STRUCTURE OF DOG INTESTINAL FORSSMAN HAPTEN AND PURIFICATION AND PARTIAL CHARACTERIZATION OF FORSSMAN HAPTEN HYDROLASE (α-N-ACETYLGALACTOSAMINIDASE EC. 3.2.1.49) FROM PORCINE LIVER

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Structure of Dog Intestinal Forssman Hapten and Purification and Partial Characterization of Forssman Hapten Hydrolase (α-N-Acetylgalactosaminidase EC. 3.2.1.49) from Porcine Liver

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ABSTRACT

STRUCTURE OF DOG INTESTINAL FORSSMAN HAPTEN AND
PURIFICATION AND PARTIAL CHARACTERIZATION OF
FORSSMAN HAPTEN HYDROLASE
(α-N-ACETYLGALACTOSAMINIDASE EC. 3.2.1.49) FROM PORCINE LIVER

By

Sun-Sang Joseph Sung

The structure of a pentaglycosylceramide from canine intestines was shown by gas chromatography, gas chromatography-mass spectrometry and stepwise stereospecific glycosidase degradation to be N-acetylgalactosaminyl-(α1→3)-N-acetylgalactosaminyl-(β1→3)-galactosyl-(α1→4)-galactosyl-(β1→4)-glucosyl-(1→1)-ceramide.

The hydrolysis of this glycosphingolipid by α-N-acetylgalactosaminidase from porcine liver was studied. The enzyme was purified 3,300-fold with respect to p-nitrophenyl-α-N-acetylgalactosaminidase activity and 19,600-fold with respect to Forssman hydrolysing activity. Steps in the purification included acid precipitation, ammonium sulphate precipitation, and chromatography on DEAE-cellulose, Con A-Sepharose, DEAE-cellulose (ampholine elution), Sephadex G-150 and hydroxylapatite. Purity was judged to be greater than 90% when analysed by SDS gel electrophoresis. The subunit molecular weight was 52,000 daltons and that of the native enzyme was 102,000 daltons. Porcine α-N-acetylgalactosaminidase is a glycoprotein; the carbohydrate moiety was found to consist of mannose and N-acetylglucosamine which together accounted for 7% of

the total weight of the enzyme. The amino acid composition of the enzyme was obtained. Isoelectric focussing gave eight enzymatically active peaks with isoelectric points between 5 and 6.5 pH units. The kinetic behavior and enzyme mobility on native polyacrylamide gels of the eight forms were similar.

The kinetic properties of purified porcine α-N-acetylgalactosaminidase towards p-nitrophenyl-α-N-acetylgalactosaminide, Forssman hapten, Forssman oligosaccharide, N-[1-^{14}C]-acetyl-sphingosyl-Forssman-oligosaccharide, porcine submaxillary mucin and GalNAc-(α1→6)-1,2:3,4-di-isopropylidene-galactose were examined. The enzyme hydrolysed these substrates with K_M values of 2.9×10^{-3}, 2.6×10^{-4}, 1.0×10^{-2}, 2.5×10^{-4}, 2.3×10^{-6}, and 4.8×10^{-3} M, respectively. The V_{max} values were 14.1, 4.2, 1.7, 0.81, 0.42 and 1.1 μmoles/min/mg for each of these substrates, respectively. p-Nitrophenyl-α-N-acetylgalactosaminide was hydrolysed at pH optimum of 4.5. The hydrolysis of Forssman hapten was optimal at a taurocholate concentration of about 4 mg/ml and a pH of 3.9. The hydrolysis of N-[1-^{14}C]-acetyl-sphingosyl-Forssman-oligosaccharide at pH 3.9 was also optimal at a detergent concentration of 4 mg/ml but with a much sharper peak. However, taurocholate at a concentration of 4 mg/ml inhibited the hydrolysis of Forssman-oligosaccharide. Hydrolysis of porcine submaxillary mucin was optimal at pH 4.2. The enzyme also hydrolysed three different human red blood cell A^+ glycosphingolipids and a dog intestinal A^+ blood group glycolipid.

The hydrolysis of Forssman hapten was slightly inhibited by the alkaline earth metals. The transition metals inhibited the enzyme hydrolysis much more strongly; the most potent of these inhibitors were

Co^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, and Pb^{2+}. N-Acetyltalosamine-(α1→6)-1,2:3,4-di-isopropylidene-galactose inhibited the hydrolysis of Forssman hapten competitively with a K_i of 1.5 mM. Half of the Forssman hapten hydrolysing activity was lost after 0.4 minutes at 60° and 6 minutes at 50°.

STRUCTURE OF DOG INTESTINAL FORSSMAN HAPTEN AND PURIFICATION AND PARTIAL CHARACTERIZATION OF FORSSMAN HAPTEN HYDROLASE (α-N-ACETYLGALACTOSAMINIDASE EC. 3.2.1.49) FROM PORCINE LIVER

By

Sun-Sang Joseph Sung

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dedicated

to

YANG LI and my PARENTS

without WHOM

this work would not have been completed

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I would like to express my sincere gratitude to those who strived to contribute to a better society, thus making this work possible. My special thanks go to all my friends who have made my five and more years of life here so thoroughly enjoyable and to Dr. Charles C. Sweeley, whose patience, understanding and erudition have made this a most enlightening experience.

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LIST OF ABBREVIATIONS*

Chromatography

GC-MS	Gas-Liquid Chromatography-Mass Spectrometry
GLC	Gas-Liquid Chromatography
MS	Mass Spectrometry
TLC	Thin-Layer Chromatography

Glycosphingolipids

GL-1a	Glucosylceramide, Glucocerebroside
GL-1b	Galactosylceramide, Galactocerebroside
GL-2	Lactosylceramide
GL-3	Galactosyl-(α1→4)-galactosyl-(β1→4)-glucosyl-(1→1)-ceramide
GL-4	Globoside, N-Acetylgalactosaminyl-(β1→3)-galactosyl-(α1→4)-galactosyl-(β1→4)-glucosyl-(1→1)-ceramide
GL-5	Forssman hapten, Forssman antigen, Forssman glycolipid
G_{M1}	Gal-(β1→3)-GalNAc-(β1→4)-Gal-[3←2αNeuAc]-(β1→4)-Glc-(1→1)-Cer
G_{M2}	GalNAc-(β1→4)-Gal-[3←2αNeuAc]-(β1→4)-Glc-(1→1)-Cer
G_{M3}	NeuAc-(α2→3)-Gal-(β1→4)-Glc-(1→1)-Cer

* Standard abbreviations have been defined in J. Biol.Chem.251, 1-10 (1976)

Miscellaneous

Con A	Concanavalin A
pNP	p-Nitrophenyl
SDS	Sodium dodecyl sulphate
RBC	Red blood cell
BSA	Bovine serum albumin
a.m.u.	Atomic mass unit
DCE	1,2-dichloroethane
Cer	Ceramide
TalNAc	N-Acetyltalosamine, N-acetyltalosaminyl
PPO	2,5-Diphenyloxazole
Dimethyl-POPOP	1,4-*bis*-[2-(4-Methyl-5-phenyloxazolyl)]-benzene

INTRODUCTION

A glycosphingolipid with a partial structure of N-acylgalactosaminyl-N-acylgalactosaminyl-galactosyl-galactosyl-glucosyl-ceramide was reported to be a major glycolipid in dog intestines (1). Judging from the sequence of sugar residues it was suggested that this glycosphingolipid belonged to the globoside series, with an additional N-acetylgalactosamine moiety. When the structural studies were completed, it was found that the glycosphingolipid indeed belonged to the globoside series (2) and had the same structure as horse spleen Forssman hapten (3).

Sweeley and Klionsky were the first to show that a lipidosis (Fabry's disease) was caused by the accumulation of complex neutral glycosphingolipids (4); it was shown subsequently that accumulation of this glycosphingolipid resulted from the deficiency of an acid α-galactosidase (5,6). It is now recognized that deficiency of any of a variety of lysosomal glycosidases gives rise to a specific glycosphingolipidosis (7). Although these inherited diseases cannot be cured by present technology, it may be possible to use enzyme replacement therapy, which involves the injection of an active enzyme preparation into the patient in a form that could reduce the level of the accumulating glycosphingolipids, to alleviate the symptoms of the disease. A thorough study of the properties of lysosomal glycosidases should precede their introduction into patients so that the effectiveness of the therapy and possible side effects, including immunological rejection, can be evaluated.

The human blood group A^+ glycosphingolipids have terminal α-N-acetylgalactosamine residues (8). A deficiency in the enzyme α-N-acetylgalactosaminidase might therefore result in the accumulation of blood group A^+ glycosphingolipids and expression of some clinical symptoms of this lipidosis. No such disease has yet been described.

The enzyme properties of α-N-acetylgalactosaminidase were studied in the hope that the development of a reliable assay for the enzyme could render the disease more readily identified. Forssman hapten can be purified readily from dog intestines. Its hydrolysis by an α-N-acetylgalactosaminidase can be used as a model for studies of the mechanism of enzymatic glycosphingolipid hydrolysis by lysosomal hydrolases. The enzyme from pork liver was chosen because it was partially purified previous to this work (9) and it gave a high specific activity *in vitro* with Forssman hapten as the substrate.

LITERATURE REVIEW

Forssman Hapten

In 1911, Forssman (10) demonstrated the formation of sheep blood hemolysins after parenteral administration of extracts from guinea pig organs into rabbits. The antigens that induce the formation of these hemolysins have been called Forssman antigens in recognition of his poineering efforts. They occur in many species of animals and bacteria but not in plants (11). Landsteiner (12) showed that Forssman antigens consist of a specific alcohol-soluble component which he called the hapten and a nonspecific protein component. Brunius (11) observed subsequently that the purified Forssman hapten from horse kidney was a galactosamine-containing lipid and Papirmeister and Mallette (13) reported that the Forssman hapten from sheep erythrocytes contained hexose, hexosamine, fatty acid and a base. Makita *et al.* (14) were the first to assign a structure, N-acetylgalactosaminyl-(α1→3)-galactosyl-(?1→4)-galactosyl-(β1→4)-glucosylceramide, for the Forssman hapten from horse kidney and spleen. In agreement with these results, Mallette and Rush (15) concluded that the Forssman hapten from sheep erythrocytes was also a tetrahexosylceramide, with the same composition as that reported for the horse Forssman hapten (14). Siddiqui and Hakomori (3) arrived at a different structure on the basis of results with the Forssman hapten of horse spleen, and proposed that the Forssman hapten has the structure N-acetylgalactosaminyl-(α1→3)-galactosylaminyl-(β1→3)-galactosyl-(α1→4)-galactosyl-(β1→4)-glucosyl-(1→1)ceramide. This controversy was later

settled when Fraser and Mallette (16) and Makita, Yokoyama and Takahashi (17) reinvestigated their Forssman glycolipids and found that their results agreed with the proposed structure of Siddiqui and Hakomori (3).

Vance, Shook and McKibbin (1) described a pentaglycosylceramide from dog intestine which they proposed to be N-acetylgalactosaminyl-N-acetyl-galactosaminyl-galactosyl-galactosyl-glucosyl-ceramide. Sung, Esselman and Sweeley (2) completed the anomerity and linkage studies of this glycolipid and showed that the oligosaccharide moiety was identical to that of the horse spleen Forssman hapten. An identical structure was proposed for Forssman hapten from goat erythrocyte (18) and guinea pig (19). To date, there is only one exception to this widely accepted structure for mammalian organs. This anomalous Forssman hapten was isolated from hamster fibroblast NIL cells and has the structure of N-acetylgalactosaminyl-(α1→3)-N-acetylgalactosaminyl-(β1→3)-galactosyl-(α1→4)-galactosyl-(β1→1)-ceramide (20). Bacterial Forssman hapten activity has not yet been characterized chemically.

The immunological specificity of Forssman hapten probably resides in the oligosaccharide moiety, since the oligosaccharide product after ozonolysis and reduction of the N-acetylsphingosyl-Forssman hapten-oligosaccharide retains its haptenic activity (21). The fact that reduction of the oligosaccharide structure from a pentaglycosylceramide, that of horse spleen, to a tetraglycosylceramide, as occurs in NIL cells, does not affect the immunological specificity of the hapten indicates that the nature of the sugar residue proximal to ceramide may not play a major role in the antibody-antigen interaction. Ceramide, with its hydrophobic nature, is probably important *in vivo* for the hapten to anchor itself in cellular membranes and in the formation of micelles.

Glycolipids are believed to be located on the surface of plasma membranes (22-28). For example, Forssman hapten of erythrocytes is probably located primarily on the erythrocyte membrane because it has been isolated in quantity from washed stroma (15). However, glycosphingolipids are also found in substantial quantities in intracellular membranes such as smooth endoplasmic reticulum (29), Golgi apparatus (30) and primary and secondary lysosomes (31). Smith and McKibbin (32) have examined the subcellular distribution of glycolipids of dog intestinal mucosal glycolipid and found that Forssman hapten is located in a crude nuclear fraction, which also contains the cellular debris and in the microsomal fraction, which also contains the plasma membrane.

Some possible functions of Forssman hapten in plasma membranes are cell-cell interaction, adhesion and recognition, cellular membrane structural organization, and action as a cell surface receptor. None of these functions has yet been definitely proven, but a vast amount of work has been done which implicates this glycolipid in some way in cell transformation and malignancy. Forssman hapten, while absent in normal human organs, has been reported to appear in a case of human metastatic tumour of biliary adenocarcinoma in liver (33). Polyoma-induced hamster kidney tumours were also shown by complement fixation to have an increased concentration of Forssman antigen (34), and a number of cultured cell lines were shown to have altered levels of cell surface Forssman antigen after infection with different viruses.

O'Neill (35) has shown that Forssman antigen reactivity was induced in polyoma and Rous virus-transformed baby hamster (BHK) cell lines, and found that the presence of this antigen was dependent upon the maintenance of the transformed state. In another cell line (NIL) , however,

both normal and polyoma-transformed cell lines exhibited Forssman reactivity. Utilizing heterologous rabbit antisera and immunofluorescent techniques, Robertson and Black (36) detected a new antigenic activity on the surfaces of SV40- and polyoma-transformed BHK cells, which was characterized as that of the Forssman antigen; no such activity was detected on adenovirus-transformed BHK cells. The increase in Forssman reactivity on virus-transformed cell surfaces may result from induced synthesis of the antigen or by the unveiling of a previously cryptic antigen by changes in membrane conformation. Studies were performed to distinguish between these processes in transformation. Burger (37) treated normal BHK cells briefly with proteases and showed that these cells have the same amount of Forssman antigen as was found in polyoma-transformed-BHK cells not treated with protease. He concluded that the Forssman receptor was not the result of new synthesis or induction of antigenic activity in the tumour cells but rather represented a receptor which is present in a cryptic form in certain cell lines and is exposed in the course of transformation. Makita and Seyama (38) similarly demonstrated that the trypsin-treated plasma membrane fraction of non-transformed BHK cells contains previously undetectable Forssman activity which is similar in intensity to the activity of the polyoma-transformed BHK plasma membrane fraction that can be obtained without trypsin treatment. Trypsin treatment of polyoma-transformed BHK plasma membranes does enhance the reactivity to a small extent, however.

The carbohydrate content of the plasma membrane was decreased as a result of transformation. These results are consistent with the hypothesis that there is decreased carbohydrate incorporation into membrane but increased exposure of cryptic Forssman active sites following polyoma

transformation.

By using NIL 2 hamster cell lines, Robbins and Macpherson (39,40) and Sakiyama, Gross and Robbins (41) studied the *de novo* synthesis of glycolipids by normal and virus-transformed cell lines based on the incorporation of [^{14}C]-palmitate. In general, it was found that the more complex neutral glycosphingolipids, including Forssman hapten, were no longer synthesized after transformation with adeno 7/SV40 hybrid virus or hamster sarcoma virus. The synthesis of Forssman hapten in tissue culture was also density-dependent. Its rate of synthesis in NIL cell lines increased as the cells grew from sparse to dense cultures (42, 43). However, when these cells were transformed by hamster sarcoma or polyoma virus, the density dependent synthesis of the more complex neutral glycolipids ceased (41-44). Moreover, when cell contact was prevented by growing normal NIL cells in a spinner culture (45), there was no appreciable increase in Forssman hapten. Dibutyryl adenosine-3', 5'-cyclic monophosphate was employed to arrest cell growth, to examine whether the failure of hamster sarcoma virus transformed NIL cells to synthesize Forssman hapten was a result of continuous cell growth or a direct result of transformation (46). The data did not support the notion that lack of Forssman hapten synthesis was due to continuous cell growth, since transformed cells did not regain their ability to synthesize Forssman hapten after arrest of cell growth. The cryptic nature of Forssman hapten in cultured cells may be related to the cell cycle, because transformed cells resemble mitotic cells in that they are also rapidly dividing cells. Their analogy extends to exposed Forssman hapten (47), which is found to be fully exposed in mitotic cells and becomes cryptic after division.

Blood Group A Active Substances

The ABO system is the first blood group system discovered. Landsteiner (48) published an article on human blood group typing in 1900 and the importance of this system for blood transfusion was soon recognized. A whole family of blood group substances has since been isolated, purified and characterized from various sources. They are identified as glycosphingolipids, glycoproteins, or oligosaccharides. All blood group A substances have the following immunological determinant,

GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→4)-GlcNAc,

while in blood group B substances a gal-(α1→3) residue substitutes for the N-acetylgalactosamine at the nonreducing end. Blood group O substances are antigens known as H substances that are thought to be precursors of the A and B blood group substances, the immunological determinant is

Fuc-(α1→2)-Gal-(β1→4)-GlcNAc.

Among the blood group A substances, the most thoroughly studied are probably those of the human red blood cell. Hakomori, Stellner and Watanabe (49) have characterized four antigenic variants of blood group A-active glycosphingolipid, with the structures shown in Table 1.

Recently, an A-active megaloglycolipid (polyglycosylceramide) with 20-40 sugar residues per ceramide was isolated from human RBC (50). This megaloglycolipid was thought to comprise a major portion of the A-active antigenic sites on the red cell suuface. This view is contrary to the commonly accepted concept that the red cell A-antigens are mostly glycoproteins (51-63) but is supported by the work of Yamato, Handa and Yamakawa (64) who have determined that the blood group A activity of the glycoprotein preparation on a dry weight basis was only about one-two hundredth of that of highly purified glycolipids. The A activity of the

glycoprotein fraction was mainly associated with PAS I (54, 64).

The blood group A-active glycosphingolipids in hog gastric mucosa have also been characterized (65-70). They vary in complexity from a hexa- to an octadeca- glycosylceramide and are similar in structure to human RBC A^a glycosphingolipid (49).

The saliva of certain individuals, known as secretors, are also A^+. The immunologically active mucin was purified from hog submaxillary mucin (71, 72) and subjected to alkaline borohydride treatment (73). The resulting A^+ oligosaccharide was shown to have the structure shown in Table 1. Dogs also possess a blood group A active glycolipid (74), the structure of which was proposed to be

$$\begin{array}{l} \text{GalNAc-}(\alpha 1\rightarrow 3)\text{-Gal-}(\beta 1\rightarrow 4)\text{-GlcNAc-}(\beta 1\rightarrow 3)\text{-Gal-}(\beta 1\rightarrow 4)\text{-Glc-Cer} \\ \qquad\qquad\quad | \ \alpha 1\rightarrow 2 \\ \qquad\qquad\quad \text{Fuc} \end{array} \qquad (75)$$

An oligosaccharide with blood group A activity has also been demonstrated to occur in the human urine (76).

The occurrence of ABO blood group substances is widespread. They are present as cell surface components of many tissue cells, including the red blood cell, and in saliva, gastric juice, duodenal juice, bile, spermatic fluid, vaginal secretions, amniotic fluid, milk, sweat, tears, urine and meconium (77). Speculations about the functions of these potent antigens are still premature, although there are quantitative and qualitative changes of blood group activity in various carcinomas and transformed cells. Complete or partial loss of blood group A and B antigens has been observed frequently in tumours arising from urinary epithelium (78). Correlation with other properties of the tumour revealed increased frequency of antigen loss, particularly among the more pleomorphic, anaplastic, infiltrating and rapidly fatal tumours. In a large

number of cases of gastrointestinal carcinoma, A, B and H blood group activity was not detectable in all anaplastic cells that ceased to secrete mucus while in mucinous carcinomas, there were both ABO positive and negative neoplastic cell types (79). The same phenomenon of progressively increasing loss of ABH antigens from carcinomas *in situ* to anaplastic, invasive, and metastatic carcinomas was also demonstrated in 355 primary carcinomas of the uterine cervix, lung, pancreas and stomach, 578 metastatic carcinomas of these organs (80) and 12 cases of oral squamous cell carcinomas (81). The incorporation of fucose into normal and virus-transformed cells has also been examined. By the use of RE2 Sprague-Dawley rat and the murine sarcoma-murine lukemia transformed RE2 (MSV-RE2) cell lines, Steiner, Brennar, and Melnick demonstrated that there was a sharp increase in fucose incorporation into the larger fucosylglycolipid and a corresponding rise in radioactivity in the more mobile and probably simpler fucosylglycolipids (82). Similar results were obtained with human cell lines transformed with malignant melanoma, rhabdomyosarcoma and lung carcinoma (83). By the use of a cold-sensitive mutant of murine sarcoma virus, it was shown that alterations in fucosyl-glycolipid metabolism were related directly to the expression of the transformed state, and were not simply the result of murine sarcoma virus infection (84). Although Stellner and Hakomori (85) showed that the loss of A and B glycolipids in carcinomas may be due to the loss of the transferase activities that convert H to A and B glycolipids,the relationship between carcinoma and loss of ABH antigens is still not clear.

Table 1. Potential Substrates of α-N-Acetylgalactosaminidase

Compound	Source	Structure	Ref.
Forssman hapten	horse spleen	GalNAc-(α1→3)-GalNAc-(β1→3)-Gal-(α1→4)-Gal-(β1→4)-Glc-(1→1)-Cer	3
	dog intestine		2
	goat erythrocyte		18
	guinea pig		19
	sheep erythrocytes		16
	hamster NIL cell	GalNAc-(α1→3)-GalNAc-(β1→3)-Gal-(α1→4)-Gal(1→1)-Cer	20
Blood group A Glycosphingo-lipid	human erythrocyte membrane	A^a GalNAc-(1→3)-Gal[-(2←1)-Fuc]-(1→4)-GlcNAc-(1→3)-$(Gal)_n$-(1→4)-Glc-Cer	49
		A^b GalNAc-(1→3)-Gal[-(2←1)-Fuc]-(1→4)-GlcNAc-(1→3)-Gal-(1→4)-GlcNAc-(1→3)-$(Gal)_n$-(1→4)-Glc-Cer	
		A^c Fuc(1→2)↘ Gal-(1→4)-GlcNAc-(1→3)↘ / GalNAc-(1→3)↗ Gal-(1→4)-[GlcNAc-(1→3)-Gal]$_n$-(1→4)-Glc-Cer / GalNAc...↘ Gal-(1→4)-GlcNAc-(1→6)↗ / Fuc(1→2)↗	
		A^d Similar to A^c but with excessive GlcNAc and with an additional branching structure	
Blood group A Glycosphingo-lipids (Megaloglyco-lipid)	human erythrocyte membrane	30-50 sugar residues/mole of ceramide	50

Table 1 (Continued)

Compound	Source	Structure	Ref.
Blood Group A Glycosphingo-lipid	hog gastric mucosa	Ceramide Heptasaccharide	
		(L) GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→3)-GlcNAc-(β1→3)-Gal-(β1→4)-Gal-(β1→4)-Glc-(β1→1)-Cer	66
		(U) GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→4)-GlcNAc-(β1→3)-Gal-(β1→4)-Gal-(β1→4)-Glc-(β1→1)-Cer	
		Ceramide Hexasaccharide	
		GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→3)-Gal-(β1→4)-Gal-(β1→4)-Glc-(1→1)-Cer	67
		Difucosyl glycolipid	
		GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→4)-GlcNAc[-(3←1)-Fuc]-(β1→3)(β1→3)-Gal-(β1→4)-Glc-(1→1)-Cer	68
		Fucolipid I	
		GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→4)-Gal-(β1→3) Gal-(β1→4)- GalNAc-(α1→3)-Gal-(β1→6) Glc-(β1→1)- Cer	70
		Fucolipid II	
		GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→3) Gal-(β1→4)-Gal-(β1→4)- GalNAc-(α1→3)-Gal-(β1→6) Glc-(β1→1)-Cer	

Table 1 (Continued)

Compound	Source	Structure	Ref.
		Fucolipid III GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→4)-Gal-(β1→3) Gal-(β1→4)- GlcNAc-(β1→4)-Gal-(β1→6) Glc-(β1→1)- Cer	69
		Complex Fucolipid I GalNAc-(α1→3)-Gal-(1→4)-GlcNAc-(1→3) Gal-(1→4)-GlcNAc- GalNAc-(α1→3)-Gal[-(2←1)-Fuc]-(1→4)-GlcNAc-(1→6) I(4←1) (1→4)-GlcNAc- GlcNAc (1→3)-Gal- I(4←1) [-(6←1)-GlcNac- GlcNAc (4←1)-Gal-(4←1)- GlcNAc]-(1→4)- Glc-Cer	
		Complex Fucolipid. II GalNAc-(α1→3)-Gal[-(2←1)-Fuc]-GlcNAc-(1→3) Gal-(1→4)-GlcNAc-(1→4)- GlcNAc-(1→4)-GlcNAc-(1→6) GlcNAc-(1→3)-Gal[-(6←1)- GlcNAc]-(1→4)-Glc-Cer	
Blood Group A Oligosaccharide	human urine	GalNAc-(α1→3)-Gal[-(2←1)-Fuc]-Gal-(β1→4)-Glc-[(3←1α)-Fuc]	76
A^+ Porcine submaxillary mucin	porcine submaxillary glands	GalNAc-(α1→3)-Gal[-(2←1α)-Fuc]-(β1→3)-Gal[-(6←2)-NGNA]	73
		GalNAcα-Thr or Ser	73
Ovine submaxillary mucin	Ovine submaxillary glands	GalNAcα -Thr or Ser	101

Metabolism of Neutral Glycosphingolipids

The glycosphingolipids are believed to be metabolized in a stepwise fashion by a family of specific exoglycosidases each of which catalyzes the hydrolysis of one sugar residue at the nonreducing end (86). The degradation of some of the glycosphingolipid substrates of α-N-acetylgalactosaminidase is shown in Figure 1.

The sources and properties of some of the glycosidases are summarized below.

α-N-Acetylgalactosaminidases

α-N-Acetylgalactosaminidases have been demonstrated in extracts of *Trichomonas foetus* (87,88), *Helix pomatia* (89), *Busycon* liver (90), *Aspergillus niger* (91), rat, porcine, and bovine livers (92) and rat brain and kidney (93). A partially purified preparation of α-N-acetylgalactosaminidase from human liver has also been characterized (94). Cleavage of the O-glycosidic linkage between N-acetylgalactosamine and the hydroxy amino acids in ovine and bovine submaxillary mucins is also due to α-N-acetylgalactosaminidases (95-101), which have been isolated from ox spleen, *Lumbricus terrestris*, and rat liver and kidney. None of these enzyme preparations has been purified to homogeneity and consequently little information is available about the physical and chemical nature of the α-N-acetylgalactosaminidases, nor of their substrate specificities. It has been noted that α-N-acetylgalactosaminidase from bovine liver dissociates in dilute enzyme solutions (102); reassociated form of the enzyme has a molecular weight of 155,000 and the monomer has a molecular weight between 30,000 and 42,000.

Using phenyl-α-N-acetyl-D-galactosaminide as substrate, it was

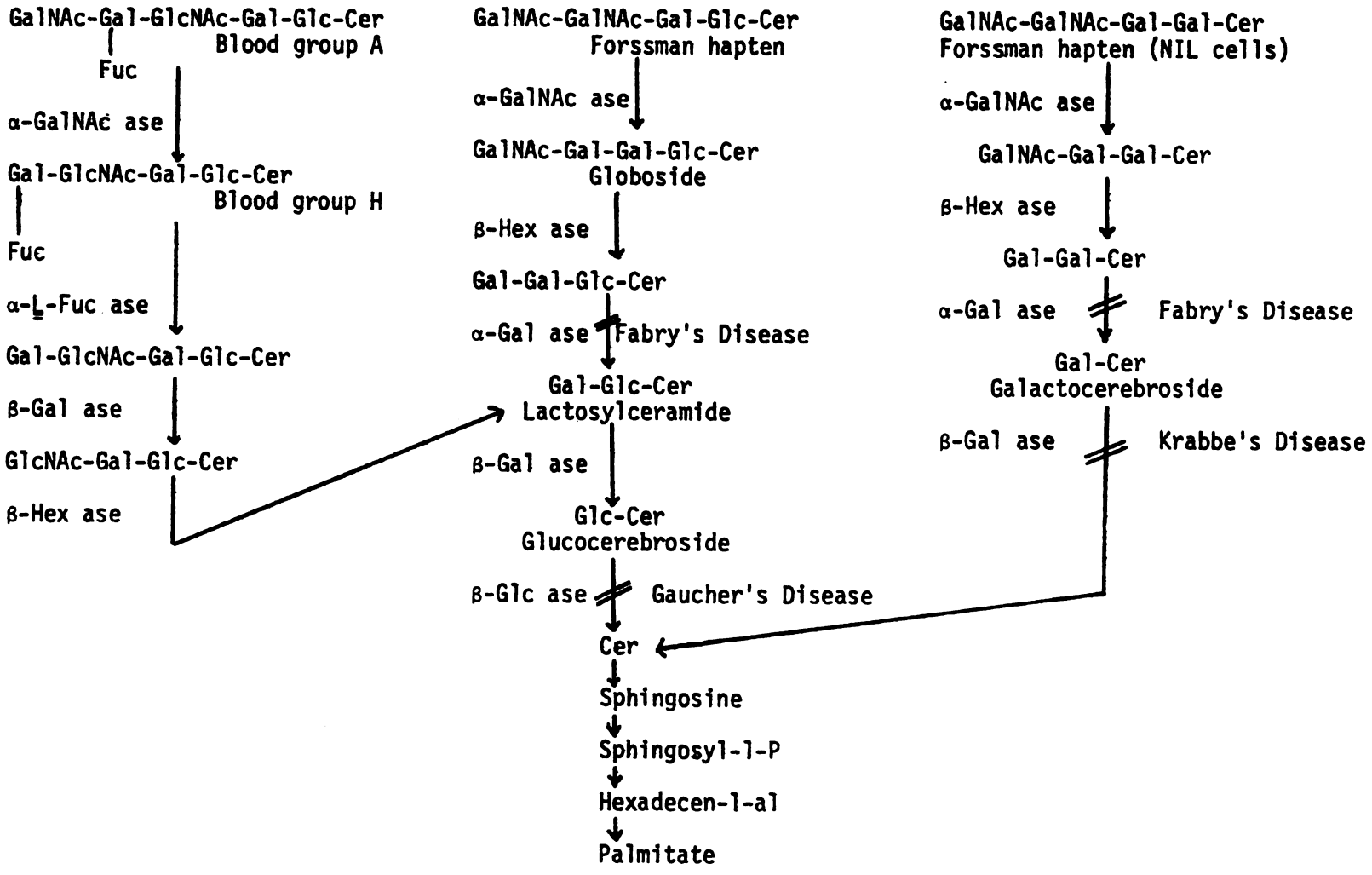


Figure 1. Metabolism of Glycosphingolipid Substrates of α-N-Acetylgalactosaminidase.

shown that the pig enzyme had a pH optimum at 4.3 and an apparent K_M of 6.4-6.6 mM. N-Acetylgalactosamine was a competitive inhibitor with a K_i of 10.1 mM. Copper sulphate and cysteine hydrochloride at 1 mM produced 10% inhibition. The beef enzyme had a pH optimum of 4.7 and a K_M of 25 mM at 38°C. Crude preparations from rat brain and kidney were shown to be able to hydrolyse Forssman hapten with a K_M of 1.0×10^{-4} M and 3.5×10^{-4} M at pH 4.4 with a taurocholate concentration of 1.5-2 mg/ml. The human α-N-acetylgalactosaminidase was found to be markedly thermostable. It maintained its maximum activity at 50° for at least 4 hours.

The optimum for hydrolysing p-nitrophenyl-α-N-acetylgalactosaminide was at pH 4.3 with K_M values of 3.5 mM for the liver enzyme. Both N-acetylgalactosamine and galactose are inhibitors of this enzyme with a K_i of 2.5 mM for the former monosaccharide.

β-N-Acetylhexosaminidases

β-N-Acetylhexosaminidase is the most studied glycosphingolipid hydrolase because of its deficiency in Tay-Sach's and Sandhoff's diseases. It has been shown to exist in four forms, A, B, C, and D. The A and B forms have been purified to homogeneity from human placenta (103,104), beef spleen (105), human plasma (106) and hen oviduct (107). Partially purified fractions were also obtained from rat cerebral cortex (108, 109) calf and human brain (110), human serum (111), rat testis (112), ram testis and epididymis (113),equine kidney (114), porcine kidney (115), rat kidney (116, 117) and bovine liver, spleen and kidney (118). Hexosaminidase C has been obtained in a partially purified form from human placenta (119).

Human placental β-D-N-acetylhexosaminidase (103) A has a pI of

5.4 while the B form has a pI of 7.9. The A form is distinguishable from the B form in terms of heat lability, and the two forms can also be distinguished by immunological means. Both forms hydrolyse 4-methylumbelliferyl-β-D-N-acetylgalactosaminide with a pH optimum of 4.4, K_M of 0.5 mM, and heat of activation of 10,500 cal (120). Cupric ion (5 mM), p-hydroxymercuribenzoate (1 mM) and acetate (30 mM) inhibit the hydrolysis of the synthetic substrate. The molecular weights of the hexosaminidases A and B are between 100,000 and 140,000. Hexosaminidase A dissociates into one major subunit corresponding to a molecular weight of 17,000 to 18,000, while hexosaminidase B dissociates in sodium dodecyl sulphate into three subunit species with molecular weights of 17,000 to 18,000, 35,000 and 55,000 daltons. Urea-starch gel electrophoresis of the subunits suggests that hexosaminidases A and B may share one common subunit. Both hexosaminidases A and B have blocked NH_2-terminal groups and differ in the COOH- terminal amino acid, hexosaminidase A having serine as the COOH-terminal amino acid while hexosaminidase B has aspartic acid or asparagine. The two forms are somewhat different in amino acid composition.

Geiger and Arnon (104) have shown that the human placental hexosaminidase A contains 1.65 residues of sialic acid while the B form has none. By reduction, alkylation and dissociation in 5 M guanidine hydrochloride, the A form can be separated into 2 types of subunits by ion-exchange chromatography, while the B form produces only 1 type of subunit. Based on these data, it was postulated that hexosaminidase A is a tetramer, $\alpha_2\beta_2$, where α and β donate the subunit species, and hexoaminidase B is composed of $\alpha_2\alpha_2$ tetramers.

The beef hexosaminidases were found to be similar to those of human placenta, with optimum for pNP-β-acetylgalactosaminide hydrolysis at pH 4.5, K_M values of about 0.9 mM and V_{max} of 30 μmoles/min/mg for both forms. The molecular weights were about 137,000 to 146,000 daltons, with the molecular weight of the smallest subunit at 56,000 daltons. The hen oviduct hexosaminidases had molecular weights of 118,000 for the A form and 158,000 for the B form.

Hexosaminidase C has been purified to apparent homogeneity from human placenta (120). It is found in the cytosol, with an estimated molecular weight of 190,000 and a pI of 5.7, compared to pI values of 5.0 and 7.3 for hexosaminidases A and B, respectively. The pH optimum for the hydrolysis of 4-methylumbelliferyl-β-N-acetyl-D-galactosaminide was found to be 7.0, with a K_M of about 0.83 mM. This form is not thermostable.

Three forms of β-hexosaminidases denoted A, A' and B were purified from equine kidney (114). G_{M2} ganglioside was hydrolysed by either A or A' but not by B. On the other hand, globoside I was hydrolysed by A' or B but not by A. When the oligosaccharides of the glycolipids were used it was found that A or A' hydrolysed G_{M2} oligosaccharide but not B while G_{M2} and globoside 2 oligosaccharides were hydrolysed by all three components, A, A' or B.

A hexosaminidase C with only β-N-acetyl-D-glucosaminidase activity has been shown to be present in bovine brain (121). It did not elute from Sephadex G-200 or DEAE-Sephadex, but came off in the void volume from Bio-gel P-200, indicating a molecular weight equal to or larger than 200,000 daltons. Another form, denoted hexosaminidase D, was shown to be present in bovine brain preparations. It was specific for

β-N-acetylgalactosaminides. The report is in agreement to that of Frohwein and Gatt (122) and Hooghwinkel *et al.* (123), which described the presence of a β-N-acetylglucosaminidase and a β-N-acetylgalactosaminidase.

A partially purified fraction of β-N-acetylhexosaminidase from hog epididymus was also shown to hydrolyse globoside I and asialo-G_{M2} while no activity toward G_{M2} was observed. The best detergent examined was sodium cholate at an optimum concentration of 2 mg/ml. The pH optimum for the hydrolysis of globoside I was 4.2 and the K_M was 3.7×10^{-3} M (124).

α-Galactosidases

α-Galactosidase was shown to occur in dog, human, ox, pig, rabbit and rat (125). Ceramide trihexosidase (globotriaosylceramide hydrolase) was purified from normal human plasma by affinity chromatography (126-128). This enzyme has also been purified to near homogeneity by conventional methods from human placenta (129,130). Partially purified fractions have been obtained from human liver (131,132) and kidney (133). Ceramide trihexosidase activity has been found in concentrated human urine (134).

The plasma enzyme was shown to consist of two forms called A-1 and A-2. They had a molecular weight of about 95,000, with subunit molecular weights of 22,000. The A form had a K_M value toward GL-3 of 4.5×10^{-4} M with 0.03 M sodium taurocholate and 0.15 M sodium chloride. In the presence of optimal sodium taurocholate and sodium chloride, the enzyme was inhibited competitively by digalactosylceramide and GL-3 oligosaccharide. The A-2 form had a K_M of 5×10^{-4} M toward GL-3, was inhibited by galactose, GL-2, myoinositol and

digalactosylceramide but not by pNP-α-galactoside or 4-methylumbelliferyl-α-galactoside (127). The human placental α-galactosidase also consists of two forms, A and B (129). The A form probably corresponds to the A-1 form and the B-form may be equivalent to the A-2 form of Mapes and Sweeley. The A form had a molecular weight of 150,000, K_M of 3.4 mM towards 4-methylumbelliferyl-α-D-galactopyranoside, and 40.6 mM for melibiose. The B form did not hydrolyse melibiose. The pH optimum for both forms was 4.5, with the A form showing a broader pH activity curve. The A form was inhibited by myoinositol, while the B form was not. The isoelectric points were at pH 4.7 and 4.42 for the A and B forms, respectively. Antibodies towards these two placental forms of α-galactosidase did not cross react.

The human liver α-galactosidase hydrolysed GL-3 with a pH optium of about 3.5, was activated by a mixture of sodium taurocholate and Triton X-100 and was inhibited by high concentrations of bovine serum albumin. It had a K_M of about 5×10^{-5} M (131) towards GL-3. The human kidney α-galactosidase had pI values of 4.5 and 4.3 for the A and B forms, respectively. In agreement with Beutler and Kuhl (130), it was shown that the A form was thermolabile while the B form was thermostable. The K_M values for the A and B forms were 6.0×10^{-3} M and 13.3×10^{-3} M towards pNP-α-D-galactopyranoside and 5.7×10^{-4} M and 4.7×10^{-4} M toward GL-3, respectively. The activity of ceramide trihexosidase was shown to be depressed by high concentrations of protein (133). The thermostabilities of the human liver α-galactosidase B (130,131) and α-N-acetylgalactosaminidase (94) in addition to the inhibition of human liver α-N-acetylgalactosaminidase by both N-acetylgalactosamine and galactose support the hypothesis of Dean, Sung and Sweeley (135) that

α-galactosidase B and α-N-acetylgalactosaminidase in human liver are the same enzyme.

