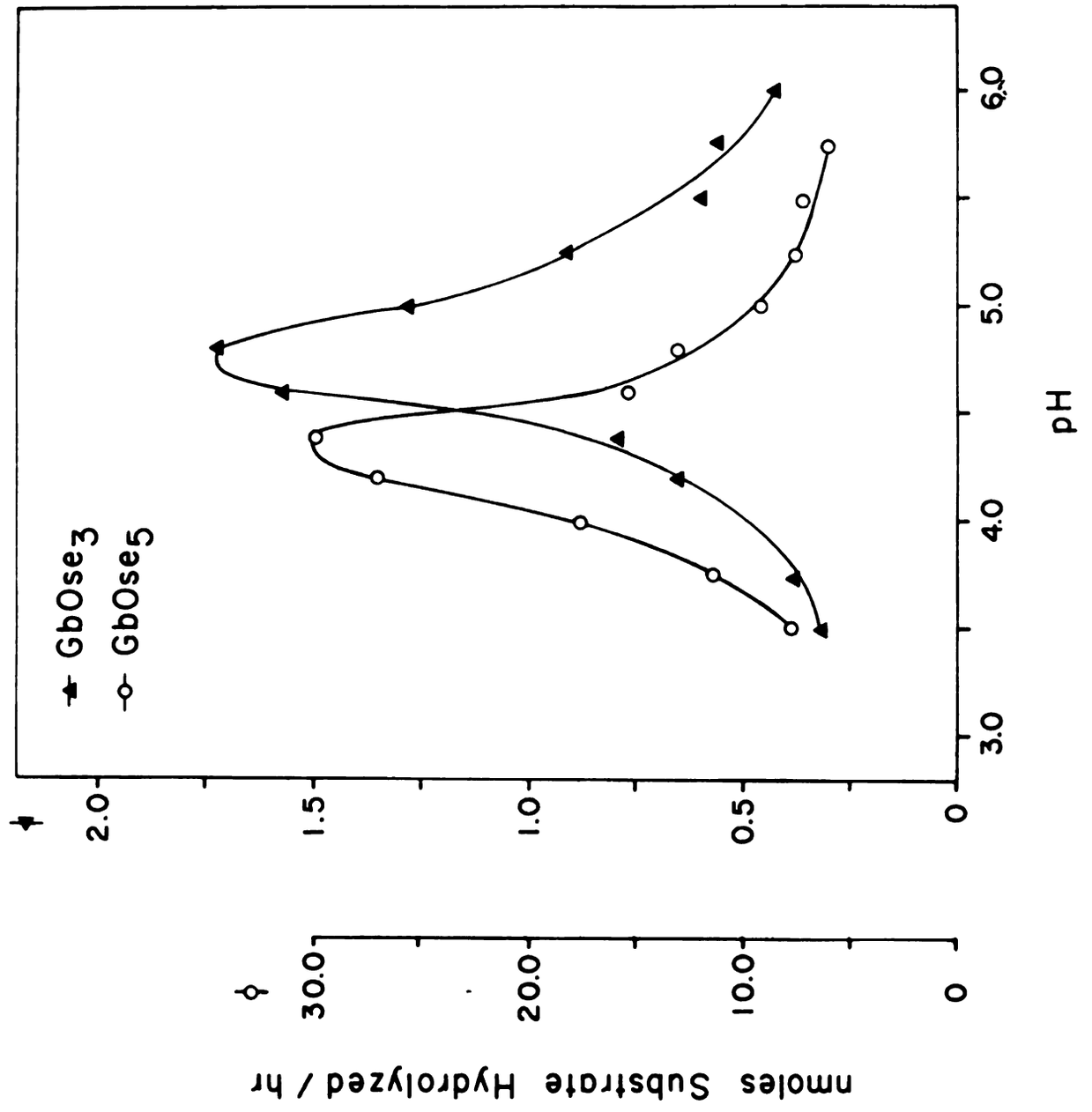

GbOse3
GbOse5
2.0
1.5
1.0
0.5
0
30.0
20.0
10.0
0
3.0
4.0
5.0
6.0
pH
nmoles Substrate Hydrolyzed / hr

Figure 55. Lineweaver-Burk Plot for the Hydrolysis of $GbOse_5$ by α-Galactosidase B

The K_m for the hydrolysis of $GbOse_5$ was 3.7 mM and the V_{max} was 9.24 μmoles/min·mg. The error in the velocity measurements was approximately ±0.5 nmoles/hr.

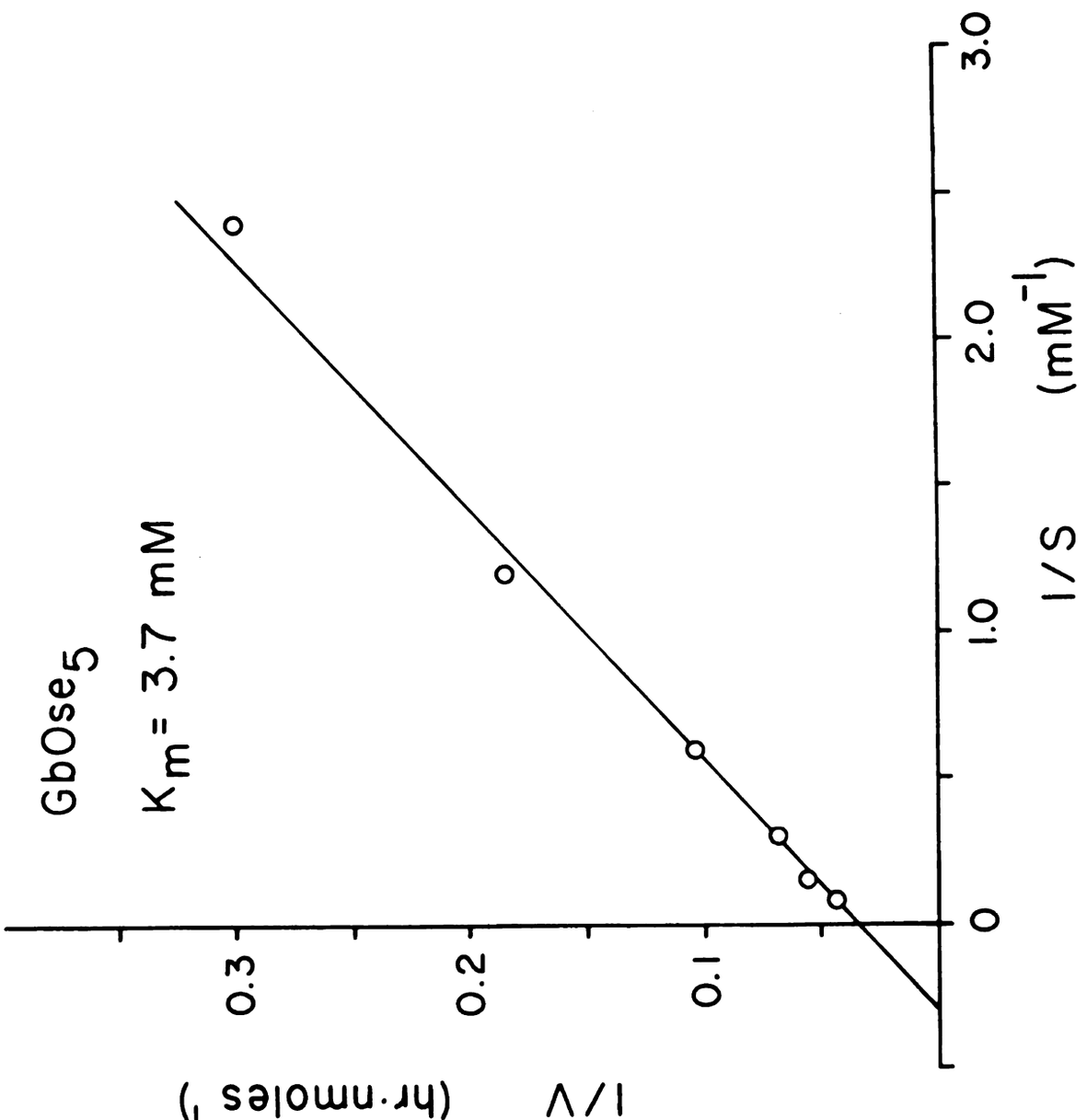

GbOse5
Km= 3.7 mM
1/V (hr·nmoles-1)
0.3
0.2
0.1
0
1.0
2.0
3.0
1/S (mM-1)

Figure 56. The Hill Plot of $GbOse_5$ Hydrolysis by α-Galactosidase B

The Hill plot of $GbOse_5$ hydrolysis was linear, with a slope of 1.0.

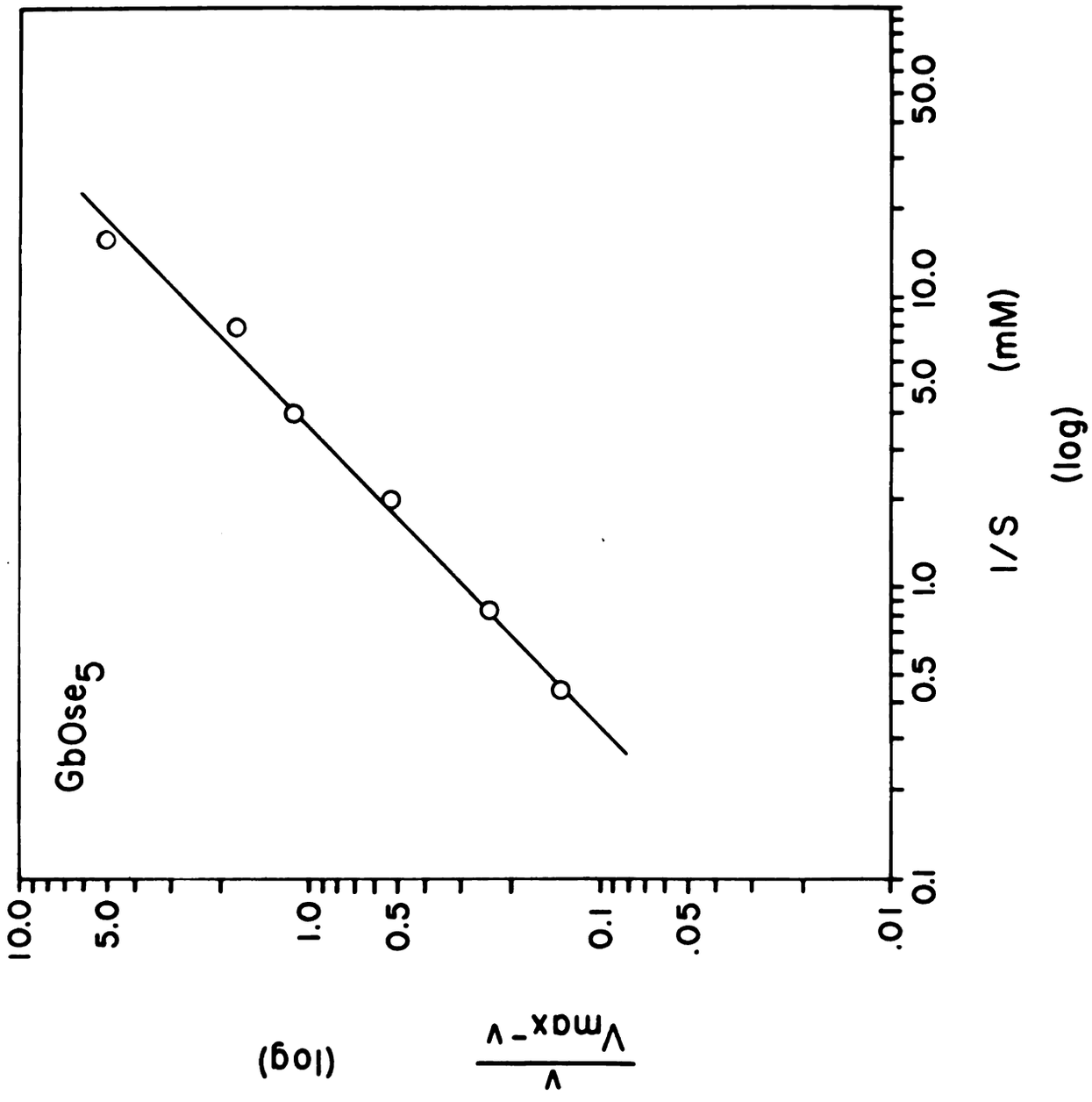

GbOse5
10.0
5.0
1.0
0.5
0.1
.05
.01
0.1
0.5
1.0
5.0
10.0
50.0
1/S (mM)
(log)
v / vmax - v
(log)

Figure 57. Lineweaver-Burk Plot of $GbOse_3$ Hydrolysis by α-Galactosidase B

The K_m for the hydrolysis of $GbOse_3$ by α-galactosidase B was 9.1 mM and the V_{max} was 4.07 μmoles/min·mg. The error in the velocity measurements was approximately ±0.5 nmoles/hr.