It was suggested that neuraminidase treatment converted the A form into a mixture of 14 enzymatically active products as revealed by isoelectric focussing, while a crude kidney sialyltransferase preparation could be used to incorporate CMP-[1-^{14}C]-N-acetylneuraminic acid or UDP-N-acetyl-[1-^{14}C]-glucosamine into the B form, resulting in the formation of a form similar in electrophoretic behaviour to the A form (136). This observation was disputed by Romeo *et al.* (132) who showed by the combined use of isoelectric focussing, DEAE-chromatography and enzyme kinetic parameters that no conversion of α-galactosidase A into B or *vice versa* could be detected after neuraminidase treatment.

In Fabry's disease, only the B form was found, accounting for about 2 % of the normal 4-methylumbelliferyl-α-D-galactopyranoside hydrolysing activity in the kidney and 20% of the normal activity in the liver (137, 138). Fibroblast cultures from patients with Fabry's disease also exhibited the presence of the B form while the A form was absent (139); the residual activity accounted for 10-30% of the normal activity towards the artificial substrate (140).

β-Galactosidases

The action of β-galactosidase on four natural glycosphingolipid substrates has been examined. The substrates are lactosylceramide, galactosylceramide, G_{M1}-ganglioside and asialo-G_{M1}-ganglioside. There is evidence that two of these β-galactosidases have different substrate specificities towards the glycosphingolipids. One form is specific for galactosylceramide and the other for G_{M1}-ganglioside, while both forms can hydrolyse lactosylceramide under certain assay

conditions. Suzuki and his group have examined the β-galactosidases of rat brain and human liver.

The human liver preparation, purified 250-fold, was active towards G_{M1}-ganglioside, asialo-G_{M1}-ganglioside, and lactosylceramide but inactive towards galactosylceramide when the Suzuki assay system was used (141). The enzyme preparation was stimulated by chloride ion and unstable upon dilution. When a liver preparation was subjected to isoelectric focussing, three β-galactosidase peaks, denoted α, β,and γ, were resolved; their pI values were at pH 4.1-4.2, 4.5-4.6 and 4.8-4.9, respectively. Gel filtration also gave three peaks of activity, the second of which corresponded to the β form and the third to the γ form. Lactosylceramidase activity was present in all three peaks, α, β, and γ, while only β and γ had G_{M1}-ganglioside and galactosylceramide hydrolysing activity (142). In globoid cell leukodystrophy, a disease with accumulation of galactosylcermide, isoelectric focussing patterns of tissue β-galactosidase activities toward lactosylceramide and asialo-G_{M1} were normal, while the 4-methylumbelliferyl-β-D-galactopyranoside and galactosylceramide hydrolysing activities existed as a single, broad but diminished peak in the range of pH 4.6 to 4.8 (143). There were two types of isoelectric focussing patterns for β-galactosidase in tissues from patients with G_{M1} gangliosidosis. One had a shifted pI to a lower pH for the two galactosylceramide β-galactosidase activity peaks while in the other type these peaks were normal. In both types of G_{M1} gangliosidosis, there was no residual asialo-G_{M1}-ganglioside hydrolysing activity and only one 4-methylumbelliferyl-β-galactosidase peak that appeared between the β and γ forms (144). The above data with globoid cell leukodystrophy and G_{M1} gangliosidosis did not give

definitive results as to the nature of the deficiency. It was hinted that there may be structural and functional interrelationships between galactosylceramide- and G_{M1}-ganglioside- β-galactosidases, perhaps similar to but more complex than the structures of the human placental hexosaminidases, which were $\alpha_2\beta_2$ for the A form and $\beta_2\beta_2$ for the B form (104). Rat brain β-galactosidase was partially purified and shown to have the following properties: pH optimum 3.9-4.2, 4.9, 3.9 and 4 for 4-methylumbelliferyl-β-galactopyranoside, lactosylceramide, asialo-G_{M1} ganglioside and G_{M1} ganglioside hydrolysing activities, respectively. The optimal taurocholate concentration was 1.5 mg/ml for the asialo-G_{M1} ganglioside and G_{M1} ganglioside β-galactosidases. The apparent K_M at optimal activities were 2.2×10^{-5} M, 2.0×10^{-4} M and 4.3×10^{-4} M for lactosylceramide-, asialo-G_{M1} ganglioside- and G_{M1}-ganglioside- β-galactosidases. Huge amounts of oligosaccharides were required to cause appreciable inhibition of asialo-G_{M1}-ganglioside- and G_{M1}-ganglioside-β-galactosidases (145). A rat brain preparation also hydrolysed galactosylsphingosine with a K_M of 1.1×10^{-5} M and pH optimum of 4.2 to 4.5 (146). Human brain preparations gave a K_M of 2×10^{-5} M for both galactosylceramide and lactosylceramide (147). A G_{M1}-ganglioside-β-galactosidase A was purified to homogeneity from human liver (148). It had a molecular weight of 65,000 to 75,000 and 72,000 by SDS gel filtration of the reduced and carboxymethylated enzyme. This enzyme also cleaved β-D-fucoside and α-L-arabinoside linkages. Ho *et al.* isolated two forms of G_{M1}-ganglioside-β-galactosidases, one of which was membrane bound and the other soluble (149). The apparent K_M was 2.8×10^{-5} M and 7.7×10^{-5} M for the two forms, respectively. G_{M1}-ganglioside-β-galactosidase was partially purified from rabbit brain. It eluted in one

single peak from Sephadex G-200. The pI was at pH 6.3. With G_{M1}-ganglioside as substrate, the pH optimum was 4.3 and the K_M 7.8×10^{-5}M while with lactosylceramide the pH optimum was 4.5 and the K_M was 1.7×10^{-5} M (150). γ-D-Galactonolactone, lactose and galactose (151) were competitive inhibitors of the hydrolysis of the natural substrates. Rat and pig brains were shown to contain galactosylceramide-β-galactosidase activity (152). The activity was activated by the N-decanoyl-derivative of 2-amino-2-methyl-1-propanol by 34% at 0.15 mM concentration (153) while N-decanoyl-DL-erythro-3-phenyl-2-aminopropanediol inhibited competitively with a K_i of 0.4 mM (154). A lactosylceramide-β-galactosidase that did not exhibit any G_{M1}-ganglioside or galactosylceramide hydrolysing activity was also purified from porcine thymus tissue (155). Two activity peaks could be resolved by isoelectric focussing, with pI values at pH 6.3 and 7.0. The pH optimum was at 4.6 and the apparent K_M was 2.3×10^{-5}M.

Glucocerebrosidase

Glucocerebrosidase activity was demonstrated to be present in spleen (156). The enzyme consisted of a heat labile, membrane bound factor C and a heat stable soluble acidic glycoprotein called factor P. In the absence of factor P, factor C could be activated by sodium taurocholate to hydrolyse glucocerebroside (157). The activation of factor C by factor P also required the presence of acidic phospholipid to give an active enzyme (158). A model was postulated in which the effector (factor P) and catalytic protein (factor C) were depicted to combine in the presence of 0.15 mM acidic phospholipid in a ratio of 1:1 at low effector concentration, and exist in an equilibrium of P_2C, PC and P when the effector was in excess (159).

The enzyme has also been purified to apparent homogeneity from human placenta. It was composed of four catalytically active subunits of 60,000 daltons each. The K_M for glucosylceramide was 6.5×10^{-5} M at an optimal pH of 5.0 (160).

Deficiency of glucocerebrosidase activity was shown to be characteristic in Gaucher's disease (161-163).

In Vivo Metabolism of Glycosphingolipids

The glycolipid hydrolysing enzymes have been shown to be lysosomal in origin (86). They have pH optima in the range of 4-5, which is known to be characteristic of lysosomal enzymes. It is believed that lysosomal hydrolases are synthesized on membrane-associated polysomes and gain access to the endoplasmic reticulum cisternae, through which they are transported to other areas of the cell (164, 165). Primary lysomes package the lysosomal hydrolases and eject them into digestive vacuoles to form secondary lysosomes. They are thought to originate either from the Golgi apparatus or GERL, a region of the smooth endoplasmic reticulum that has an intimate structural relationship with the innermost Golgi element. In the neurons of dorsal root ganglia the innermost Golgi element is composed of anastomosing tubules that enclose polygonal compartment, in each of which a tubule of endoplasmic reticulum, probably GERL, is found. There are three ways that lysosomes digest endogenous material. This process is known as autophagy. The primary lysosomes fuse with intracellular vacuoles and the hydrolases are released into the vacuole form a so called type 1 vacuole. In another type of autophagy, vacuole 2 appears to form by enlargement and twisting of GERL. The membrane internalizes to form inner tubules as the vacuole enlarges, enclosing bits of cytoplasm in the process. The third process,

known as microautophagy, is believed to be occurring in a similar manner as the type 1 vacuole, only at a much lower scale and with less easily identifiable manifestations. Exogenous materials can also be digested in a similar manner of fusion with the primary lysosomes. They enter the cells by either phagocytosis or pinocytosis. All the above digestive vacuoles are known as secondary lysosomes. They can also fuse with other vacuoles to form another secondary lysosome. When the process of digestion is completed, electron opaque materials are left enclosed in the original lysosomal membrane. These structures are then called residual bodies.

The process of digestion of glycosphingolipids may be similar to the endocytosis processes cited above. The glycolipids may be digested as part of a membrane fragment, or by association with certain lipid carrier proteins. The detergent requirement of hydrolysis of glycosphingolipid *in vitro* may mimick the hydrolysis of membrane associated glycosphingolipids *in vivo*.

An activator for the hydrolysis of glucocerebrosidase was found in human spleen (166). It is a heat stable glycoprotein and associates with the catalytic subunit of the enzyme in the presence of phospholipids to form an enzymatically active entity (157,158). It was contended that a similar activator from human spleen was present in molar quantities of only 1/25 of the amount of gludocerebrosidase. The presence of an eight-fold in excess of the physiological amount did not result in activation of glucocerebrosidase activity. An activator that can substitute for detergents was also demonstrated (168, 169). A heat-stable protein factor that activates the hydrolysis of cerebroside sulphatase was purified 2,000-fold and found to have a molecular weight of 21,500

with the pI at pH 4.3. The activating effect of this protein was about half as effective as taurodeoxycholate at their optimal concentrations. The amount of activator protein required for maximum enzyme activity was however only 1/300 that of taurodeoxycholate in molar amounts (170). It is likely that activator proteins along with phospholipid may play significant roles in the *in vivo* metabolism of glycosphingolipids.

Enzyme Therapy of Glycosphingolipidoses

In cases of genetic defects where certain acid hydrolase activities are absent, glycosphingolipid accumulation in residual bodies results. A whole family of such disease called sphingolipidoses has been discovered (7). The strategy for alleviating the symptoms is to purify the enzyme and administer it in a form that would result in minimal immune responses and yet active in hydrolysing the accumulated glycosphingolipids. The packaging of the enzyme in lysosomes and the coating of the lysosome surface with organ specific marker molecules may be a plausible approach to enzyme replacement therapy. Pioneering work in genetic engineering to introduce β-galactosidase genes into G_{M1}-gangliosidosis fibroblasts were also done (171). Such work should be approached with prudence because any unforeseeable mistake would result in the irreparable damage to the genetic make-up of the individual.

Animal models for studying the physiological impairment and its possible cure are used. Numerous reports of animal gangliosidoses have appeared (172). Artificial induction of lipidoses by injection of glycosphingolipids into small animals were successful in producing cells that have lipid containing residual bodies (173-175). The addition of anti-lysosomal enzyme antibodies to cell cultures also resulted in

dramatic increases in the number and size of residual bodies (176, 177). The inhibitory effect of N-hexyl-O-glucosylsphingosine on glucocerebrosidase was used to produce cell strains that exhibit the accumulation of glucocerebroside (168,178,179). These cell cultures can also be very useful in studying the physiological effects of glycolipid accumulations.

The studies of storage diseases have made possible the early diagnoses of these diseases by amniocentesis. Tay-Sachs disease was diagnosed by hexosaminidase A deficiency in amniotic fluid and cells (180). This represents a great step forward in combating inherited sphingolipidoses.

Glycosidases in Transformed Cells

The role of glycosidases in transformed cells is not well understood. Their changes in activities may be a primary trigger of the transformed state, or more likely, one of the secondary events that sets in after the cell is transformed from the normal state. N-Acetyl-β-glucosaminidase and β-glucuronidase were increased in platelet-free plasma from patients with malignancies (181). β-Glucosidase and N-acetyl-β-glucosaminidase activities were likewise increased in oncogenic virus-transformed fibroblasts (182). Glycosidase levels of polyoma virus-transformed BHK cells and murine sarcoma virus- and Rous sarcoma virus- transformed 3T3 cells were also shown to be increased over the levels in normal cells (183-185). Elevated levels of proteases and glycosidases were found in human breast and colon tumours (186). They are thought to be released to modify the cell surface so that the transformed state can be maintained.

MATERIALS AND METHODS

Materials

The following solvents were of reagent grade: methanol, chloroform, pyridine, dimethylformamide, toluene, acetic anhydride, dichloromethane, cyclohexane, 1-pentanol, 1-propanol, 2-propanol, benzene, 1-butanol, acetic acid, acetone, hexanes, tetrahydrofuran (Mallinckrodt, St. Louis, Mo.); dimethylsulphoxide, 1-octanol, 1,2-dichloroethane (Fisher, Fair Lawn, N. J.); acetonitrile (Aldrich, Milwaukee, Wis.). All solvents were redistilled before use.

Chemicals and their sources are as follows: NaH from Alfa Inorganics (Beverly, Mass.); G-150, G-25, LH-20 and Sepharose 4B from Pharmacia (Piscataway, N. J.); DE-52 from Whatman (Chifton, N. J.); DEAE-cellulose, Bio-bead P-2 from Bio-Rad (Rockville Center, N. Y.); silicic acid and hydroxylapatite from Clarkson (Williamsport, Pa.); N-acetylgalactosamine, α-methyl glucoside, α-methyl mannoside, glucosaminic acid, galactose, N-acetylglucosamine, glucose, glucosamine hydrochloride, galactosamine hydrochloride from Pfanstiehl (Waukegan, Ill.); nitrosyl chloride adduct of galactal from Raylo (Edmonton, Alberta, Canada); porcine submaxillary glands from Pel-Freez (Rogers, Ark.); ampholytes from LKB (Chicago, Ill.); Concanavalin A and chondrosine from Miles (Kankakee, Ill.); sodium taurocholate from Calbiochem (La Jolla, Ca.); silica gel G TLC plates from Analtech (Newark, Del.); [^{3}H]-$NaBH_4$, and acetyl-l-[^{14}C]-acetic anhydride from New England Nuclear (Boston, Mass.); galactose

oxidase and horseradish peroxidase from Worthington (Freehold, N. J.); molecular weight calibration proteins from Boehringer-Mannheim (Indianapolis, Ind.); anti-A and anti-B antisera from Ortho Diagnostics (Raritan, N. J.); Bio-Solv BBS-3 and scintillation grade toluene from Beckman (Fullerton, Ca.); PPO and dimethyl-POPOP from Research Product International (Elk Grove Village, Ill.); p-nitrophenyl-α-N-acetyl-galactosaminide, p-nitrophenyl-β-N-acetylgalactosaminide, p-nitrophenyl-α-N-acetylglucosaminide, and p-nitrophenyl-β-N-acetyl-glucosaminide from Koch Light (Colnbrook, Buckinghamshire, England); 3% SE-30 on Supelcoport (80-100 mesh), 3% SP-2100 on Supelcoport (80-100 mesh), 0.2% EGS, 0.2% EGA and 1.4% XE-60 on Gas Chrom Q (80-100 mesh), 3% ECNCSS-M on Supelcoport (80-100 mesh), 3% OV-210 on Gas Chrom Q (80-100 mesh) from Supelco (Bellefonte, Pa.); mercaptoethanol, and borane-tetrahydrofuran from Aldrich (Milwaukee, Wis.); sodium borohydride, Dowex 1, Dowex 50, galactose, glucose, fucose, melibiose, stachyose, raffinose, N-acetylneuraminic acid, hexamethyldichlorosilazane, trimethylchlorosilane, trifluoroacetic acid, neuraminidase, cetyltrimethyl ammonium bromide, tris-(hydroxymethyl)-aminomethane, glycine, α-naphthol, bromophenol blue and chitin from Sigma (St. Louis, Mo.); dialysis tubings from A. H. Thomas (Philadelphia, Pa.); iodomethane, cyanogen bromide, phenylmethylsulphonyl fluoride and sodium cyanoborohydride from Pflatz and Bauer (Stamford, Conn.); 90% nitric acid, nitric acid, bromine, ammonium persulphate and orcinol from Fisher (Fair Lawn, N. J.); acrylamide, and N, N'-methylene bisacrylamide from Canalco (Rockville, Md.); N,N, N',N'-tetramethyl-ethylenediamine and Photo-Flow 600 from Eastman Kodak (Rochester, N. Y.); silver carbonate, phenol, citric acid, EDTA, urea, and sodium citrate from Mallinckrodt (St. Louis, Mo.);

xylene brilliant cyanine G from ICN Pharmaceuticals (Cleveland, Ohio); 100% ethanol from Commericial Solvents (Terre Haute, Ind.); Hydrochloric acid, sulphuric acid and iodine, from Baker (Philipsburg, N. J.); and Folin Ciocalteu reagent from Harleco (Phila., Pa.).

Methods

(I) Substrate Preparation

(1) Forssman Hapten

Forssman hapten from dog intestines was purified by the method of Saito and Hakomori (187). Dog intestines (6 Kg) were rinsed in distilled water, cut up into small pieces with scissors, and homogenized with 12 liters of chloroform/methanol 2/1 in a Waring blender. The homogenate was filtered with a Büchner funnel. Homogenization and filtration were repeated with 12 liters each of chloroform/methanol 2/1, 1/1 and 1/2. The pooled filtrate was evaporated to dryness *in vacuo* on a rotary evaporator. The lipid was dried by evaporation of traces of water with 100% alcohol and the residue was acetylated overnight in a mixture of 450 ml of dry pyridine and 300 ml of acetic anhydride. The acetylation solution was evaporated to dryness *in vacuo* with repeated addition of large amounts of toluene. The acetylated lipid was loaded onto a 1000 g Florisil column (100 x 7.5 cm) packed with 1,2-dichloroethane (DCE). After washing the column with ten column volumes of DCE, the acetylated glycolipids were eluted with 10 column volumes of DCE/acetone (1/1). The eluate was evaporated to dryness *in vacuo* and dissolved in 500 ml of chloroform/methanol 2/1. A solution (250 ml) of 0.5% sodium methoxide in methanol was added and the mixture was allowed to stand at room temperature for several hours. A large volume of

ethyl acetate was added to neutralize the base and the solution was evaporated *in vacuo* . The de-O-acetylated glycolipid was then dispersed in water by sonication, dialysed overnight against distilled water, and lyophilized. The lyophilized glycolipids were dissolved in a small volume of chloroform/methanol 2/1 and loaded onto a 100 g DEAE-cellulose column (70 x 4.5 cm) prepared in chloroform. The neutral glycolipid fraction was eluted with 10 column volumes of chloroform/methanol (7/3), evaporated to dryness, and loaded onto a 1.2 Kg silicic acid column (100 x 7.5 cm) packed in chloroform. A gradient of 4 liters of chloroform and 3 liters of methanol was used to elute the column. Fractions of 20 ml were collected. The glycolipids were monitored using TLC with the solvent system chloroform/methanol/water 100/42/6. The Forssman hapten (GL-5) fractions were pooled into 5 fractions and evaporated to dryness, the residues were dissolved in known volumes of chloroform/methanol 2/1. About 1.8 g of crude GL-5 was thus isolated.

(2) Labelling of GL-5 with the Galactose Oxidase-Tritiated Sodium Borohydride Method (Figure 2).

About 100 mg of crude GL-5 was purified with preparative thin layer chromatography using 500 micron plates developed with solvent system chloroform/methanol/water (100/42/6). The GL-5 bands were visualized with iodine vapor, scraped off and eluted with 20 volumes each of chloroform/methanol 2/1, 1/2, methanol and finally chloroform/methanol/water 50/50/15. The pooled eluate was dried *in vacuo*, dissolved in 10 ml of chloroform/methanol 2/1 and washed with 2.5 ml of 20% sodium thiosulphate, after which the lower phase was washed three times with water, and dried under nitrogen. The glycolipid was labeled with galactose oxidase-sodium borotritiide according to the method of Suzuki and Suzuki with

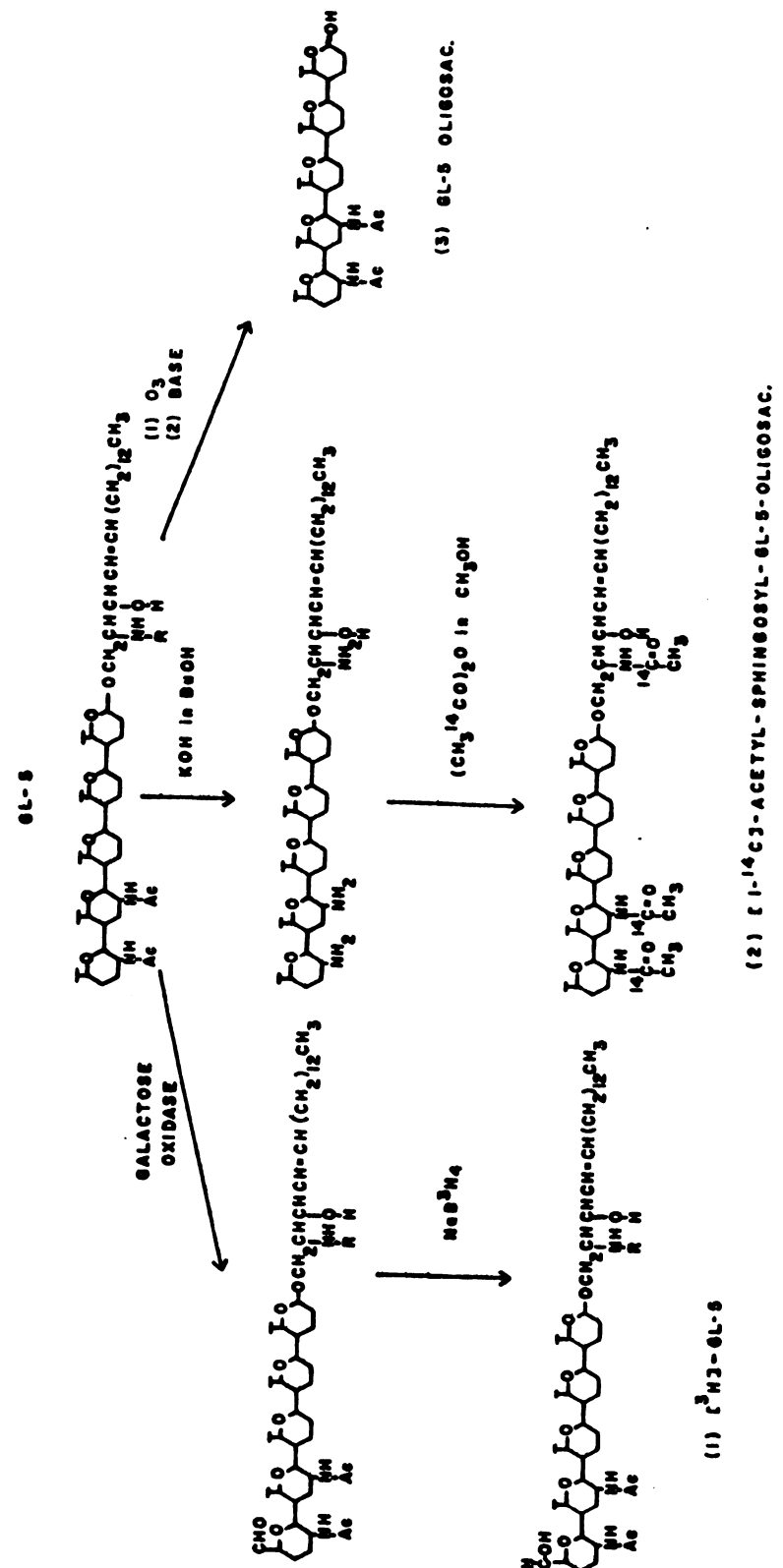


Figure 2. Preparation of [^{3}H]-GL-5 and Its Derivatives

modifications (188). The purified GL-5 was dissolved in 4 ml of 0.1 M phosphate buffer, pH 7.0. Approximately 330 units of horseradish peroxidase (3324 I. U./mg) and 0.5 ml galactose oxidase (854 units/ml) were added and shaken for 4 hours at 37°C and 0.5 ml more of the galactose oxidase solution was added. The incubation was performed overnight. The oxidation reaction was terminated with 20 ml of chloroform/methanol 2/1. The lower phase was dried and the GL-5 dissolved in 5 ml of tetrahydrofuran with 5 µl 5 N potassium hydroxide. The oxidized GL-5 was reduced with 2.3 mg $NaBT_4$ (14 mCi) overnight. The excess $[^3H]$-$NaBH_4$ was destroyed with several drops of glacial acetic acid added in a fume hood. The solution was dried under nitrogen and the residue was partitioned in 10 ml chloroform/methanol/water 8/4/3. After the lower phase was dried under nitrogen, 30 mg of $NaBH_4$ in 3 ml water was added to the GL-5 to ensure complete reduction. After allowing to stand overnight at 4°, the excess $NaBH_4$ was again decomposed with several drops of glacial acetic acid. Solvent (12 ml of chloroform/methanol 2/1) was added and the lower phase was removed and washed once again with 6 ml of theoretical upper phase. The $[^3H]$-GL-5 was further purified by TLC developed with chloroform/methanol/water 65/45/8. The GL-5 was visualized with iodine vapor, scraped, eluted with chloroform/methanol 2/1, 1/2, methanol and chloroform/methanol/water 50/50/15 as before. The eluate was dried, dissolved in 10 ml chloroform/methanol 2/1, washed with 20% $Na_2S_2O_3$, twice with theoretical upper phase, and dried again. The $[^3H]$-GL-5 product was dissolved in 25 ml chloroform/methanol 2/1 and quantitated by GLC analysis of methanolysis products. The 48 mg of $[^3H]$-GL-5 thus obtained was diluted with cold purified GL-5 to a final yield of 105.5 µmoles of diluted $[^3H]$-GL-5

with a specific activity of 446,000 cpm/μmole. To find the specific activity of the non-reducing terminal N-acetylgalactosamine residue of the [^{3}H]-GL-5, 150 mmoles of GL-5 was hydrolysed with excess purified porcine liver α-N-acetylgalactosaminidase until the release of radioactivity counts levelled off. This count was taken as the total count on the terminal N-acetylgalactosamine of [^{3}H]-GL-5. The specific activity (terminal residue) was thus calculated to be 360,000 cpm/μmole.

(3) Synthesis of GalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-galactose and TalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-galactose

N-Acetylgalactosamine-(α1→6)-1,2:3,4-di-O-isopropylidene-galactose was synthesized by the method of Lemieux, James and Nagabhushan (189). The reaction is shown in Figure 3. Dimeric tri-O-acetyl-2-deoxy-2-nitroso-α-D-galactopyranosyl chloride (13.0 g) was coupled with 8.0 g of 1,2:3,4-di-O-isopropylidene-galactose in dimethylformamide as described. After working up the mixture, 12.0 g of 6-O-(tri-O-acetyl-2-oximino-α-D-lyxo-hexopyranosyl)-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose was obtained; m.p., 171-172°. The compound (20 mmoles) was acetylated and reduced in borane-tetrahydrofuran, acetylated and de-O-acetylated. The reduced disaccharides were applied to a 80 x 3.8 (i.d.) cm column with 453 g of Dowex 1-X2 (200-400 mesh, OH^- form) which was prewashed with 1 N NaOH, water, acetone and 1 N HCl. Elution was performed with water, and 18.4 ml fractions were collected. An aliquot of each fraction (10 μl) was spotted on a TLC plate and sprayed with 2% α-naphthol (in ethanol) and sulphuric acid. The carbohydrate-positive fractions were analysed by GLC after methanolysis. Fractions 44 to 51 were pooled to give GalNAc-(α1→6)-1,2:3,4-di-isopropylidene-Gal and fractions 57 to 107 for TalNAc-(α1→6)-1,2:3,4-di-isopropylidene-Gal. The pooled

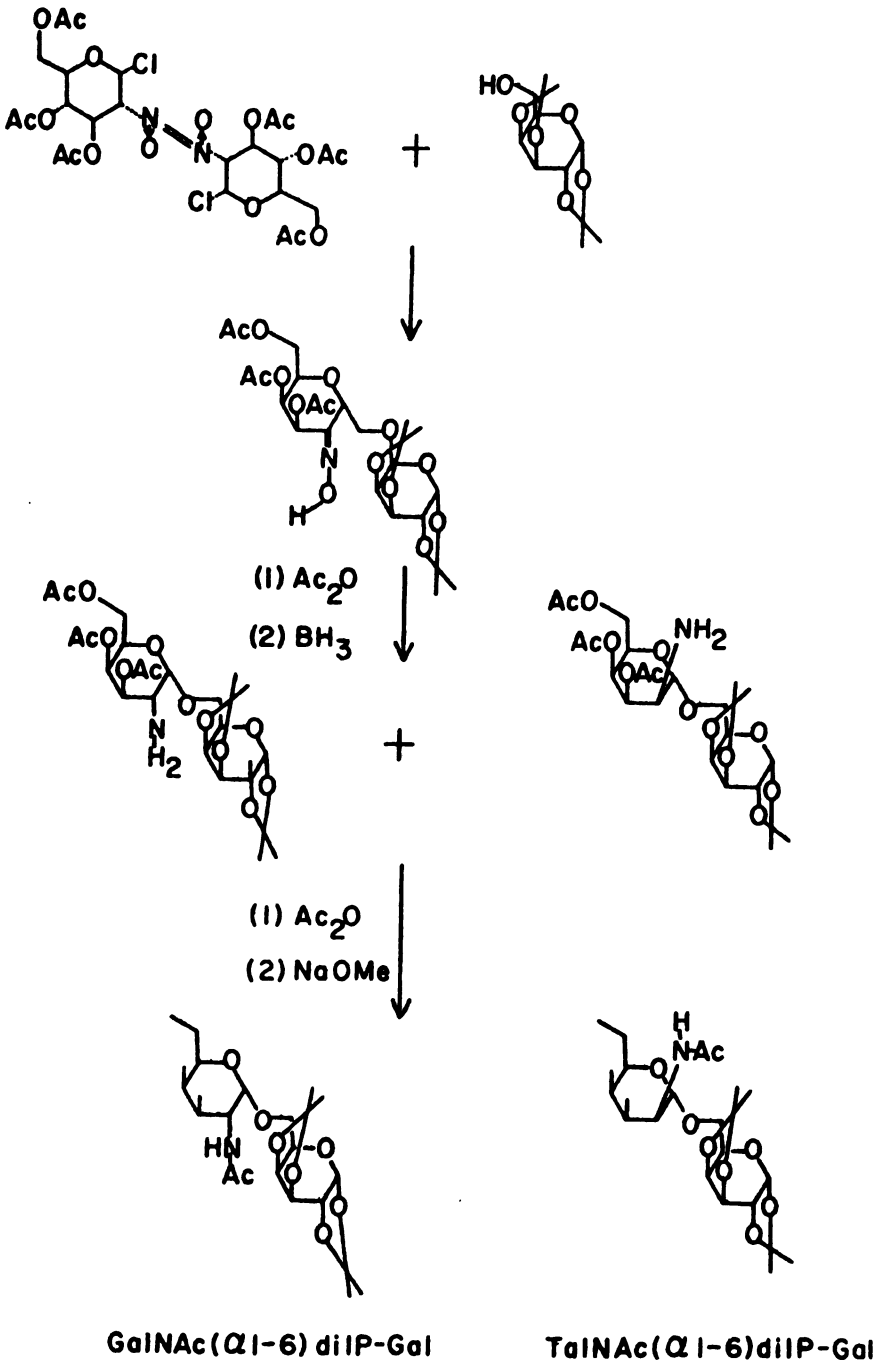


Figure 3. Synthesis of N-Acetylgalactosaminyl-(α1→6)-1,2:3,4-di-O-isopropylidene-Galactose and N-Acetyltalosaminyl-(α1→6)-1,2:3,4-di-O-isopropylidene-Galactose.

fractions were lyophilized and recrystallized, the former from ethyl acetate-hexane and the latter from water. The yield of the GalNAc dissacharide was 0.85 g. The ratio Galactose:N-acetylgalactosamine was 1:0.91. The isopropylidene groups were hydrolysed off by trifluoro-acetic acid. TalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-galactose was obtained from Dowex 1-X2 dolumn as a by product. The yield was 2.97 g.

(4) Preparation of A^+-Porcine Submaxillary Mucin

Blood group A activity of individual glands was determined by the haemagglutination inhibition reaction (64). Small pieces of the glands were minced in 100 µl of saline and dilutions of the homogenate was allowed to react with 4 haemagglutination units of anti-A antiserum for 30 minutes, followed by addition of 50 µl of 2% washed human A^+-red blood cells in isotonic saline solution. Haemagglutination inhibition was read after some wells showed a tight button of red cells.

A^+-Porcine submaxillary mucin was extracted from the pooled A^+-glands by a method described previously (72). Pooled A^+-glands (446 g) were used and the total amount of lyophilized major mucin isolated was 4.7 g.

(5) Isolation of A^+-Glycosphingolipid from Human Red Blood Cell Membrane

Twenty-four units of outdated A^+ human red blood cells were centrifuged at 5,000 x g to sediment the red blood cells, which were then lysed with an equal volume of 0.2% acetic acid. The packed red cell and membrane fractions were homogenized with 3 liters each of the following solvents, chloroform/methanol 2/1 (twice), 1/1, 1/2 and then refluxed with 3 liters methanol overnight. The pooled solvents were dried *in vacuo* and the glycolipids were acetylated with 150 ml of acetic

anhydride/pyridine 1/2 at room temperature overnight. The solution was dried with the aid of toluene and the residue applied in DCE to a 100 g column of Florisil packed in DCE. After washing with 1 liter of DCE, the glycolipids were eluted with 1 liter of DCE/acetone 1/1, dried, dissolved in 50 ml chloroform/methanol 2/1, deacetylated with 10 ml 2% sodium methoxide, dried again and dialysed in water. The lyophilized glycolipids were then loaded onto a 5 g column of washed DEAE-cellulose packed in chloroform/methanol 7/3, and eluted batchwise with 200 ml each of chloroform/methanol 7/3, 1/2 and methanol. The majority of the blood group A^+-glycolipids were obtained in the chloroform/methanol 1/2 fraction. The glycolipids in this fraction were separated and purified by TLC using the solvent system chloroform/methanol/water/acetic acid 55/45/5/5. Blood group A activity of the individual bands was determined by haemagglutination inhibition. The carbohydrate composition of each of the glycolipids was determined by GLC after methanolysis.

(6) Preparation of $[^3H]$-GL-5-Pentasaccharide (Figure 2)

A solution of 2.8 μmole of $[^3H]$-GL-5 in 2 ml of methanol was ozonolyzed in a Supelco ozonolyzer. The evolving gas from the glycolipid solution was monitored by a 2% solution of KI. When the KI solution turned brown, the reaction was stopped and the GL-5 solution was evaporated under nitrogen. One milliliter of 0.2 N Na_2CO_3 solution was added and the mixture was left at room temperature for 14 hours. After neutralization with acetic acid and evaporation of the solvent under nitrogen, the residue was partitioned in 5 ml of chloroform/methanol/water 8/4/3. The lower phase was washed with 2.5 ml of theoretical upper phase. The pooled upper phase was dried and applied to a 50 ml Bio-Gel P-2 column (1 x 60 cm) packed in water. The fractions

(0.83 ml/fraction) were monitored with radioactivity and TLC with solvent system butanol/acetic acid/water 100/50/50 using galactose, melibiose, raffinose and stachyose as reference compounds. A yield of 1.17 μmoles of [^{3}H]-GL-5-pentasaccharide was obtained.

(7) Preparation of N-[1-^{14}C]-Acetyl-Sphingosyl-Forssman-Pentasaccharide

Forssman hapten (354 mg) was deacylated by the method of Taketomi *et al.* (18). The glycolipid was dissolved in 30 ml of 90% aqueous butanol containing 1 N KOH and the solution was refluxed for 2.5 hours. The solution was then dialysed for 2 days evaporated *in vacuo* with the aid of absolute ethanol, dissolved in 3 ml chloroform/methanol 2/1 and centrifuged. The supernatant fraction was poured into 30 ml of acetone. The precipitate was collected by centrifugation at 1,200 x g for 3 minutes and dissolved in a known volume of chloroform/methanol 2/1.

Deacylated GL-5 (30 mg) was dissolved in 4 ml of dry methanol. Acetic-[1-^{14}C]-anhydride (5.8 mg, 4.4 mCi/mM) in 1.5 ml of thiophene-free dry benzene was added to the solution and the mixture was left at room temperature overnight. After evaporation of the acetic anhydride under nitrogen in a fume hood, the glycolipid was analysed by radio-scanning TLC. Complete acetylation was achieved with the addition of 0.5 ml of acetic anhydride to the glycolipid dissolved in 5 ml of methanol and allowed to stand at room temperature overnight. A yield of 11.2 mg of N-[1-^{14}C]-acetyl-sphingosyl-GL-5-pentasaccharide was obtained. It was further purified by preparative TLC using the solvent system butanol/acetic acid/water 100/50/50.

(II) Enzyme Assays

(1) Assay with Forssman Glycolipid

Forssman glycolipid was assayed by dissolving 150 nmoles of [^{3}H]-glycolipid in 50µl of sodium taurocholate (12 mg/ml) with sonication. To this solution were added 50 µl of 0.3 M sodium citrate buffer, pH 3.9, and 50 µl of enzyme solution; incubation was for 30 minutes at 37°. The reaction was terminated by adding 4 ml of chloroform/methanol 2/1 and 0.85 ml water. After thorough mixing, the samples were centrifuged. The lower phase was pipetted out and the upper phase was washed with 2.5 ml of theoretical lower phase. The upper phase was then pipetted into scintillation vials, dried and counted in 0.5 ml of water and 10 ml of scintillation cocktail (7 g PPO, 0.6 g dimethylPOPOP, 100 ml Bio-Solv BBS-3 and 1 liter of toluene).

(2) Assay with p-Nitrophenyl-α-N-acetylgalactosaminide

The assay mixture contained 100 µl of 2 mM p-nitrophenyl-α-N-acetyl-galactosaminide, 50 µl of 0.3 M sodium citrate buffer pH 4.3 and 50 µl of enzyme solution. After incubation at 37° for 15-30 minutes, the reaction was terminated with 3 ml of 0.6 M potassium borate pH 10.4. Absorbance was read at 410 nm.

(3) Assay with GL-5-pentasaccharide Hydrolysis

The incubation mixture contained in a final volume of 25 µl, 25 nmoles of GL-5-pentasaccharide in 5 µl of 0.3 M sodium citrate buffer, pH 3.9 and 10 µl of enzyme solution. The solution was incubated at 37° for 30 minutes and terminated by boiling in a water bath for 3 minutes. The samples were spotted on silica gel G plates with 100 µg each of galactose and stachyose standards on the same plate. After

chromatography in butanol/acetic acid/water 100/50/50, the standards were sprayed with orcinol-sulphuric acid and heated at 100° for 5-10 minutes until the carbohydrate spots were visualized. The area corresponding to the monosaccharide region were scraped off and counted directly in 0.5 ml water and 10 ml toluene-based scintillation fluid. Samples were hydrolysed with pure enzyme until the amount of liberated monosaccharide levelled off and the total amount of radioactivity liberated by this exhaustive digestion was taken as the total counts of 25 nmoles of N-acetylgalactosamine liberated. The specific activity thus found was 400 cpm/nmole.