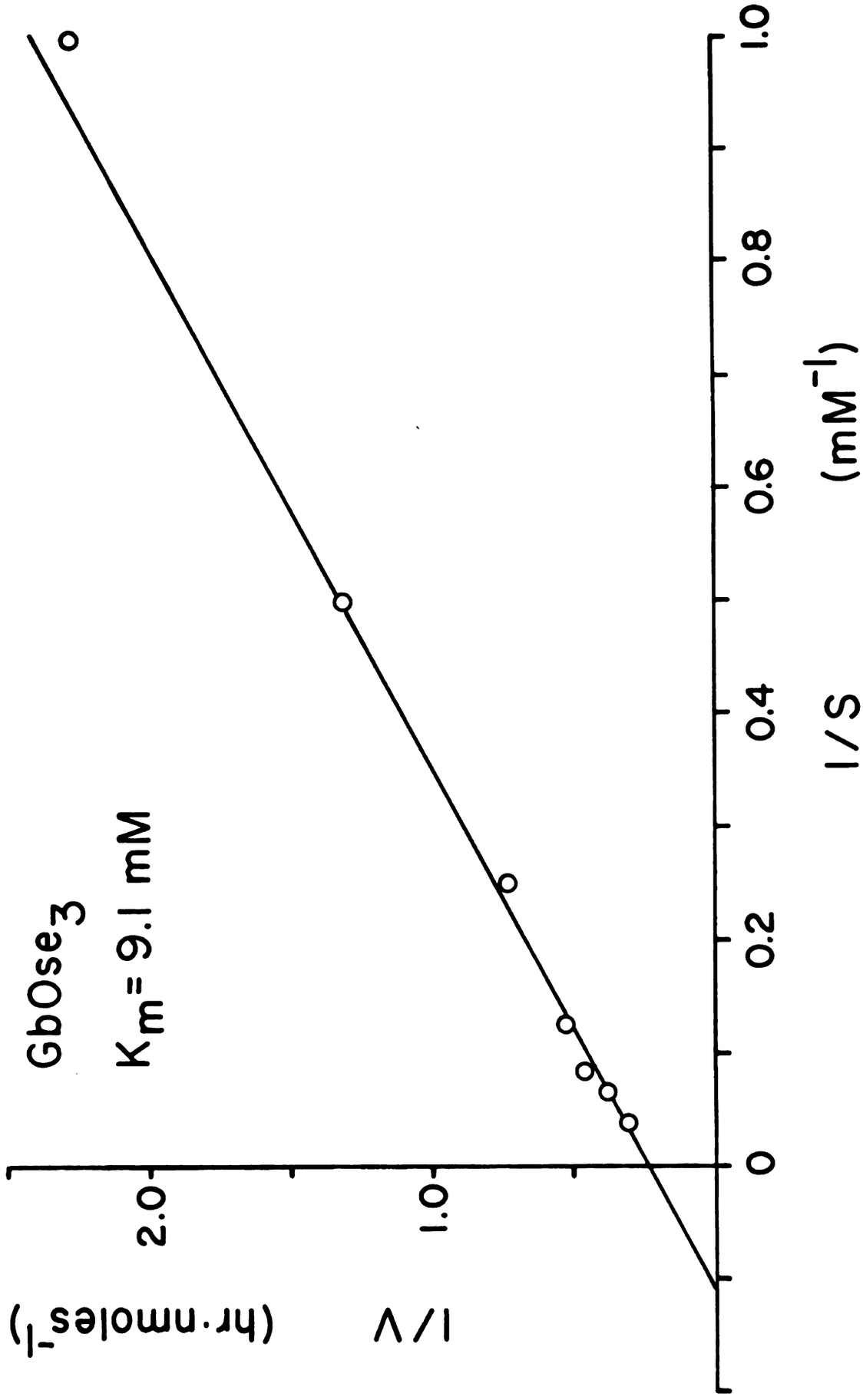
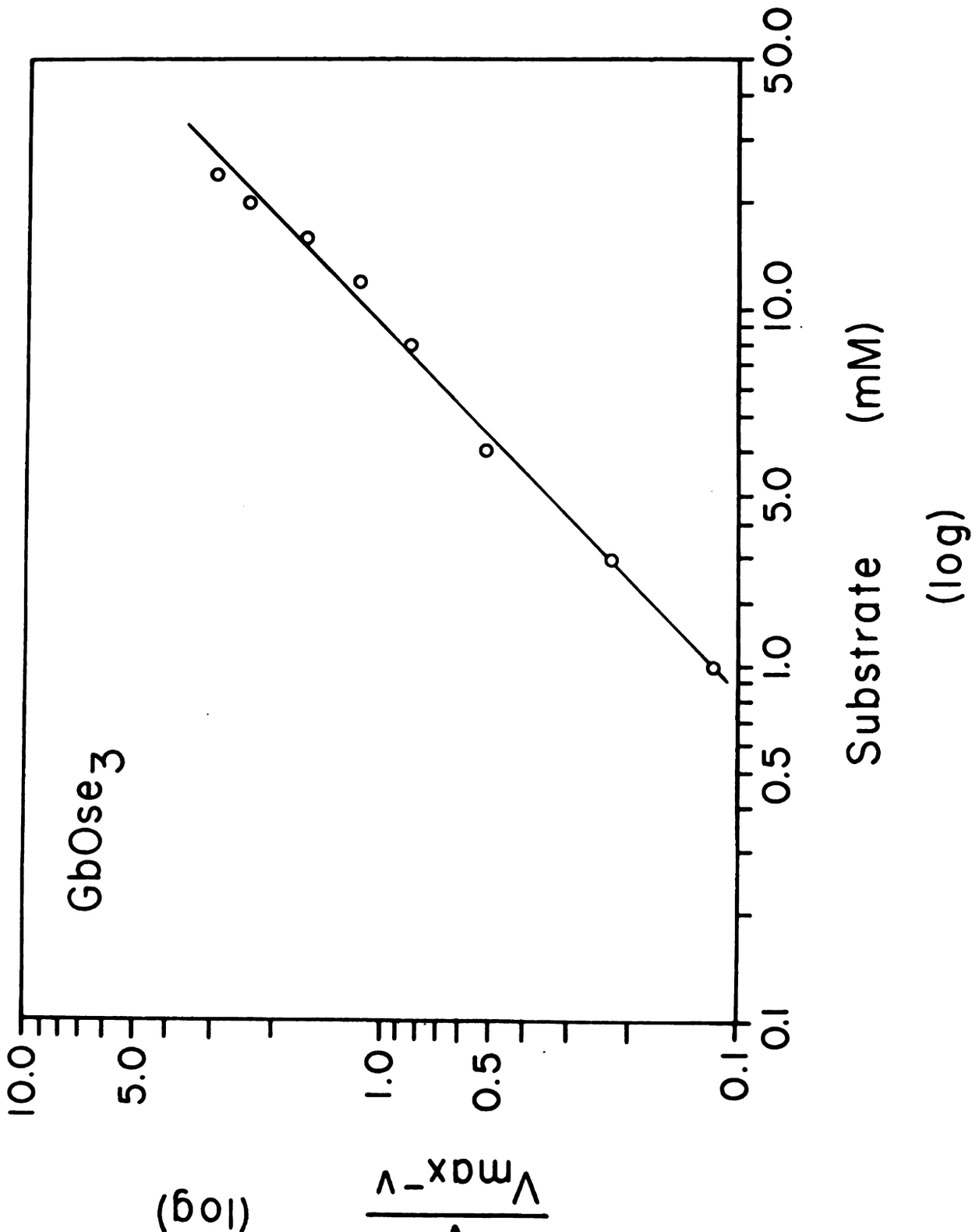

GbOse3
Km= 9.1 mM
1/V
(hr·nmoles-1)
2.0
1.0
0
0.2
0.4
0.6
0.8
1.0
1/S
(mM-1)

Figure 58. The Hill Plot of $GbOse_3$ Hydrolysis by α-Galactosidase B

The Hill plot of $GbOse_3$ hydrolysis by α-galactosidase B was linear, with a slope of 1.0.


GbOse3
10.0
5.0
1.0
0.5
0.1
0.1
0.5
1.0
5.0
10.0
50.0
Substrate (mM)
(log)
v / Vmax-v
(log)

V_{max} was 4.07 μmoles/min·mg. The Hill plot of $GbOse_3$ hydrolysis was linear, with a slope of 1.0 (Figure 59).

Glycolipid Substrates. The thin-layer chromatogram of [^{3}H]$GbOse_5Cer$ is shown in Figure 59. The specific activity of the [^{3}H]-labeled glycolipid was 12,177 cpm/nmole. The hydrolysis of this glycolipid was linear with respect to time (up to 4 hours) and enzyme concentration (up to 100 μg with 20 minute incubations). A thin-layer chromatogram of $GbOse_5Cer$ hydrolysis by α-galactosidase B is shown in Figure 60. The hydrolysis of this glycolipid was optimal at pH 3.9 (Figure 61) with 7.4 mM sodium taurocholate (Figure 62). The Lineweaver-Burk plot of $GbOse_5Cer$ hydrolysis was sigmoidal, resembling the plot of $GbOse_3Cer$ hydrolysis by α-galactosidase A. The K_m with $GbOse_5Cer$ was 0.59 mM (Figure 63) and the V_{max} was 0.27 μmoles/min·mg. The Hill plot of this activity also deviated from linearity (Figure 64), but approximated a line with a slope of 1.0.

α-Galactosidase B also catalyzed the hydrolysis of $GbOse_3Cer$, however prolonged incubation periods were required to detect activity with this substrate. The hydrolysis of $GbOse_3Cer$ was linear with respect to time (up to 10 hours) and enzyme concentration (up to 180 μg). The hydrolysis of this substrate was optimal at pH 4.3 (Figure 61) with 9.3 mM sodium taurocholate (Figure 62). Unlike the Lineweaver-Burk plots of the hydrolysis of other glycolipids by α-galactosidases A and B, the Lineweaver-Burk plot of $GbOse_3Cer$ hydrolysis was linear (Figure 65). The K_m with $GbOse_3Cer$ was 0.35 mM and the V_{max} was 0.18 μmoles/min·mg. The Hill plot was linear, with a slope of 1.1 (Figure 66).

The enzymatic properties of α-galactosidases A and B are summarized in Table 2.