(4) Assay of N-[1-^{14}C]-Acetyl-sphingosyl-GL-5-pentasaccharide Hydrolysis

Hydrolysis of 50 nmoles of N-[1-^{14}C]-acetyl-sphingosyl-GL-5-pentasaccharide was measured in a final volume of 50 μl; components were 10 μl sodium taurocholate (20 mg/ml), 10 μl of 0.3 M sodium citrate buffer, pH 3.9, and 10 μl enzyme solution. After at 37° for 30 minutes, the reaction was terminated in a boiling water bath for 3 minutes. The incubated mixtures were spotted on silica gel G plates and chromatographed with solvent system chloroform/methanol/acetic acid/ water 55/45/5/5. At the end of the TLC run, the lanes were scanned by a Berthold radio-scanner. The radioactive N-[1-^{14}C]-acetyl-galactosamine spots on the plates were located by matching the thin layer plates with the radioactive scanning traces. The areas corresponding to the liberated N-acetylgalactosamine were marked, scraped into scintillation vials and counted with 0.5 ml of water and 10 ml of toluene-based scintillation cocktail. The total radioactivity of 50 nmoles of liberated N-acetylgalactosamine was found by exhaustive hydrolysis of the substrate with pure α-N-acetylgalactosaminidase and counting the area corresponding to

the liberated monosaccharide. The specific activity of the substrate thus found was 499 cpm/nmole.

(5) Assay of Porcine Submaxillary Mucin Hydrolysis

A solution (200 µl) of major A^+ porcine submaxillary mucin (10 mg/ml) was hydrolysed by 25 µl of enzyme with 25 µl 0.3 M sodium citrate buffer, pH 4.2, at 37° for 30 minutes. The reaction was terminated by the addition of 750 µl of absolute ethanol. The precipitated porcine submaxillary mucin was centrifuged at 3 minutes at 1200 x g in a Sorvall GLC-1 centrifuge. The supernatant fraction was pipetted out and the precipitate was rinsed with 1 ml of absolute ethanol. The pooled supernatant was dried in a vial under nitrogen, with the addition of 20 nmoles of mannitol as the internal standard. A mixture (50 µl) of pyridine/hexamethyldisilazane/trimethylsilane 10/4/2 was then added to trimethylsilylate the sugars. After sonication and standing for at least 30 minutes, 2-5 µl aliquots of the samples were injected into a 6 ft 5% SE-30 column on Supelcoport (80-100 mesh) at 185°. The amount of liberated N-acetylgalactosamine was quantitated by comparing its area with the area of the peak for the mannitol. The relative detector response of N-acetylgalactosamine as compared to mannitol was determined by comparing the areas of known amounts of the two sugars and was found to be 0.84:1.

(6) Assay of N-Acetylgalactosamine-(α1→6)-1,2:3,4-di-O-isopropylidene-Gal Hydrolysis

The hydrolysis of GalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-Gal was performed in a final volume of 150 µl, consisting of 100 µl substrate, 25 µl of 0.3 M sodium citrate buffer, pH 4.3, and 25 µl of an appropriate dilution of the purified enzyme. The solution was incubated at 37° for

one hour, terminated by boiling in water for 2 minutes, evaporated to dryness after the addition of known amounts of mannitol and trimethylsilylated with 50 µl of pyridine/hexamethyldisilazane/trimethylsilane 10/4/2 for at least 30 minutes. The product (1 to 2 µl aliquot was analysed by GLC with a 3% SP-2100 column on Supelcoport (100-200 mesh) temperature programmed from 180° to 230° at 3°/min. The liberated N-acetylgalactosamine was quantitated as in the porcine submaxillary mucin hydrolysis assay.

(7) Hydrolysis of Human Red Blood Cell and Dog Intestinal Blood Group A Glycolipids

Blood group A-active glycolipids were hydrolysed in a final volume of 150 µl, a solution with 4 mg/ml sodium taurocholate and 0.1 M sodium citrate, pH 3.9. After incubating at 37° overnight the reaction was stopped by boiling for 2 minutes in water and chromatographed by TLC using chloroform/methanol/acetic acid/water 55/45/5/5 as the solvent system. The products were visualized by iodine vapor or orcinol-sulphuric acid.

(III) Enzyme Purification

Fresh liver was put on ice after excision from the animals. All subsequent steps were performed at 4° unless otherwise indicated. The liver (6 Kg) was homogenized in a Waring blendor twice for intervals of 15 seconds in 3 volumes of 1 mM phenylmethylsulphonyl fluoride and 0.1 mM EDTA. The homogenate was centrifuged and adjusted to pH 3.7 with 1 M citric acid. After stirring for 15 minutes the solution was readjusted to pH 4.8 with 1 M sodium citrate and quickly centrifuged. Ammonium sulphate was added to the supernatant fraction to 30% of

saturation and stirred for one hour before centrifugation. The 30% supernatant fraction was then mixed with ammonium sulphate to 55% of saturation and allowed to stand for another hour. The 30-55% ammonium sulphate precipitate was redissolved in water and dialysed exhaustively against 5 mM sodium citrate buffer, pH 6.0, with several changes of water. The supernatant fraction of the dialysed, centrifuged enzyme solution was then loaded onto a DEAE-cellulose column (40 x 4.5 cm i.d.) packed with 200 g of DE-52 and preequilibrated with 5 mM sodium citrate buffer, pH 6.0. After the column was washed overnight with 5 mM sodium citrate buffer, pH 6.0, the enzyme activity was eluted with 2 liters of 0.6 M NaCl in the starting buffer and quickly precipitated with ammonium sulphate to 70% saturation. The precipitate was dialysed against 0.05 M sodium citrate buffer, pH 6.0, and applied at room temperature to a 200 ml Con A-Sepharose column (30 x 3.5 cm i.d.) which was equilibrated in 0.05 M sodium citrate buffer, pH 6.0. The column was washed with five column volumes of the equilibrating buffer after which the activity was eluted with 500 ml of 0.5 M α-methyl mannoside in 0.05 M citrate buffer, pH 6.0. The solution was immediately concentrated and dialysed exhaustively and applied to a small 10 g DE-52 column (40 x 1.5 cm i.d.) equilibrated with 5 mM sodium phosphate buffer pH 6.8. α-N-Acetylgalactosaminidase activity was eluted with 200 ml of 1% ampholine pH 4-6 in water. The eluted enzyme activity was concentrated, dialysed against 0.05M sodium citrate, pH 6.0, and applied to a 700 ml Sephadex G-150 column (1200 x 25 cm i.d.) equilibrated with 0.05 M sodium citrate buffer, pH 6.0. Fractions of 12.7 ml were collected. The enzyme activity peak (fractions 24-28) was pooled and dialysed against 1 mM sodium phosphate, pH 6.8. A 10 g

hydroxylapatite column (16 x 1 cm i.d.) equilibrated in 1 mM sodium phosphate buffer, pH 6.8, was used for the final purification step. The enzyme was loaded and eluted with a linear gradient of sodium phosphate buffer, with 220 ml of 1 mM and 200 ml of 0.4 M sodium phosphate buffers, pH 6.8, in the gradient chambers. The final pure enzyme preparation was concentrated and stored frozen in 0.05 M sodium citrate buffer, pH 6.0.

(IV) Preparation of Con A-Sepharose

Con A-Sepharose was prepared according to the method of Lloyd (190). Packed Separose 4B (250 ml) was activated according to the method of March, Parikh and Cuatrecasas (191). Con A (2 g) was added to a 500 ml suspension of the activated Sepharose beads with 2.2 g of $NaHCO_3$ at 4° and after two days with occasional swirling, the beads were packed into a column and washed with 1 liter 0.07 M $NaHCO_3$, and 4 liters each of 0.1 M sodium borate, pH 8.5, in 1 M NaCl, 0.1 M sodium acetate, pH 4.1, in 1 M NaCl and finally 1 M NaCl. The Sepharose was then suspended in 1 M NaCl in a final volume of 700 ml. The amount of Con A coupled to Sepharose was 7.61 mg, as determined by the direct Lowry determination (192) of an aliquot of the beads.

(V) Methanolysis

Methanolysis of glycolipids and carbohydrates were performed by the method of Vance and Sweeley (193). Samples were hydrolysed in 3 ml of 0.75 M methanolic HCl in sealed ampules for 24 hours at 82°. Fatty acid methyl esters were extracted three times with equal volumes of hexane. Silver carbonate was added until the solution is neutral to a wetted pH paper. Acetic anhydride (0.3 ml) was added to re-N-acetylate

the amino-sugars for at least 6 hours at room temperature. After the addition of mannitol and 2 drops of water, the sample was centrifuged and the supernatant fraction removed. The residue was washed four times with 2 ml aliquots of methanol. The pooled supernatant fraction was dried under nitrogen and derivatized with about 100 µl of pyridine/hexamethyldichlorosilanzane/trimethylchlorosilane 10/4/2. Trimethylsilylated methyl glycosides were analysed on an F & M Model 402 gas chromatograph with a 3% SE-30 or SP-2100 on Supelcoport (80-100 mesh).

(VI) Permethylation for Linkage Studies

Permethylation of carbohydrates was performed by the method of Hakomori (194). All the permethylation operations were done under dry nitrogen. Hexane was dried by refluxing with BaO (20 g/liter) for 2 hours. The redistilled dried hexane was stored over sodium. Dimethylsulphoxide was dried by refluxing with BaO (50 g/liter) for 2 hours, redistilled and stored over molecular sieves. All the other solvents used were redistilled. A sample of NaH (0.9 g of 57% oil emulsion) was washed 7 times with 15 ml aliquots of dried redistilled hexane. Dry redistilled dimethylsulphoxide (10 ml) was added and allowed to react at 65-70° until bubbling of hydrogen ceased (approximately 90 minutes). The dimethylsulphinyl ion solution (0.5 ml) was added to samples (0.5 mg) dissolved in 0.5 ml dimethylsulphoxide and allowed to react for 30 minutes with periodic sonications. Redistilled iodomethane (2 ml) was then slowly added and allowed to stand for 2 hours at room temperature. The solutions were then dissolved in 5 ml of chloroform and washed twice with 5 ml water, once with 5 ml 20% $Na_2S_2O_3$ and thrice with water. The samples were dried under nitrogen with the aid of absolute alcohol and hydrolysed in 0.5 ml 0.5 N H_2SO_4 in

95% acetic acid for 24 hours at 85°. Water (0.5 ml) was then added and allowed to react for an additional 5 hours at 85°. A small column with 2 ml of Dowex 1 x 8, acetate form (50-100 mesh) was used to adsorb the sulphate. The column was washed with 2-3 ml of acetic acid. The hydrolyzate was evaporated to dryness under nitrogen and reduced with 0.5 ml of $NaBH_4$ (10 mg/ml) for 2 hours. Reduction was terminated with the addition of several drops of glacial acetic acid. The solution was dried under nitrogen. Borate was removed as its methyl ester by 1-2 drops of acetic acid and 2 ml methanol, heating in a boiling water bath for 5 minutes, and evaporating under nitrogen. The esterification procedure was repeated three more times. The dried sample was acetylated in 0.5-1 ml acetic anhydride for 60-90 minutes at 100°. After drying under nitrogen with the aid of toluene, dissolved in 2 ml of CH_2Cl_2, washed three times with 1-2 ml water and drying under nitrogen again, the partially methylated alditol acetates were ready for analyses by GLC or GC-MS. Column packings used for these analyses were 3% ECNSS-M on Supelcoport (100-200 mesh), 3% OV-210 on Gas Chrom Q (80-100 mesh) or 0.2% EGS/0.2% EGA/1.4% XE-60 on Chromosorb P (100-200 mesh). The mass spectra were interpreted according to Bjorndal *et al.* (195).

(VII) Polyacrylamide Gel Electrophoresis

The method of Gabriel was used for native gel electrophoresis (196). Gels were stained with coomassie blue fast stain (197). p-Nitrophenyl-α-N-acetylgalactosaminidase activity on gels was monitored by incubation of the gel in 3 ml of p-nitrophenyl-α-N-acetylgalactosaminide solution in a final concentration of 2 mM substrate and 0.1 M sodium citrate, pH 4.3, at 37° for 15-30 minutes. Liberated p-nitrophenol was visualized with 0.6 M potassium borate, pH 10.4. The activity was

scanned immediately at 410 nm on a Gilford spectrophotometer equipped with a linear transport unit.

(VIII) General Procedures

Isoelectric focussing was performed by the method of Vesterberg (198) using ampholytes at pH 3-10, 4-6 and 5-8 and an LKB isoelectric focussing unit with a capacity of 110 ml.

A Sorvall RC 2-B centrifuge was used for enzyme purification procedures. Either a GSA or SS-34 rotor was spun at 23,000 x g for 20 minutes. A Sorvall GLC-1 centrifuge was used for all centrifugation procedures that did not require high speed. A centrifugal force of 1200 x g for 3 minutes was used. Protein was determined by the method of Lowry *et al.* (192) using BSA as standards. pH measurements were performed at 25°. Solutions used in enzyme purifications were prepared in glass-distilled water. The purified enzyme used for assays was diluted with 10 mg/ml BSA solutions. The final protein concentrations in enzyme assays were maintained above 0.4 mg/ml. Dialysis tubing was boiled with ethanol and washed by a published procedure before use (199). α-Galactosidase from ficin and β-galactosidase from Jack bean were purified by procedures described previously (200-202). The conditions for enzymatic and partial acid hydrolyses have been published (203,204). Periodate oxidation was performed by the method of Siddiqui and Hakomori (3). Amino acid composition was analysed as described (205). The carbohydrate composition was determined by the method of Chambers and Clamp (206). Molecular weights were determined by SDS polyacrylamide gel electrophoresis as described by Segrest and Jackson (207) using cytochrome C, aldolase, catalase, chymotrypsinogen A, BSA, ovalbumin, and ferritin as standards. The molecular weight of the native enzyme

was estimated by Sephadex G-150 chromatography using the same protein standards (208).

Sialic acid in the purified α-N-acetylgalactosaminidase was analysed by incubating 59 µg of enzyme with 0.05 units of neuraminidase from *Clostridium perfringens* in a final volume of 100 µl, containing 0.05 M acetate buffer,pH 5.0, for 2 hours at 37°. After terminating the reaction with 250 µl of 5% phosphotungstic acid the liberated sialic acid was quantitated by the thiobarbituric acid assay (209).

Glycolipids and carbohydrates on TLC plates were visualized by heating in a 110° oven for 10 minutes after spraying with 2% α-naphthol in 95% alcohol and sulphuric acid or 0.5% orcinol in 4 N sulphuric acid. Glycolipids were also visualized by exposing the plates to iodine vapour in a TLC tank.

RESULTS

Characterization of the Pentaglycosylceramide in Dog Intestines

A thin-layer chromatogram of the mixture of glycosphingolipids from canine kidney and intestine is shown in Figure 4. Monoglycosylceramide (GL-1), triglycosylceramide (GL-3), and hematoside (G_{M3}) were the major components of kidney, while GL-3 and the pentaglycosylceramide (GL-5) were the major constituents of intestine (Table 2). Gas chromatographic analysis of the trimethylsilyl derivatives of methyl glycosides from GL-5 (Figure 5) indicated that the oligosaccharide moiety consisted of glucose, galactose, and N-acetylgalactosamine in a molar ratio of 1.0: 2.1:2.0. Colorimetric analysis of the long-chain base fractions, liberated from GL-5 by methanolysis, indicated approximately 1 mole of base per mole of glucose.

Partial acid hydrolysis of GL-5 liberated glucosylceramide and a diglycosylceramide with a 1:1 molar ratio of galactose to glucose, indicating a probable partial internal sequence of galactosylglucosylceramide. Enzymatic hydrolysis of GL-5 was carried out by the following mixtures of glycosidases on a scale (200 μg each) sufficient for analysis by thin-layer chromatography (Figure 6): (a) α-N-acetylhexosaminidase alone, and (b) α-N-acetylgalactosaminidase plus β-N-acetylhexosaminidase. The GL-3 derived from partial acid hydrolysis or enzymatic hydrolysis was treated with (a) α-galactosidase, and (b) α-galactosidase plus β-galactosidase. The products were a tetraglycosylceramide, a

Figure 4. Thin-Layer Chromatography of Glycosphingolipids of Canine Intestine (Lane I) and Kidney (Lane K). Lane S: Standards from Porcine Erythrocytes.

The solvent system was chloroform/methanol/water 100/42/6 and 0.25 mm Silica gel H plates were used. Plates were visualized by iodine vapor.

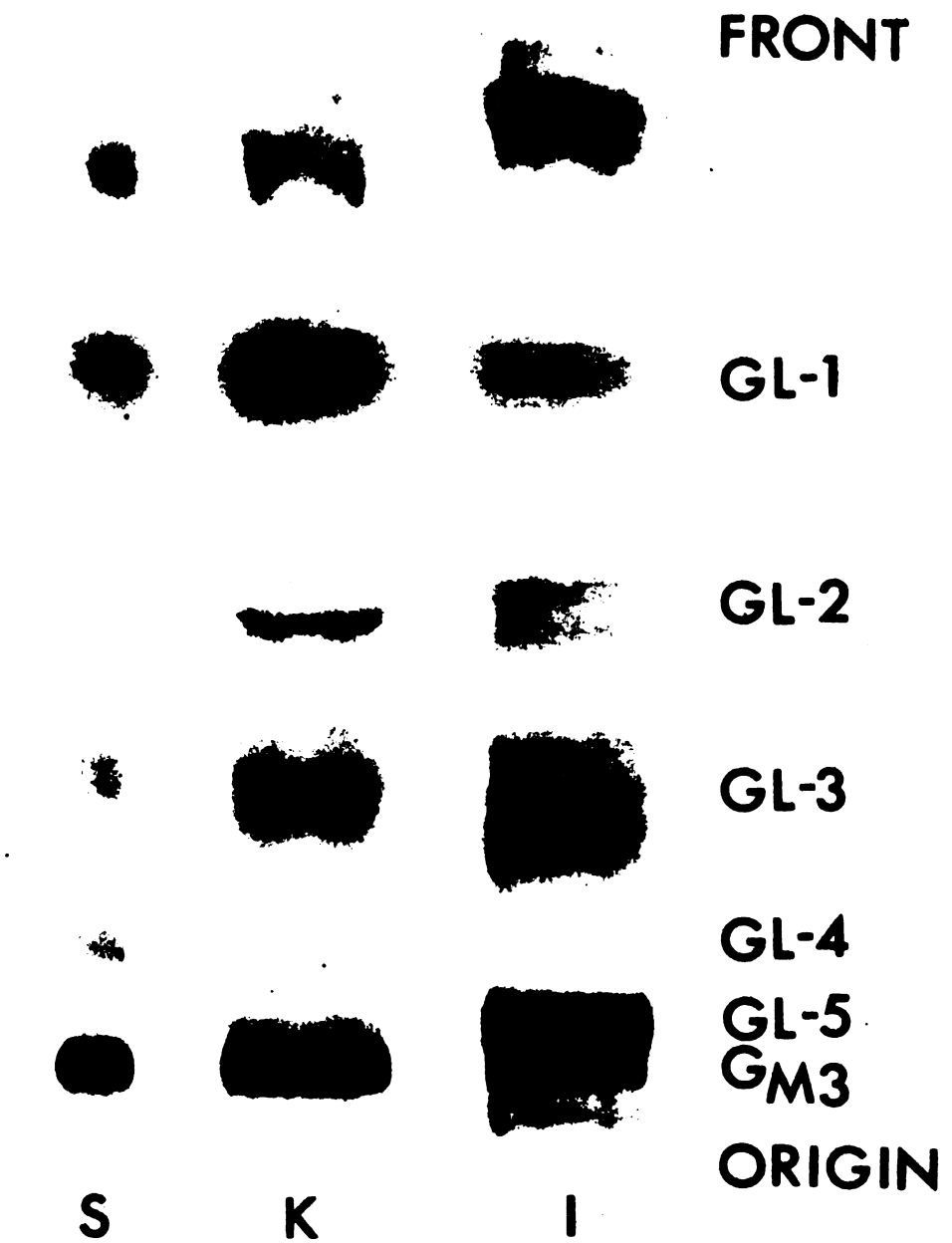


Figure 4.

Table 2. Yields of Glycosphingolipids from Canine Intestines.

Glycosphingolipids	Amount (μmoles/g wet weight)
Glucosylceramide	0.052
Lactosylceramide	0.050
Triglycosylceramide	0.160
Tetraglycosylceramide	trace
Forssman hapten	0.110
Hematoside	0.028

Figure 5. Gas-Liquid Chromatography of Trimethylsilylated Methyl Glycosides of Standard Sugars (Top), Canine Forssman Hapten (Center) and Porcine Globoside (Bottom).

A: methyl galactosides; B: methyl glucosides; and C: methyl N-acetylgalactosaminides.

Analyses were performed on a 2 m x 2 mm column packed with 3% SE-30 on 80-100 mesh Supelcoport and programmed from 160°-210° at 2°/min with nitrogen as carrier gas.

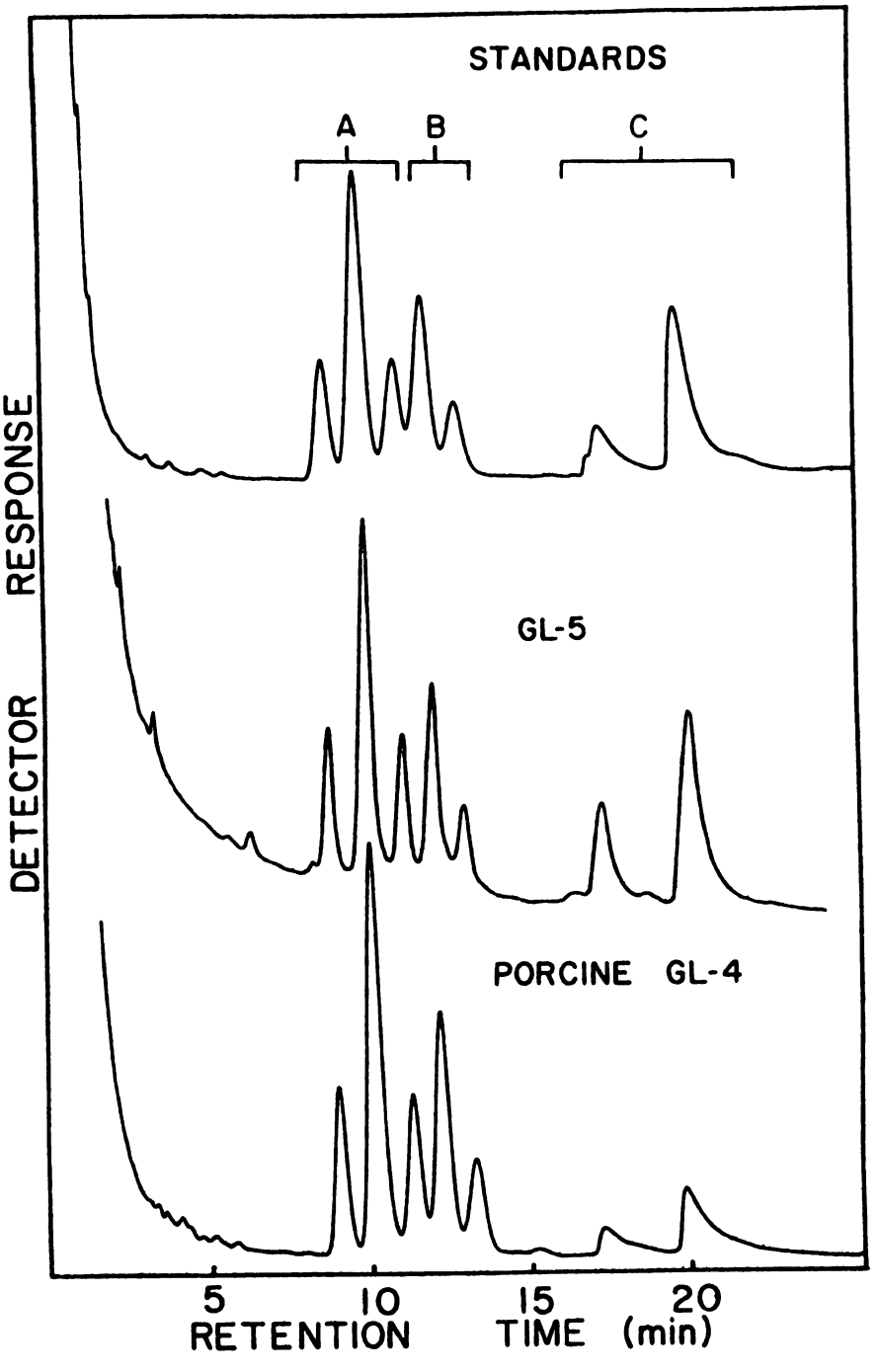

STANDARDS
A
B
C
GL-5
PORCINE GL-4
DETECTOR RESPONSE
5
10
15
20
RETENTION TIME (min)

Figure 5.

Figure 6. Thin-Layer Chromatography after Enzymatic Hydrolyses of Forssman Hapten.

Lane 1: Canine intestinal GL-5;
Lane 2: GL-5 treated with α-N-acetylgalactosaminidase;
Lane 3: GL-5 treated with α-N-acetylgalactosaminidase and β-N-acetylgalactosaminidase, could be visualized by α-naphthol-sulphuric acid;
Lane 4: GL-3 obtained from partial hydrolysis of GL-5;
Lane 5: GL-3 treated with α-galactosidase;
Lane 6: GL-3 treated with α-galactosidase and β-galactosidase;
Lane 7: Horse kidney glycolipid standards.

TLC conditions are the same as Figure 4.

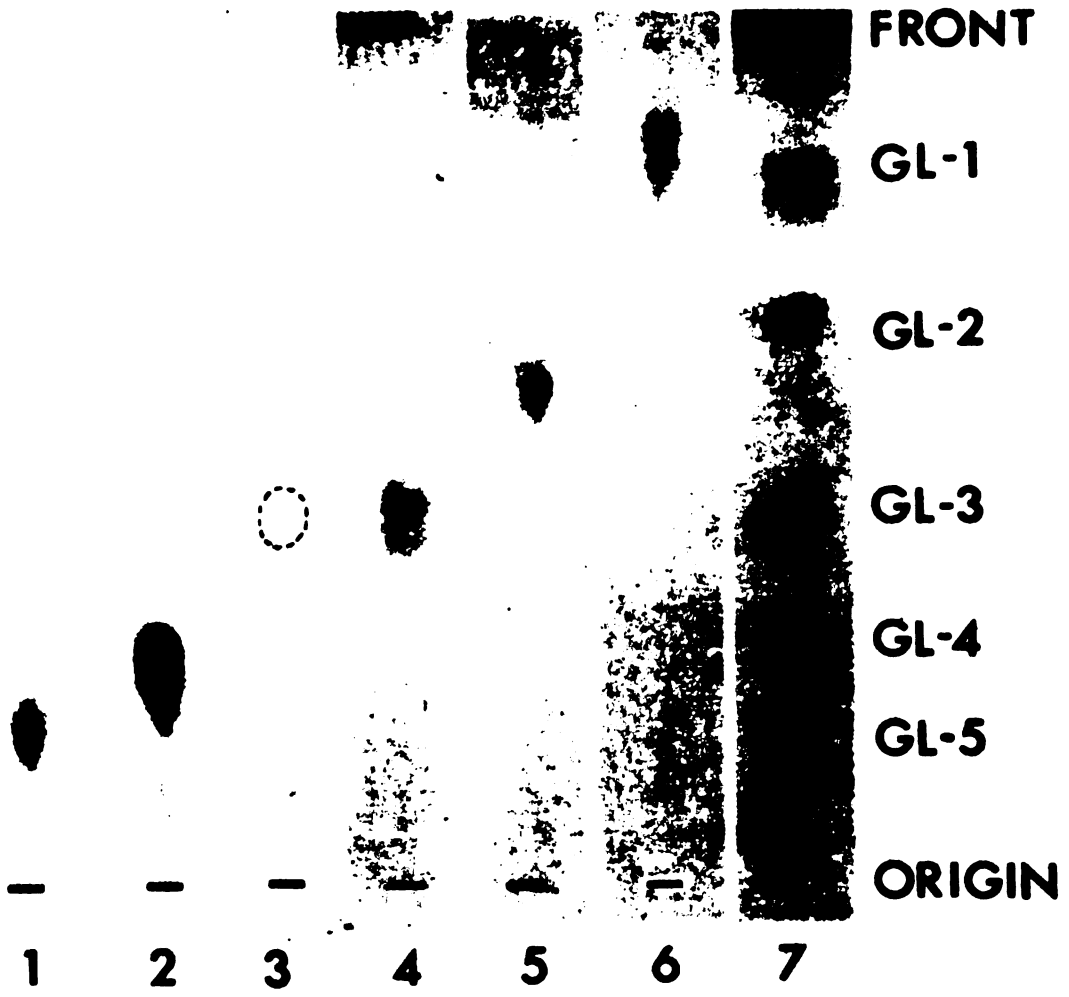


Figure 6.

triglycosylceramide, a diglycosylceramide, and a monoglycosylceramide, respectively. The sequence and stereochemistry of the glycosidic linkages were therefore (α-N-acetylgalactosaminyl)-(β-N-acetylgalactosaminyl)-(α-galactosyl)-(β-galactosyl)-(glucosyl)-ceramide. These results confirm the arrangement of carbohydrate units proposed by Vance *et al.* (1).

Gas chromatograms of partially methylated alditol acetates, obtained from the neutral sugars of canine GL-5 and porcine globoside, are shown in Figure 7. The three peaks from GL-5 corresponded in their relative areas and retention times to those of porcine globoside. Mass spectra provided evidence that they were 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol (peak A), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol (peak B), and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol (peak C), respectively. Enzymatic degradation of 5 mg of GL-5 by a mixture of α-N-acetylgalactosaminidase and β-N-acetylhexosaminidase (as described above) gave a triglycosylceramide which was isolated by preparative thin-layer chromatography. Permethylation and mass spectral analysis of the products after acid hydrolysis, borohydride reduction, and acetylation gave evidence for the presence of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-acetylgalactitol, and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol. The internal likages were therefore galactosyl-(1→4)-galactosyl-(1→4)-glucosyl(1→1)-ceramide. When considered along with the results of permethylation of the intact GL-5 presented above, it was concluded that the penultimate N-acetylgalactosamine residue must have a 1→3 glycosidic linkage to galactose.

Gas-liquid chromatography of the partially methylated alditol

Figure 7. Gas-Liquid Chromatography of Neutral Partially Methylated Alditol Acetates from Canine Forssman Hapten (upper) and Porcine Globoside (lower).

The carbohydrate derivatives were analysed on a 2 m x 2 mm column with a mixture of liquid phases containing 0.2% ethylene glycol succinate, 0.2% ethylene glycol adipate and 1.4% XE-60 on 100-120 mesh Gas-chrom P. The temperature was programmed from 155° to 210° at 2°/min. Retention times are in minutes.

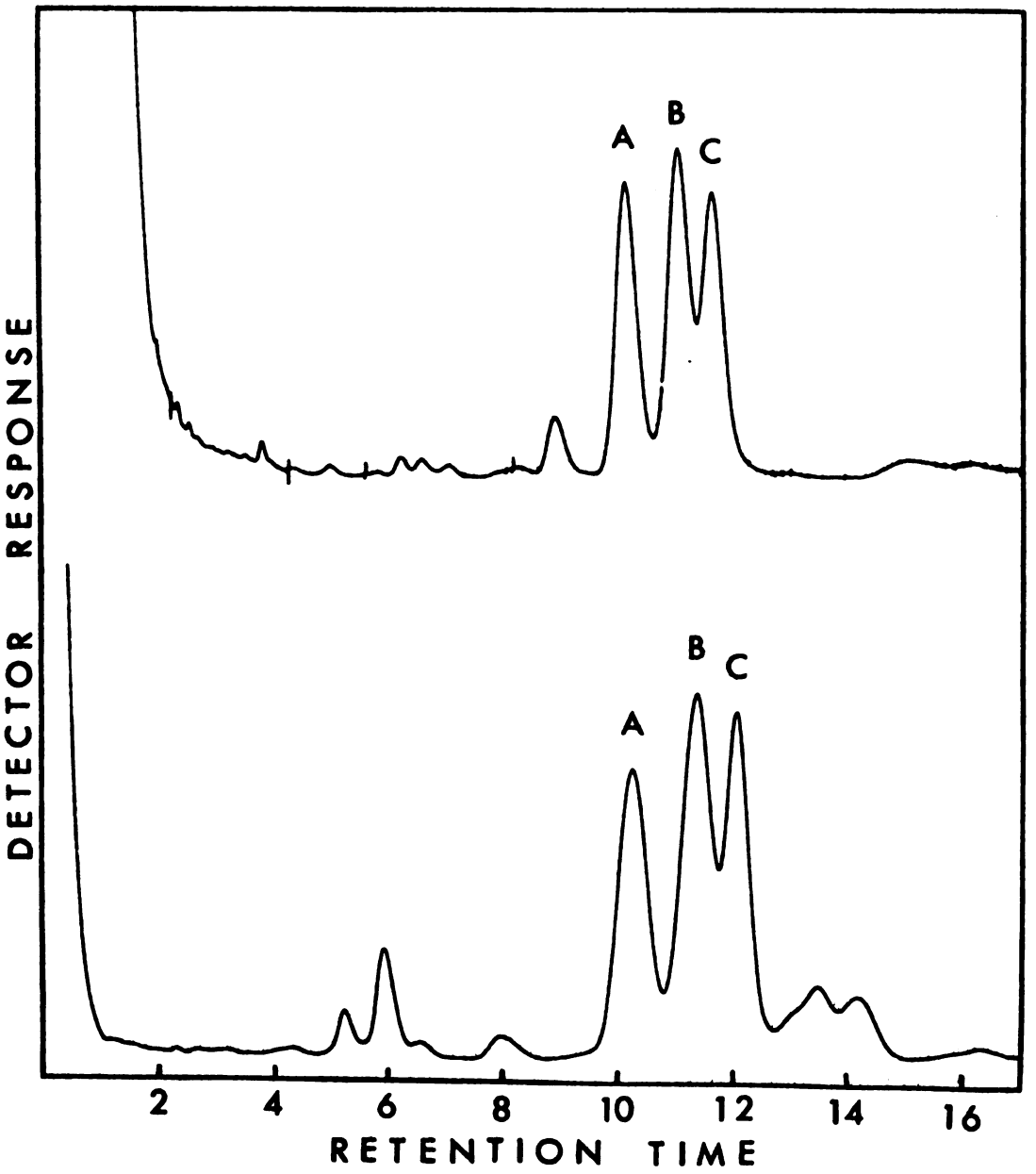


Figure 7.

acetates derived from the neutral and amino sugars from GL-5 is shown in Figure 8. Peak C is identical in retention time with the aminosugar derived from globoside, N-acetyl-N-methyl-1,5-di-O-acetyl-3,4,6-tri-O-methylgalactosaminitol (Structure 1, Figure 9). Mass spectra of the authentic sample, from globoside, and that from the unknown are shown in Figure 10. Primary fragment ions at m/e 158,161, 202 and 205 can be found in both specta. The base peak at m/e 116 is probably derived from m/e 158 by the loss of ketene (42 a.m.u.). Prominent peaks at m/e 98, 142 and 145 are assumed to be formed from m/e 158, 202 and 205, respectively, by the loss of acetic acid (60 a.m.u.). A significant peak at m/e 129 is related to m/e 202 by the loss of either CH_2OAc or N-methylacetamide (73 a.m.u.).

Peak D (Figure 8) had the same retention time as N-acetyl-N-methyl-1,3,5-tri-O-acetyl-4,6-di-O-methylgalactosaminitol (Structure 2, Figure 9) derived from N-acetylchondrosine, and mass spectra of the two peaks, shown in Figure 11, B and C, indicate their identity. Significant peaks from primary ions were found at m/e 158 and 318 (M^+-73). The base peak was at m/e 116, as observed with other partially methylated alditol acetates of aminosugars permethylated by the Hakomori procedure as outlined by Björndal *et al.* (195). Secondary fragments were found at m/e 170, assumed to be formed from the ion at m/e 230 by the loss of acetic acid; m/e 242 derived from m/e 346 by the loss of acetic acid and ketene; and m/e 272, derived from m/e 346 by the loss of ketene and methanol.

The mass spectrum (Figure 11) of N-acetyl-N-methyl-1,4,5-tri-O-acetyl-3,6-di-O-methylglucosaminitol (Structure 3, Figure 9), prepared from chitin, is clearly different from Peak D derived from GL-5. A similar pattern of ions was observed at m/e 74, 98, 116 and 158, but

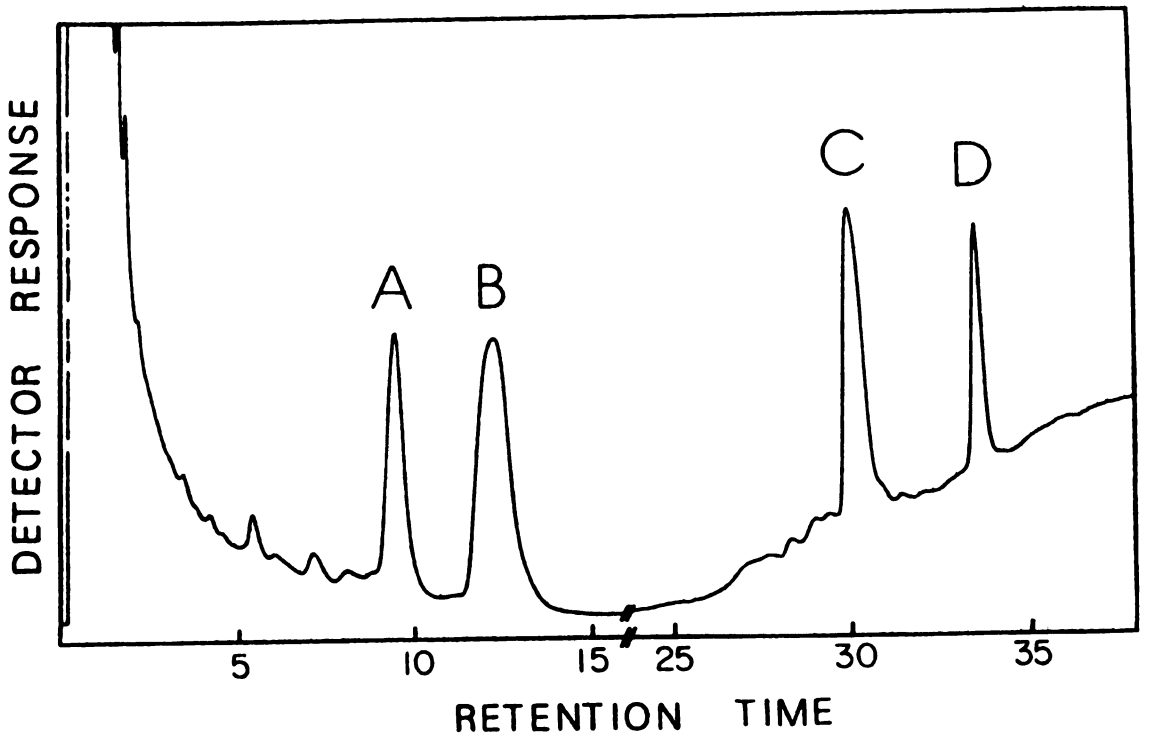


Figure 8. Gas-Liquid Chromatography of Partially Methylated Alditol Acetates of Neutral and Aminosugars from Forssman Hapten.