III. Characterization of α-Galactosidases A and B

The molecular weights of α-galactosidases A and B were estimated by gel filtration on Sephadex G-150, as shown in Figure 67. The molecular weight of α-galactosidase A was approximately 104,000 daltons and α-galactosidase B (α-*N*-acetylgalactosaminidase) was 90,000 daltons. The isoelectric point of α-galactosidase A was 4.7 (Figure 68), and α-galactosidase B was 4.5, as previously shown (Figure 45).

Figure 59. Thin-Layer Chromatogram of [^{3}H]Globopentaglycosylceramide

The thin-layer chromatogram of [^{3}H]globopentaglycosylceramide was obtained on a silica gel G thin-layer plate. Canine intestinal neutral glycolipids were used as a standard. The radioactive glycolipid was located by scanning with a Varian Aerograph Berthold Radio Scanner, and had a specific activity of 12,177 cpm/nmole. The glycolipids were visualized with orcinol.

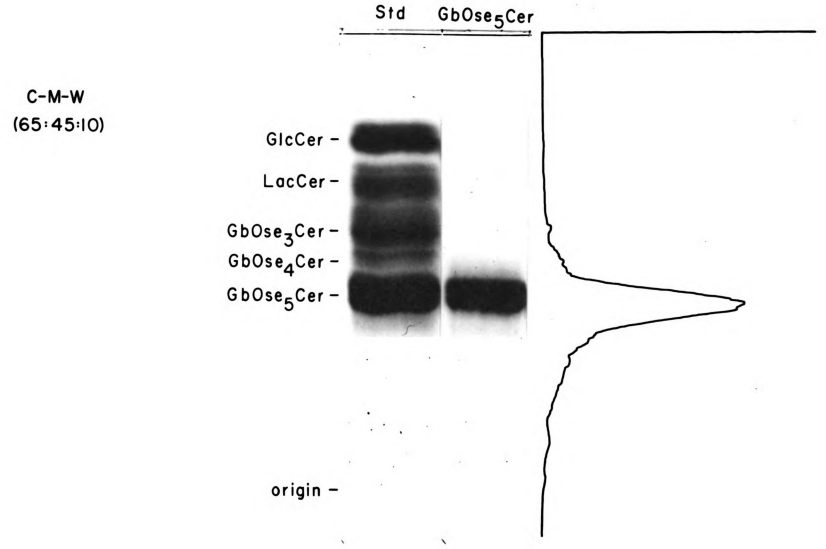

Std
GbOse5Cer
C-M-W
(65:45:10)
GlcCer –
LacCer –
GbOse3Cer –
GbOse4Cer –
GbOse5Cer –
origin –

Figure 60. Thin-Layer Chromatogram of $GbOse_5Cer$ Hydrolysis by α-Galactosidase B

The hydrolysis of $GbOse_5Cer$ by α-galactosidase B was examined by thin-layer chromatography. Canine intestinal neutral glycolipids were used as a standard. The standard contained glucosylceramide (GL-1), lactosylceramide (GL-2), globotriglycosylceramide (GL-3), globotetraglycosylceramide (GL-4) and globopentaglycosylceramide (GL-5).

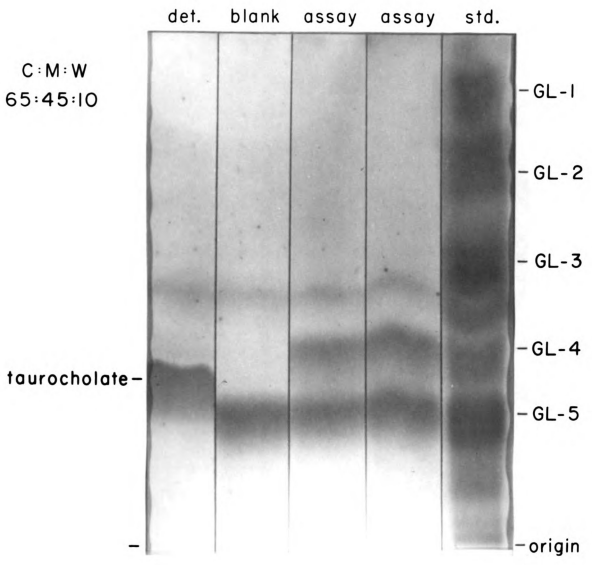

det.
blank
assay
assay
std.
C:M:W
65:45:10
GL-1
GL-2
GL-3
GL-4
GL-5
taurocholate
origin

Figure 61. The pH Optima for the Hydrolysis of $GbOse_3Cer$ and $GbOse_5Cer$ by α-Galactosidase B

The pH optima for the hydrolysis of $GbOse_3Cer$ and $GbOse_5Cer$ were pH 4.3 and pH 3.9, respectively. The reaction mixtures with $GbOse_3Cer$ contained 9.3 mM sodium taurocholate and Gomori citrate-phosphate buffers (275). The reaction mixtures with $GbOse_5Cer$ contained 7.4 mM sodium taurocholate and 50 mM sodium citrate buffers.

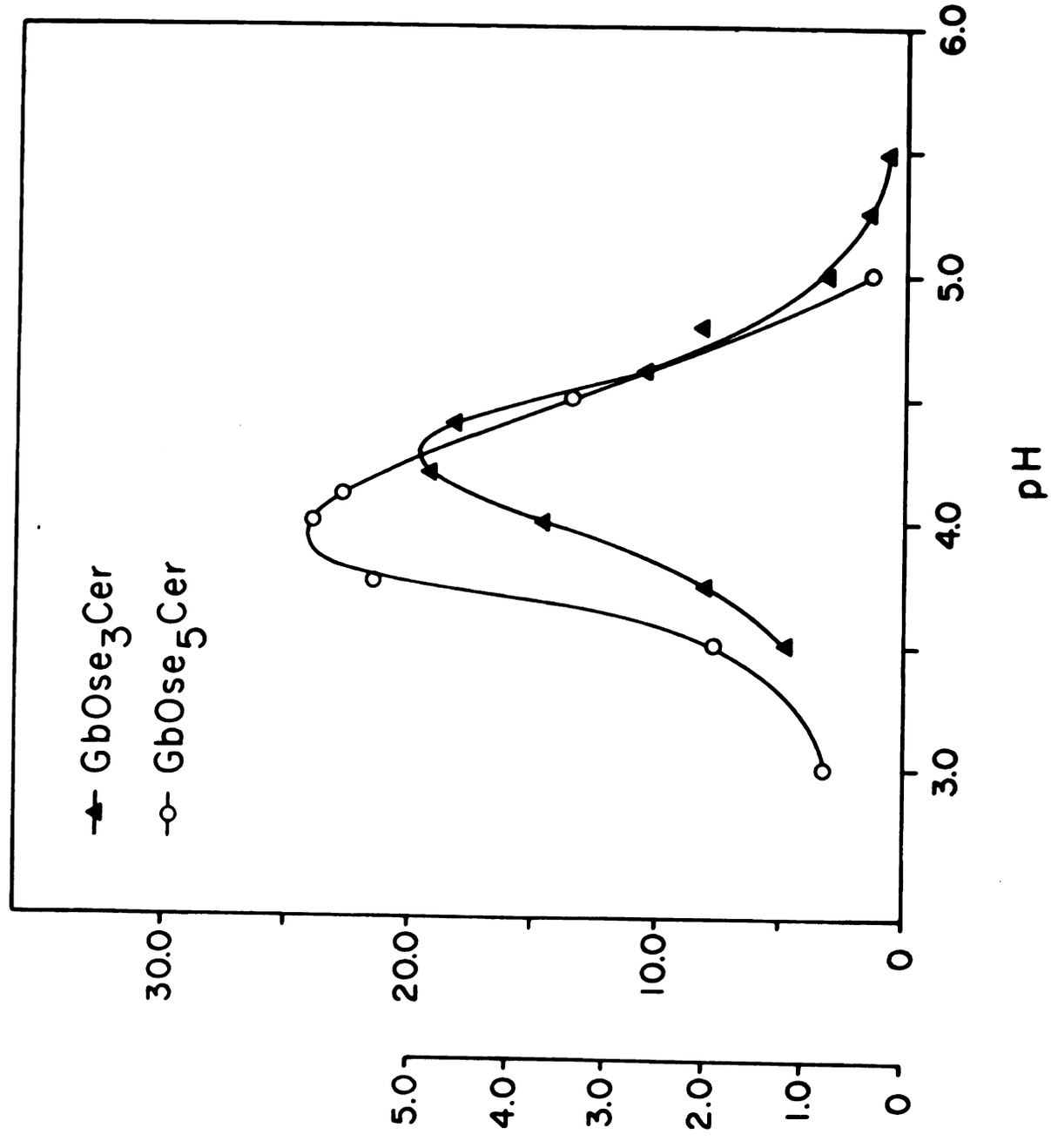

GbOse3Cer
GbOse5Cer
30.0
20.0
10.0
0
5.0
4.0
3.0
2.0
1.0
0
3.0
4.0
5.0
6.0
pH

Figure 62. The Sodium Taurocholate Optima for the Hydrolysis of $GbOse_3Cer$ and $GbOse_5Cer$ by α-Galactosidase B

The hydrolysis of $GbOse_3Cer$ and $GbOse_5Cer$ were optimal with 9.3 mM and 7.4 mM sodium taurocholate, respectively. The reaction mixtures with $GbOse_3Cer$ contained Gomori citrate-phosphate buffer, pH 4.3 (275). The reaction mixtures with $GbOse_5Cer$ contained 50 mM sodium citrate, pH 3.9.