Analysis was performed on a 2 m x 2 mm column of 3% OV-210 on 80-100 mesh Supelcoport operated isothermally for 15 minutes at 160° and then programmed from 160° to 250° at 5° per minutes with nitrogen as carrier gas. Retention times are in minutes. A: 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol; B, a mixture of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol and glucitol. The mass spectra of peaks C and D are shown in Figures 10 and 11.

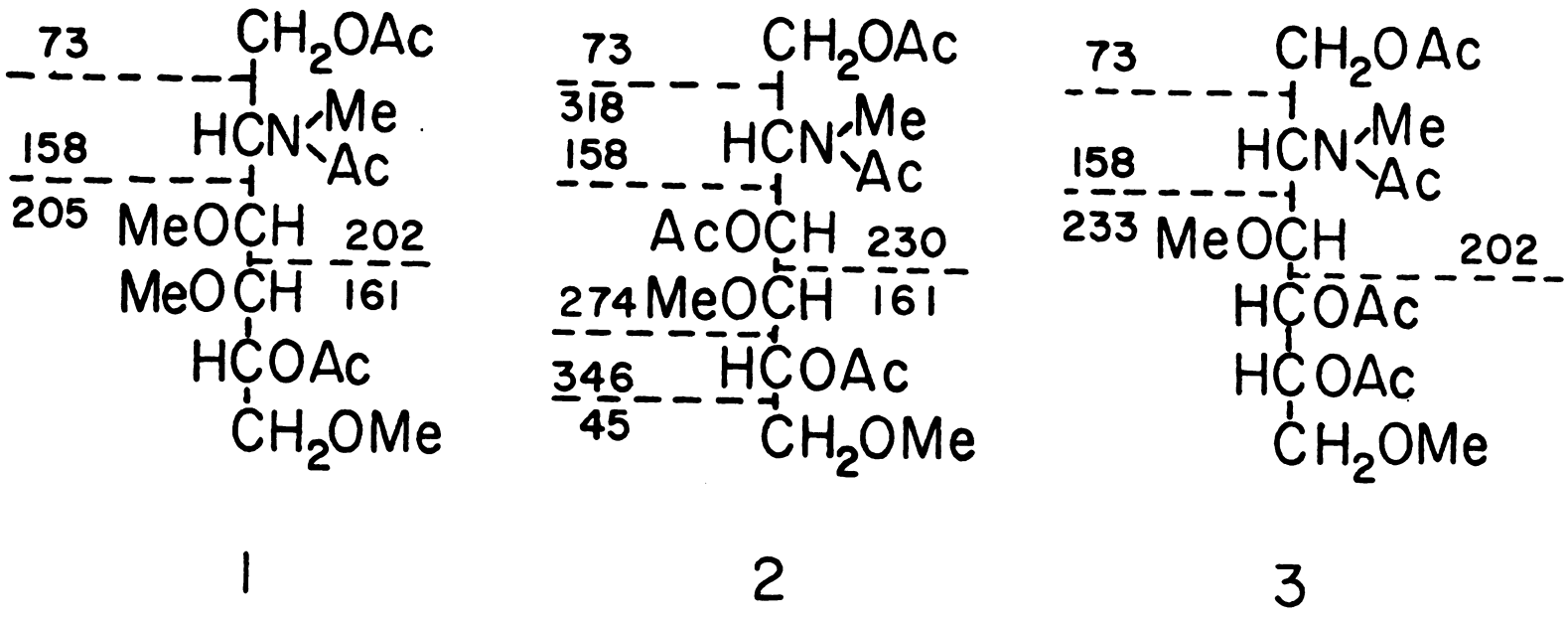


Figure 9. Primary Fragments of Partially Methylated Alditol Acetates of Aminosugars.

Structure 1: N-acetyl-N-methyl-1,5-di-O-acetyl-3,4,6-tri-O-methylgalactosaminitol;
Structure 2: N-acetyl-N-methyl-1,3,5-tri-O-acetyl-4,6-di-O-methylgalactosaminitol; and
Structure 3: N-acetyl-N-methyl-1,4,5-tri-O-acetyl-3,6-di-O-methylglucosaminitol.

Figure 10. Mass Spectrum of Partially Methylated Alditol Acetate of Terminal Aminosugar of Forssman Hapten Compared with a Standard.

(A) N-acetyl-N-methyl-1,5-di-O-acetyl-3,4,6-tri-O-methylgalactosaminitol (Strucure 1 in Figure 9) from porcine globoside;
(B) peak C in Figure 8.

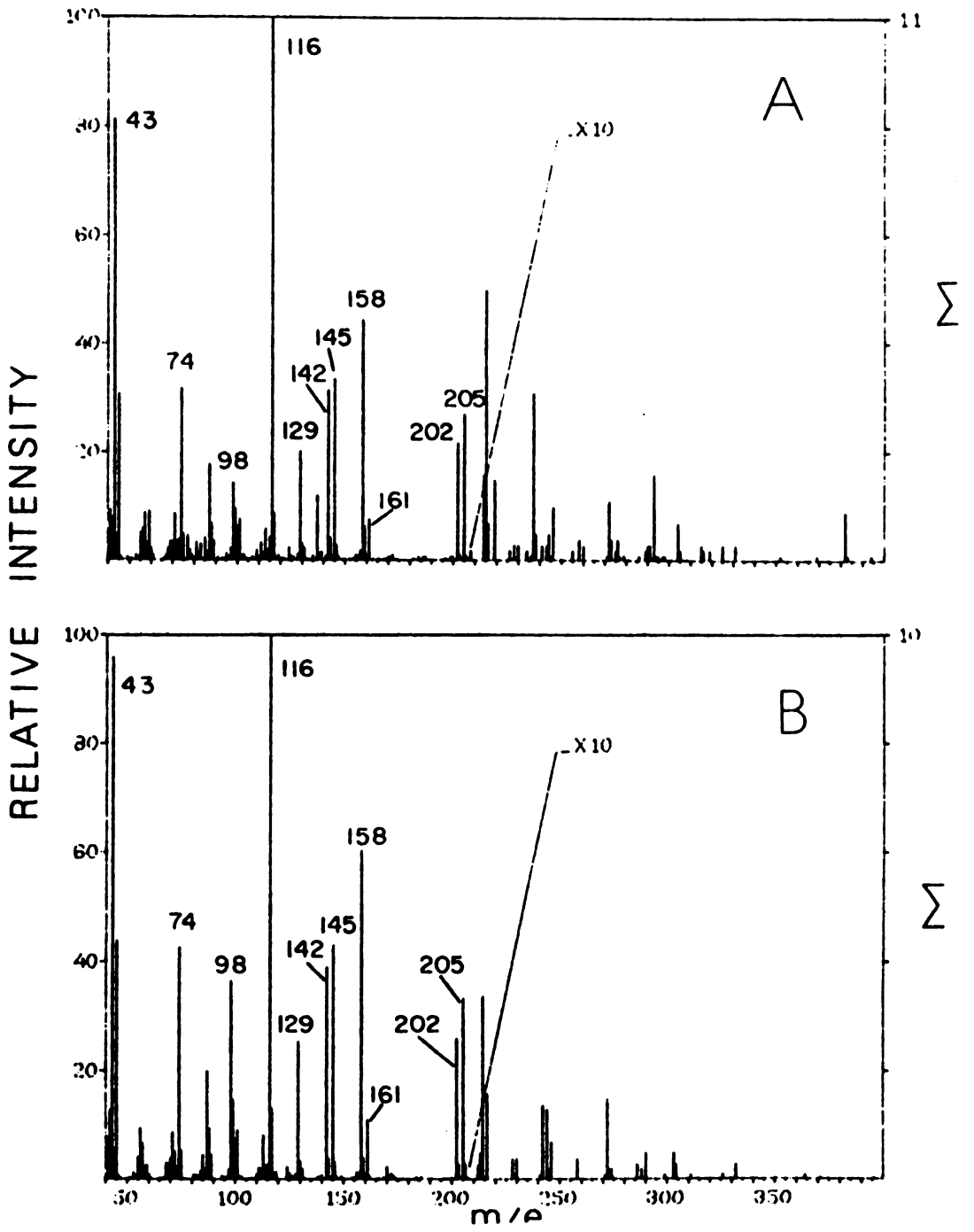

RELATIVE INTENSITY
A
B
X10
X10
43
116
74
98
129
142
145
158
161
202
205
Σ
Σ
m/e
50
100
150
200
250
300
350

Figure 10.

Figure 11. Mass Spectrum of Partially Methylated Alditol Acetate of the Internal Aminosugar of Forssman Hapten Compared with Standards.

(A) N-acetyl-N-methyl-1,4,5-tri-O-acetyl-3,6-di-O-methylglucosaminitol from chitin;
(B) N-acetyl-N-methyl-1,3,5-tri-O-acetyl-4,6-di-O-methylgalactosaminitol from N-acetylchondrosine;
(C) Peak D of Figure 8.

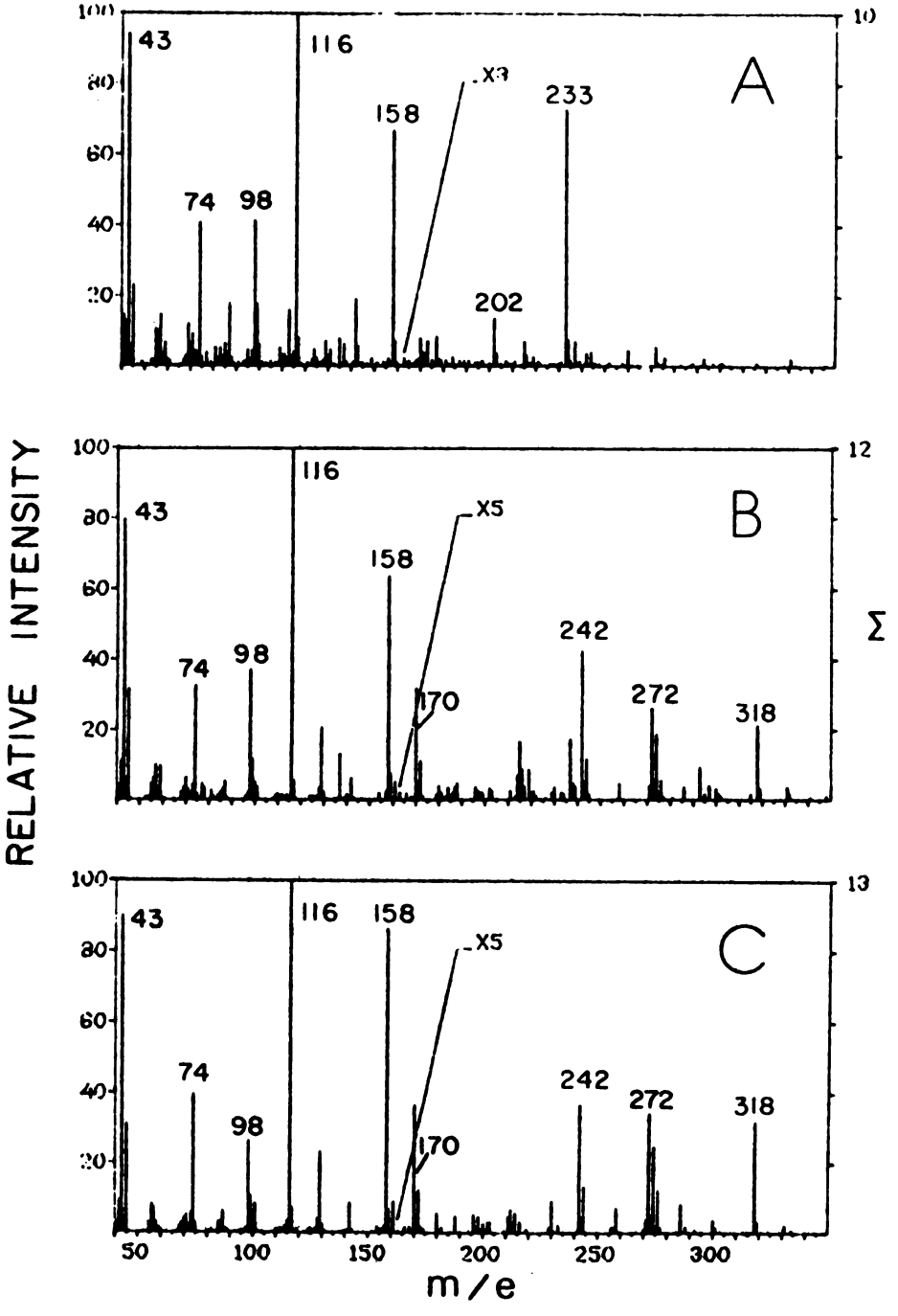

RELATIVE INTENSITY
A
B
C
43
116
158
74
98
202
233
242
272
318
170
X3
X5
m/e
50
100
150
200
250
300

Figure 11.

significant ions characteristic of this amino sugar were observed at m/e 202 and 233. Furthermore, fragment ions at m/e 161, 170, 242, 272 and 318 in the spectrum of Structure 2 were either absent or of very low intensity in this spectrum.

Glucose, galactose, and N-acetylgalactosamine in a molar ratio of 1:1:1 were recovered after periodate oxidation of GL-5, showing that the terminal N-acetylgalactosamine residue is not linked 1→6 to the penultimate sugar. The glucose of GL-5 is periodate-resistant under these particular condition (3). It can thus be concluded from analyses of permethylation and periodate oxidation products that GL-5 has a 1→3 glycosidic linkage between the two N-acetylgalactosamine residues.

The pentaglycosylceramide from kidney and intestine had strong Forssman activity when tested by the complement fixation test (210). Hydrolysis with α-N-acetylgalactosaminidase gave a product which showed globoside activity by Ouchterlony double diffusion and provided additional evidence for the assignment of the configurations of the internal glycosidic linkages of GL-5. A single precipitation band was observed when GL-5 was tested by Ouchterlony double diffusion with anti-Forssman antiserum prepared against horse spleen Forssman antigen. Furthermore, the precipitation band was fused with the horse spleen Forssman precipitation band with no overlap.

With the data presented above, it was demonstrated that canine GL-5 isolated from both kidney and intestine is a true Forssman hapten, which is chemically and immunologically identical with that of horse spleen Forssman hapten (3), that is, N-acetylgalactosaminyl-(α1→3)-N-acetylgalactosaminyl-(β1→3)-galactosyl-(α1→4)-galactosyl-(β1→4)-glucosyl-(1→1)-ceramide.

Purification of α-N-Acetylgalactosaminidase from Porcine Liver

The method and results of purification are summerized in Table 3. The pNP-α-N-acetylgalactosaminidase activity was purified 3,300-fold with a 2% recovery while the Forssman hapten hydrolysing activity was purified 20,000-fold with a 10% recovery. A protease inhibitor,phenylmethylsulphonyl fluoride, was used to prevent proteolytic degradation of the enzyme (211). In the acid precipitation step, the Forssman hydrolysing activity increased almost two-fold. This may be due to precipitation of unknown inhibitors in the homogenate. Steps subsequent to DEAE-cellulose chromatography should be performed quickly so that the enzyme is not inactivated due to dilution. The Con A-Sepharose step is the most potent single step in the purification scheme, giving a 23-fold purification for the pNP-α-N-acetylgalactosaminidase activity and a 47-fold purification for Forssman hapten hydrolase activity. The enzyme was eluted by α-methyl mannoside in a skewed peak, as shown in Figure 12 for a typical run. A large number of fractions had to be pooled because of the tailing of the peak and consequently a batchwise method was adopted. The DEAE-ampholine elution step is shown in Figure 13. There were shoulders of α-N-acetylgalactosaminidase activities, indicating not only that the enzyme preparation was chromatographically heterogeneous, but also the high resolution of the ampholine elution procedure. The amount of purification obtained by this step is also evident in the native gel electrophoresis of enzyme preparations in Figure 14. A major contamination band which ran slightly slower than α-N-acetylgalactosaminidase on native gel electrophoresis (Figure 14) was removed by Sephadex G-150 column chromatography. This major impurity band could also be seen in the Sephadex G-150 elution profile shown in Figure 15. It eluted before

Table 3. Purification of α-N-Acetylgalactosaminidase from Porcine Liver

Fraction	Total Protein	Total Activity pNP	Total Activity Forssman	Specific Activity pNP	Specific Activity Forssman	Purif. Factor pNP	Purif. Factor Forssman	Recovery pNP	Recovery Forssman
	(mg)	(μmol/min)		(μmol/mg protein/min) x 10^3		(-fold)		(%)	
Homogenate	493920	702	47.5	1.42	0.096	1	1	100	100
Acid pptn	152100	547	90.3	3.6	0.594	2.5	6.2	78	190
$(NH_4)_2SO_4$ 30-55%	80010	363	58.3	4.54	0.73	3.2	7.6	52	123
DEAE cellulose	4472	152	19.8	34.0	4.43	23.9	46	22	41.6
Con A-Sepharose	87	68.2	18.2	784	209.2	553	2180	9.7	38.3
Ampholine Elution	42.9	52.5	16.8	1224	392	863	4050	7.5	35.4
Sephadex G-150	15.8	39.5	11.4	2500	723	1760	7580	5.6	24
Hydroxylapatite	2.6	12.3	5.0	4730	1920	3310	19600	1.75	10.4

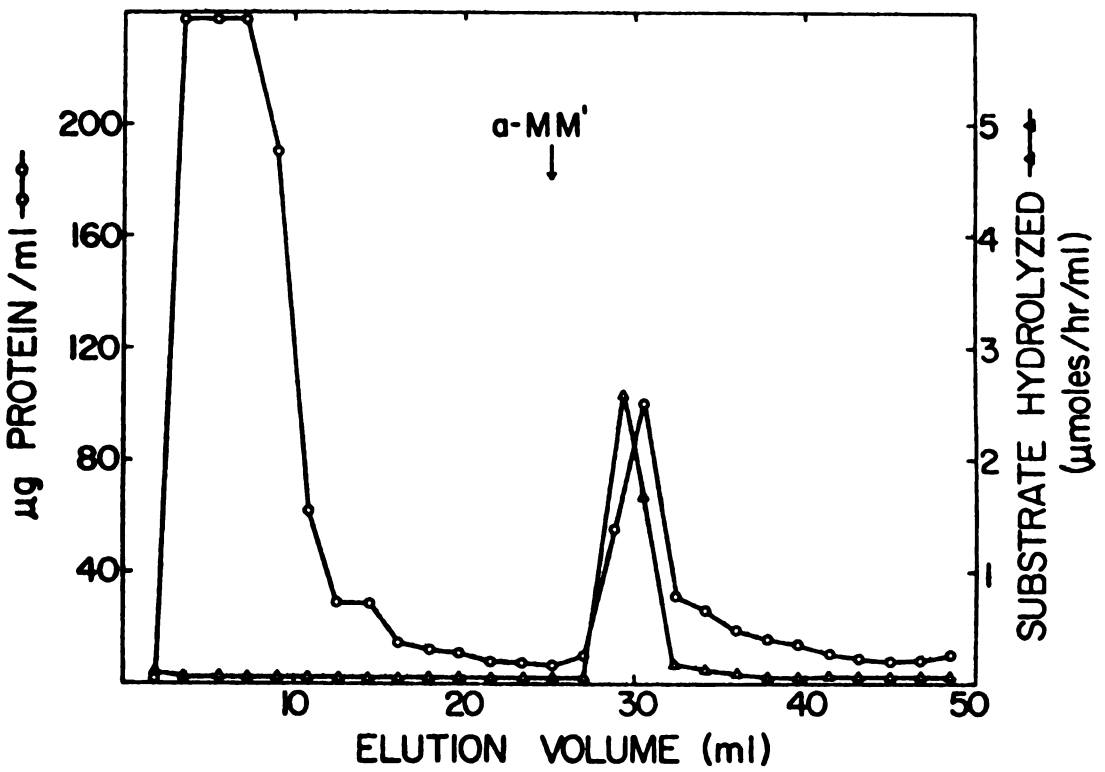


Figure 12. A Typical Elution Pattern of α-N-Acetylgalactosaminidase from Con A-Sepharose Column.

9 mg of DEAE eluate was loaded onto 2 ml of Con A-Sepharose. Arrow indicates elution with 0.5 M α-methylmannoside. The substrate used was pNP-α-GalNAc.

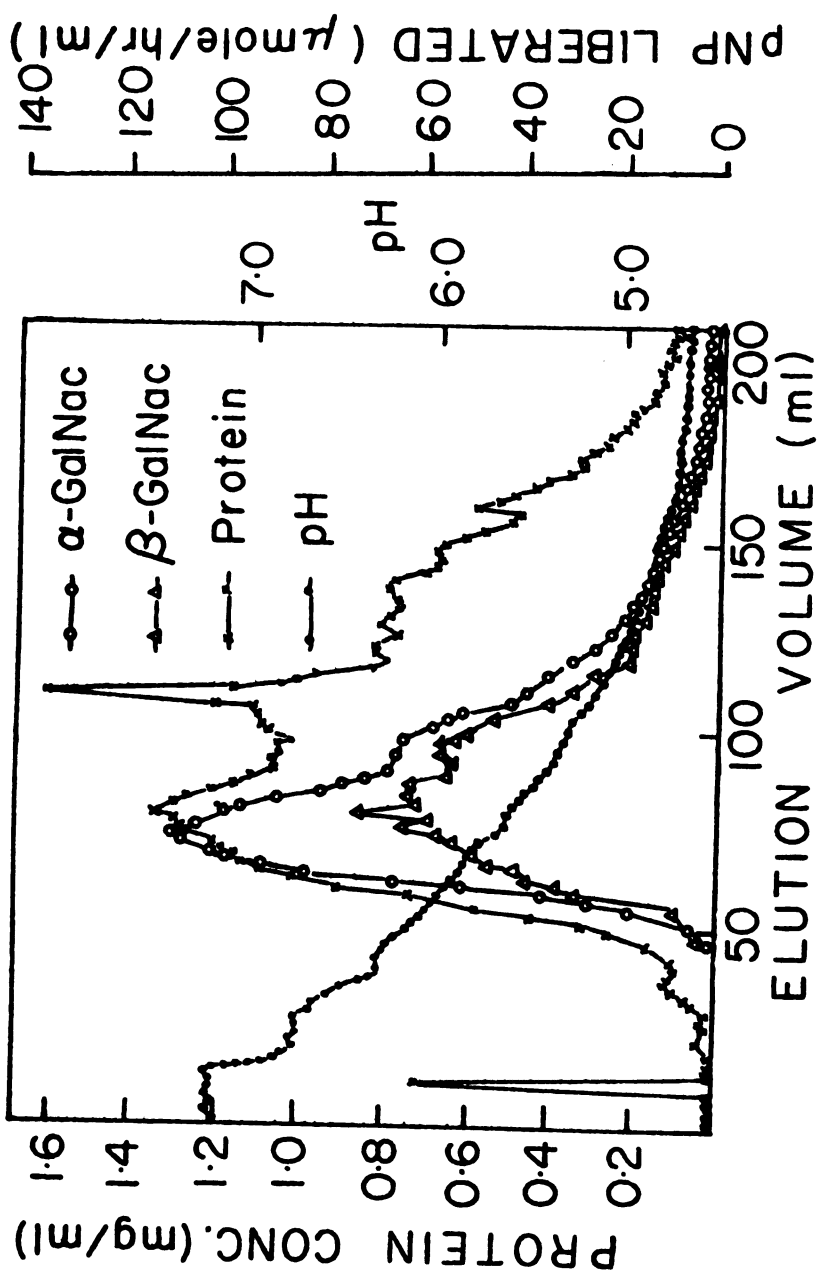


Figure 13. DEAE-Ampholine Elution in the Purification of α-N-Acetylgalactosaminidase.

The chromatography was performed on a 10 g DE-52 column (40 x 1.5 cm i.d.) equilibrated with 5 mM sodium phosphate buffer, pH 6.8. The activity was eluted with 1% ampholine in water, pH 4-6.

Figure 14. Native Gel Electrophoresis of Enzyme Preparations in the Purification of α-N-Acetylgalactosaminidase.

Electrophoresis was performed at pH 8.3 in 7% gels at 2 mA/tube.

(A) Blank gel;
(B) 100 μg of Con A-Sepharose eluate;
(C) 60 μg of DEAE-ampholine elution eluate;
(D) 27 μg of Sephadex G-150 eluate.

Figure 14.

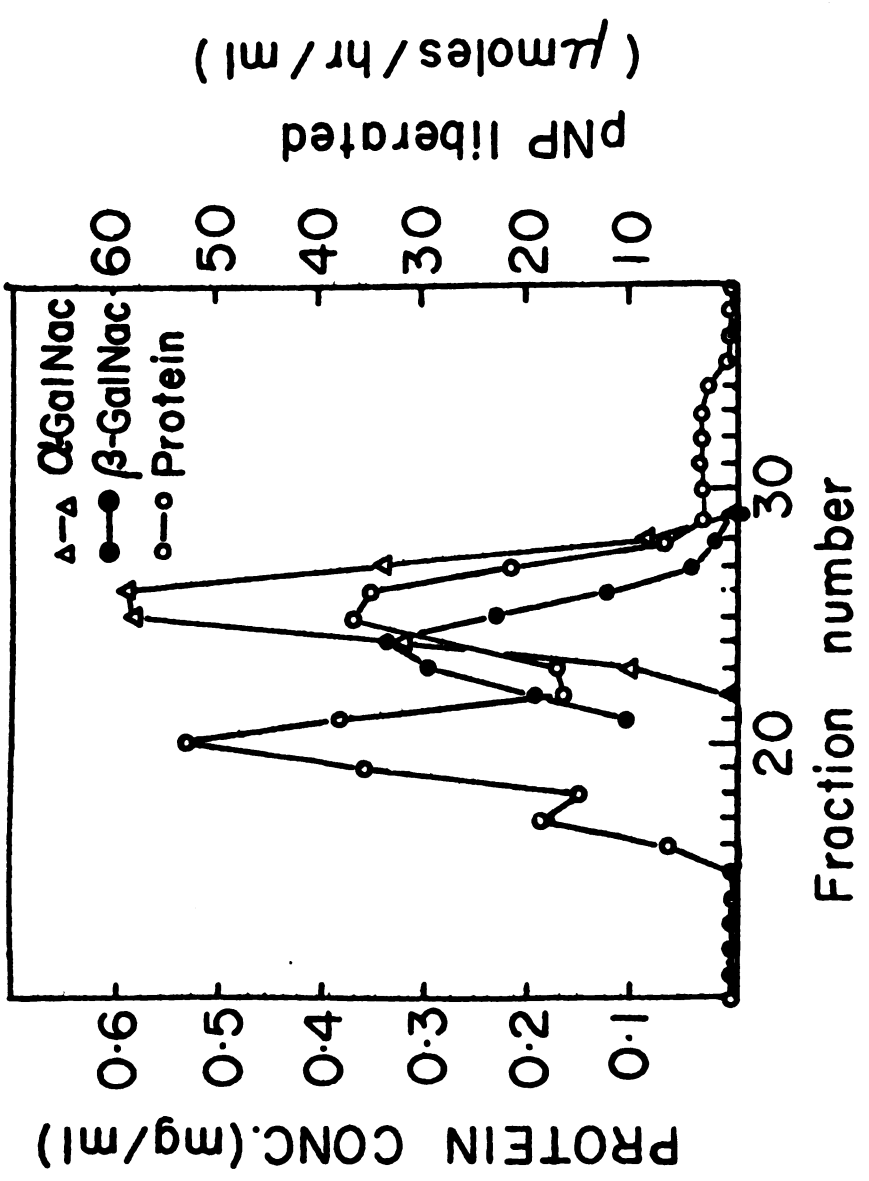


Figure 15. Sephadex G-150 Elution Profile in the Purification of α-N-Acetylgalactosaminidase.

Chromatography was performed with a 1200 x 25 cm i.d. column with a bed volume of 700 ml. Enzyme activity was eluted with 0.05 M sodium citrate buffer, pH 6.0, at a flow rate of 16 ml/hr. Fractions of 12.7 ml were collected.

the enzyme activity peak.

β-N-Acetylgalactosaminidase eluted slightly before the α-N-acetylgalactosaminidase activity. An attempt to separate the two enzyme further by repeating the chromatography on the Sephadex G-150 column was not successful. In Figure 16, α-N-acetylgalactosaminidase activity eluted with the protein peak and the β-N-acetylgalactosaminidase eluted with it as well. If the β-N-acetylgalactosaminidase activity were eluted in a Gaussian distribution, it would have been eluted before instead of coinciding with the α-N-acetylgalactosaminidase activity. This implied that the β-N-acetylgalactosaminidase in porcine liver was heterogeneous in exclusion behaviour.

Some slight contamination persisted after Sephadex G-150 column chromatography and can be seen as a faint band slightly above the two enzyme bands in Figure 14. This faint band was removed by hydroxylapatite chromatography (Figure 17). This step gave a two-fold purification of the pNP-α-GalNAc hydrolysing activity and 2.6-fold purification of the Forssman hydrolysing activities but only 30% of the applied pNP-α-N-acetylgalactosaminidase and 43.5% of the Forssman hydrolysing activity were recovered. The hydroxylapatite step was also necessary for the elimination of the final traces of β-N-acetylglucosaminidase activities.

Physical and Chemical Characterization of Enzyme Preparation

The final enzyme preparation was electrophoresed as the native enzyme (Figure 18). The scan of protein stain at 550 nm indicated that there were two protein bands (lower scan). The activity stain (upper scan) showed that both of these bands are enzymatically active against

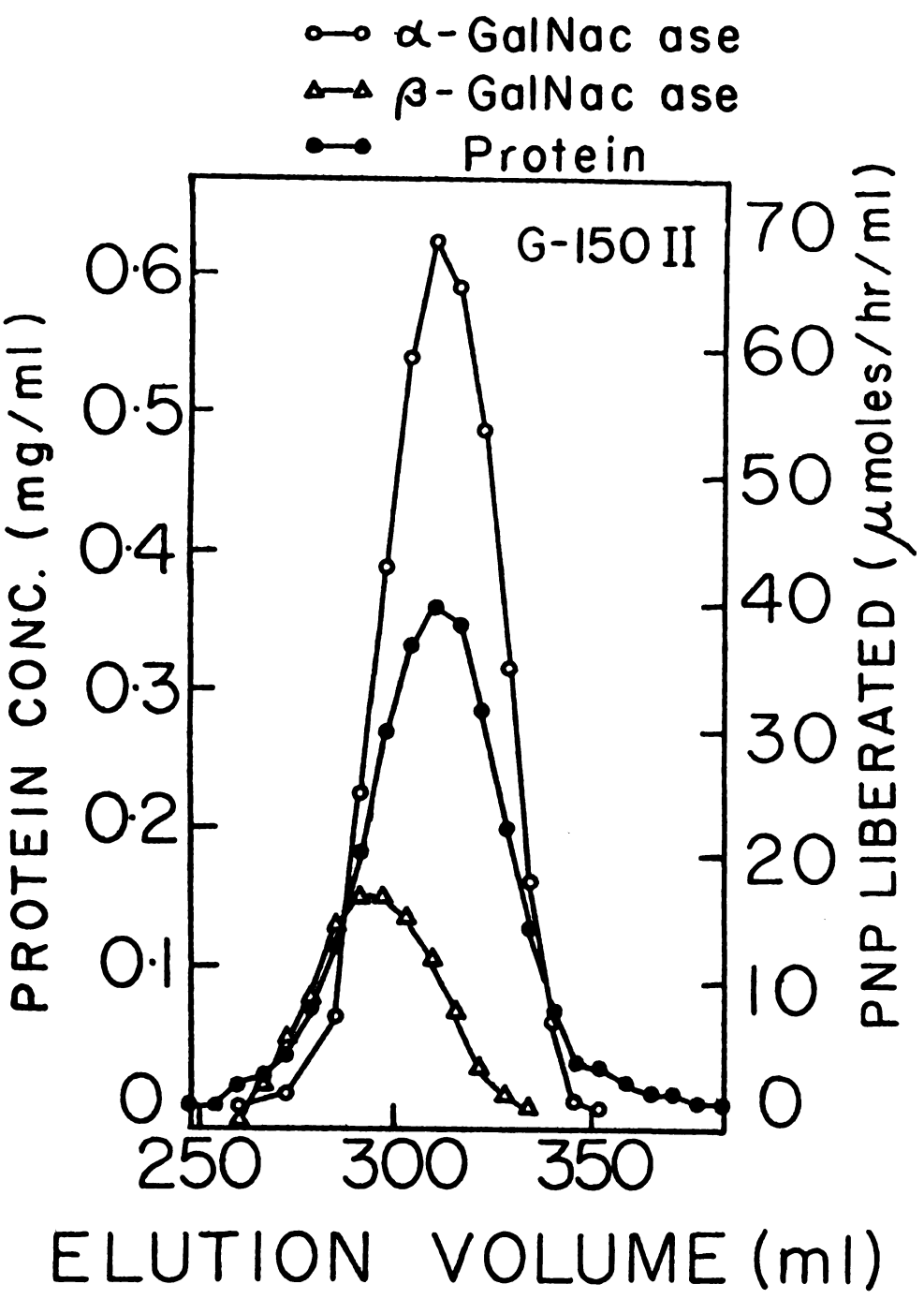


Figure 16. Repeated Sephadex G-150 Column Chromatography of α-N-Acetyl-galactosaminidase.

The conditions were the same as Figure 15.

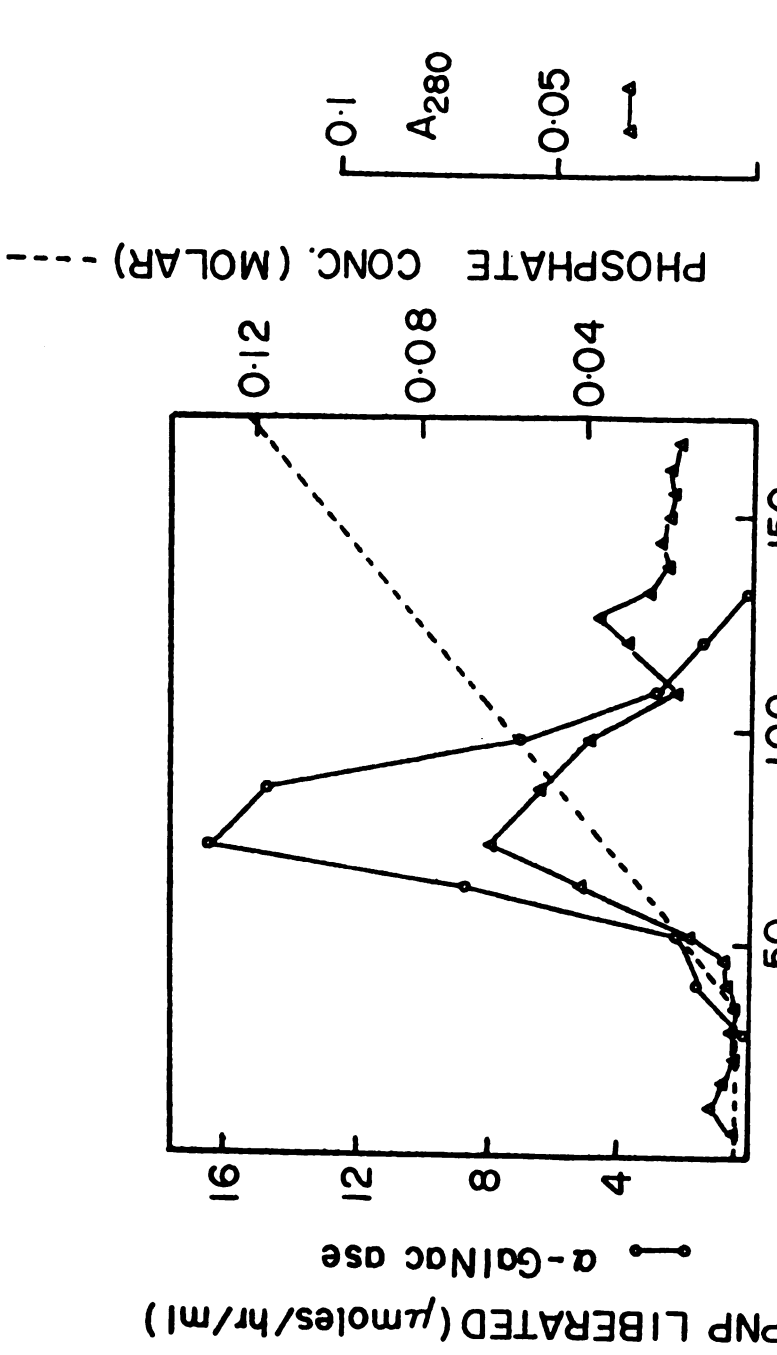


Figure 17. Hydroxylapatite Chromatography in the Purification of α-N-Acetylgalactosaminidase.

Chromatography was performed in a 16 x 1 cm i.d. column with 10 ml hydroxylapatite preequilibrated with 1 mM phosphate buffer, pH 6.8. Elution was achieved with a linear gradient of 220 ml of 1 mM sodium phosphate, pH 6.8, and 200 ml of 0.4 M sodium phosphate buffer at the same pH.

Figure 18. Scans of Native Gel Electrophoresis of Purified α-N-Acetylgalactosaminidase Preparation from Hydroxylapatite Column.

22.5 μg of the purified enzyme preparation was used for each gel. The electrophoresis conditions are the same as Figure 14. The upper scan is the activity scan at 410 nm and the lower scan the protein stain scanned at 550 nm.

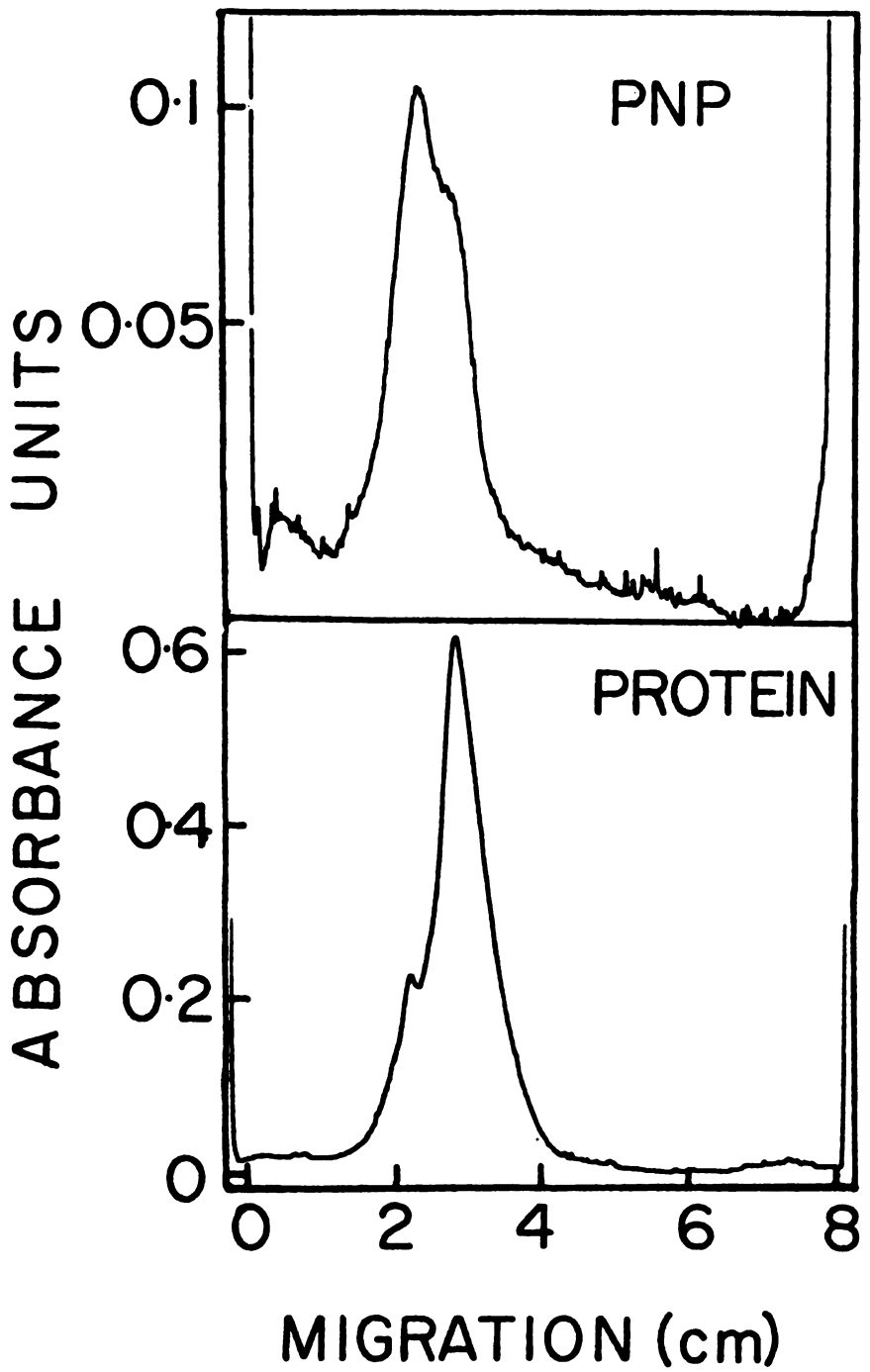

PNP
PROTEIN
0·1
0·05
0·6
0·4
0·2
0
0
2
4
6
8
ABSORBANCE UNITS
MIGRATION (cm)

Figure 18.

the p-nitrophenyl-α-GalNAc substrate. The higher molecular weight species may be an aggregation of the lower molecular weight form.