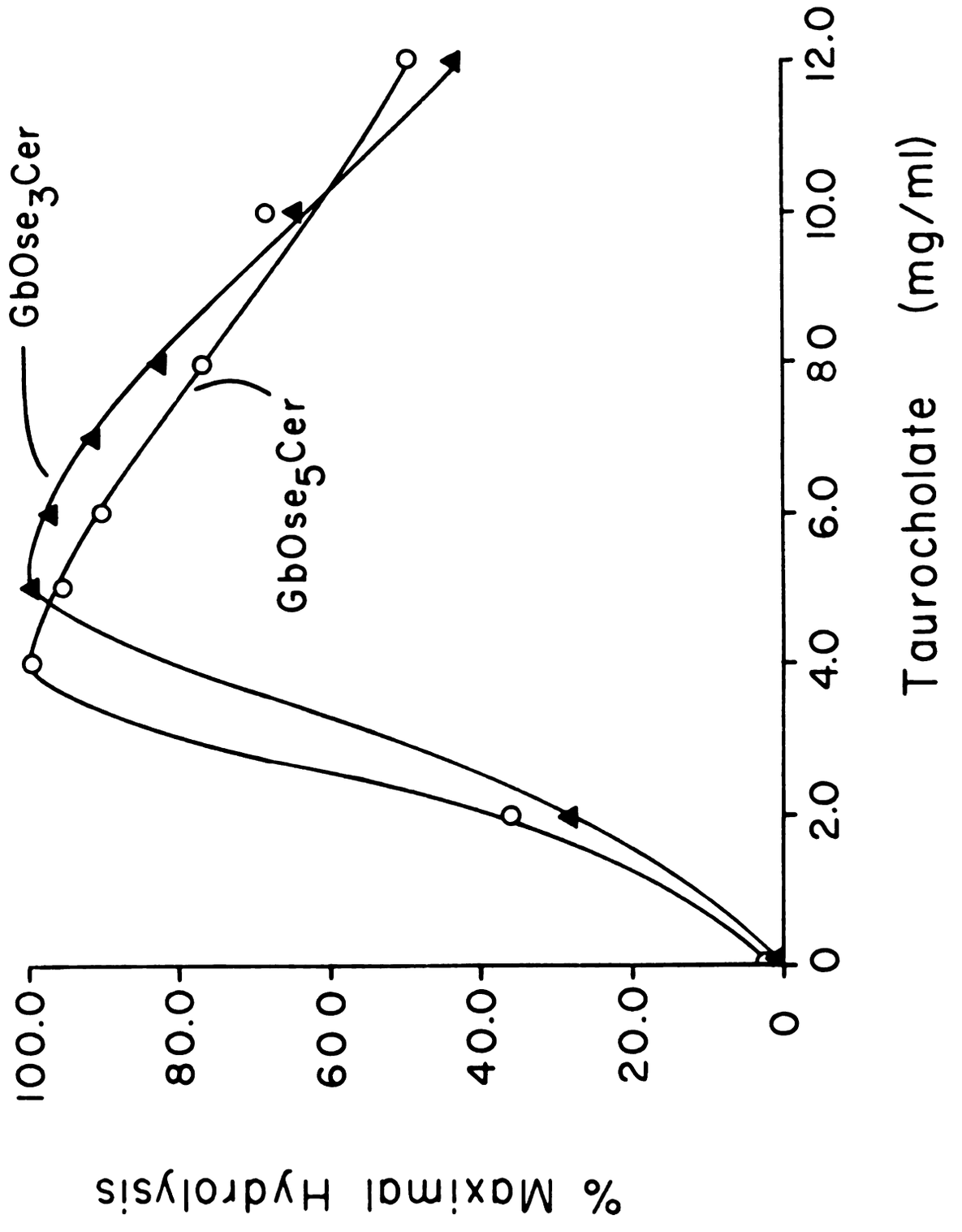

% Maximal Hydrolysis
100.0
80.0
60.0
40.0
20.0
0
0
2.0
4.0
6.0
8.0
10.0
12.0
Taurocholate (mg/ml)
GbOse3Cer
GbOse5Cer

Figure 63. Lineweaver-Burk Plot of the Hydrolysis of $GbOse_5Cer$ by α-Galactosidase B

The Lineweaver-Burk plot of $GbOse_5Cer$ hydrolysis by α-galactosidase B was sigmoidal, with an apparent K_m of 0.59 mM and an apparent V_{max} of 0.27 μmoles/min·mg. The error in the velocity measurements was approximately ±0.5 nmoles/hr.

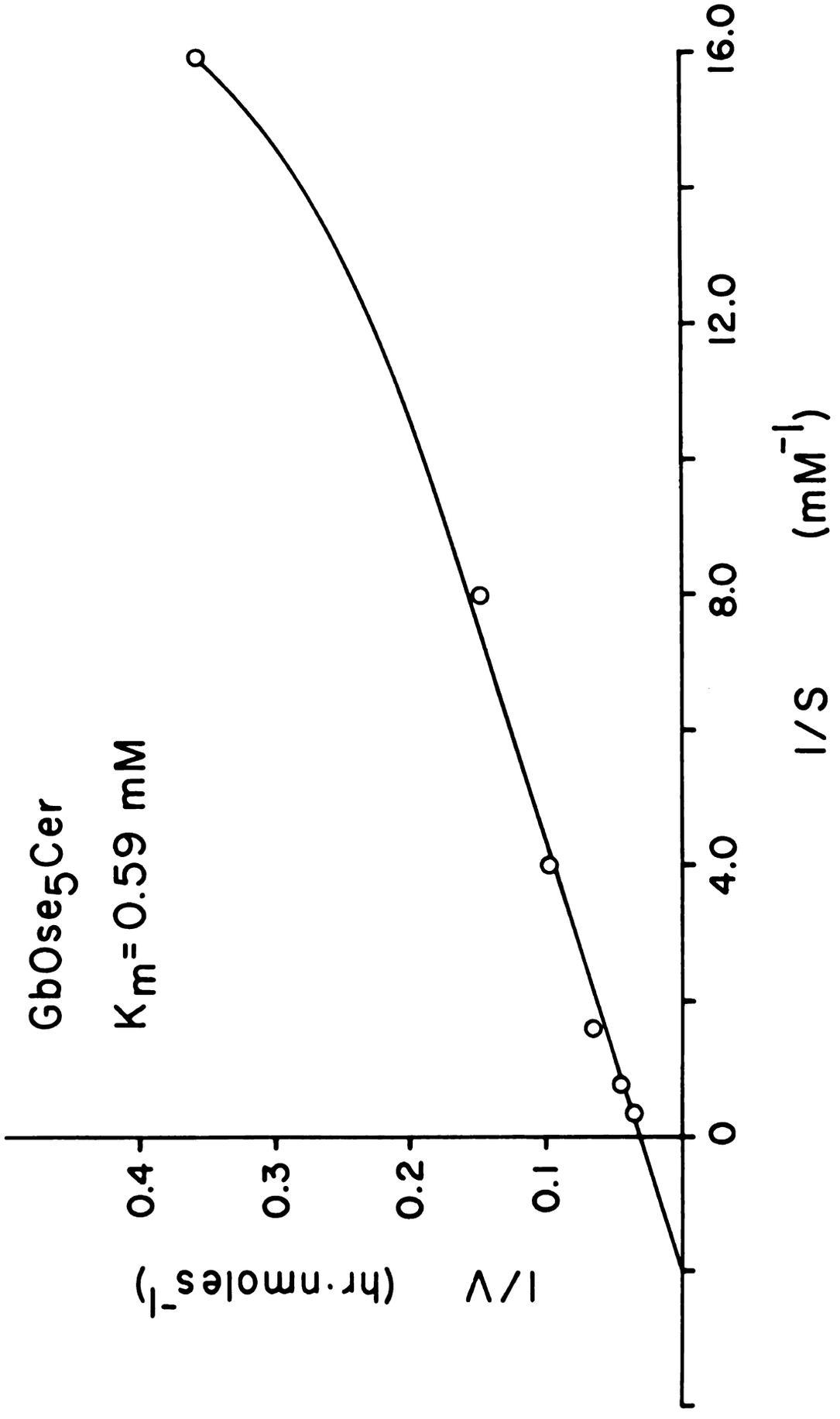

GbOse5Cer
Km= 0.59 mM
1/V (hr·nmoles-1)
0.4
0.3
0.2
0.1
0
4.0
8.0
12.0
16.0
1/S (mM-1)

Figure 64. The Hill Plot of $GbOse_5Cer$ Hydrolysis by α-Galactosidase B

The Hill plot of $GbOse_5Cer$ hydrolysis was not linear, but approximated a line with a slope of 1.0.

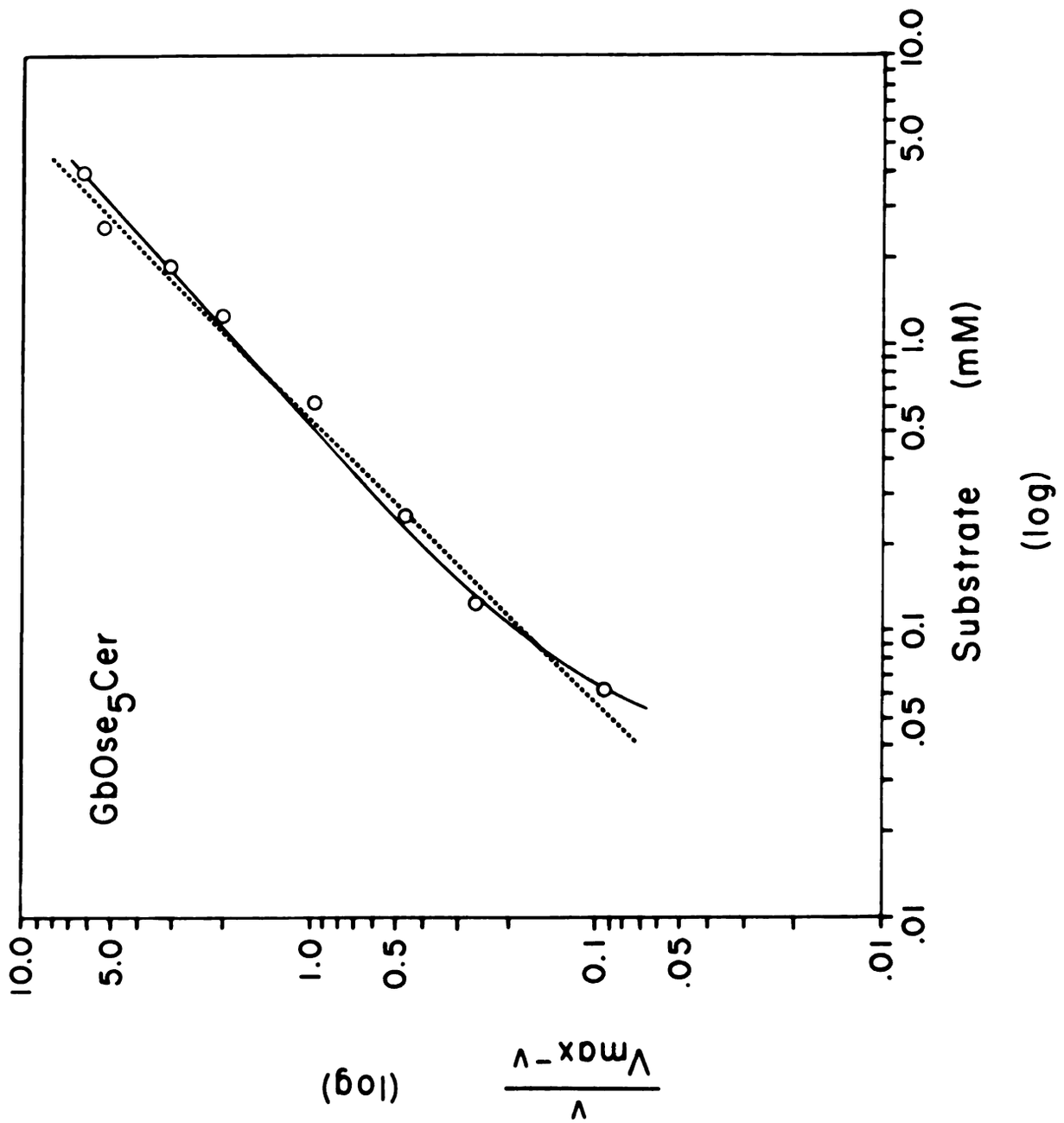

GbOse5Cer
Substrate (mM)
(log)
v / Vmax - v
(log)
10.0
5.0
1.0
0.5
0.1
0.05
.01
.05
0.1
0.5
1.0
5.0
10.0

Figure 65. Lineweaver-Burk Plot of $GbOse_3Cer$ Hydrolysis by α-Galactosidase B

The K_m for the hydrolysis of $GbOse_3Cer$ by α-galactosidase B was 0.35 mM and the V_{max} was 0.18 μmoles/min·mg. The error in the velocity measurements was approximately ±0.5 nmoles/hr.