The enzyme was reduced with dithiothreitol and electrophoresis was carried out in SDS with 5%, 7%, 10% and 12.5% gels. Figure 19 shows the SDS gel electrophoresis scan of the purified α-N-acetylgalactosaminidase in 10% gel. One major protein peak was found. A small shoulder that migrated slightly faster than the enzyme band can be seen. The molecular weight was estimated by comparing the relative migration of the reduced enzyme with that of standard proteins. In Figure 20 only the curves for 10% and 12.5 % gels are shown. The enzyme was found to have an apparent molecular weight in SDS gels of about 52,000 daltons.

The molecular weight of the native enzyme was determined by gel filtration on Sephadex G-150. A molecular weight of about 102,000 daltons was found. The native enzyme is probably composed of 2 subunits of the 52,000 molecular weight species.

The carbohydrate composition was found by GLC after methanolysis of 500 μg of the purified enzyme in 1 N methanolic HCl. The GLC trace of the carbohydrates from α-N-acetylgalactosaminidase is shown in Figure 21. The top trace (I) is of the trimethylsilylated methyl glycosides derived from the enzyme: A, B and C are methyl mannosides, mannitol and methyl N-acetylglucosaminides, respectively. Although A did not coincide exactly with peaks A in trace II, they had almost identical retention times relative to mannitol, 0.621 compared to 0.612. Likewise, the retention times of C relative to B were 1.24 and 1.26 for traces I and II, respectively. X was probably derived from citric acid. Y and Z were not found in the traces when the sample was injected 30 minutes after derivatization, indicating that they had groups

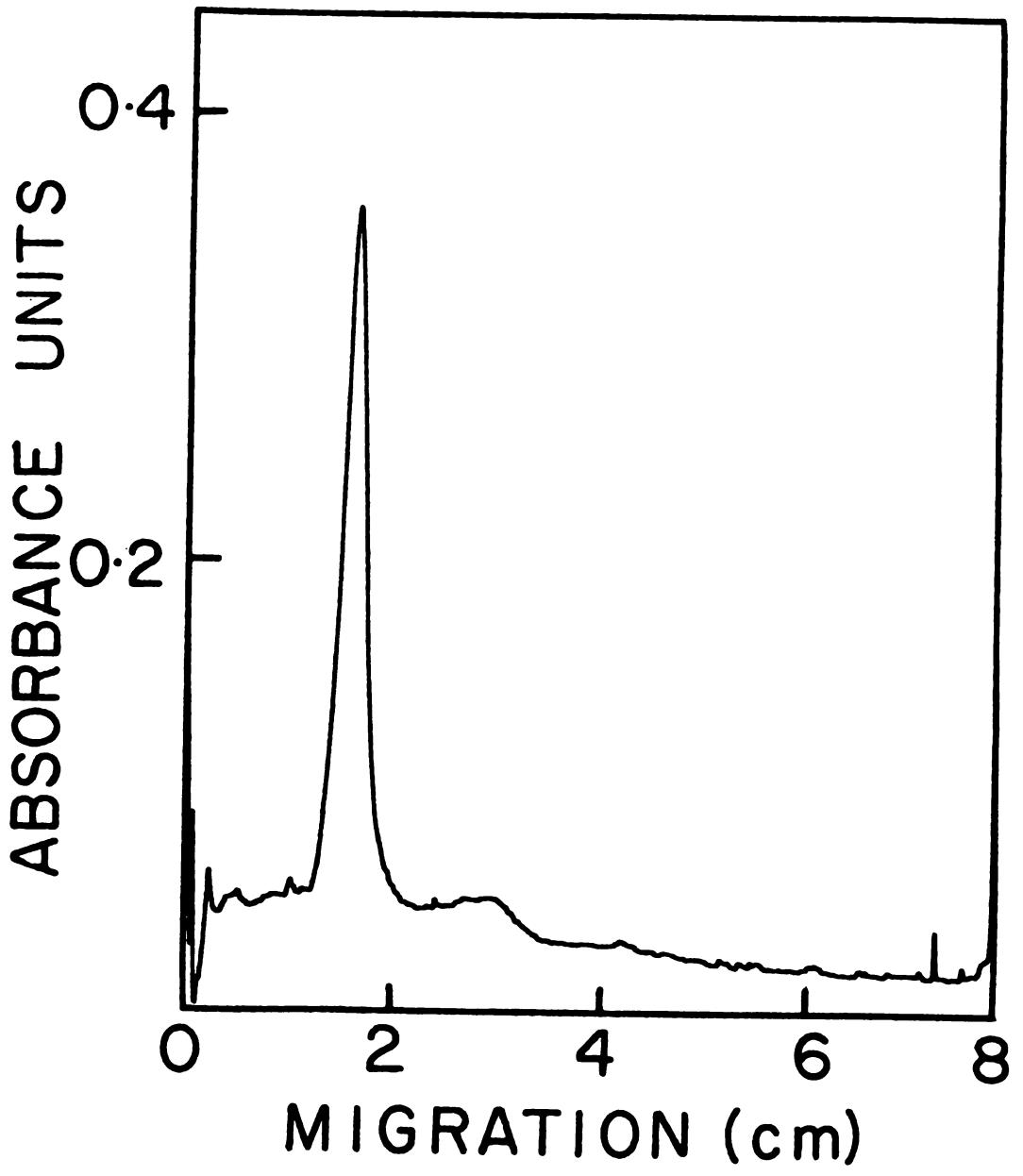


Figure 19. Scan of SDS Gel Electrophoresis of Purified α-N-Acetyl-galactosaminidase from Hydroxylapatite Column.

22.5 μg of the purified enzyme was electrophoresed in 10% gel in 0.1 M sodium phosphate, pH 7.1, with 0.1% SDS at 8 mA/tube. The scan was at 550 nm with a scan speed of 0.5 cm/min.

Figure 20. Molecular Weight Determination of the Subunits of Purified α-N-Acetylgalactosaminidase in SDS Polyacrylamide Gels; a semi-log plot of molecular weight vs the relative mobility of the protein.

The electrophoresis conditions were the same as Figure 19. The crosses on the curves indicate the relative mobility of the enzyme.

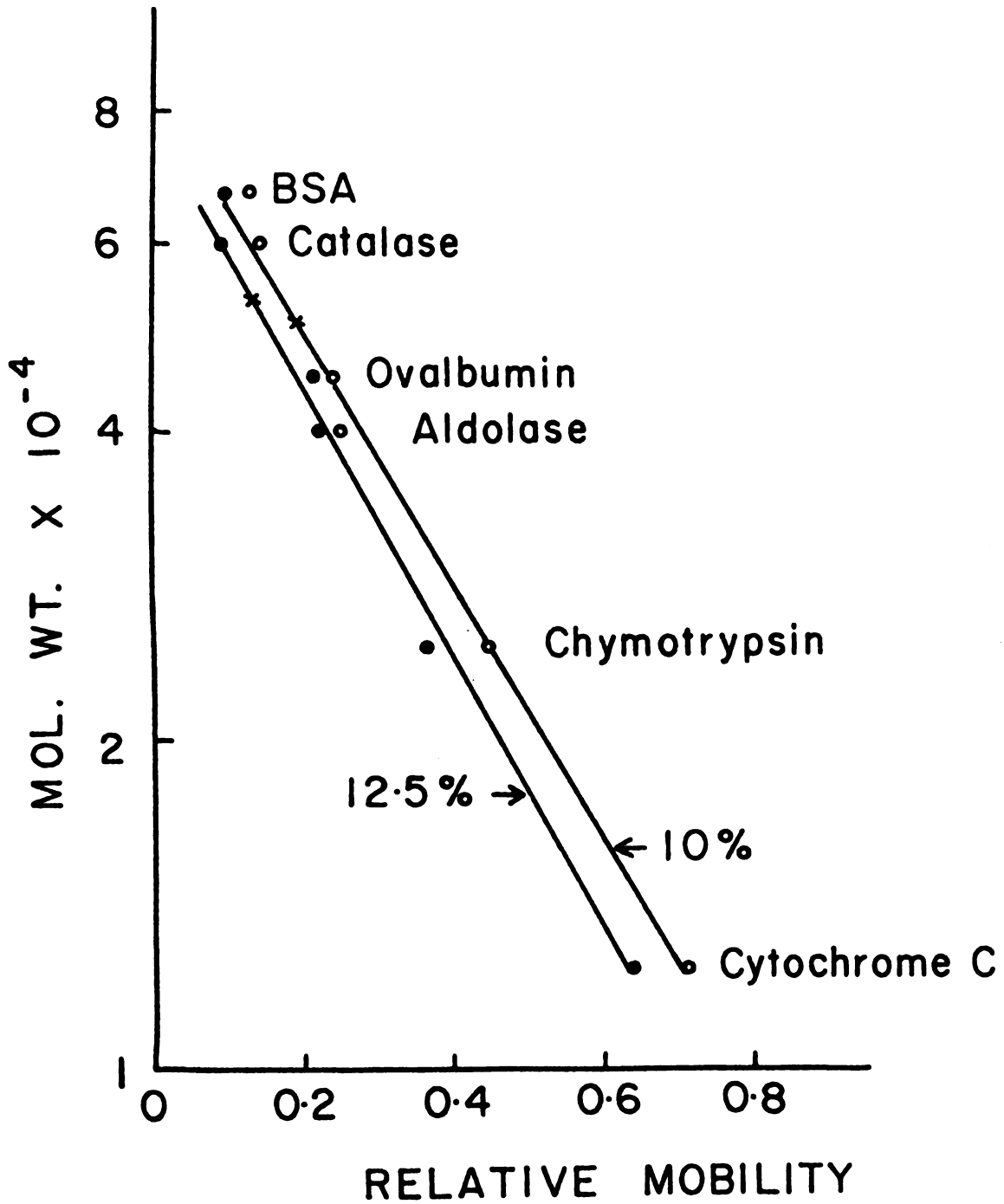

8
6
4
2
1
MOL. WT. X 10^{-4}
BSA
Catalase
Ovalbumin
Aldolase
Chymotrypsin
12·5% →
← 10%
Cytochrome C
0
0·2
0·4
0·6
0·8
RELATIVE MOBILITY

Figure 20.

Figure 21. Gas-Liquid Chromatography Trace of Carbohydrate Analysis of α-N-Acetylgalactosaminidase.

I is the trace of sugars from α-N-acetylgalactosaminidase, II and III are standard sugar runs.

A: mannose; B: mannitol; C: N-acetylglucosamine; D: NeuAc; E: galactose; F: glucose; G: N-acetylgalactosamine.

The sugars are trimethylsilylated methyl glycosides. X, Y and Z are unknown impurities. GLC was performed on a 3% SP-2100 on Supelcoport (80-100 mesh) with nitrogen at a flow rate of 45 ml/min. Temperature was programmed from 150°-230° at 3°/min.

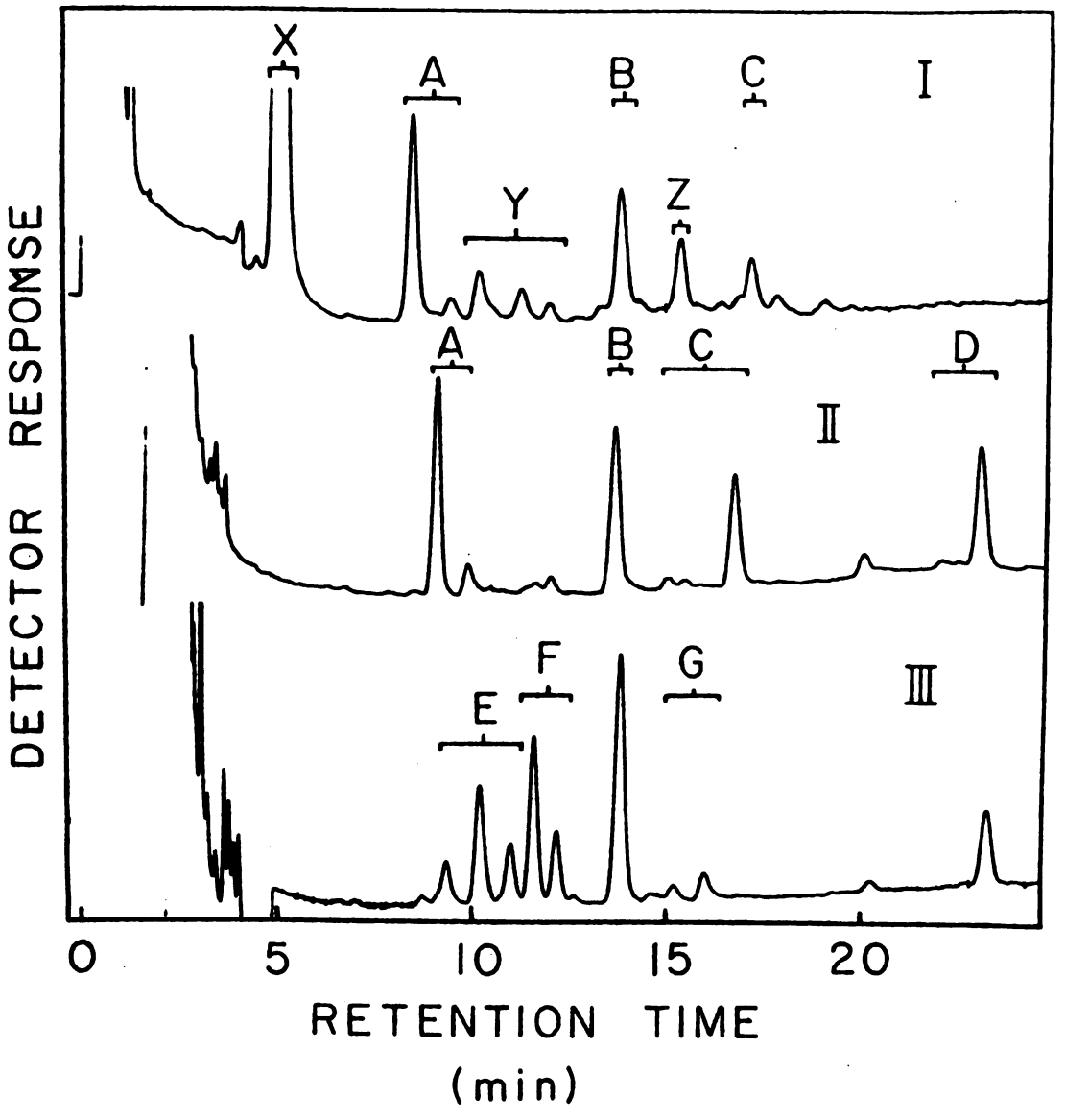

X
A
B
C
I
Y
Z
A
B
C
D
II
F
G
E
III
DETECTOR RESPONSE
0
5
10
15
20
RETENTION TIME
(min)

Figure 21.

(e.g. COOH or NH_2) that were more difficult to derivatize than OH-groups. Besides, these peaks did not coincide with peaks of known trimethylsilylated methyl glycosides. The carbohydrate composition data also agreed with the fact that the enzyme was bound by Con A, which specifically binds with α-mannoside or α-glucoside residues (212). Mass spectrometry of peaks at A, B and C also confirmed the identities of these peaks. The carbohydrate composition of α-N-acetylgalactosaminidase is shown in Table 4. Mannose and N-acetylglucosamine were present in a ratio of 2.4:1 and the two sugars constitute about 7.4% of the total weight of the enzyme. It is worth noting that there is no galactose, fucose, glucose, N-acetylgalactosamine or sialic acid in this enzyme.

The amino acid composition of the enzyme is shown in Table 5. Aspartic acid and glutamic acid comprised 19.6%, while serine and threonine constituted 14.2% of the total amino acid. These two types of amino acids are known to be involved in glycosidic linkages with oligosaccharides in glycoproteins. The non-polar amino acids made up about 42% of the amino acids and basic amino acids shared about 14% of the total amino acids.

Isoelectric Focussing of the Purified Enzyme Preparation

Weissmann and Hinrichsen (9) found that a partially purified preparation of α-N-acetylgalactosaminidase from porcine liver could be separated by isoelectric focussing into eight bands, all of which were active toward phenyl-α-N-acetylgalactosaminide. These fractions had isoelectric points which varied from pH 5 to 6.5. Isoelectric focussing of the more highly purified enzyme obtained by our procedure showed the same general pattern of multiple peaks (Figure 22). Forssman hapten

Table 4. Carbohydrate Composition of α-N-Acetylgalactosaminidase

Sugar	Amount (nmoles/μg protein)
Mannose	0.290
N-Acetylglucosamine	0.121

Table 5. Amino Acid Composition of α-N-Acetylgalactosaminidase

Amino Acid	Percentage
Cysteic Acid	--
Methionine Sulfone	--
Aspartic Acid	8.68
Threonine	6.06
Serine	8.14
Glutamic Acid	10.91
Proline	0.33
Glycine	12.27
Alanine	10.19
Valine	0.71
Methionine	0.24
Isoleucine	6.20
Leucine	12.94
Tyrosine	3.68
Phenylalanine	5.92
Lysine	4.78
Histidine	2.99
Arginine	5.97
Total	100

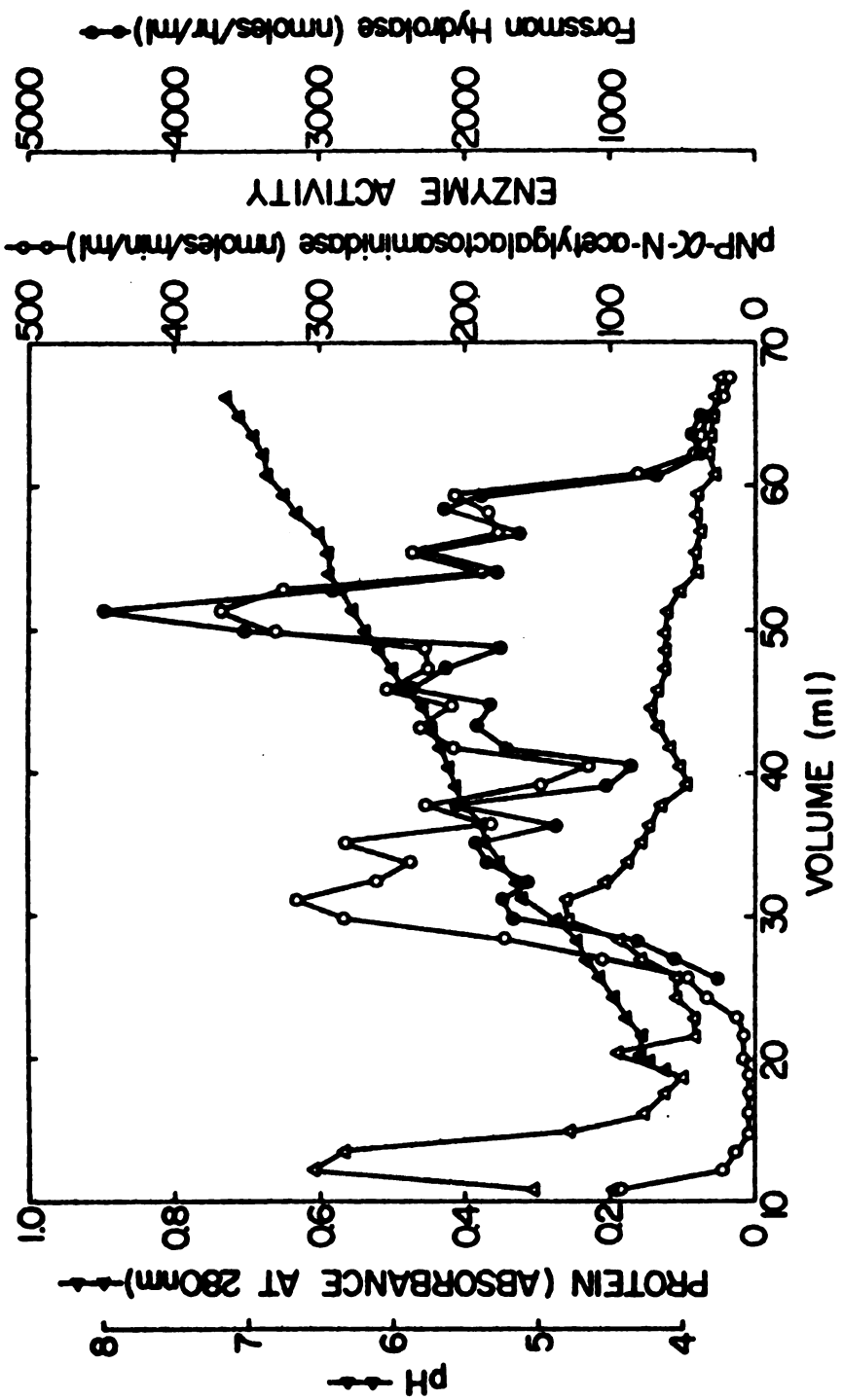


Figure 22. Isoelectric Focussing of Partially Purified α-N-Acetylgalactosaminidase.

Isoelectric focussing was performed in a 110 ml apparatus with pH between 5 and 8 for 48 hours at 600 V. About 0.8 mg of the Con A-Sepharose fraction was used.

hydrolase activity generally coincided with the peaks of activity toward artificial substrate, although the ratio of glycolipid to artificial substrate activity increased slightly with increasing pI of the multiple forms.

Native polyacrylamide gel electrophoresis of the fractions separated by preparative isoelectric focussing is shown in Figure 23. Two protein bands were observed, which coincided with the pNP-α-N-acetylgalactosaminidase activities (Figure 24). The mobilities of the forms were all similar and thus no differences could be shown by polyacrylamide gel electrophoresis.

The K_M values of three of the peaks with pNP-α-N-acetylgalactosaminides and Forssman hapten were compared (Table 6). The K_M values with pNP-α-GalNAc increased with increasing pI of the enzyme while the K_M values with Forssman hapten as substrate were almost the same for the three peaks.

Kinetic Properties of the p-Nitrophenyl-α-N-acetylgalactosaminide Activity

The hydrolysis of pNP-α-N-acetylgalactosaminide proceeded linearly for at least 60 minutes. When the final concentration of enzyme was less than 0.6 ng/μl the enzyme activity was less than that at higher concentrations (Figure 25). At concentrations above 0.8 ng/μl, the enzyme activity was linear up to at least a concentration of 4 ng/μl. The pH optimum for the hydrolysis of pNP-α-N-acetylgalactosaminide was broad (Figure 26). The highest activity was at pH 4.5 and the range of the optimum was from 4.3 to 4.7. A lineweaver-Burk plot of 1/v vs 1/S is shown in Figure 27. The K_M value was found to be 2.9 mM and V_{max} was 14.1 μmoles/min/mg.

Figure 23. Native Polyacrylamide Gel Electrophoresis of Isoelectric Focussing Peaks of α-N-Acetylgalactosaminidase.

The enzymes were electrophoresed in 7% gel in tris-glycine buffer, pH 8.3. The numerals indicate the number of the enzymatic peak in Figure 22, starting from the peak with the lowest pI.

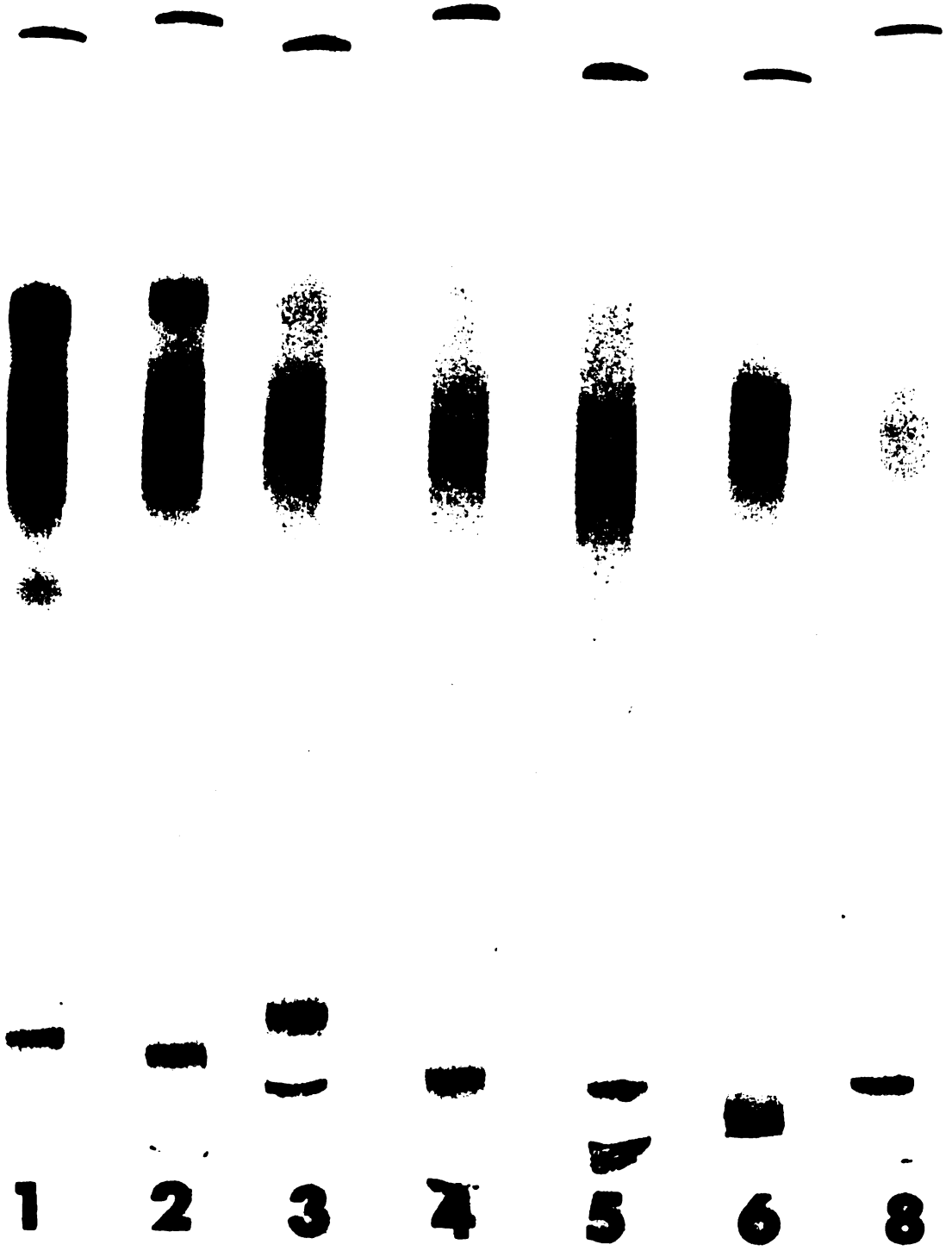


Figure 23.

Figure 24. Scan of Native Polyacrylamide Gel Electrophoresis of the Isoelectric Focussing Peak of α-N-Acetylgalactosaminidase.

Scan at 550 nm are for protein stains and 410 nm for pNP-α-N-acetylgalactosaminidase activity stains. The electrophoresis conditions are the same as in Figure 23.

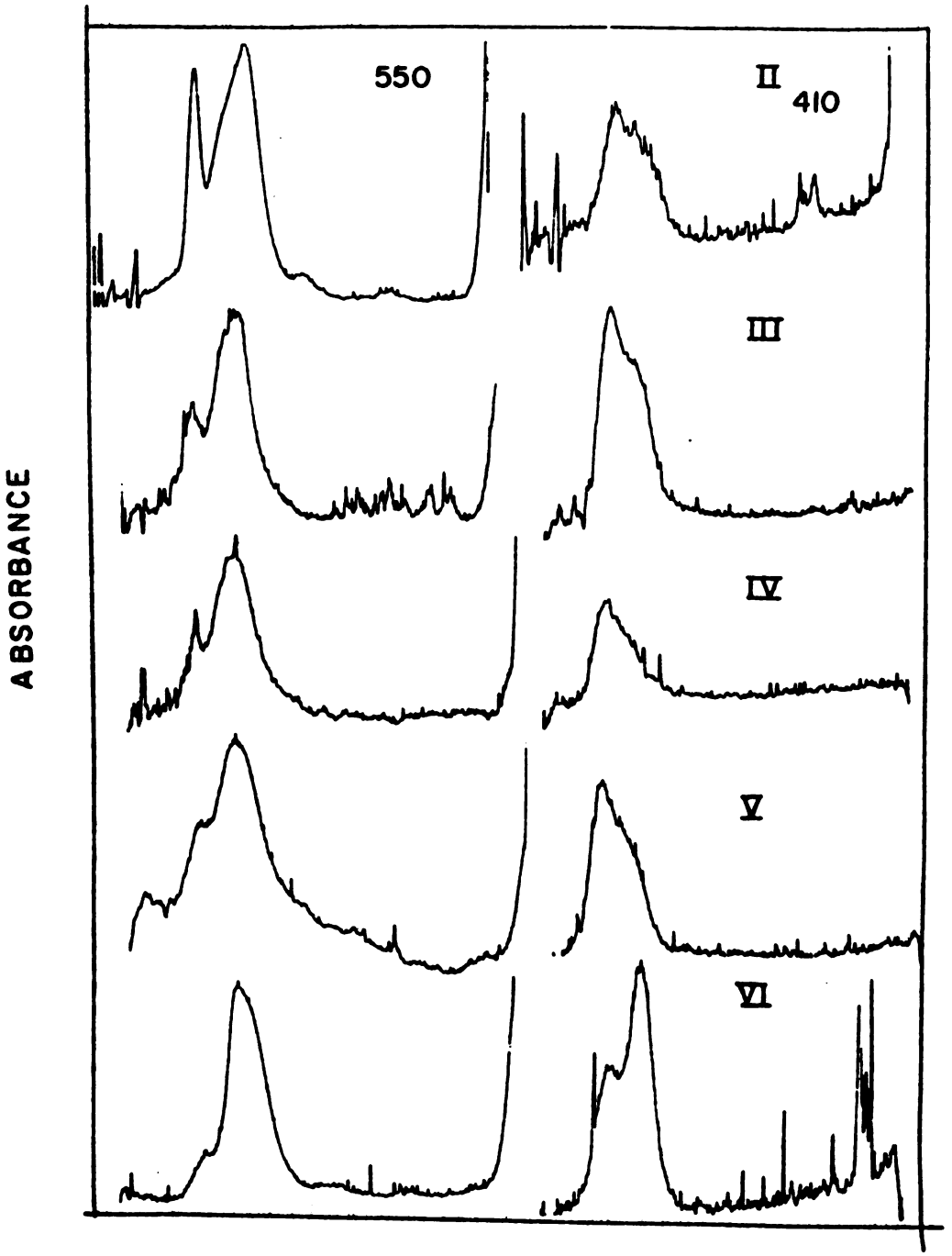


Figure 24.

Table 6. Specificities of the Multiple Isoelectric Focussing Forms of α-N-Acetylgalactosaminidase

pI	K_M (mM)		
	phenyl-α-GalNAc*	p-nitrophenyl-α- GalNAc	Forssman hapten
6.00	6.8	5	0.53
6.25	--	12.5	0.83
6.50	--	20	0.71

*Data from Weissmann, B. and Hinrichsen, D. F Reference 9.

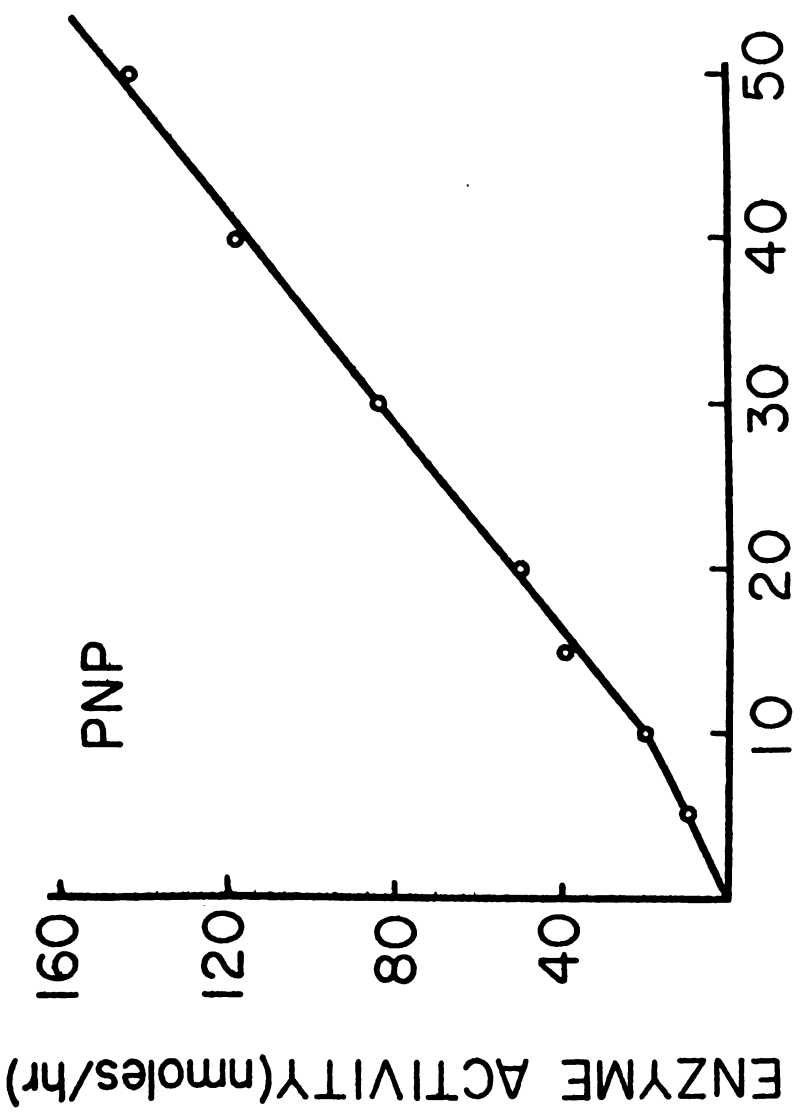


Figure 25. Enzyme Activity of pNP-α-N-Acetylgalactosaminidase vs Enzyme Concentration. The concentration of enzyme was 11.8 ng/μl.

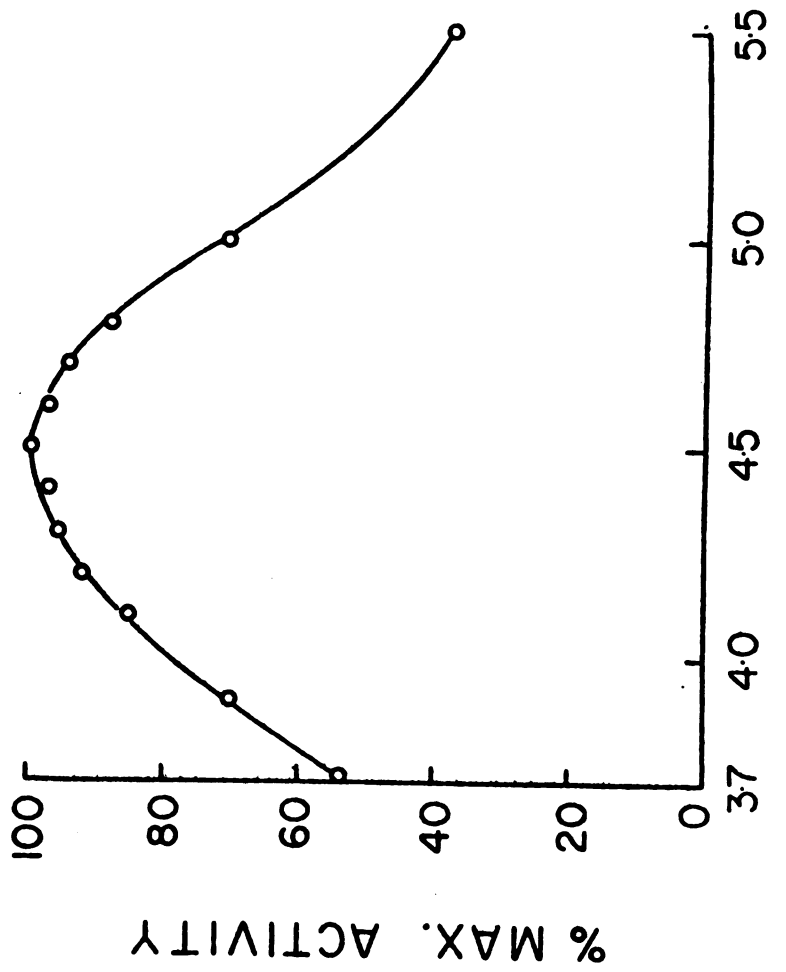


Figure 26. Rate of Hydrolysis of pNP-α-N-Acetylgalactosaminide vs pH.
0.3 µg of enzyme was used in each assay.