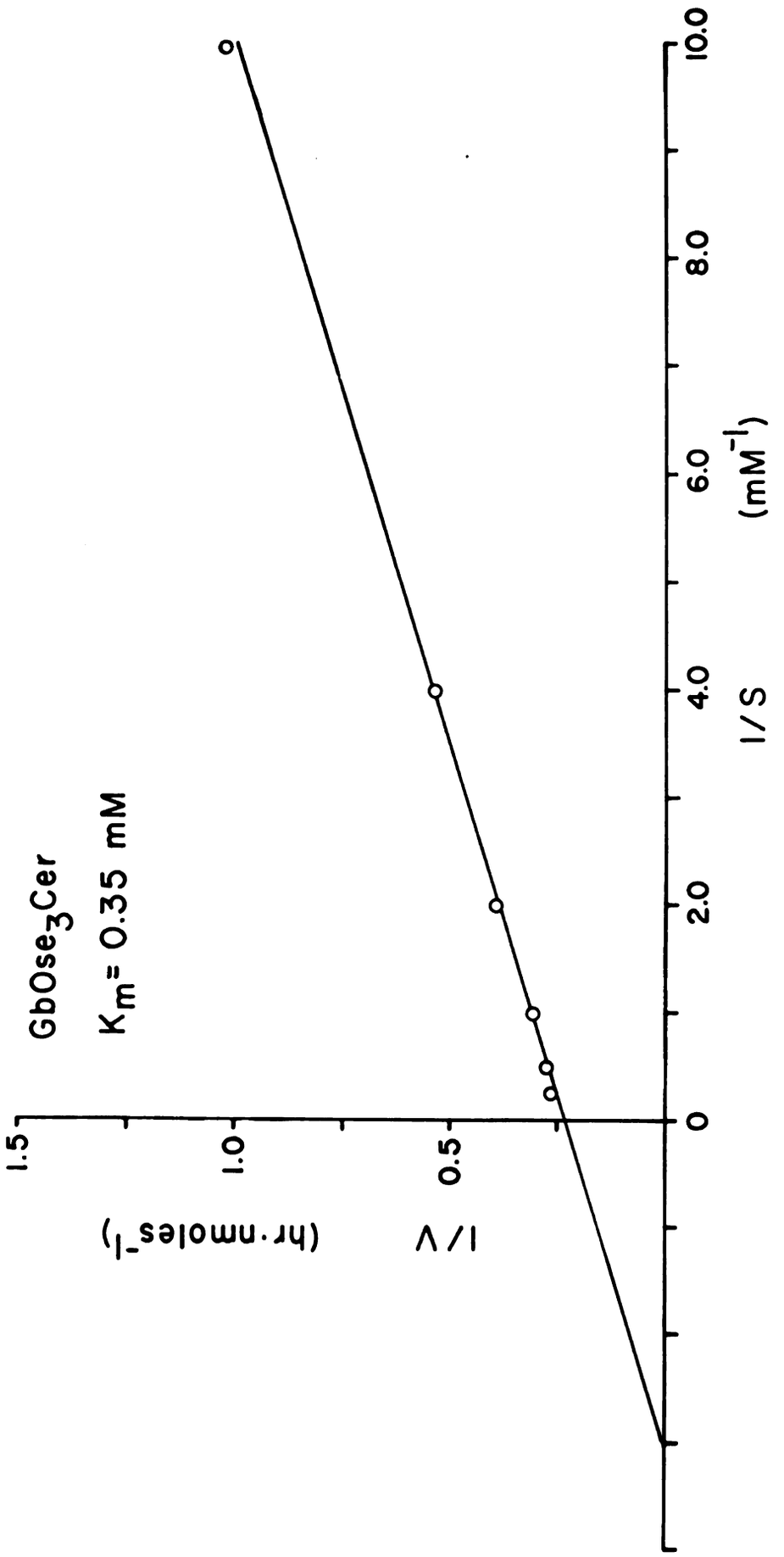

GbOse3Cer
Km= 0.35 mM
1/V
(hr.nmoles-1)
1/S
(mM-1)
1.5
1.0
0.5
0
2.0
4.0
6.0
8.0
10.0

Figure 66. The Hill Plot of $GbOse_3Cer$ Hydrolysis by α-Galactosidase B

The Hill plot of $GbOse_3Cer$ hydrolysis was linear, with a slope of 1.1.

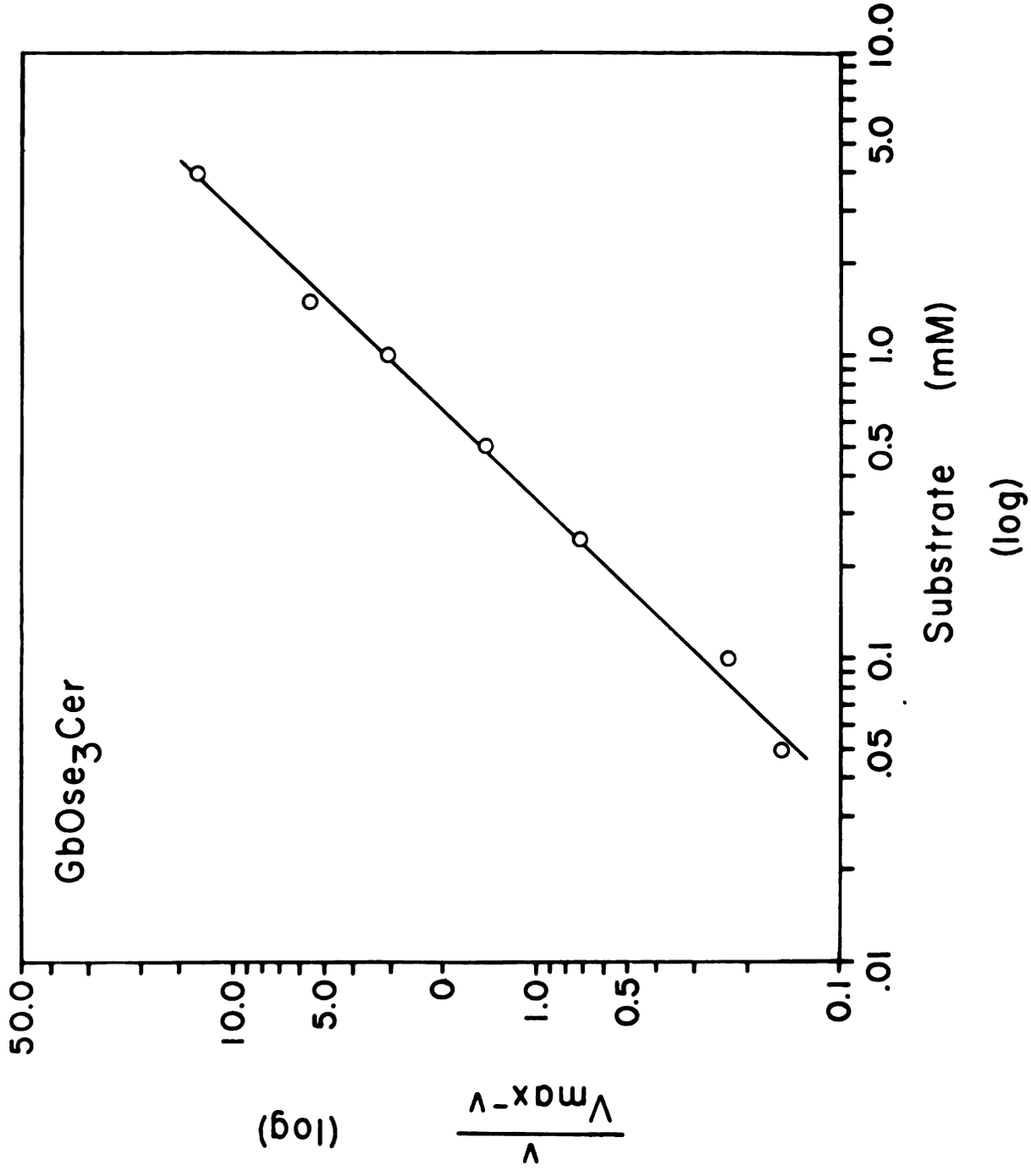

GbOse3Cer
50.0
10.0
5.0
0
1.0
0.5
0.1
.01
.05
0.1
0.5
1.0
5.0
10.0
Substrate (mM)
(log)
v / Vmax-v
(log)

Table 2. Summary of the Enzymatic Properties of α-Galactosidases A and B

	α-Galactosidase A			α-Galactosidase B		
substrate	pH optimum	K_m (mM)	V^*_{max}	pH optimum	K_m (mM)	V^*_{max}
$GaOse_2Cer$	4.1	0.28	1.09	ND[1]	ND	ND
$GbOse_3Cer$	4.1	0.18	1.16	4.3	0.35	0.18
$GbOse_5Cer$	NH[2]	NH[2]	NH[2]	3.9	0.59	0.27
$GaOse_2$	4.5	5.5	15.76	ND	ND	ND
$GbOse_3$	4.5	4.1	13.76	4.8	9.1	4.07
$GbOse_5$	NH[3]	NH[3]	NH[3]	4.4	3.7	9.24
4-MU-α-Gal	4.6	2.9	28.68	4.7	6.8	18.73
o-NP-α-GalNAc	NH[4]	NH[4]	NH[4]	4.3	1.3	59.95
p-NP-2-deoxy-α-Gal	4.6	8.2	37.40	4.7	14.7	215.70
o-NP-α-D-Fuc	NH[5]	NH[5]	NH[5]	4.7	8.8	0.16

* μmoles/min·mg [1] not determined [2] hydrolysis not detected with 2.0 mM $GbOse_5Cer$ after 3 hours at 37°C [3] hydrolysis not detected with 5.0 mM $GbOse_5$ after 2 hours at 37°C [4] hydrolysis not detected with 10.0 mM o-NP-α-GalNAc after 1 hour at 37°C [5] hydrolysis not detected with 10.0 mM o-NP-α-D-Fuc after 1 hour at 37°C

Figure 67. Estimation of the Molecular Weights of α-Galactosidases A and B by Gel Filtration

The molecular weights of α-galactosidases A and B were estimated by gel filtration on Sephadex G-150 (3.0 x 110 cm column).

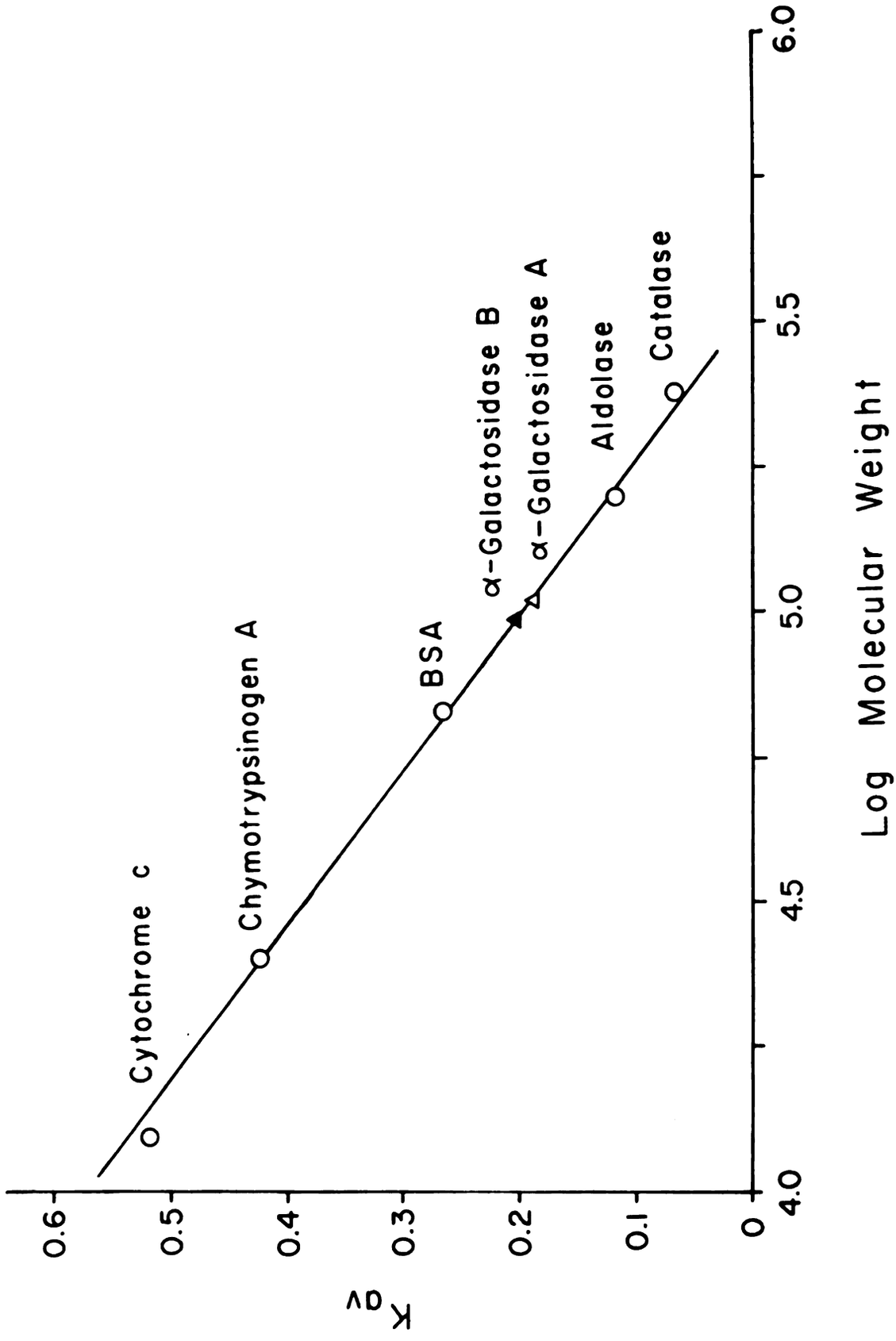
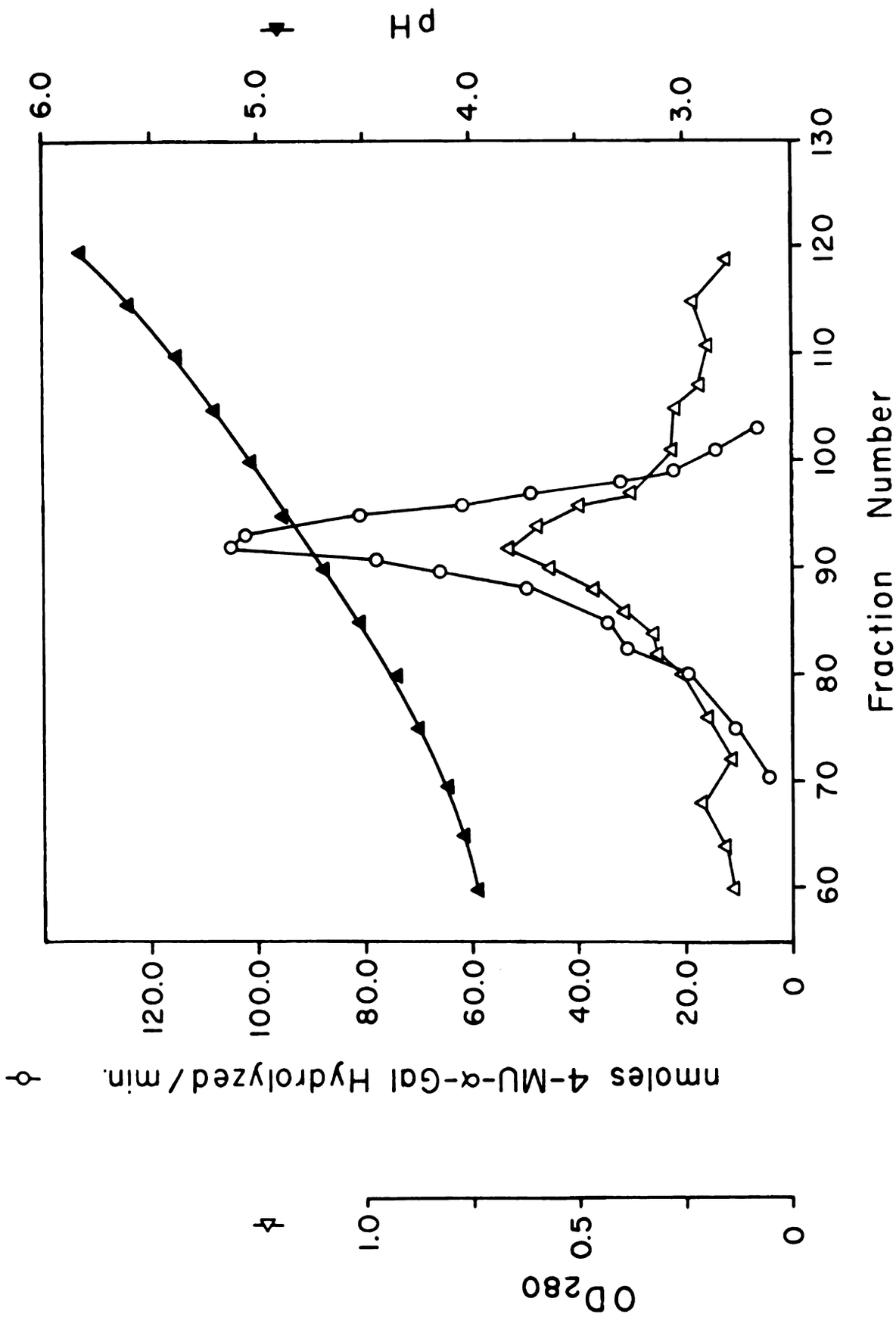

Cytochrome c
Chymotrypsinogen A
BSA
α-Galactosidase B
α-Galactosidase A
Aldolase
Catalase
0.6
0.5
0.4
0.3
0.2
0.1
0
4.0
4.5
5.0
5.5
6.0
K_{av}
Log Molecular Weight

Figure 68. Isoelectric Focusing of Purified α-Galactosidase A

Isoelectric focusing of purified α-galactosidase A was performed in an LKB 8101 isoelectric focusing column with LKB carrier ampholytes, pH 3-5, as described by Vesterberg (279). α-Galactosidase A had pI = 4.7.


OD280
1.0
0.5
0
nmoles 4-MU-α-Gal Hydrolyzed / min.
120.0
100.0
80.0
60.0
40.0
20.0
0
pH
6.0
5.0
4.0
3.0
60
70
80
90
100
110
120
130
Fraction Number

DISCUSSION

The salient results of the investigations described in this thesis are: (i) the purification of α-galactosidases A and B to higher specific activities than previously reported, (ii) the characterization of the enzymatic properties of α-galactosidase A with several artificial and natural substrates, and the effect of nonionic and anionic detergents on the hydrolysis of these substrates, (iii) the identification of α-galactosidase B as an α-N-acetylgalactosaminidase, and the characterization of the enzymatic properties of this enzyme with several artificial and natural substrates and (iv) the preparation of two novel artificial substrate analogs, p-nitrophenyl-2-deoxy-α-D-galactopyranoside and o-nitrophenyl-6-deoxy-α-D-galactopyranoside (o-nitrophenyl-α-D-fucopyranoside), and the use of these substrate analogs to investigate the carbohydrate-binding specificities of α-galactosidases A and B. These findings are discussed in greater detail below.

I. Purification of α-Galactosidases A and B

The purification of α-galactosidases A and B described here achieved higher specific activities with 4-MU-α-Gal than values previously reported by Romeo *et al.* (211) (2,867 nmoles/min·mg and 246 nmoles/min·mg for α-galactosidases A and B, respectively), Mayes and Beutler (222) (8,500 nmoles/min·mg for α-galactosidase A) and Kusiak *et al.* (223) (4,683 nmoles/min·mg and 4,517 nmoles/min·mg for α-galactosidases A and B, respectively). The success of this purification is primarily due to the use of ampholyte displacement chromatography. This procedure was first described by Leaback and Robinson (82) for the purification of isozymes of β-N-acetylhexosaminidase B. These workers reported that ampholyte displacement chromatography gave greater resolution of β-N-acetylhexosaminidase B isozymes than did isoelectric focusing, and could be carried out in only a few hours, in contrast with the periods of several days often required for isoelectric focusing. Mayes and Beutler (222) and Kusiak *et al.* (223) employed Concanavalin A Sepharose chromatography in their purifications of α-galactosidases. This step was not included in the purification scheme described here,

because of the poor recoveries of glycosidases from Con-A sepharose that were reported when this support first became available (77). However, the recoveries of glycosidases from Con-A sepharose chromatography that were reported by Mayes and Beutler (222) and Kusiak et al. (223) are acceptable, and this may be a useful step to employ in future purifications of these enzymes. Octyl-Sepharose chromatography (39) may also be a useful step to employ in future purifications of these enzymes.

II. Characterization of the Enzymatic Properties of α-Galactosidases A and B

A. α-Galactosidase A

α-Galactosidase A catalyzed the hydrolysis of several artificial and natural substrates, including 4-MU-α-Gal, $GaOse_2Cer$, $GbOse_3Cer$, $GaOse_2$, $GbOse_3$ and p-NP-2-deoxy-α-Gal. The glycolipids were not freely soluble in the aqueous reactions mixtures, and the anionic detergent sodium taurocholate was used to solubilize them as glycolipid-taurocholate mixed micelles (283). The hydrolysis of the water-soluble artificial substrate 4-MU-α-Gal was optimal at pH 4.6, however the pH optimum for the hydrolysis of the glycolipid substrates was lower, at pH 4.1. Furthermore, the Michaelis constants and maximal velocities with these substrates were approximately one order of magnitude smaller than those observed with 4-MU-α-Gal (Table 2).

The differences in the pH optima, Michaelis constants and maximal velocities with 4-MU-α-Gal and the glycolipid substrates were investigated. The hydrolysis of the water-soluble oligosaccharides derived from $GaOse_2Cer$ and $GbOse_3Cer$ by ozonolysis and treatment with base was examined, and the hydrolysis of 4-MU-α-Gal was examined in the presence of sodium taurocholate and Triton X-100. The hydrolysis of $GaOse_2$ and $GbOse_3$ was optimal at pH 4.5, corresponding with the pH optimum for the hydrolysis of 4-MU-α-Gal. Similarly, the Michaelis constants with these oligosaccharides approximated the Michaelis constant with 4-MU-α-Gal, however the maximal velocities with $GaOse_2$ and $GbOse_3$ were approximately 50% of that observed with 4-MU-α-Gal. This difference probably reflects the relative lability of the glycosidic bond of the artificial substrate due to the aromatic group it is linked to, rather than a difference in the catalytic ability of the enzyme with these different substrates. The differences in pH optima and kinetics with water-soluble substrates and glycolipid substrates, therefore, did not appear to

be related to any differences in the interaction of the enzyme with the carbohydrate portion of these molecules.

Because the differences in the pH optima and kinetics of the hydrolysis of water-soluble and glycolipid substrates did not appear to be related to the carbohydrate portion of the molecules, the effect of detergents on the hydrolysis of 4-MU-α-Gal was investigated. The nonionic detergent Triton X-100 had no effect on the pH optimum for the hydrolysis of 4-MU-α-Gal. However, sodium taurocholate, at the same concentration used to solubilize the glycolipid substrates, had a profound effect on both the pH optimum and kinetics of 4-MU-α-Gal hydrolysis. Sodium taurocholate inhibited the hydrolysis of 4-MU-α-Gal below pH 4.8 and produced hyperbolic Lineweaver-Burk plots for the hydrolysis of this substrate below pH 4.8. The observation that the hyperbolic character of the Lineweaver-Burk plots diminished as the pH increased, suggests that the effect of sodium taurocholate on the hydrolysis of 4-MU-α-Gal may be due to an electrostatic interaction between sodium taurocholate and α-galactosidase A. This hypothesis is supported by the finding that α-galactosidase A has pI = 4.7. Below pH 4.7 the enzyme would bear a positive charge, taurocholate would be negatively charged (pK = 1.4 (284)), and there would be a strong electrostatic interaction. Above pH 4.7 the enzyme and the detergent would both bear negative charges and their interaction would be minimal.