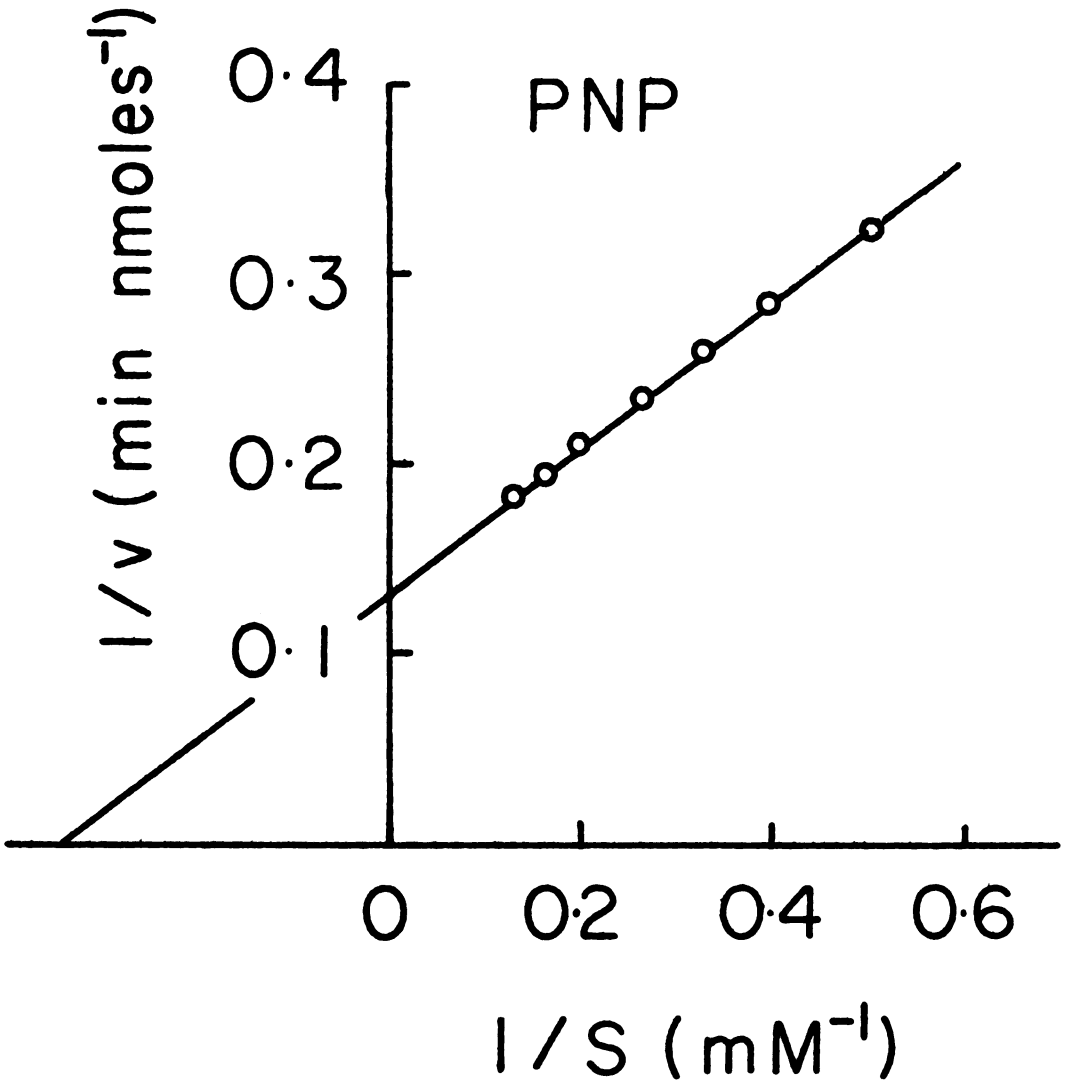


Figure 27. Lineweaver-Burk Plot for the Hydrolysis of p-Nitrophenyl-α-N-Acetylgalactosaminide by α-N-Acetylgalactosaminidase.

The amount of Enzyme used for each assay was 0.54 µg.

Hydrolysis of Forssman Hapten by α-N-Acetylgalactosaminidase

(1) Characterization of Products of Hydrolysis of Forssman Hapten

The hydrolysis product of Forssman hapten by α-N-acetylgalactosaminidase was characterized. The radioactivity scan of the TLC run of the Folch upper phase after hydrolysis of Forssman hapten by α-N-acetylgalactosaminidase is shown in Lane 1 of Figure 28. The radioactivity had the same migration as the monosaccharide (Lane 6), indicating that the radioactive production of hydrolysis in the Folch upper phase was a monosaccharide which was expected to be N-acetylgalactosamine. The heavy spot in Lane 2 turned yellow when sprayed by orcinol-sulphuric acid and was shown to be identical in migration to sodium taurocholate.

The lower phase was analysed by TLC in the solvent system chloroform/methanol/water 100/42/6. Lane 1 in Figure 29 shows the lower phase of the control sample with no enzyme added. The radioactivity scan of this lane is shown in scan a. When α-N-acetylgalactosaminidase was added to the incubation mixture, the mobility of the glycolipid product increased and was similar to that of standard GL-4. The radioactivity scan, shown in lane b, was lost from the glycolipid.

The results indicated that the [^{3}H]-GL-5 was labelled in the non-reducing terminal N-acetylgalactosamine. α-N-Acetylgalactosaminidase acted as an exoglycosidase, with the hydrolysis products identified as GL-4 and N-acetylgalactosamine.

(2) Kinetics of Hydrolysis of Forssman Hapten

The hydrolysis of Forssman hapten by α-N-acetylgalactosaminidase was linear for at least 3 hours. It was linear with respect to enzyme concentration except at very low concentrations (Figure 30).The optimal

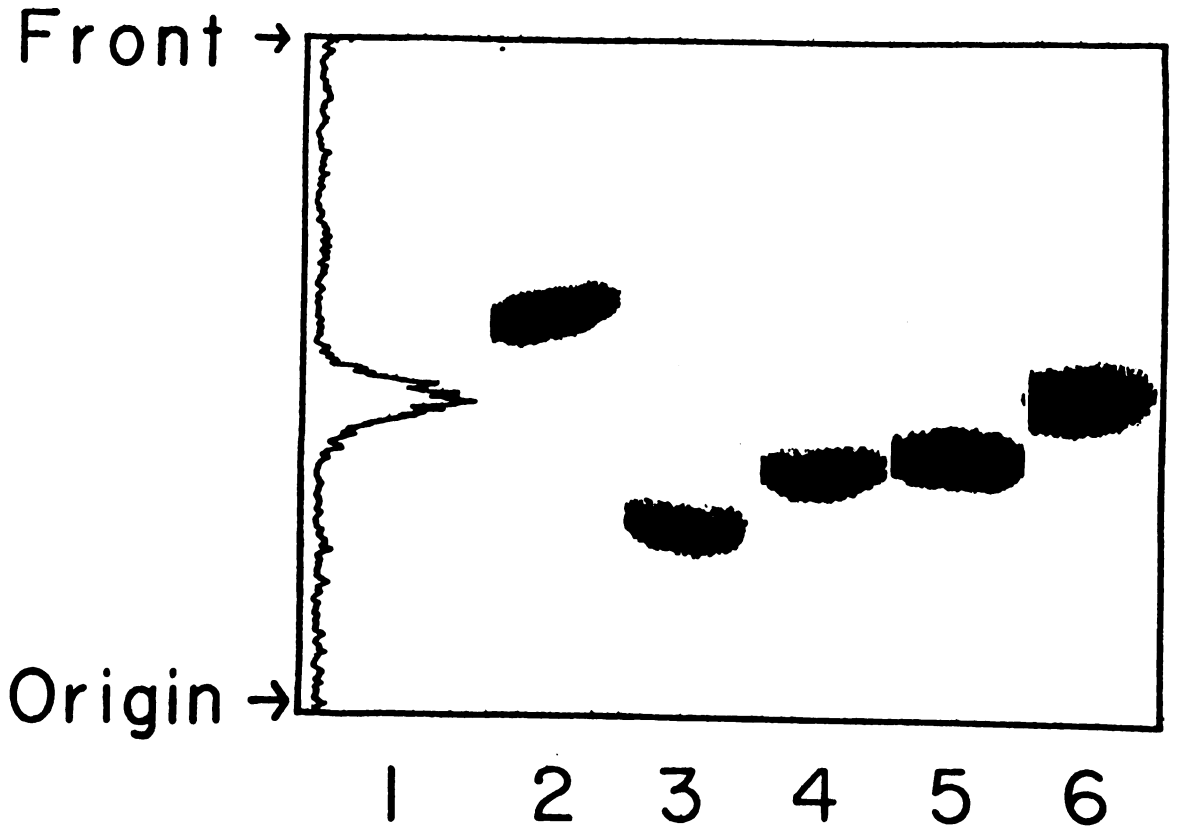


Figure 28. Radio-Thin-Layer Chromatogram and Thin-Layer Chromatography of the Folch Upper Phase Product after Hydrolysis of Forssman Hapten by α-N-Acetylgalactosaminidase.

Lane 1: Radio-thin-layer chromatogram of Lane 2;
Lane 2: hydrolysis product; Lane 3: stachyose,100 µg;
Lane 4: raffinose, 100 µg; Lane 5: melibiose, 100 µg;
Lane 6: galactose, 100 µg. The solvent system was butanol/water/acetic acid 100/50/50.

Figure 29. Radio-Thin-Layer Chromatogram and Thin-Layer Chromatography of the Folch Lower Phase Product after Hydrolysis of Forssman Hapten by α-N-Acetylgalactosaminidase.

Lane S: standard glycolipids from horse kidney;
Lane 1: Folch lower phase of control;
Lane 2: Folch lower phase of enzymatic hydrolysis product;
Lane 3: [^{3}H]-GL-5 substrate, 150 nmoles;
Lane b: radioactivity scan of Lane 2;
Lane a: radioactivity scan of Lane 1.

TLC in chloroform/methanol/water 100/42/6 on silica gel G plates. Radioactivity scan at 1900 V, gain at 50, sensitivity at 1 x 10 counts per second, time constant at 10 second, slit width at 1 x 10 mm and scan speed at 10 in/hr.

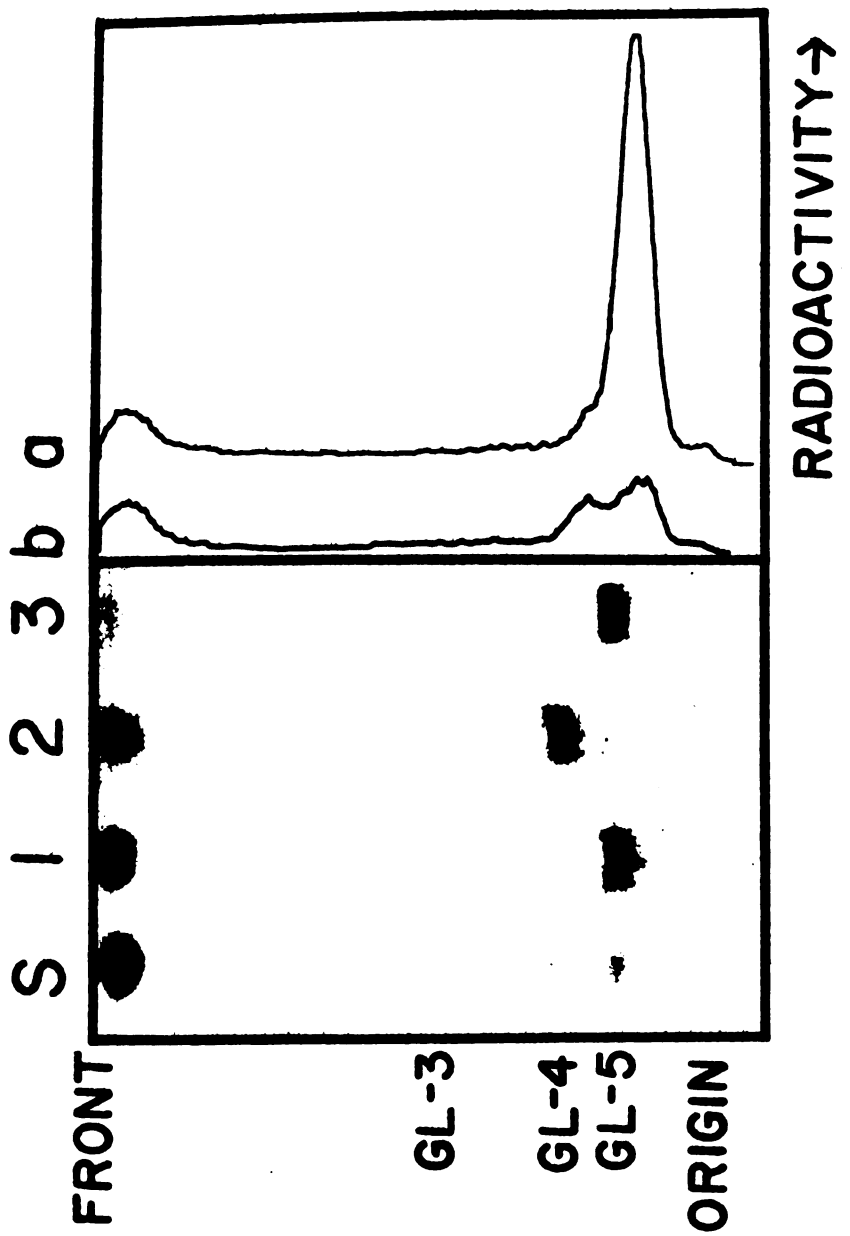


Figure 29.

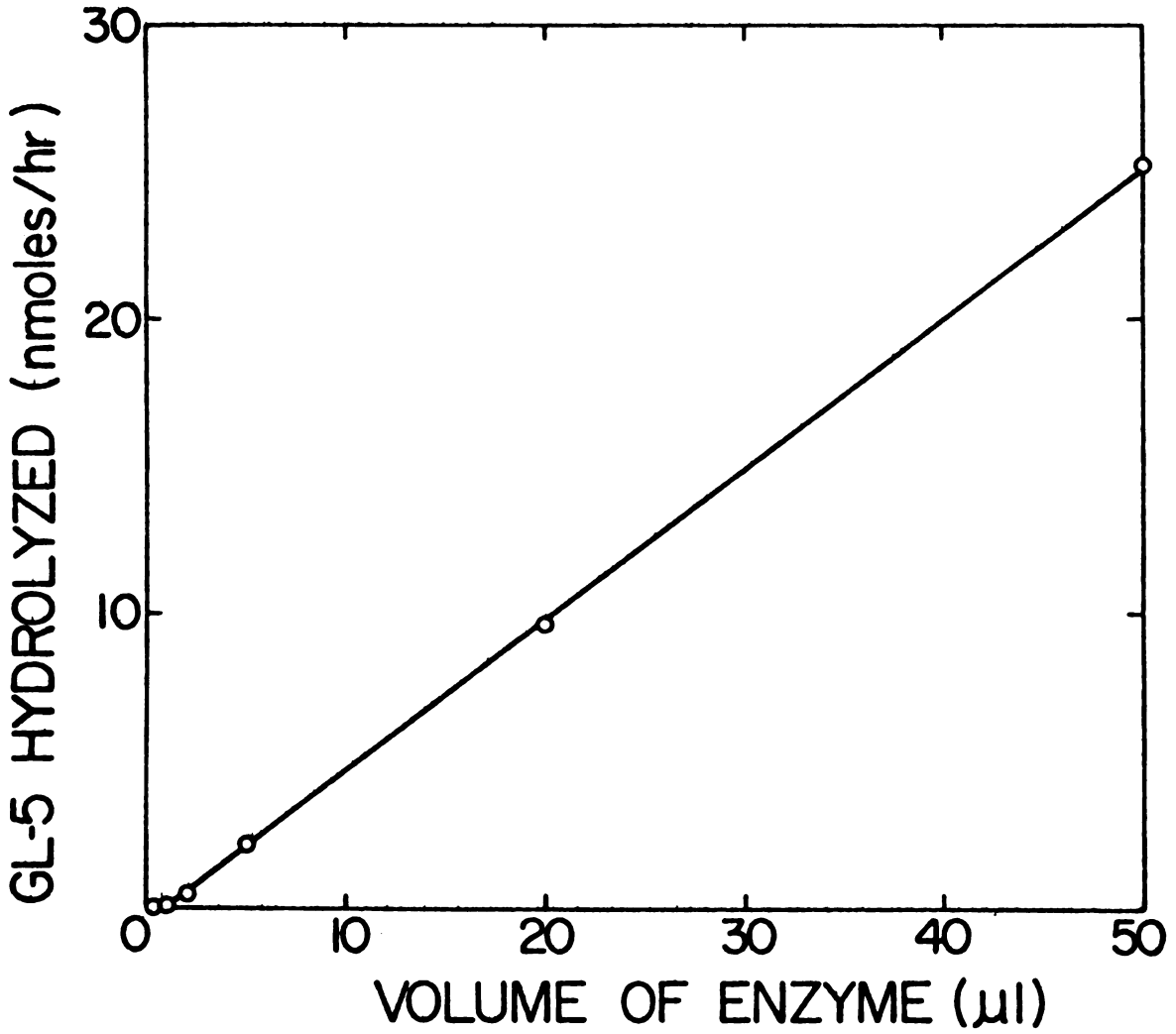


Figure 30. Rate of Hydrolysis of Forssman Hapten vs Enzyme Concentration. The concentration of enzyme used was 2.4 ng/µl.

activity was at pH 3.9 (Figure 31). No hydrolysis of Forssman hapten was found in the absence of detergent. When taurocholate was used, the activity showed a maximum at a final concentration of 4 mg/ml (Figure 32). The Lineweaver-Burk plot for the effect of change in substrate concentration on the rate of hydrolysis was linear. The phenomenon of inhibition at high substrate concentration was not observed. The K_M value of the enzyme was found to be 0.26 mM and the V_{max} was 4.2 μmoles/min/mg (Figure 33).

(3) Inhibitors of Forssman Hydrolase Activity

Metal salts at a concentration of 10 mM were used to study the effects of metal ions on the hydrolysis of Forssman hapten by α-N-acetylgalactosaminidase. Whenever available in a soluble form, chloride salts were used. The results shown in Table 7 indicate that all of transition metals studied inhibited the hydrolysis to a certain extent, the most potent being Ag^{+}, Fe^{3+}, Hg^{2+} and Pb^{2+}. Alkali metals did not affect the enzymatic hydrolysis while alkaline earth metals were slightly inhibitory.

When Cl^{-} was used as an inhibitor with increasing concentrations of NaCl from 0.01 M to 0.1 M, no inhibition of the hydrolysis of GL-5 was observed.

Of various sugars and carbohydrate derivatives tested, only N-acetylgalactosamine, GalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-Gal, and surprisingly, TalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-Gal (Table 8) were inhibitors. The hydrolysis of Forssman hapten was inhibited competitively by TalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-Gal with a K_i of 1.5 mM. The Lineweaver-Burk plot of the effects on inhibition is shown in Figure 34. The results indicated the configuration of the

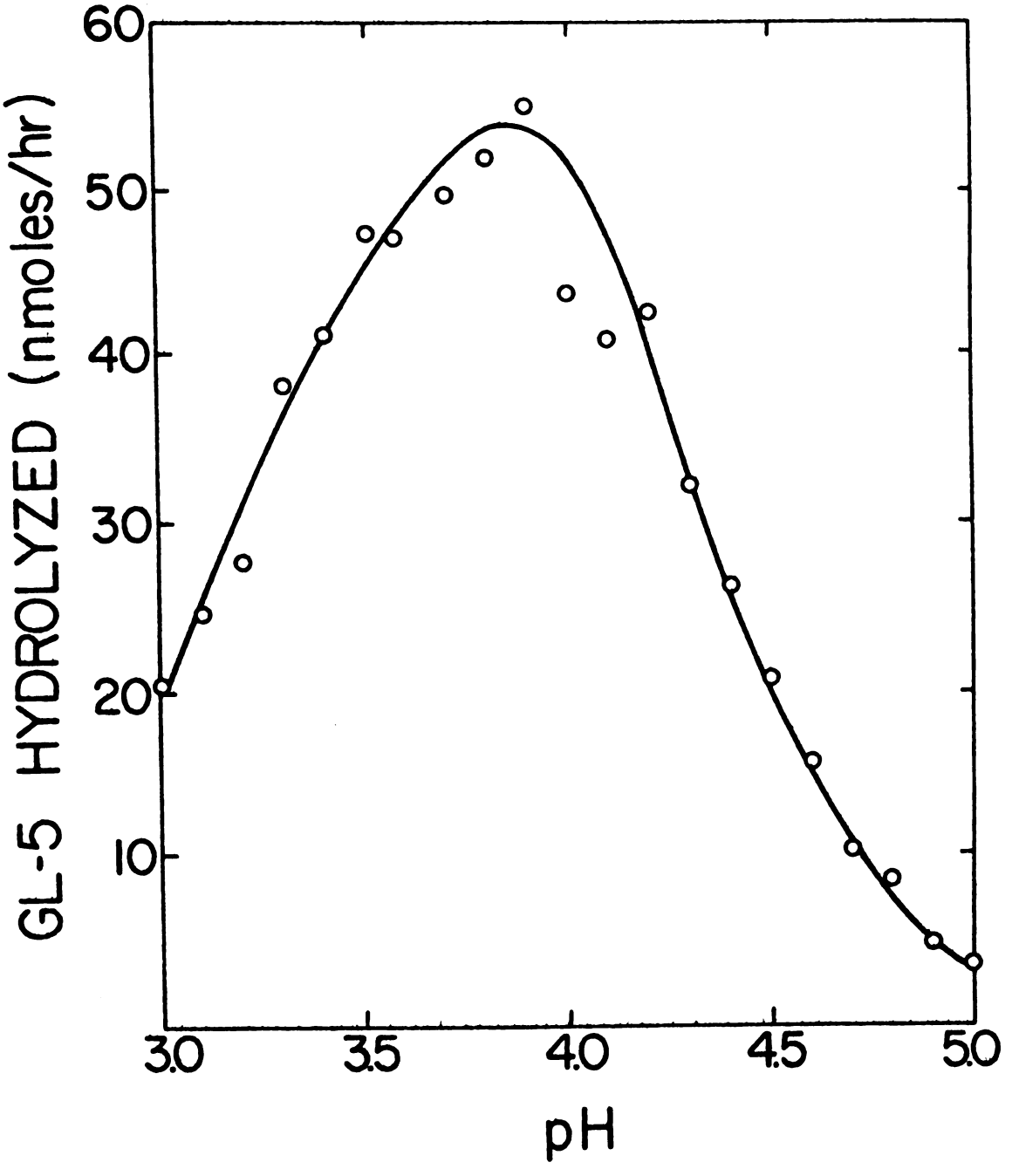


Figure 31. Rate of Hydrolysis of Forssman Hapten vs pH.

Citrate buffer was used for the entire range and 0.24 µg of enzyme was used for each assay.

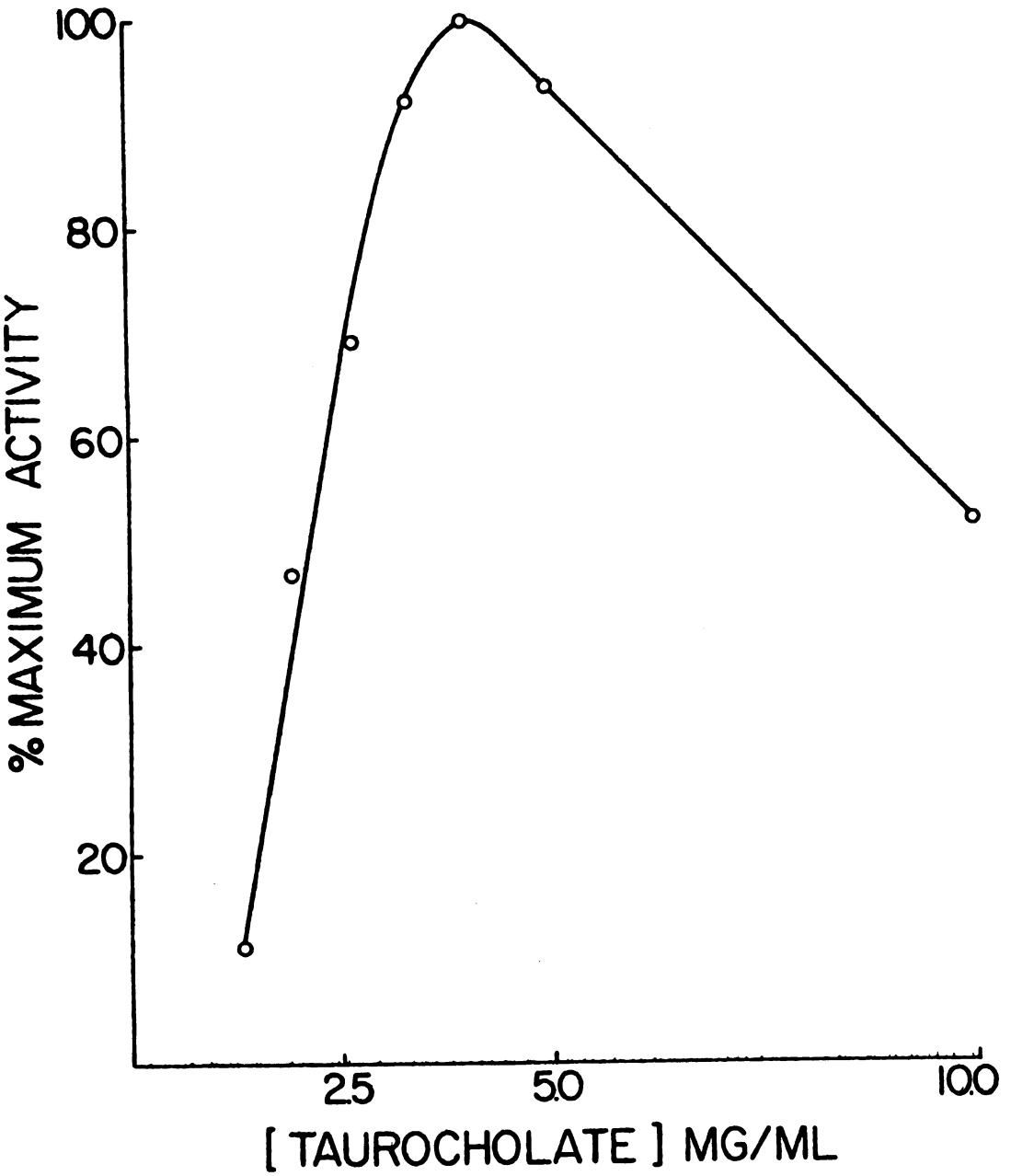


Figure 32. The Effect of Taurocholate Concentration on the Rate of Hydrolysis of Forssman Hapten

0.24 µg of enzyme was used for each assay.

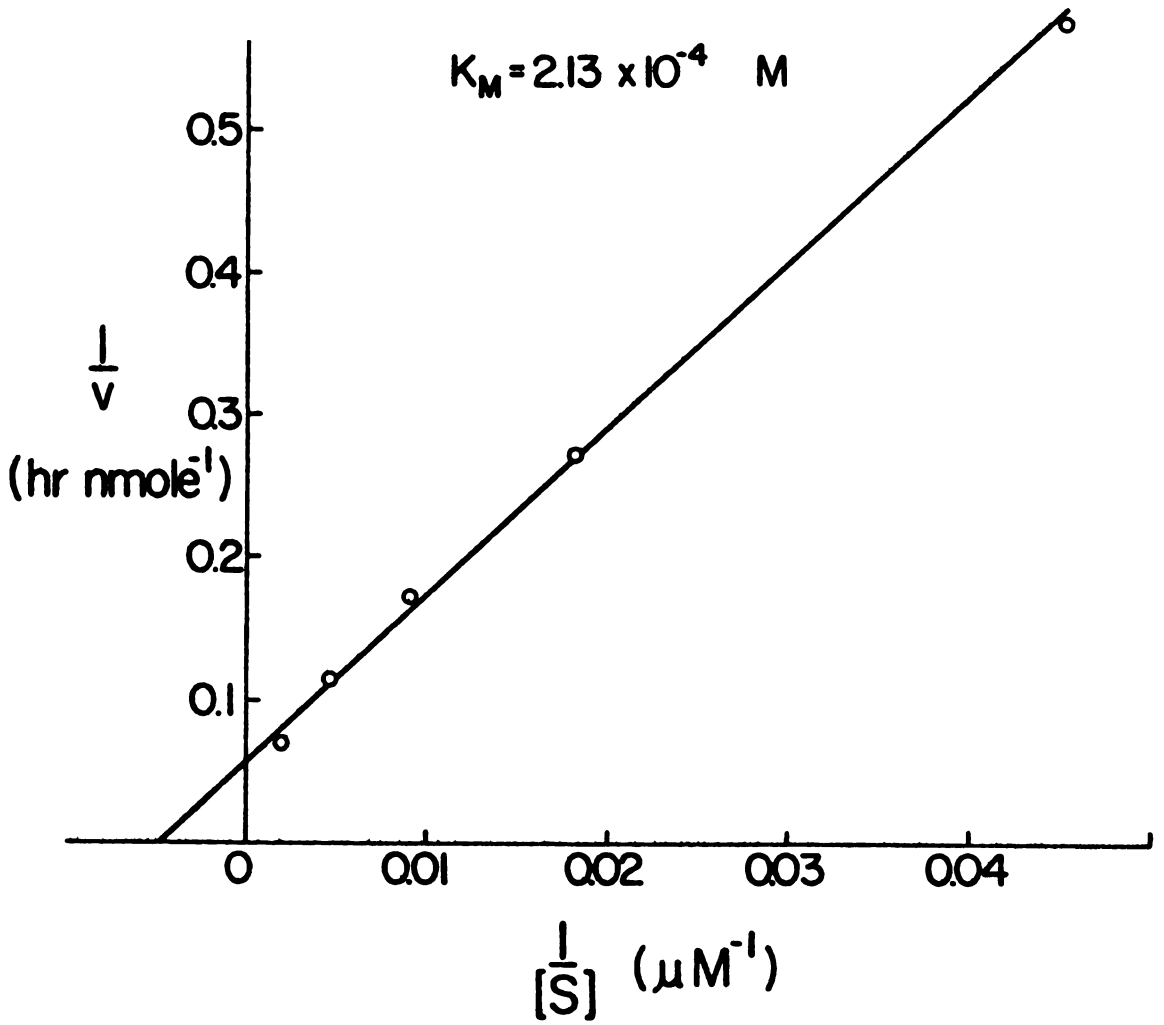


Figure 33. Lineweaver-Burk Plot for the Effect of Change of Substrate Concentration on the Rate of Hydrolysis of Forssman Hapten.

A standard assay procedure with 0.36 μg of enzyme was used for each assay.

Table 7. Metal Inhibitors of Forssman Hydrolase

Metal (10 mM Cl^- salt)	% Activity
Ag^+	0
Ba^{2+}	86
Ca^{2+}	80
Cd^{2+}	68
Co^{2+}	59
Cu^{2+}	35
Fe^{3+}	7
K^+	101
Li^+	108
Hg^{2+}	25
Mg^{2+}	60
Mn^{2+}	69
NH_4^+	103
Na^+	100
Pb^{2+}	31
Sr^{2+}	84

Table 8. Carbohydrate Derivatives in the Inhibition of Forssman Hydrolase

Carbohydrate Derivatives	Conc. (mM)	% Activity
GalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-Gal	5.6	44
TalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-Gal	6	37
GalNAc	10	54

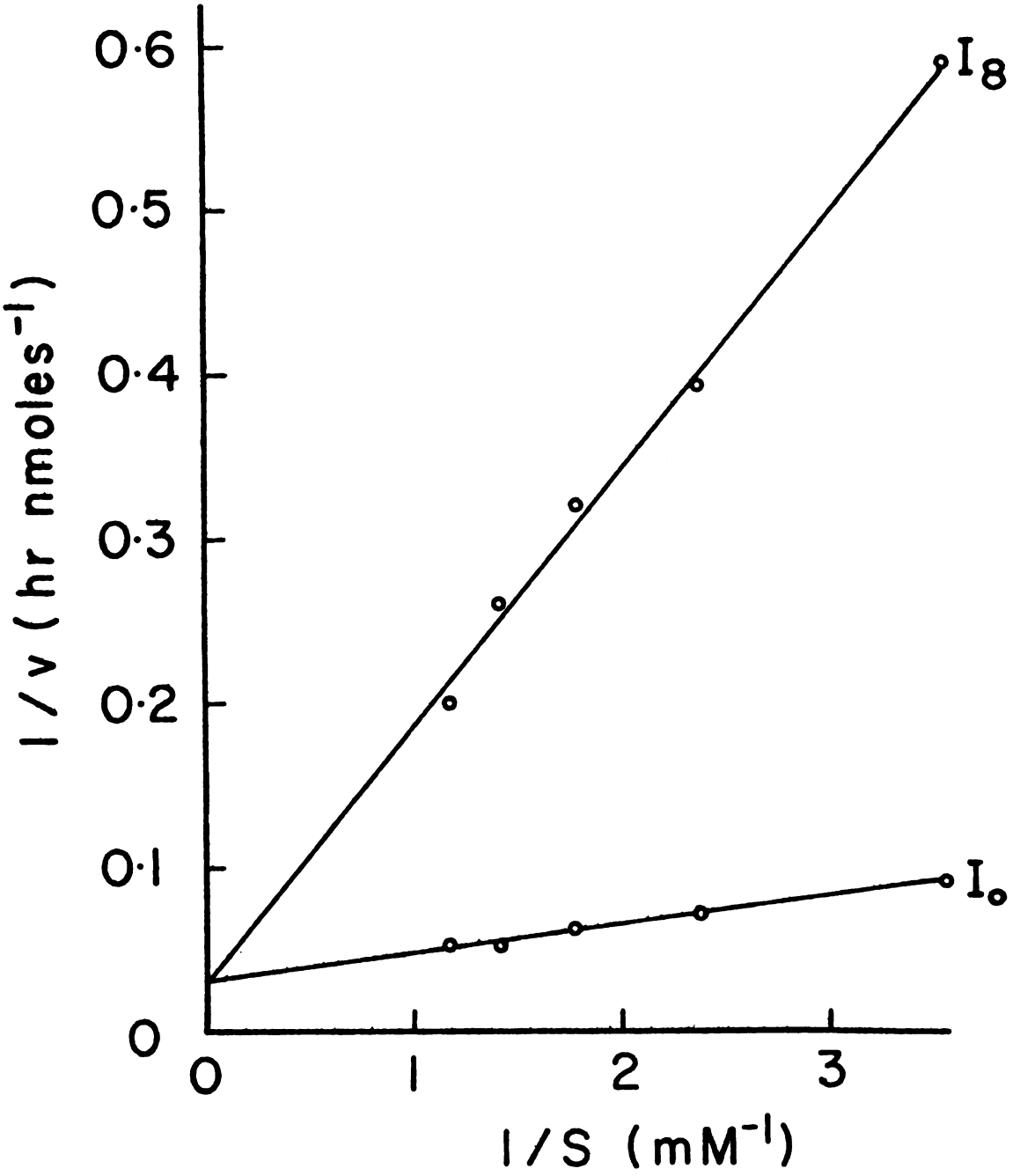


Figure 34. Lineweaver-Burk Plot of Hydrolysis of Forssman Hapten with N-Acetyltalosaminyl-(α1→6)-1,2:3,4-di-O-isopropylidene-Galactose as an inhibitor.

0.24 μg of enzyme was used for each assay. I_8, 8 mM inhibitor; I_0, no inhibitor.

entire N-acetylgalactosamine residue was important for inhibition, except for the C2-position, where an epimer could also inhibit the hydrolysis of Forssman hapten.

(4) Temperature Inactivation of Forssman Hapten Hydrolase

The enzyme activity of α-N-acetylgalactosaminidase on Forssman hapten decreased very rapidly at 60° (Figure 35). Half of the activity was lost after incubating the enzyme at that temperature for about 1.4 minutes. The activity reached zero after 20 minutes of incubation.

At 50°, the decline in activity was not so rapid. A semi-logarithmic plot of the percent activity remaining vs time did not give a straight line, implying that the enzyme activity might have existed in more than one species, which showed different degrees of stability toward temperature inactivation. The half life of the total enzyme preparation was about 6 minutes at this temperature.

Hydrolysis of Forssman Pentasaccharide by α-N-Acetylgalactosaminidase

(1) Characterization of GL-5 Pentasaccharide

The product after ozonolysis, hydrolysis and P-2 chromatography showed a product not visualized by iodine, indicating that it was not lipoidal in nature. The spot turned pink after orcinol-sulphuric acid spray, and migrated below the standard stachyose, a tetrasaccharide (Figure 36). GLC analysis of the methanolysate of an aliquot of the carbohydrate product showed that it is composed of galactose, glucose and N-acetylgalactosamine in the ratio of 2:1:2. The mode of formation of the compound with ozonolysis and alkaline hydrolysis, combined with the above results, characterized the product as GL-5-pentasaccharide with the structure: GalNAc-(α1→3)-GalNAc-(β1→3)-Gal-(α1→4)-Gal-(β1→4)-Glc.

Figure 35. Semi-logarithmic Plot of Temperature Inactivation of Forssman Hapten Hydrolysing Activity vs Time.

The enzyme was incubated in a water bath and aliquots were taken out at different time intervals and assayed for Forssman hydrolysing activity.

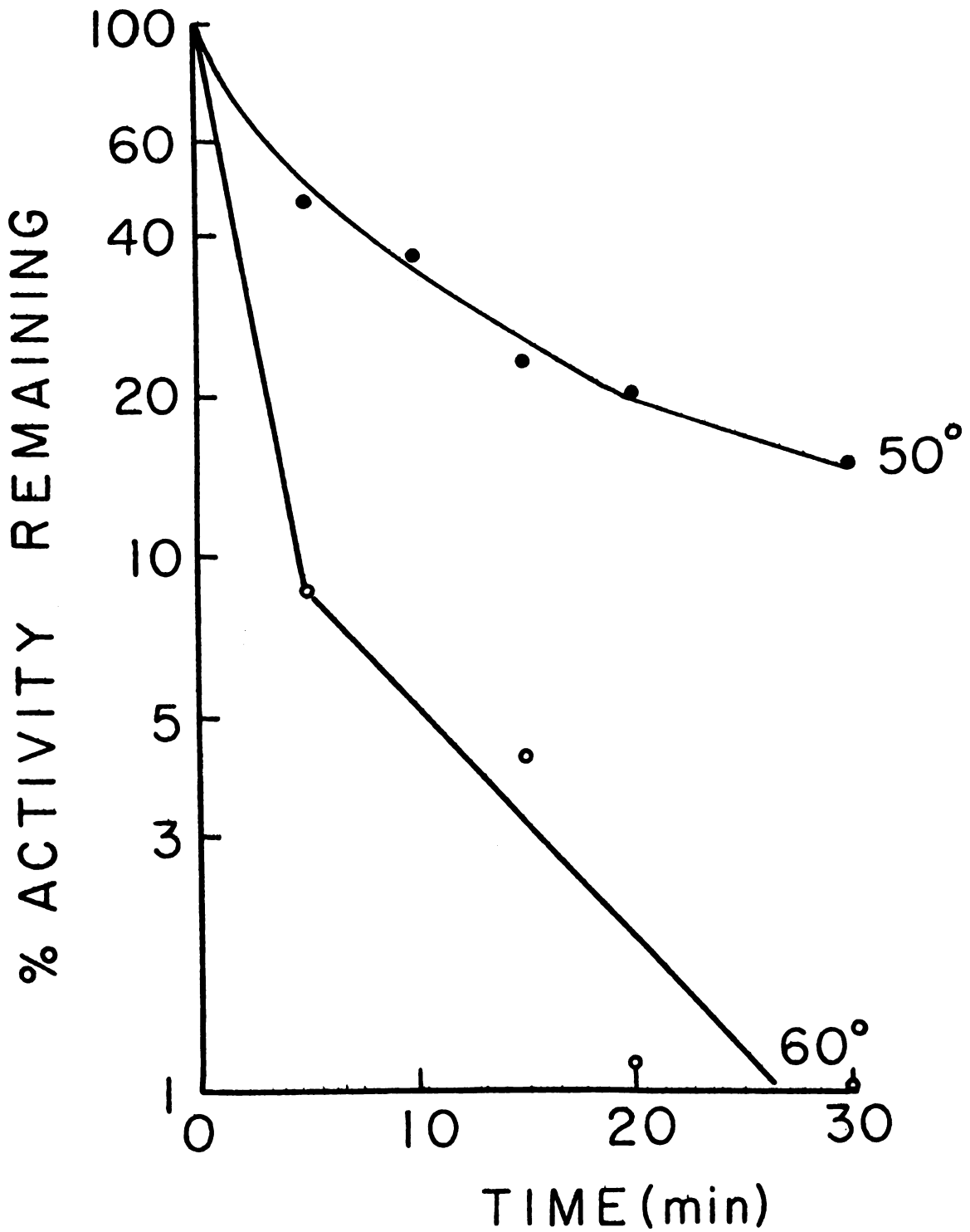


Figure 35.