In view of the fact that 4-MU-α-Gal is water-soluble, its hydrolysis by α-galactosidase A is not dependent on the interaction of the enzyme with taurocholate-substrate mixed micelles. Indeed, the hydrolysis of 4-MU-α-Gal is inhibited by sodium taurocholate at low pH. In contrast, the hydrolysis of glycolipids should be dependent on the interaction of α-galactosidase A with glycolipid-taurocholate mixed micelles. This hypothesis is supported by the lower pH optimum observed with the glycolipid substrates, and the lower apparent K_m, which probably reflects the strong electrostatic interaction between the taurocholate micelles and the enzyme, rather than the affinity of α-galactosidase A for the substrate. The lower maximal velocity observed with the glycolipid substrates may be due to allosteric interactions with the detergent, or the low pH of the reaction mixture.

The Lineweaver-Burk plots with the water-soluble substrates (4-MU-α-Gal, $GaOse_2$ and $GbOse_3$) were linear, conforming to typical Michaelis-Menton kinetics. The Hill plots with these substrates were also

linear, with slopes of ∿1.0, suggesting that there is no cooperativity in the binding of the substrate with the enzyme. In contrast, the Lineweaver-Burk plots with $GaOse_2Cer$ and $GbOse_3Cer$ were sigmoidal, as described by Gatt et al. (283) for enzymes that utilize micellar lipid substrates. The Hill plots with these glycolipids also deviated from linearity, suggesting that the detergent had an allosteric effect on the interaction of the enzyme with the substrate (285). Fung and Sweeley (286) have suggested that monomeric, rather than micellar taurocholate may be involved in these allosteric interactions.

Although it may be argued that the use of detergents to solubilize glycolipids in aqueous solutions is quite an unnatural environment for the enzyme to encounter its substrate, it should be pointed out that, in vivo, glycolipids are components of membranes which may be negatively charged, and electrostatic interactions between the enzymes of glycolipid metabolism and membranes may play an important role in bringing the enzyme and substrate together.

Li and coworkers (212-214) have isolated a glycoprotein which they describe as an 'activator' for glycolipid hydrolases. These investigators use this 'activator' protein to replace detergents in their reaction mixtures containing glycolipids. However, this protein is nonspecific, it stimulates the hydrolysis of both neutral glycolipids and gangliosides by their respective hydrolases, and appears to act as a detergent. Furthermore, these investigators have not demonstrated the presence of this 'activator' protein in the lysosome, the predominant site of glycolipid catabolism. An interesting hypothesis is that this 'activator' protein may not be involved in glycolipid catabolism at all, but may serve as a carrier protein for glycolipids during glycolipid biosynthesis. As discussed in the first section of this thesis, the biosynthesis of some glycolipids and glycoproteins, such as the blood group-active glycolipids and glycoproteins, may be carried out by a common pathway. This 'activator' protein may solubilize the glycolipids to enable then to interact with the same glycosyltransferases that carry out the biosynthesis of glycoproteins. This hypothesis has not been investigated, but poses an interesting alternative to the proposed function of the 'activator' protein.

The hydrolysis of IV^4-α-Gal-$LcnOse_4Cer$ and blood group B-active glycolipids and glycoproteins was not examined, but α-galactosidase A is probably responsible for the hydrolysis of these glycoconjugates in vivo.

The blood group B-active glycolipids accumulate in Fabry patients with this phenotype and blood group B-active glycopeptides and oligosaccharides would also be expected to accumulate in these patients. The accumulation of IV^4-α-Gal-$LcnOse_4Cer$ in Fabry patients with the P_1 phenotype has not been reported, but this glycolipid would also be expected to accumulate in these patients.

B. α-Galactosidase B

α-Galactosidase B and α-N-acetylgalactosaminidase activities co-chromatographed throughout the purification procedure. Furthermore, these hydrolase activities had coincident thermal inactivation curves and isoelectric points, and the hydrolysis of 4-MU-α-Gal was competitievly inhibited by o-NP-α-GalNAc. The K_m for the hydrolysis of the latter was approximately 5 times lower than the K_m with 4-MU-α-Gal and a V_{max} that was approximately 3 times greater than that with 4-MU-α-Gal. These findings suggest that α-galactosidase B is, in fact, an avid α-N-acetylgalactosaminidase.

This enzyme also catalyzed the hydrolysis of $GbOse_3Cer$, $GbOse_5Cer$, $GbOse_3$ and $GbOse_5$. The Michaelis constants and maximal velocities with the glycolipids were quite similar, however this was undoubtedly due to the presence of sodium taurocholate in the reaction mixtures. The Michaelis constants with the oligosaccharides derived from $GbOse_3Cer$ and $GbOse_5Cer$ approximated those observed with 4-MU-α-Gal and o-NP-α-GalNAc, respectively. The maximal velocities with these oligosaccharides were approximately 5 times lower than those observed with the artificial substrates. However, this difference probably reflects the relative lability of the glycosidic bond of the artificial substrates due to the aromatic groups that they are linked to.

The predominant physiological activity of α-galactosidase B is somewhat speculative, but glycolipids containing terminal α-galactose residues accumulate in Fabry's disease in spite of normal or even somewhat elevated levels of α-galactosidase B in these patients. It appears, therefore, that α-galactosidase B is more likely to function as an α-N-acetylgalactosaminidase rather than an α-galactosidase *in vivo*. It would therefore be more accurate to refer to it as an α-N-acetylgalactosaminidase (EC 3.2.1.49). Globopentaglycosylceramide (Forssman antigen) and blood group A-active glycolipids and glycoproteins contain terminal α-N-acetylgalactosamine

residues and are likely substrates for this enzyme.

These findings have been confirmed by Schram et al. (238), who found that antibodies prepared against α-galactosidase B precipitated both α-galactosidase B and α-N-acetylgalactosaminidase activities from a crude α-galactosidase B preparation from normal human liver. In addition, this antibody precipitated α-galactosidase activity from a crude α-galactosidase B preparation from Fabry liver (215). Purified porcine liver α-N-acetylgalactosaminidase also catalyzed the hydrolysis of 4-MU-α-Gal with a K_m that was 5 times greater than that with p-NP-α-GalNAc and a V_{max} that was more than 4 times lower than that with p-NP-α-GalNAc (60).

C. The Carbohydrate Binding Specificities of α-Galactosidases A and B

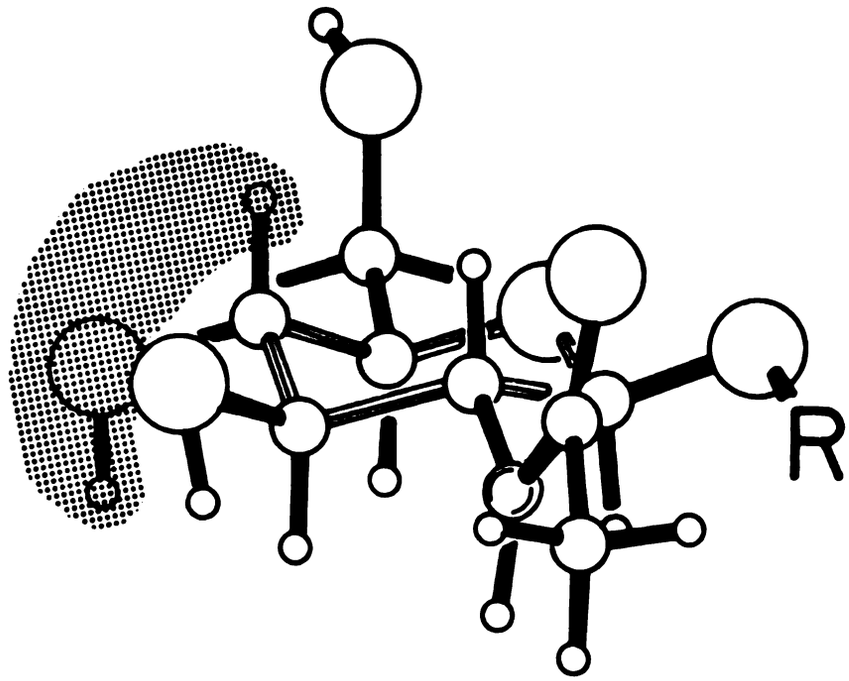
Several of the lysosomal glycosidases lack absolute specificity for the carbohydrate residues that they hydrolyze. Chester et al. (287) reported that human liver β-glucosidase also catalyzed the hydrolysis of 4-MU-β-Gal, p-NP-β-D-Fuc, 4-MU-β-Xyl and 4-MU-α-L-Ara. This suggests that β-glucosidase lacks absolute specificity for the substituent at C-5, and the conformation of the hydroxyl group (axial or equatorial) at C-4. However, all of these substrates had equatorial hydroxyl groups at C-2 and C-3, and these groups may be important in the binding of the substrate at the active site, or the mechanism of the enzymatic hydrolysis of the glycosidic bond. Wallenfels and Weil (288) have suggested that the equatorial hydroxyl group at C-2 may play a role in the mechanism of the hydrolysis catalyzed by β-galactosidase. These investigators suggested that the developing carbonium ion at C-1 may be stabilized by the formation of a 1,2-epoxide, which is subsequently hydrolyzed to yield free galactose as the product.

The β-N-acetylhexosaminidases hydrolyze the 4-methylumbelliferyl derivatives of both β-N-acetylgalactosamine and β-N-acetylglucosamine (56,57), demonstrating a lack of absolute specificity for the conformation of the hydroxyl group at C-4, as shown in Figure 69.

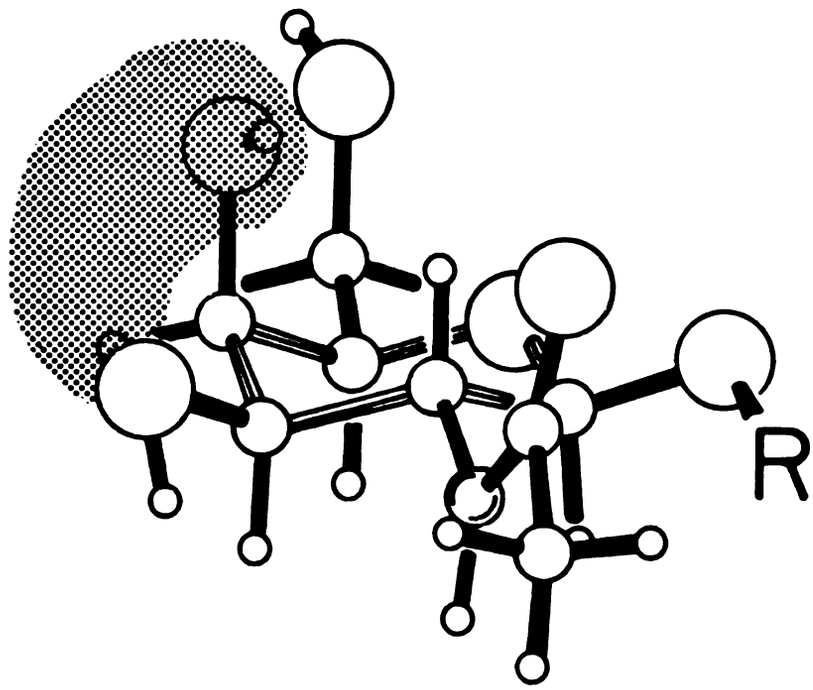
α-Galactosidase A catalyzed the hydrolysis of 4-MU-α-Gal and o-NP-2-deoxy-α-Gal, but not p-NP-α-Glc, o-NP-α-GalNAc or o-NP-α-D-Fuc. Furthermore, the hydrolysis of 4-MU-α-Gal was not competitively inhibited by o-NP-α-GalNAc, p-NP-α-Glc, D-(+)-fucose or D-galactal. These findings suggest that the hydroxyl group at C-2 does not play a significant role in the binding of the carbohydrate at the active site of the enzyme, or

Figure 69. Three-Dimensional Projections of Carbohydrate Residues Hydrolyzed by the β-N-Acetylhexosaminidases

The β-N-acetylhexosaminidases catalyze the hydrolysis of both β-N-acetylglucosamine (I) and β-N-acetylgalactosamine (II) residues. This suggests a lack of absolute specificity for the configuration of the hydroxyl group at C-4 (shaded).