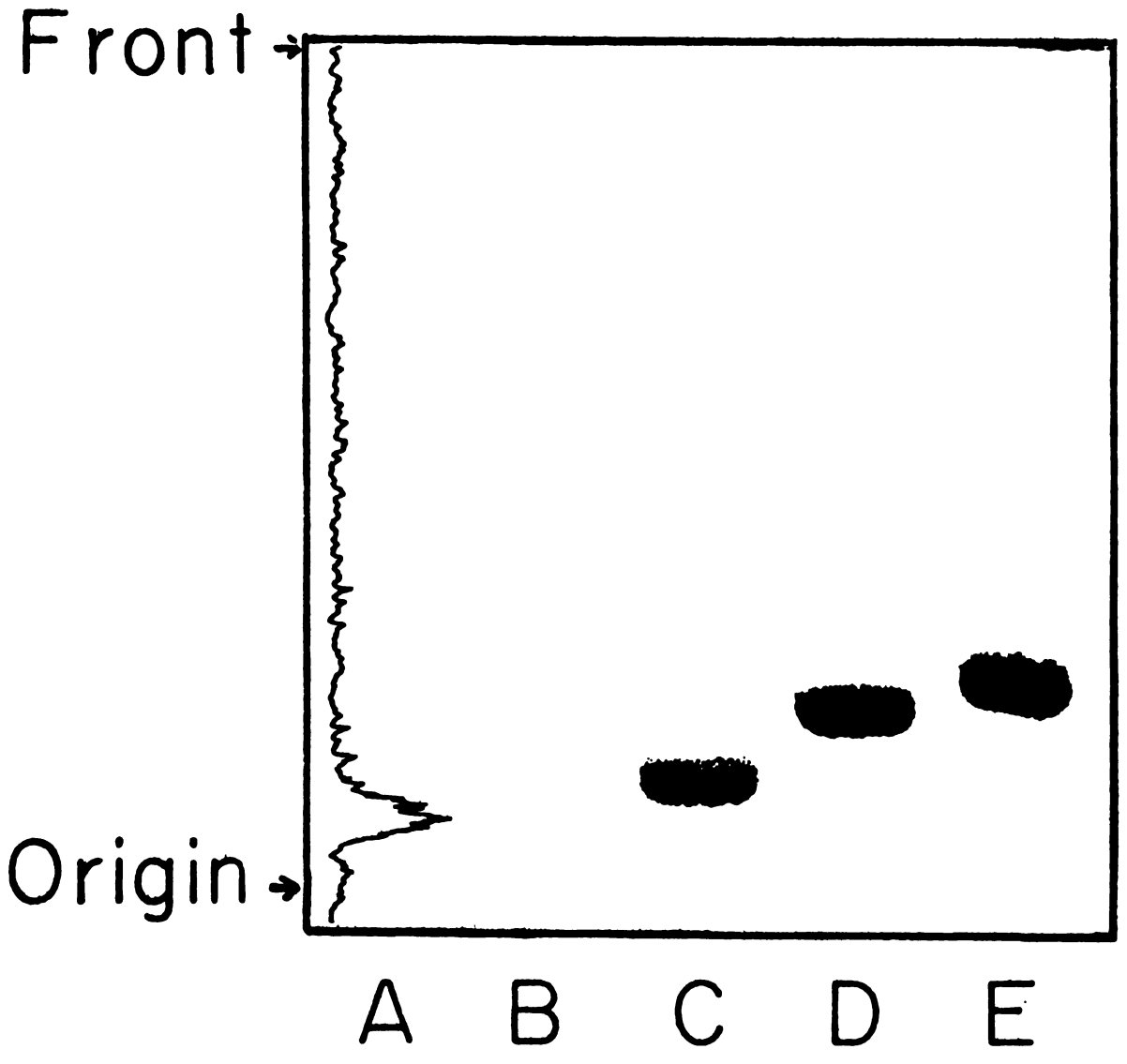


Figure 36. Thin-Layer Chromatography of Forssman Pentasaccharide.

GL-5 Pentasaccharide (Lane B) was scanned with a Berthold radio scanner (Lane A) and compared with the migrations of stachyose (Lane C), raffinose (Lane D) and melibiose (Lane E) in butanol/methanol/acetic acid 100/50/50 on silica gel G plates. The spots were visualized with orcinol-sulphuric acid.

(2) Characterization of Products after Hydrolysis of GL-5 Pentasaccharide with α-N-Acetylgalactosaminidase

The hydrolysis products after the action of α-N-acetylgalactosaminidase on GL-5 pentasaccharide are shown in Figure 37 (Lane B). There were orcinol-positive spots that migrated just a little below the standard stachyose. A faint spot charred by sulphuric acid can be found migrating in the region of the standard monosaccharide (galactose). This monosaccharide spot coincided with the liberated radioactivity (Lane A). Very little radioactivity remained in the tetrasaccharide-pentasaccharide region. The results showed that the radioactive N-acetylgalactosamine at the non-reducing end of GL-5 petasaccharide was liberated by the enzyme with the globoside-tetrasaccharide remaining intact.

(3) Kinetics of Hydrolysis of GL-5 Pentasaccharide

The hydrolysis of GL-5 pentasaccharide was linear for at least 90 minutes with 1 μg of enzyme per assay. The hydrolysis was liear with enzyme concentration up to 1.6 μg of enzyme per assay (Figure 38). The K_M was found to be 10.4 mM and the V_{max} was 1.7 μmoles/min/mg (Figure 39). The addition of taurocholate to a final concentration of 4 mg/ml inhibited the enzyme activity by 13%.

Hydrolysis of N-[1-^{14}C]-Acetyl-sphingosyl-Forssman-pentasaccharide

(1) Characterization of N-[1-^{14}C]-Acetyl-sphingosyl-Forssman-pentasaccharide

The mobility of the alkaline degradation product of GL-5 by KOH is shown in Lane 3 of Figure 40. The deacylated compound, when re-N-acetylated (Lane 2) by [1-^{14}C]-acetic anhydride, migrated faster than the

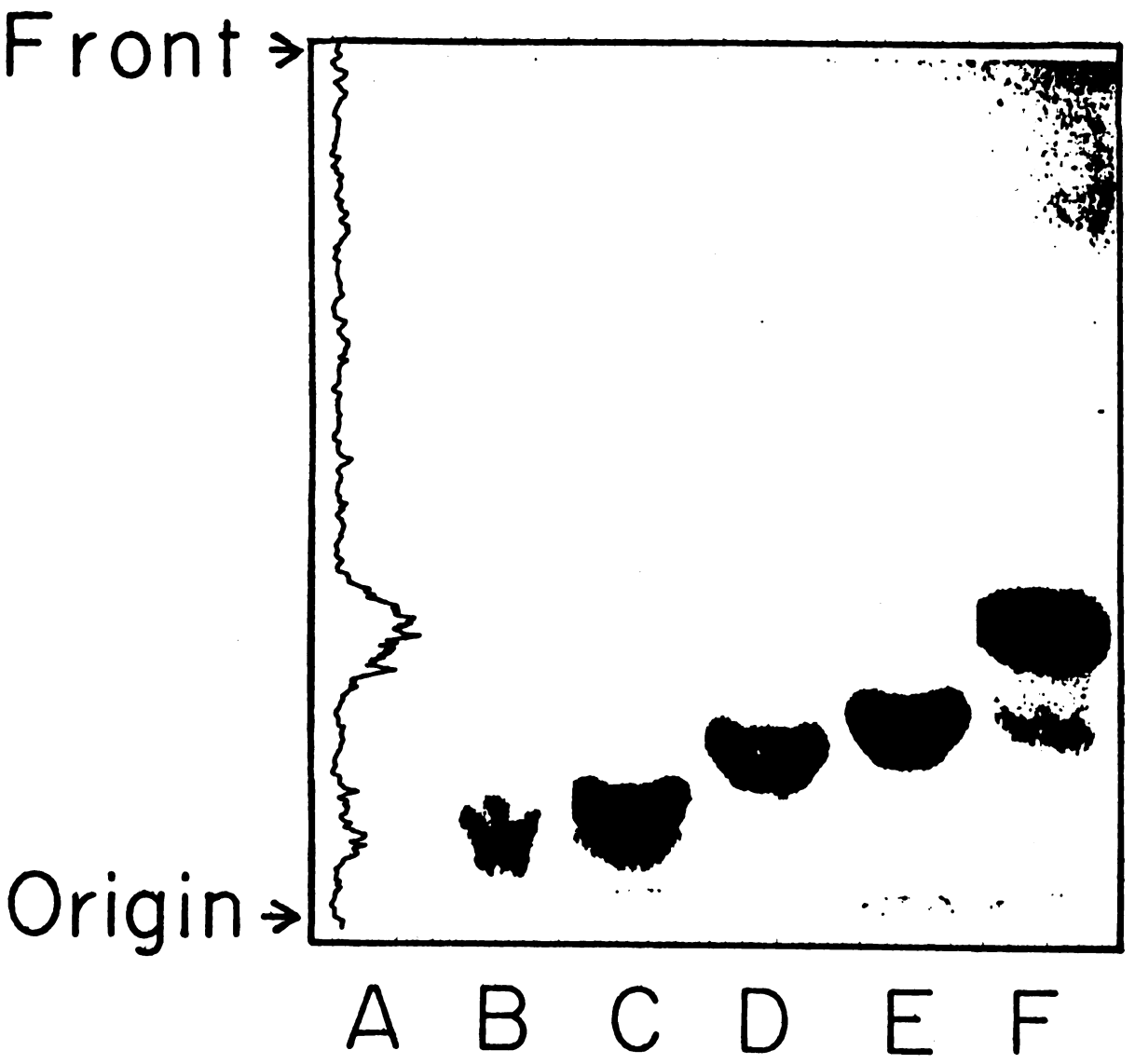


Figure 37. Thin-Layer Chromatography of Hydrolysis Products Obtained after α-N-Acetylgalactosaminidase Treatment of Forssman Pentasaccharide.

B: hydrolysis product of α-N-acetylgalactosaminidase on GL-5 pentasaccharide; A: radioactivity scan of Lane B; C: stachyose; D: raffinose; E: melibiose; F: galactose.

The chromatography conditions were the same as Figure 35. 25 nmoles of substrate was incubated with 0.47 μg of enzyme for 12 hours.

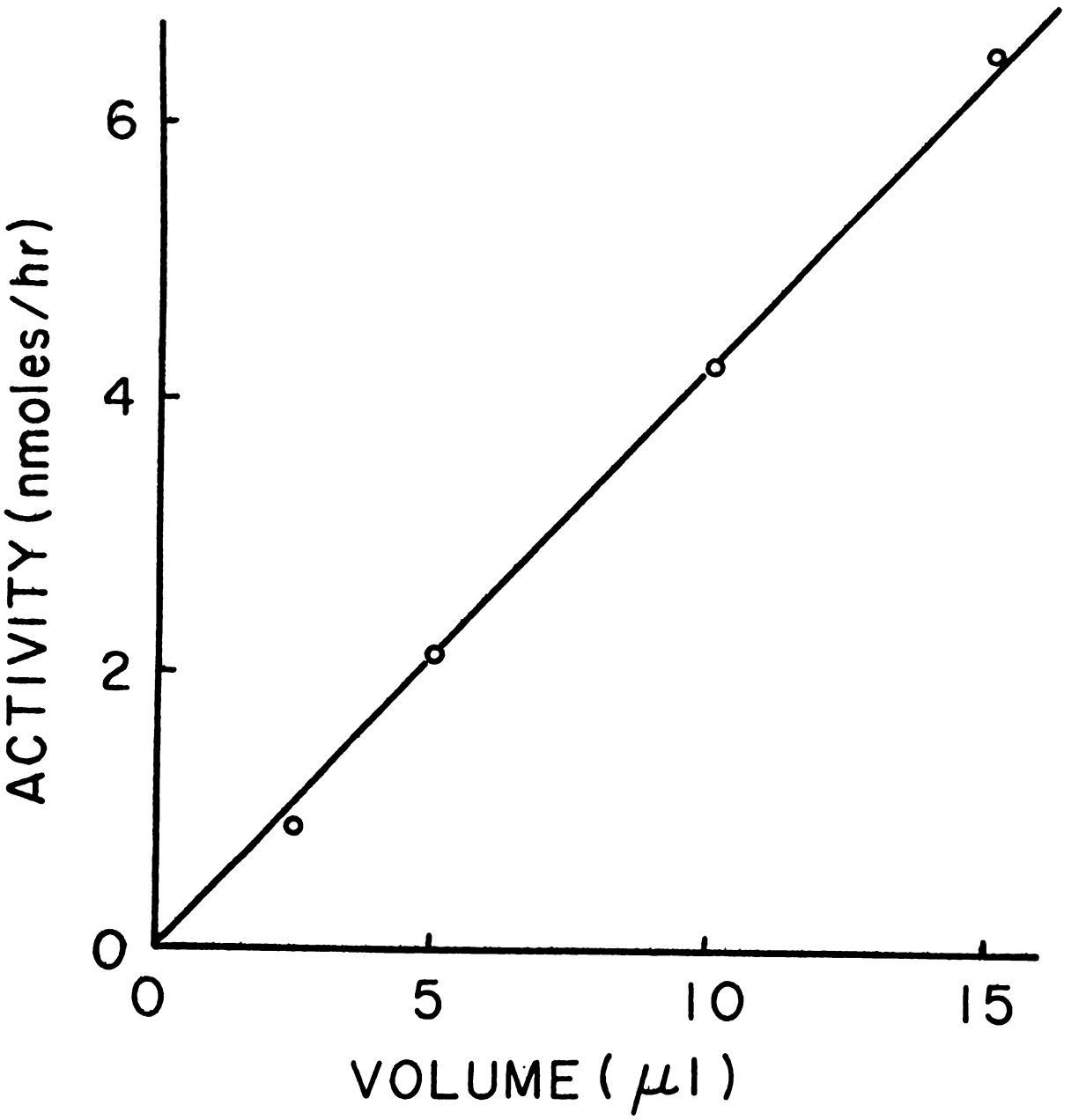


Figure 38. The Activity of Hydrolysis of Forssman Pentasaccharide vs Amount of Enzyme.

The concentration of enzyme was 0.11 μg/ml. Incubation was for 30 minutes.

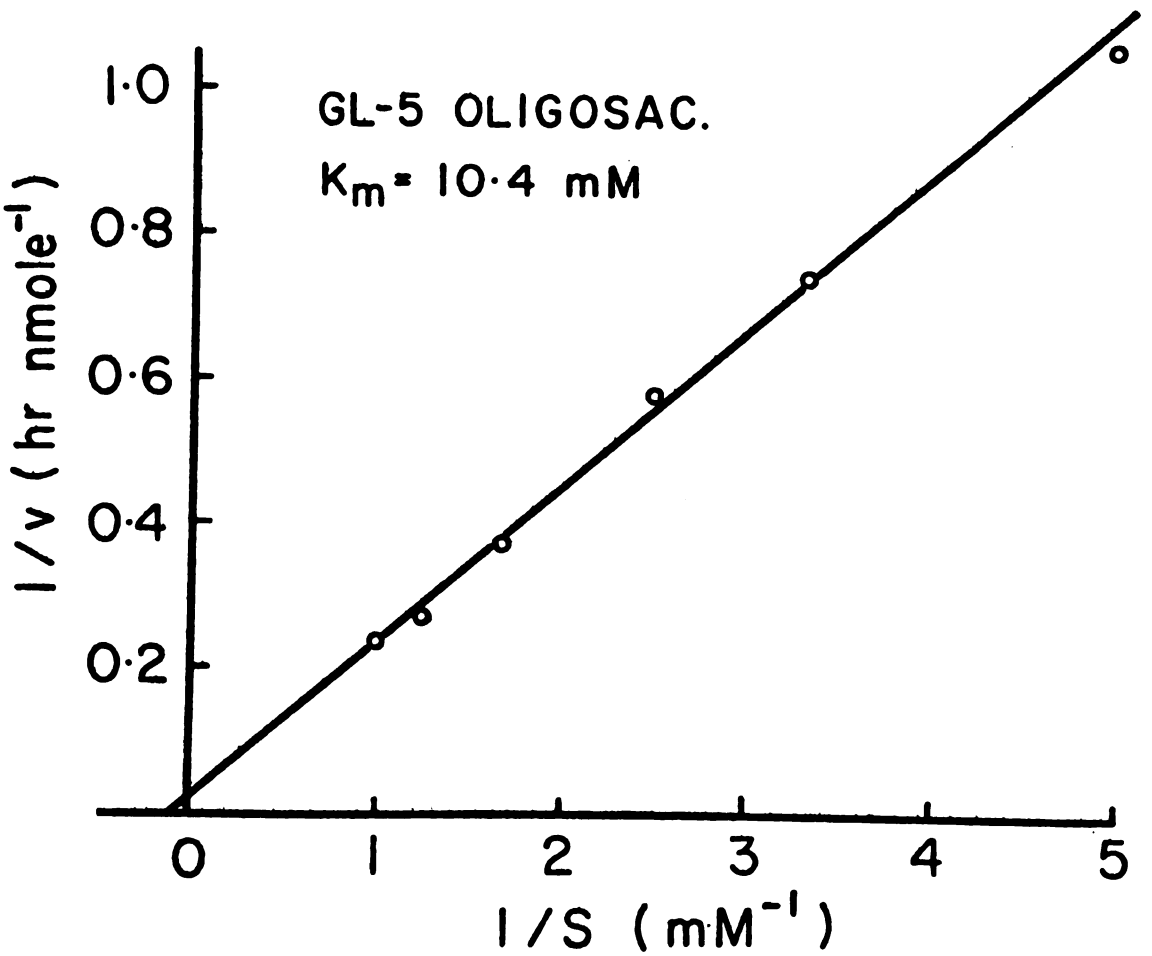


Figure 39. Lineweaver-Burk Plot of the Hydrolysis of Forssman Pentasaccharide by α-N-Acetylgalactosaminidase.

0.24 μg of enzyme was used for each assay.

Figure 40. Characterization of N-[1-^{14}C]-Acetyl-sphingosyl-Forssman-pentasaccharide.

Frame A was the TLC of deacylated (Lane 3) and Re-N-acetylated GL-5 (Lane 2). The solvent system was chloroform/methanol/water 100/42/6. Lanes 1 and 5 contained standard glycolipids from GL-5. Lane 4: starting material (GL-5). Frame B was TLC of starting material (Lane 6) and the deacylated-re-N-acetylated GL-5 in chloroform/methanol/acetic acid/water 55/45/5/5 and the radioactivity scan of Lane 7 (Lane 8).

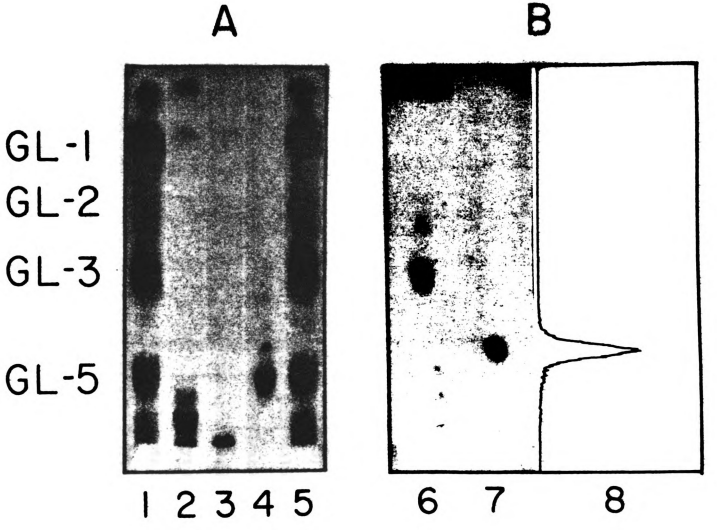


Figure 40.

deacylated GL-5 but slower than a GL-5 standard (Lane 1, 5) and the starting material (Lane 4). The N-[1 -^{14}C]-acetyl-sphingosyl-GL-5-pentasaccharide was further purified and the product (Lane 7, Figure 40) was chromatographyically homogeneous on TLC using the solvent system chloroform/methanol/water/acetic acid 55/45/5/5; its mobility coincided with the radioactivity (Lane 8) and was a little below that of the starting material (Lane 6). The carbohydrate composition of the compound was analysed by GLC of the trimethylsilylated methyl glycosides. A ratio of 2:2:1 for galactose:N-acetylgalactosamine:glucose was found.

(2) Characterization of Hydrolysis Products from α-N-Acetylgalactosaminidase Treatment of N-[1-^{14}C]-Acetyl-sphingosyl-GL-5-pentasaccharide

When N-[1-^{14}C]-sphingosyl-GL-5-pentasaccharide was hydrolysed by α-N-acetylgalactosaminidase, a lipid band (visualized by iodine vapor) was produced that migrated faster than the parent compound (Figure 41). A radioactivity scan showed two radioactive peaks (Lane 5), the faster of which corresponded in migration to standard N-acetylgalactosamine (Lane 6). The second radioactive peak had the same migration as the iodine-positive band. There were no other iodine-positive bands except the one that corresponded to taurocholic acid. GLC analysis of the iodine-positive product showed that it contained galactose, glucose and N-acetylgalactosamine in an approximate ratio of 2:1:1. These results provided evidence that the hydrolysis products were N-acetylgalactosamine (the faster radioactive band) and N-[1-^{14}C]-acetyl-sphingosyl-GL-4-tetrasaccharide (the slower radioactive band).

Figure 41. Thin-Layer Chromatography and Radioactivity Scans of the Products of Hydrolysis of N-[1-^{14}C]-Acetyl-sphingosyl-Forssman-pentasaccharide by α-N-Acetylgalactosaminidase.

1: 100 µg N-acetylgalactosamine;
2: hydrolysis product;
3: substrate without enzyme;
4: scan of lane 3;
5: scan of lane 2;
6: scan of lane 1.

TLC was with chloroform/methanol/acetic acid/water 55/45/5/5 on silica gel G plates and visualized with iodine (carbohydrate were not visualized unless presnet in large amounts). 150 nmoles of substrate were used for the assay and control.

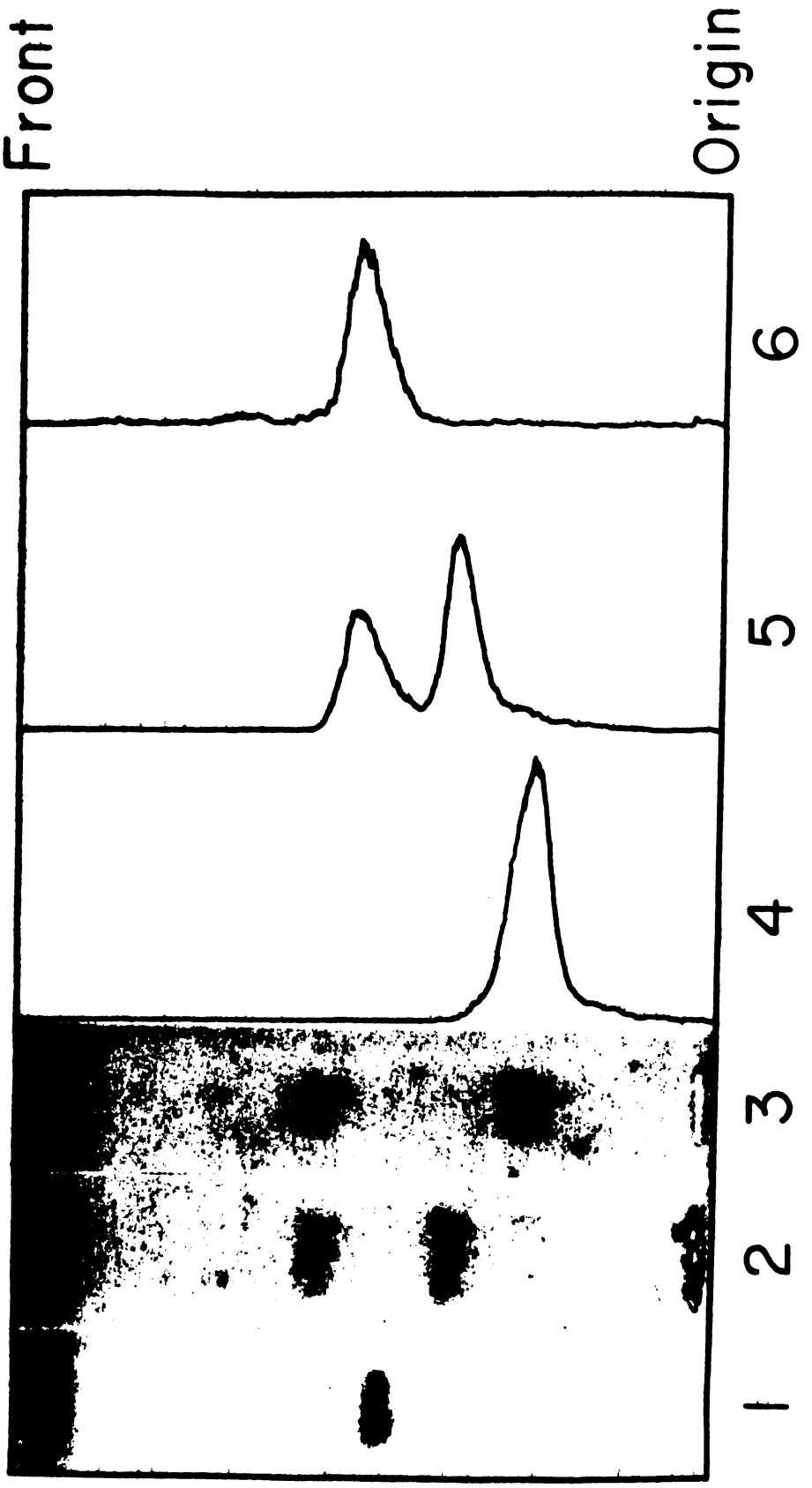


Figure 41.

(3) Kinetics of the Hydrolysis of N-[1-^{14}C]-Acetyl-sphingosyl-Forssman pentasaccharide

The hydrolysis of N-[1-^{14}C]-acetyl-sphingosyl-Forssman-pentasaccharide was optimal at a final sodium taurocholate of 4 mg/ml (Figure 42). The optimum was much sharper than the sodium taurocholate requirement curve for the hydrolysis of Forssman hapten (Figure 32). Hydrolysis of this substrate was linear for at least 30 minutes when 0.27 µg of the enzyme was used for each assay. The hydrolysis was linear up to 1.08 µg of enzyme used for each assay (Figure 43). There was no apparent lag of activity at low enzyme concentration, probably because the total volume of the assay was smaller and consequently the final concentration of the enzyme was higher. The K_M value was found to be 2.5×10^{-4} M for this substrate and V_{max} was 0.81 µmoles/min/mg(Figure 44). No substrate inhibition was found in the Lineweaver-Burk plot of the action of α-N-acetylgalactosaminidase on this enzyme.

Hydrolysis of Porcine Submaxillary Mucin by α-N-Acetylgalactosaminidase

The A^+-porcine submaxillary mucin was prepared by a method described previously (64). The product liberated from the mucin by α-N-acetylgalactosaminidase was identical in retention time on GLC to an authentic trimethylsilylated N-acetylgalactosamine sample. The hydrolysis of porcine submaxillary mucin was optimal at pH 4.2 (Figure 45). At this pH the hydrolysis was linear with respect to time for at least 60 minutes when 2.17 µg of enzyme was used for each assay. The activity was linear up to 5.34 µg of enzyme added per assay when 30 minutes incubations were used but there was a lag in the hydrolysis when the amount of enzyme used was below 1 µg per assay (Figure 46). The K_M of the enzyme in mg/ml was

Figure 42. Detergent Dependence in the Hydrolysis of N-[1-^{14}C]-Acetyl-sphingosyl-Forssman-pentasaccharide by α-N-Acetylgalactosaminidase.

The amount of enzyme used for each assay was 0.27 μg.

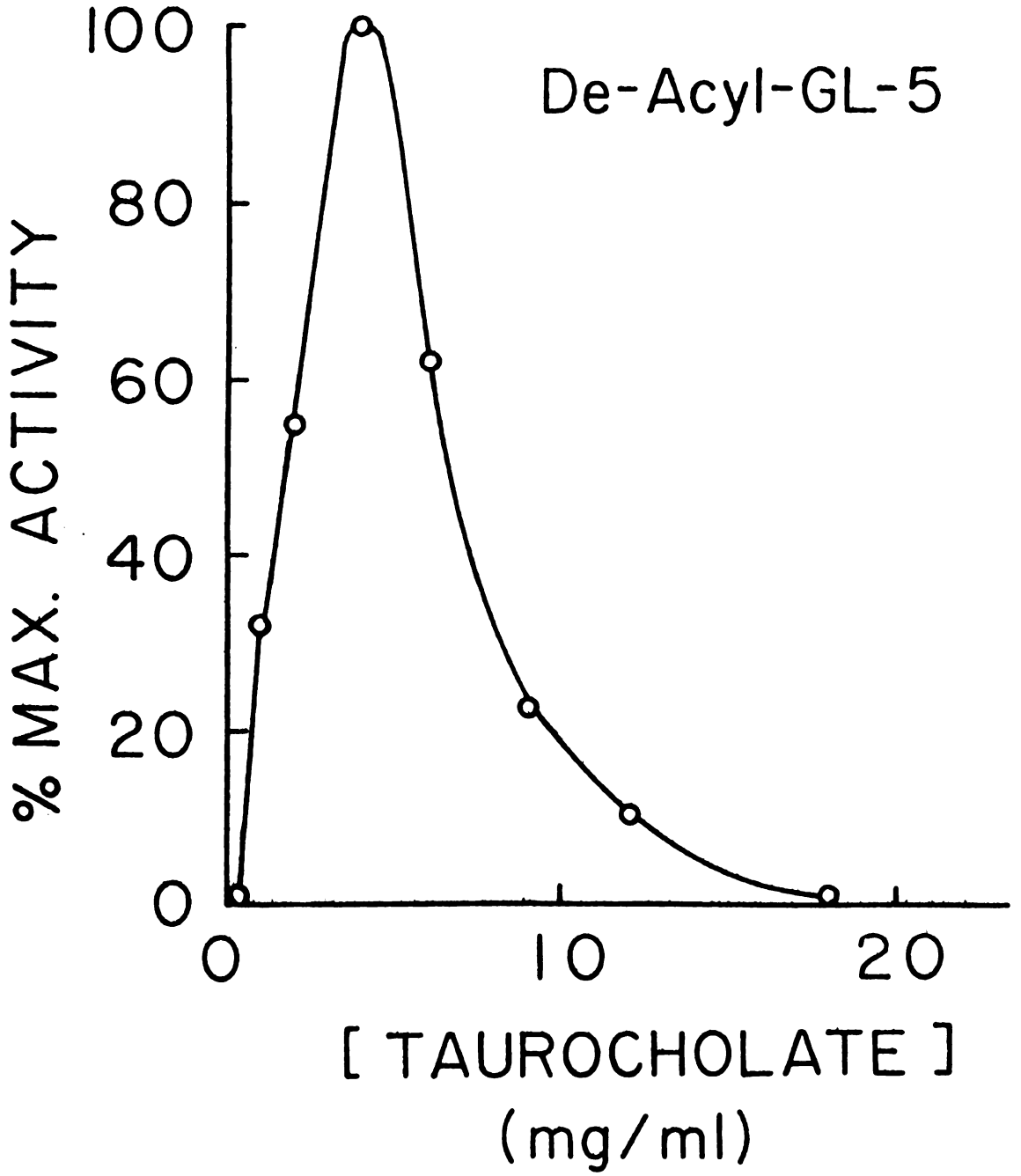

De-Acyl-GL-5
% MAX. ACTIVITY
100
80
60
40
20
0
0
10
20
[TAUROCHOLATE]
(mg/ml)

Figure 42.

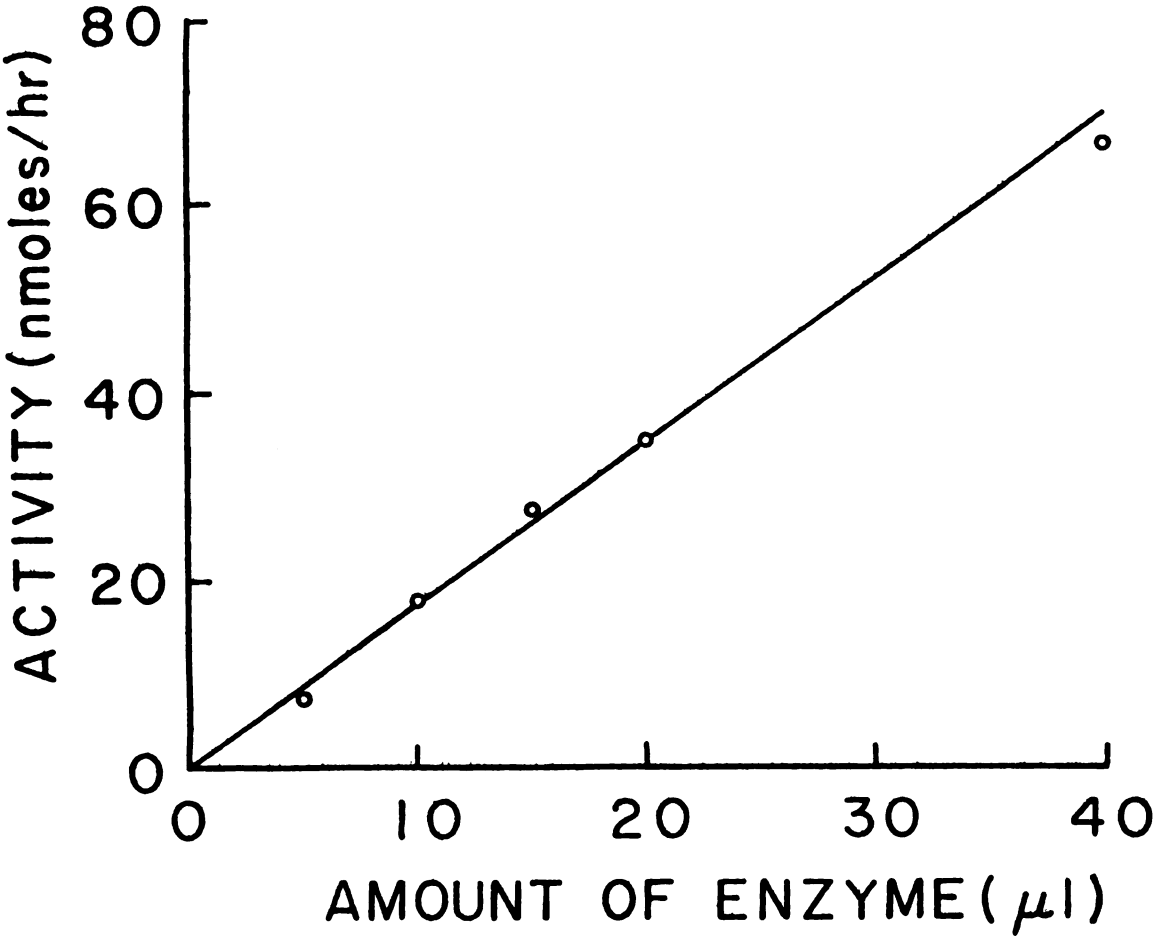


Figure 43. Hydrolysis of N-[1-^{14}C]-Sphingosyl-Forssman-pentasaccharide by Varying Amounts of α-N-Acetylgalactosaminidase.

The concentration of enzyme used was 27 ng/μl.

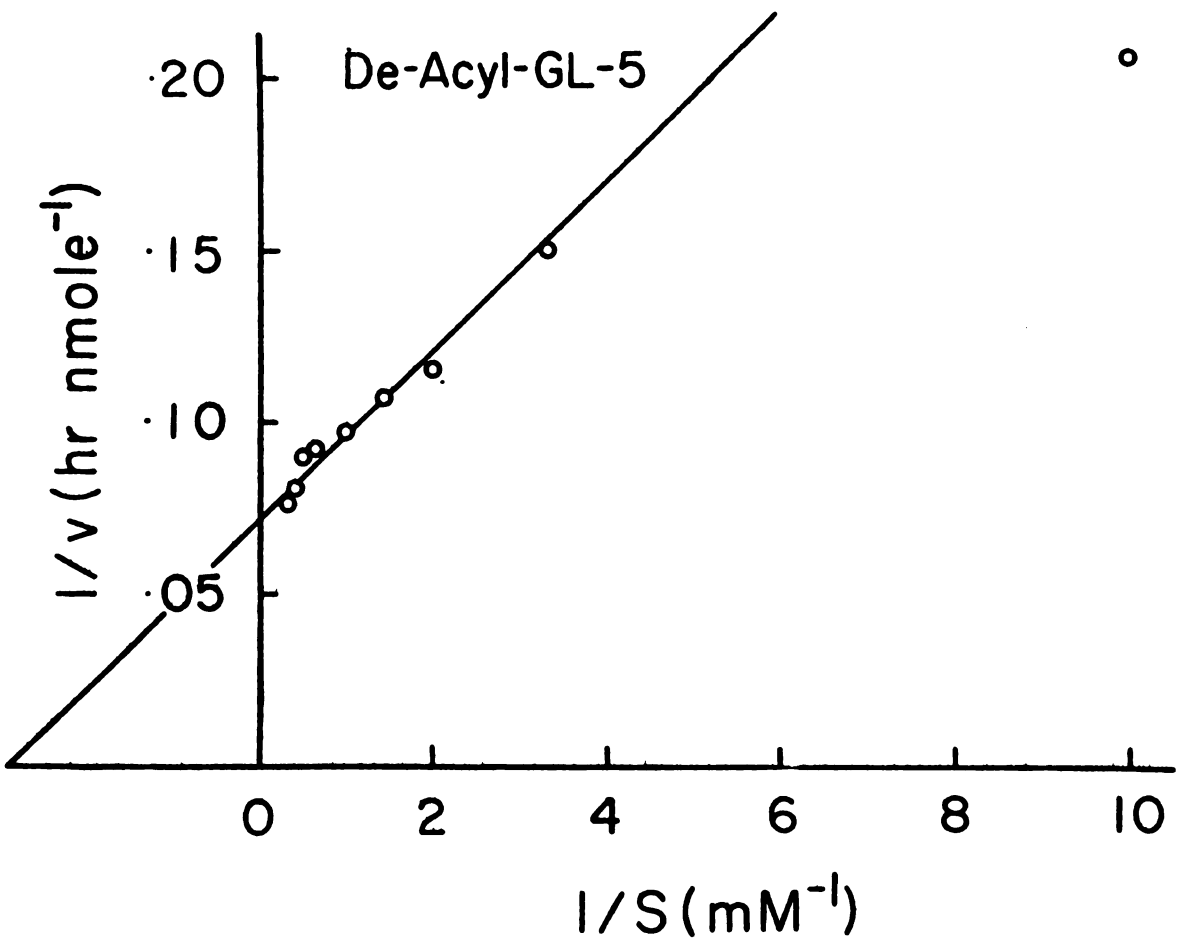


Figure 44. Lineweaver-Burk Plot of the Hydrolysis of N-[1-^{14}C]-Acetyl-sphingosyl-Forssman-pentasaccharide by α-N-Acetylgalactosaminidase.

The amount of enzyme used in each assay was 0.27 µg.