I



II

in the mechanism of the enzymatic hydrolysis of these substrates (Figure 70). However, replacement of the hydroxyl group at C-2 with an acetamido group results in a loss of activity. This may be due to steric hindrance in binding the 2-acetamido-2-deoxy-substrate at the active site of the enzyme. The failure of α-galactosidase A to hydrolyze p-NP-α-Glc and o-NP-α-D-Fuc, and the failure of 4-MU-α-Gal hydrolysis to be competitively inhibited by p-NP-αGlc and D-(+)-fucose, suggest that the axial hydroxyl group at C-4 and the hydroxyl group at C-6 may be important in binding the carbohydrate at the active site of the enzyme. These findings, together with the failure of D-galactal to inhibit the hydrolysis of 4-MU-α-Gal, suggest that the mechanism of the enzymatic hydrolysis of glycosides by α-galactosidase A may proceed through a different mechanism than that proposed by Wallenfels and Weil (288) for β-galactosidase.

α-Galactosidase B (α-N-acetylgalactosaminidase) catalyzed the hydrolysis of 4-MU-α-Gal, o-NP-α-GalNAc, p-NP-2-deoxy-α-Gal and o-NP-α-D-Fuc. These findings suggest that his enzyme lacks absolute specificity for the substituents at C-2 and C-6, as shown in Figure 71. Unlike α-galactosidase A, the B form was also an α-N-acetylgalactosaminidase, although the lower pH optimum for the hydrolysis of o-NP-α-GalNAc suggests that a change in the conformation of the enzyme may be required to accommodate the bulky acetamido substituent. In addition, it has been reported that α-N-acetylgalactosaminidase also catalyzes the hydrolysis of α-N-acetyltalosamine residues (60), as shown in Figure 71.

The preparation of artificial substrate analogs that are missing specific substituents can provide insight into the carbohydrate-binding specificities and possibly the mechanism of the reactions catalyzed by the enzymes of glycoconjugate metabolism. The use of deoxy or halogenated carbohydrates may also provide insight into the carbohydrate-binding specificities of glycosyltransferases, where they could serve as dead-end carbohydrate acceptors. Therefore, these compounds might serve as inhibitors of specific steps in glycoconjugate biosynthesis or catabolism.

III. Characterization of α-Galactosidases A and B

The molecular weight of α-galactosidase A from human liver was approximately 104,000 daltons, in agreement with molecular weight estimates for α-galactosidase A by Mapes *et al.* (231) and Kusiak *et al.* (223).

Figure 70. Three-Dimensional Projection of the Carbohydrate Residue Hydrolyzed by α-Galactosidase A

α-Galactosidase A catalyzes the hydrolysis of α-galactose and 2-deoxy-α-galactose residues, suggesting a lack of absolute specificity for the hydroxyl group at C-2 (shaded).

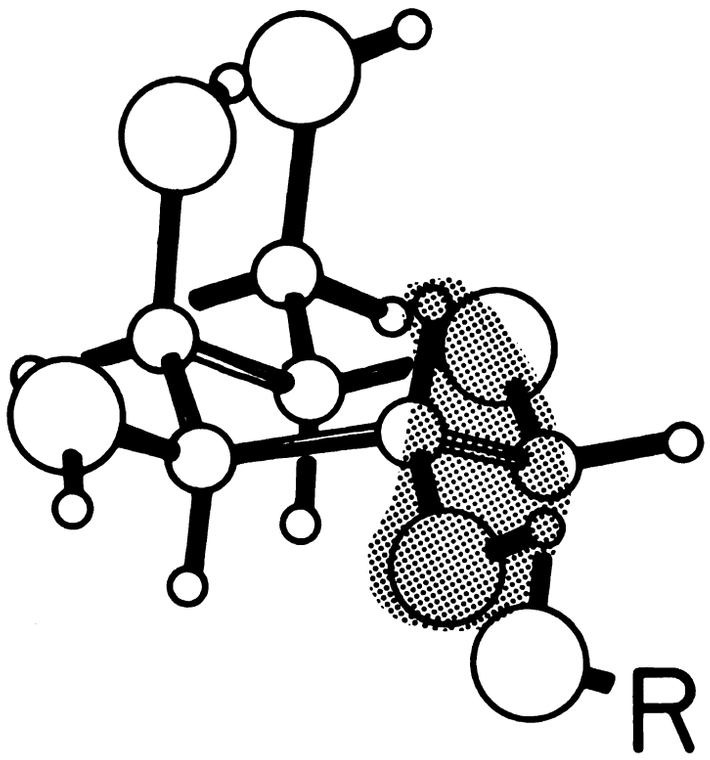
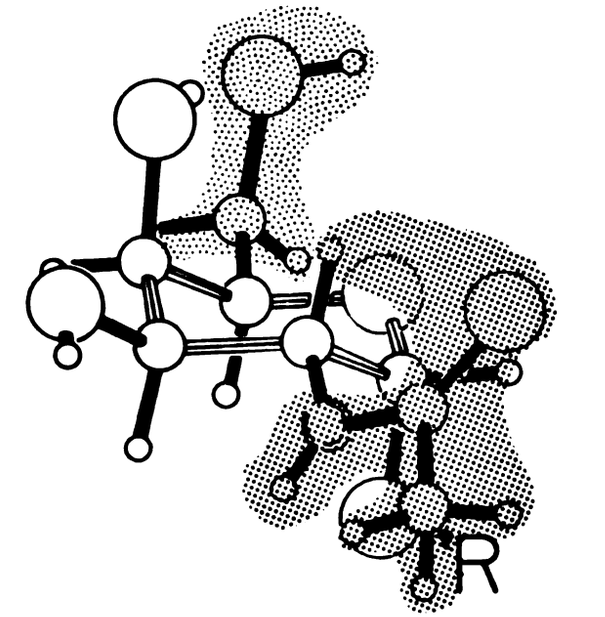
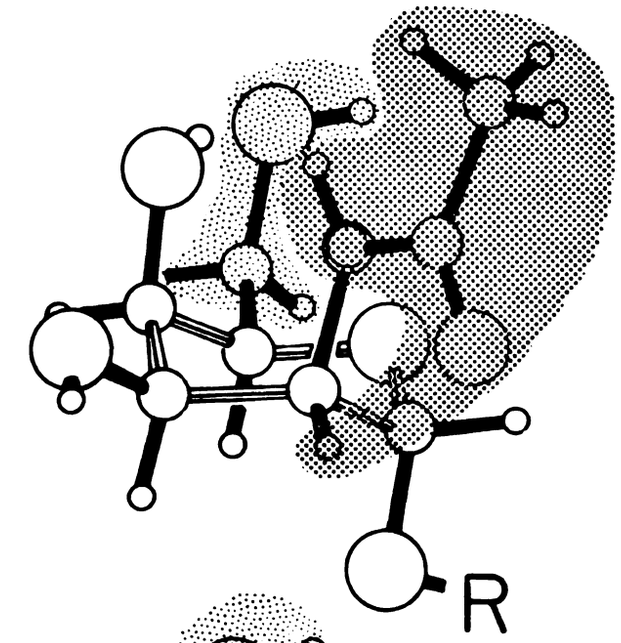

R

Figure 71. Three Dimensional Projections of Carbohydrates Hydrolyzed by α-Galactosidase B

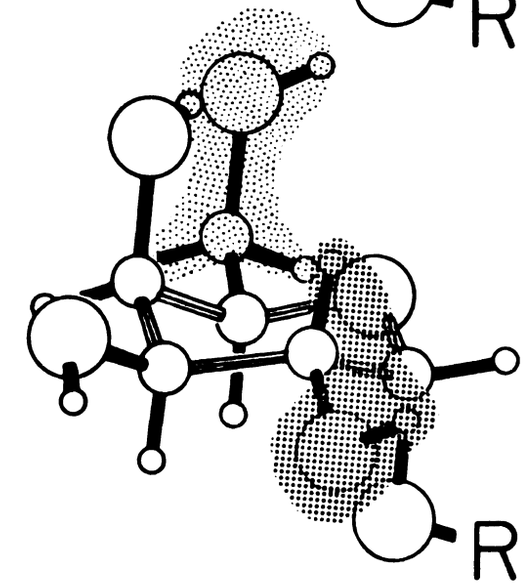
α-Galactosidase B catalyzes the hydrolysis of α-N-acetylgalactosamine residues (I), α-N-acetyltalosamine residues (II), α-galactose residues (III) and α-D-fucose residues (not shown), suggesting a lack of absolute specificity for the substituents at C-2 and C-6 (shaded).



I



II



III

Kusiak et al. (223) found that α-galactosidase A was a dimer, with subunits of 57,700 daltons. The molecular weight of the B form from human liver was 90,000 daltons, in agreement with the molecular weight determined by Kusiak et al. (223). These workers found that the B form was a dimer with subunits of 47,700 daltons.

The isoelectric points of α-galactosidases A and B were at pH 4.7 and pH 4.5, respectively, in agreement with values reported by Beutler and Kuhl (219). Bishop and Sweeley (39) reported that α-galactosidase A from normal human plasma had pI = 3.7. Treatment of this enzyme with C. perfringens neuraminidase shifted the isoelectric point to 4.3. These findings suggest that α-galactosidase A from plasma may be sialylated, with oligosaccharide substituents similar to those shown in Figure 1. However the tissue α-galactosidases, which were not susceptible to neuraminidase treatment, may a different type of oligosaccharide substituent. Sung (289) found that porcine liver α-N-acetylgalactosaminidase did not contain sialic acid, but appeared to have an oligomannose type of oligosaccharide. This finding suggests that the metabolism of tissue forms of the glycosidases may be quite different from the metabolism of secreted forms of these enzymes, such as those found in plasma. This hypothesis is supported by studies by Desnick et al. (290), who found that plasma α-galactosidase A had a much longer survival in circulation than splenic α-galactosidase A, in Fabry patients receiving these enzymes. As discussed in the first section of this thesis, the presence of terminal sialic acid residues on the carbohydrate moiety of glycoproteins prolongs their survival in circulation. However, removal of the sialic acid residues greatly reduces the survival of these glycoproteins in circulation. Henderson et al. (291) have reported the presence of a mannose-binding protein in livers of rabbits and rats. This receptor could be responsible for the rapid clearance of tissue forms of α-galactosidase A from circulation.

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APPENDIX

APPENDIX

List of Publications

Dean, KJ, Sung, S-SJ and Sweeley, CC: Purification and Partial Characterization of Human Liver α-Galactosidases: Is α-Galactosidase B an α-N-Acetylgalactosaminidase? Fed. Proc. 36, 731 (1977).

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