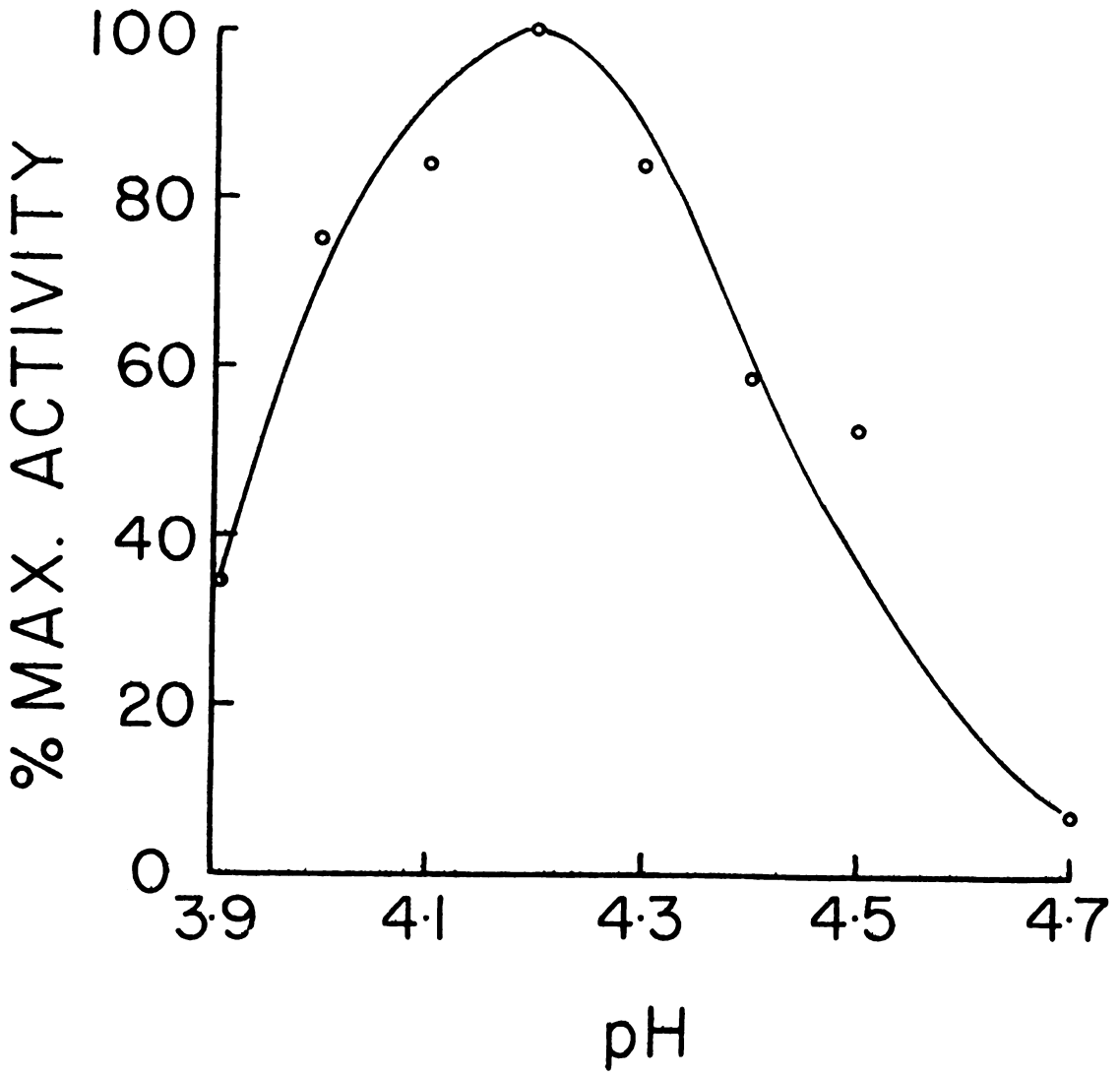


Figure 45. Effect of pH on the Hydrolysis of Porcine Submaxillary Mucin.

2.17 µg of enzyme was used for each assay and incubation was at 37° for 30 minutes.

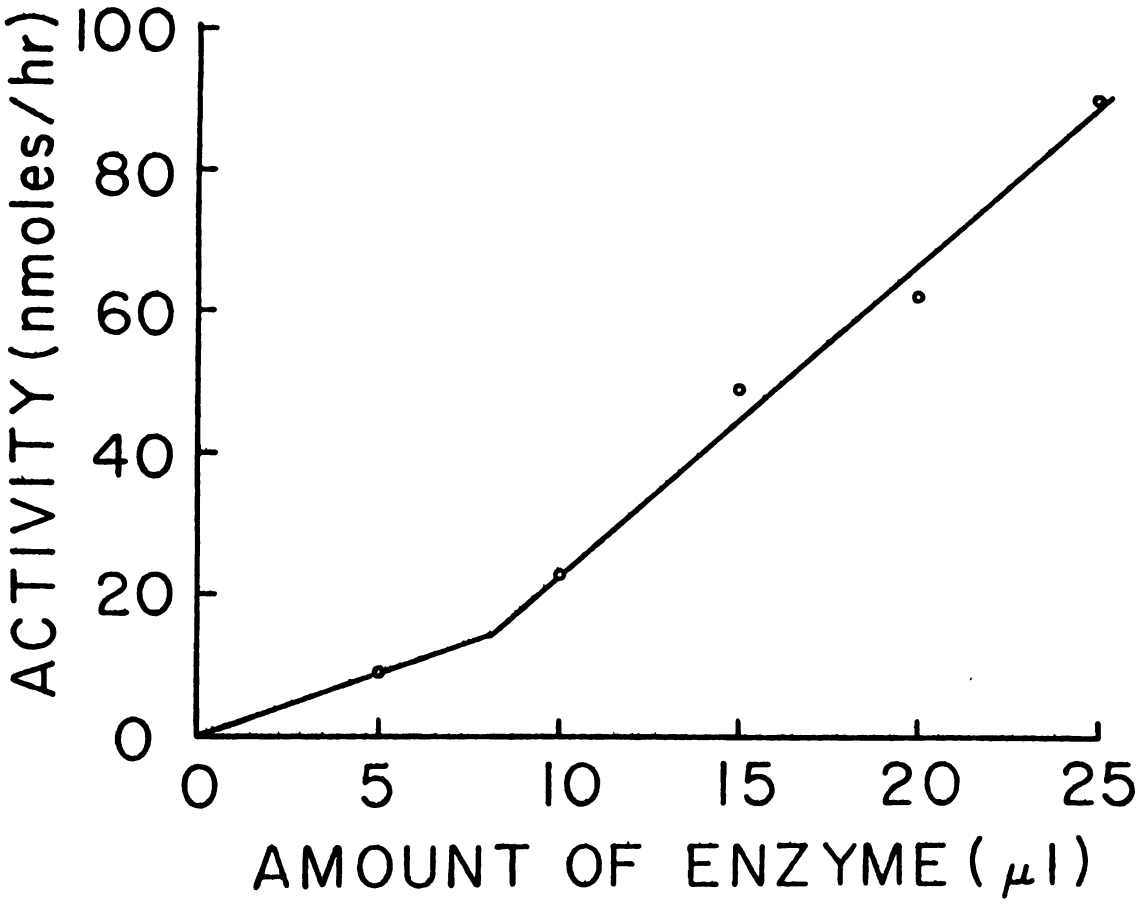


Figure 46. Hydrolysis of Porcine Submaxillary Mucin with Varying Amount of α-N-Acetylgalactosaminidase.

The concentration of enzyme was 0.22 μg/μl. Incubation was at 37° for 30 minutes.

found to be 2.31 (2.3 x 10^{-6} M) and the V_{max} was found to be 0.42 μmoles min/μg (Figure 47).

Hydrolysis of GalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-Gal

The substrate accounted for only 60% of the weight of the compound when quantitated by GLC, and on TLC, it was not homogeneous. One major and two minor bands were found (Figure 48, lane 2). When the diisopropylidene group on the compound was removed by trifluoroacetic acid, (Lane 3), the same pattern was found, with one major and two minor bands. The major band coincided with the mobility of a disaccharide, while the impurities migrated in the monosaccharide and trisaccharide regions, respectively. Analyses by GLC of the methanolysed trimethylsilylated methyl glycosides of GalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-galactose (I) and GalNAc-(α1→6)-Gal (II) are shown in Figure 49. The ratio of galactose to N-acetylgalactosamine was approximately 1:1.

One of the products obtained from the enzyme hydrolysis of GalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-galactose was identical in retention time to an authentic sample of N-acetylgalactosamine when the trimethylsilylated derivatives of the hydrolysis products were anlysed by GLC.

The Lineweaver-Burk plot of the hydrolysis of the disaccharide by α-N-acetylgalactosaminidase is shown in Figure 50. The K_M value was 4.8 x 10^{-3} M and the V_{max} was 1.1 μmoles/min/mg protein.

The linearity of activity with respect to enzyme concentration and time was not examined. The hydrolysis of the substrate, however, was limited to less than 10% of the total initial substrate present in the assay.

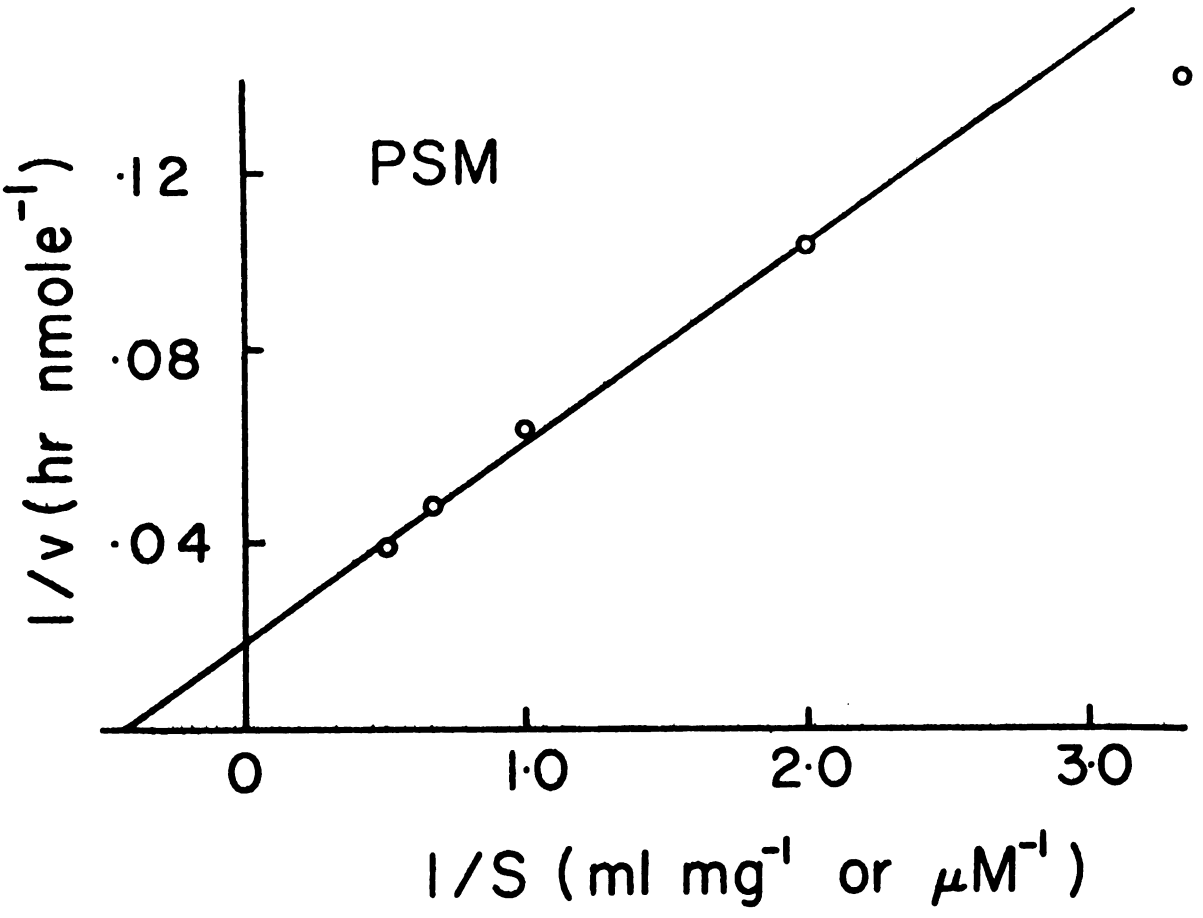


Figure 47. Lineweaver-Burk Plot of Porcine Submaxillary Mucin Hydrolysis by α-N-Acetylgalactosaminidase.

2.17 μg of enzyme was used for each assay.

Figure 48. Thin-Layer Chromatography of N-Acetyltalosaminyl-(α1→6)-1,2:3,4-di-O-isopropylidene-Galactose (lane 1), N-Acetylgalactosaminyl-(α1→6)-1,2:3,4-di-O-isopropylidene-Galactose (lane 2) and N-Acetylgalactosaminyl-(α1→6)-Galactose (lane 3).

The solvent system used was butanol/acetic acid/water 100/50/50. 100 μg of sample was used for each compound. The standards used were stachyose (lane 4), raffinose (lane 5), melibiose (lane 6), and galactose (lane 7).

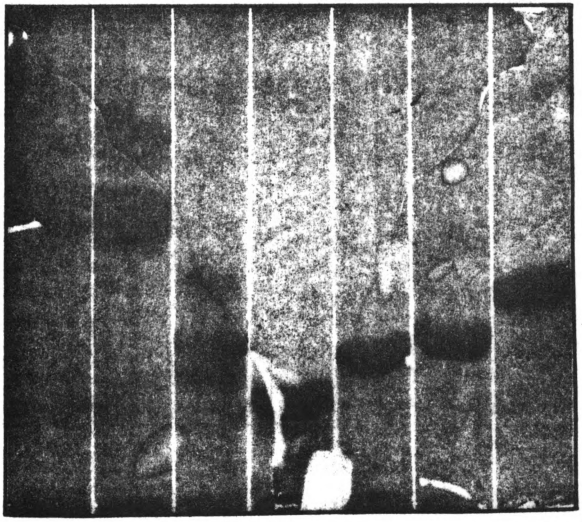


Figure 48.

Figure 49. Gas-Liquid Chromatography of Trimethylsilylated Methyl Glycosides of Methanolysed N-Acetylgalactosaminyl-(α1→6)-Galactose, N-Acetylgalactosaminyl-(α1→6)-1,2:3,4-di-O-isopropylidene-Galactose and N-Acetyltalosaminyl-(α1→6)-1,2:3,4-di-O-isopropylidene-Galactose.

They are shown in I, II and III, respectively. GLC was performed isothermally at 185° and the samples were chromatographed on 3% SP-2100 on Supelcoport (80-100 mesh) with 6 ft columns using nitrogen at 45 ml/min as carrier gas. The peaks are A: galactose; B: mannitol; C: N-acetyl-galactosamine and peaks in D are probably N-acetyltalosamine.

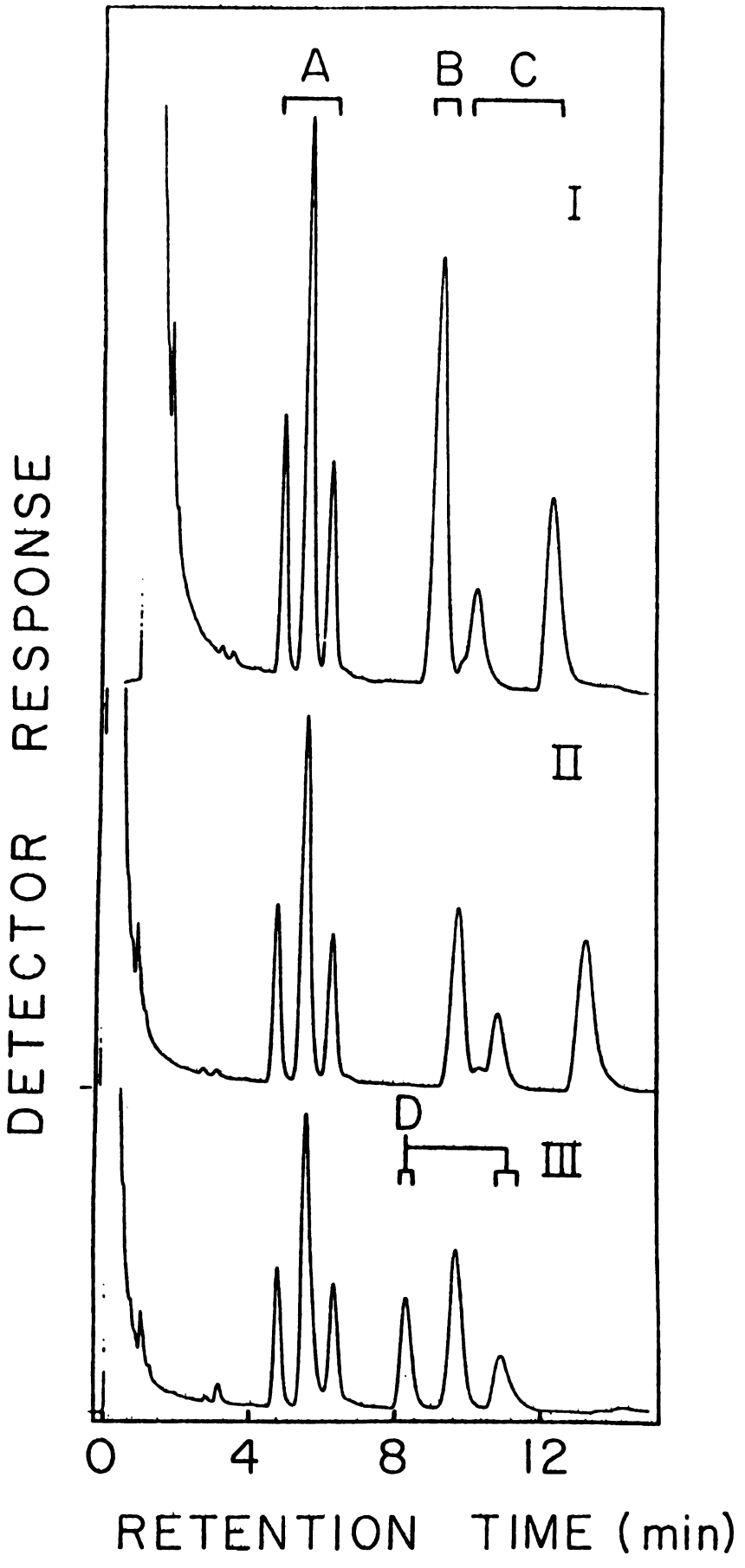


Figure 49.

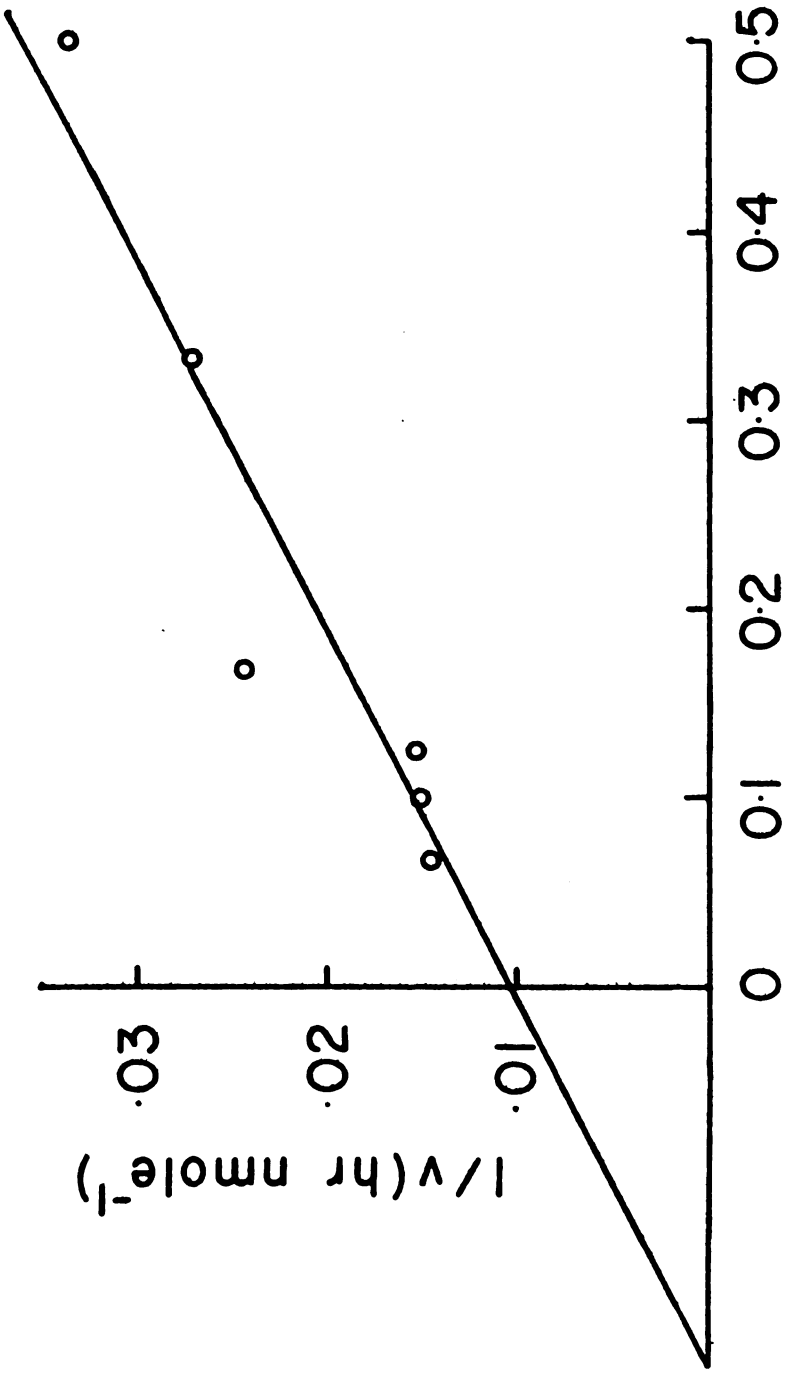


Figure 50. Lineweaver-Burk Plot of the Hydrolysis of N-Acetylgalactosaminyl-(α1→6)-1,2:3,4-di-O-isopropylidene-Galactose by α-N-AcetyTgalactosaminidase.

The amount of enzyme used in each assay was 1.36 µg.

Hydrolysis of Human Red Blood Cell and Dog Intestinal A^+-Glycosphingolipids

When the human RBC blood group A-active glycolipid (lane 1) was hydrolysed by the enzyme, TLC gave an iodine- and orcinol-sulphuric acid- positive band between the taurocholic acid and blood group A^+ band (lane 2, Figure 51).

Human red blood cell A_2 glycolipid (lane 4, Figure 51) was hydrolysed by α-N-acetylgalactosaminidase to give a product which migrated on TLC higher than the starting material (lane 3, Figure 51).

Human red blood cell A_3 glycolipid (lane 5) was also hydrolysed by the enzyme to give an orcinol-sulphuric acid- positive band (lane 6) on TLC.

Dog intestinal A^+-glycosphingolipid (lane 8) was hydrolysed by the enzyme to a product which reacted positively on TLC with orcinol as well as iodine and which migrated slightly higher than the starting material.

Figure 51. Thin-Layer Chromatography of Products of Hydrolysis of Blood Group A Glycosphingolipids by α-N-Acetylgalactosaminidase.

The samples were chromatographed on silica gel G with chloroform/methanol/acetic acid/water 55/45/5/5. The plates were visualized by iodine vapor except RBC-3, which was visualized by orcinol-sulphuric acid. S denotes starting material and P denotes the lipid products after hydrolysis with α-N-acetylgalactosaminidase. RBC-1, RBC-2, and RBC-3 were blood group A glycolipids from human red blood cell with decreasing mobility from 1 to 3; D1-F2 was A^+-blood group glycolipids from dog intestines. The dense spot at R_f 0.6 was identified as taurocholic acid.

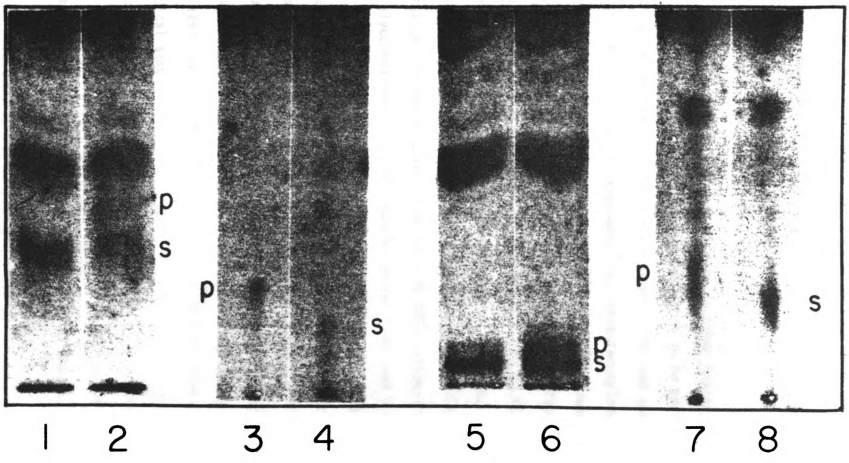


Figure 51.

DISSUSSION

Structural Determination of Forssman Hapten

The structure of Forssman hapten was shown to be smilar to that found in horse spleen (3), sheep erythrocytes (16) and goat erythrocytes (18) but different from Forssman-positive tetraglycosylceramide purified from NIL cells (20). These structural studies involved permethylation of the intact glycolipid by Hakomori's procedure (194) and subsequent reduction, acetylation and determination by GC-MS by the Lindberg technique (195) for the assignment of linkages. This permethylation technique is a very versatile procedure and has been used for linkage studies of glycolipids (2,3,20,203), oligosaccharides (213, 214), glycopeptides (215,216), and bacterial (195) and yeast (217) cell walls. Not only will the procedure indicate the linkages of the saccharide units present in an oligosaccharide, but it also gives valuable information regarding the ratios of different sugars with different linkages and the homogeneity of the sample. The stationary phase for GLC is crucial for the best resolution of the various partially methylated alditol acetates present in the sample, especially with some biological samples that are exceedingly complex. An oligosaccharide with only four kinds of sugar linked in 1,2 or 3 positions can give up to 44 peaks in a single GLC chromatogram. An example of the complexity of the chromatograms is illustrated in Figure 51, which is a GLC analysis of an accumulating decasaccharide in the urine of fucosidosis patients (214). The decasaccharide contains fucose, mannose,

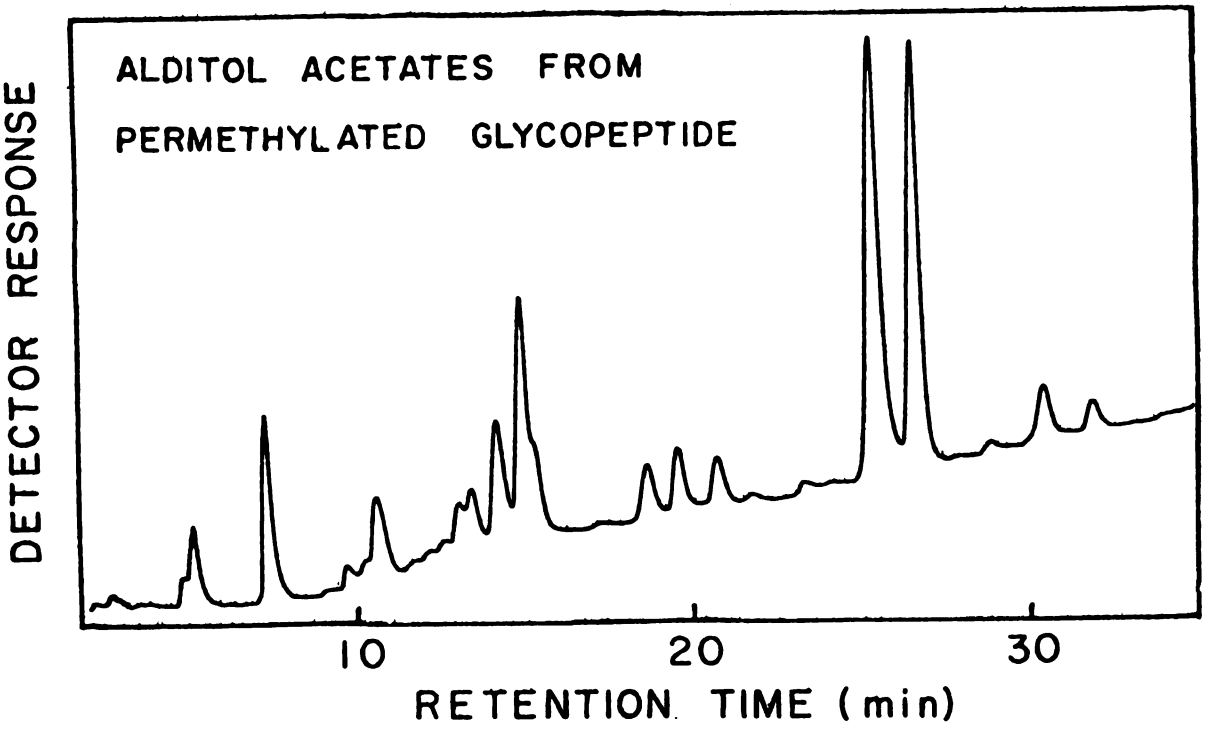


Figure 52. Gas-Liquid Chromatography of the Partially Methylated Alditol Acetate Derived from the Purified Fucodecasaccharide A from the Urine of Fucosidosis Patients.

The sugar derivatives was analysed on a 2 mm x 2 m column with 3% OV-210 on Chromosorb Q (100-120 mesh) at 125^{0} to 250^{0} programmed at 2^{0}/min with nitrogen at a flow rate of 45 ml/min.

galactose and N-acetylglucosamine.

Identification and quantitation of the individual peaks can be very difficult because of the inability of liquid phases to completely resolve the individual partially methylated alditol acetates. Single scans of the peaks sometimes give mass spectra of mixtures, which are very confusing and may lead to erroneous interpretations. This problem was resolved by taking repeated mass spectral scans of the GLC eluant at intervals of about 4 seconds and storing the data in a PDP/8I computer. When the GLC run was completed, the data were displayed by monitoring specific ions characteristic of different alditol acetates, usually at m/e 45, 59, 71, 87, 89, 99, 101, 113, 115, 116, 117, 127, 129, 131, 139, 143, 145, 161, 175, 189, 203, 205, 233, and 261. The powerful resolution of this method can be illustrated by Figure 53, where ions at m/e 189 and 117 were monitored along with the total ion intensity, which approximates the detector response of GLC analysis. The ion at m/e 189 is characteristic of 1,2-substituted hexopyranosides while the ion at m/e 117 monitors 1-substituted, 2-unsubstituted hexopyranosides. By comparing the two mass chromatograms, it can be seen that the peaks at scans 112 and 117, indicated by arrows, have an ion at m/e 189, but have little intensity at m/e 117, while an extra peak at scan 114 can be found in the chromatogram for m/e 117. The peak at scan numbers 112 and 114 were not resolved in the total ion intensity chromatogram and exist as a broad peak.

The ratio of each of the peaks can be quantitated by measuring the areas of the peaks for a specific ion and multiplying each peak by a factor which is determined by the amount of that specific ion formed from a particular alditol acetate.

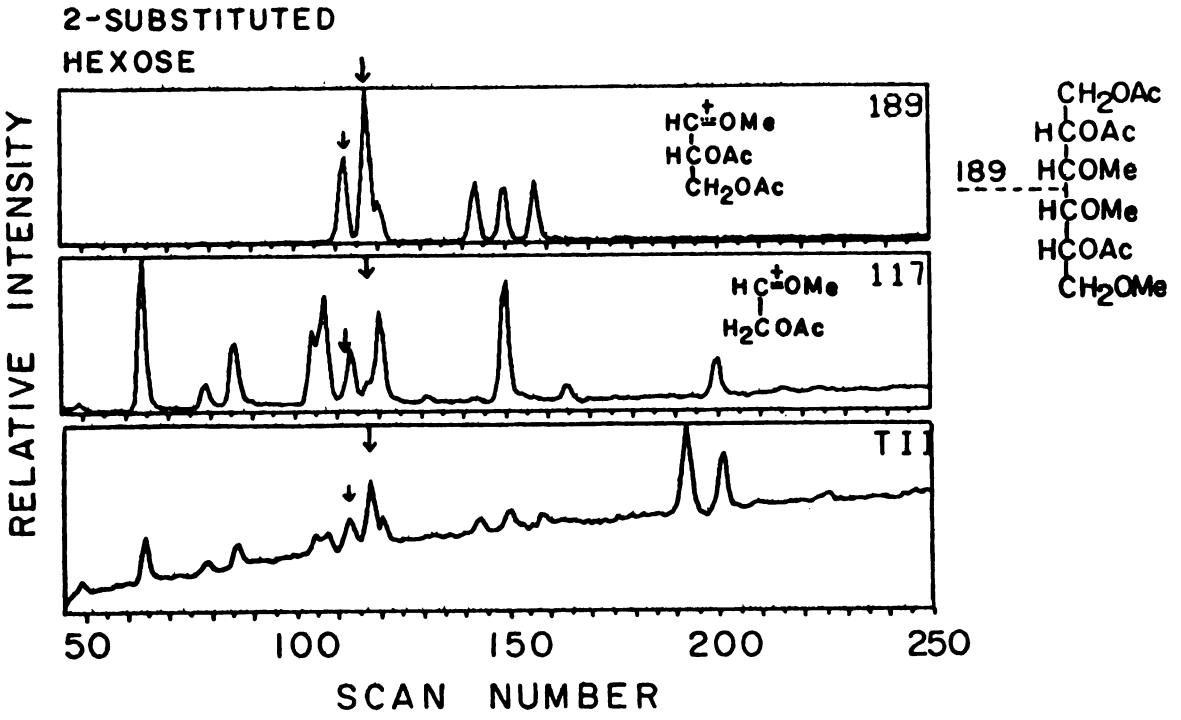


Figure 53. Mass Chromatograms of the Partially Methylated Alditol Acetates of Decasaccharide A from the Urine of Fucosidosis Patients.

The mass spectra of eluants of the GLC run of Figure 50 were taken at 5 second intervals and displayed for relative ion intensities of m/e 189 and 117. The lower scan is a chromatogram of the relative intensities of the total ion in each mass spectrum.

The most commonly used liquid phases for the GLC analysis of partially methylated alditol acetates is ECNSS-M (195). This liquid phase is not stable at temperatures above 200°C and various alternative polar liquid phases have been introduced. They are 3% OV-210, 3% OV-225, 3% OV-17 and EGS/EGA/XE-60 (0.2/0.2/1.4%). Analysis on OV-225 has been found to give a low yield of partially methylated aminosugars and is therefore not useful for general oligosaccharide analysis. We favour the use of OV-210 because this liquid phase gives a reasonable resolution of the aoditol acetates, is thermostable up to 275°C, does not absorb aminosugars, and can be used for general analysis without a change of column. For higher resolution of disubstituted hexopyranosides a polar liquid phase mixture consisting of 0.2% EGS/0.2% EGA/1.4% XE-60 has also been used with success (2). OV-17 has been utilized primarily for the analysis of partially methylated alditol acetates of amino sugars (218).

Three methods can be used to determined anomeric configuration. Stereospecific enzymatic analysis is the most useful because precise anomerity and sequence assignments can be obtained with microgram amounts of material. Purification of specific glycosidases for these investigations can be very tedious, however. Anomerities can also be determined by CrO_3 oxidation (219) or proton magnetic resonance (213). These two methods are more appropriate for simple oligosaccharides because only ratios of α and β sugar residues can be obtained for one species of monosaccharide present in the compound while the exact location of the α- or β- linkages cannot be determined. The proton magnetic resonance method suffers further from the disadvantage that mg amounts of material are required for the determination.

Purification of α-N-Acetylgalactosaminidase

A major difficulty in the purification of α-N-acetylgalactosaminidase from porcine liver lies in the lability of this enzyme at low protein concentrations. Losses of 60-80%/day at 4^{o} is not uncommon at protein concentrations below 0.1 mg/ml (9). The strategy in the purification scheme was to employ large-scale preparations in such a way that protein concentrations could be maintained above 0.1 mg/ml, even when highly purified. Steps that would result in low protein concentrations were carried out quickly and the enzyme activity was pooled and concentrated immediately after the fractions were assayed. Extensive efforts by Weissmann and Hinrichsen (9) to purifiy the enzyme further with conventional methods failed to yield a preparation with a specific activity greater than 1150 mU/mg of protein. To refine their purification sheme further, the use of affinity chromatography seemed to be a plausible solution. The problem of finding an appropriate adsorbent for coupling to a chromatographic matrix was solved when the disaccharide GalNAc-(α1→6)-Gal was synthesized by the reduction and N-acetylation of the coupled product of the nitrosyl chloride adduct of galactal with 1,2:3,4-di-isopropylidene-galactose (Figure 3). When this disaccharide was coupled to dextran-coated glass beads, α-N-acetylgalactosaminidase bound to the affinity adsorbent with such tenacity that the enzyme activity could not be eluted with 0.1% Triton X-100, 0.2 M potassium borate buffer, pH 10.0, or 50 mM GalNAc in 0.05 M potassium phosphate buffer, pH 7.0. Using a pH gradient of 0.1 M disodium citrate and 0.1 N hydrochloric acid, the activity was finally eluted at pH 3.2. At this low pH the enzyme was rapidly inactivated and the method was consequently discarded.

Nonspecific affinity chromatography on Con A-Sepharose, however, proved to be very useful in the purification of α-N-acetylgalactosaminidase. The enzyme preparation from DEAE-cellulose was purified 30-fold by this step with about 50% recovery. The discovery by Leaback and Robinson (220) that β-hexosaminidase A could be separated into α- and β- forms by elution of the enzyme from CM-cellulose by ampholytes prompted the use of a similar ampholine elution procedure to purify α-N-acetylgalactosaminidase, and the result was a further increase in specific activity of the enzyme preparation by two-fold. The powerful resolution of hydroxylapatite column chromatography was illustrated by the work Tulsiani, Keller and Touster (221). A chromatographically and electrophoretically homogeneous β-glucuronidase preparation was separated into eight enzymatically active peaks by this procedure. Indeed when this method was applied to the purification of α-N-acetylgalactosaminidase, various shoulder similar to those found on the profile of ampholine elution of α-N-acetylgalactosaminidase from DEAE-cellulose (Figure 13) was observed (not shown in data), and the preparation from this column was almost electrophoretically homogeneous.

The previous purification of α-N-acetylgalactosaminidase from porcine liver was not successful in removing the contaminating β-N-acetylgalactosaminidase activities. This would cause errors in the interpretation of results if the enzyme preparation were used in the study of anomerities of N-acetylgalactosamine residues on oligosaccharides. The enzyme preparation in this work did not contain any detectible β-N-acetylgalactosaminidase activity and is therefore more appropriate for structural work. Further improvement in the purification

scheme to remove the last traces of impurity is necessary, but it also risks the danger of further diluting the enzyme and resulting in inactivating the enzyme activity and subsequent decrease in the specific activity of the enzyme preparation.

Phenylmethylsulphonyl fluoride was introduced into the purification scheme to eliminate the possibility of forming artifactual isozyme forms by the action of proteases. With the use of this protease inhibitor, the three most acidic forms were attenuated in size but never eliminated, indicating that protease action may not be the sole source of the multiple isoelectric focussing forms. The acid precipitation step was performed in as short an interval as possible so that the possibility degradation by the lysosomal enzymes as well as inactivation or acid hydrolysis can be reduced.

Characterization of the Kinetic Properties of α-N-Acetylgalactosaminidase

In this work, α-N-acetylgalactosaminidase from pig liver was purified to near homogeneity so that more insight could be obtained into the kinetic and structural aspects, substrate specificity and mode of action of the enzyme on glycolipid substrates. This enzyme preparation was able to act as an exo-α-N-acetylgalactosaminidase on all the potential substrates examined, namely pNP-α-N-acetyl-D-galactosaminide, Forssman hapten, N-acetyl-sphingosyl-Forssman pentasaccharide, Forssman pentasaccharide, human blood group A glycolipids, dog blood group A glycolipids, porcine submaxillary mucin, and N-acetylgalactosamine-(α1→6)-1,2:3,4:-di-O-isopropylidene-galactose. The kinetic parameters were examined except for cases where availability

of the potential substrate was the limiting factor.

The hydrolysis of a glycolipid substrate by porcine α-N-acetyl-galactosaminidase is best examined by comparing the hydrolysis of Forssman hapten, N-acetyl-sphingosyl-Forssman pentasaccharide and Forssman pentasaccharide. Forssman hapten has a large hydrophilic head group with five sugar residues and a forked hydrophobic tail group made up of the sphingosine and the fatty acid chains. No hydrolysis of this molecule by the enzyme preparation was found without the use of detergents. The same detergent requirement was also observed with N-acetyl-sphingosyl-Forssman pentasaccharide, which had the same head group but a tail group with only one prong made up of sphingosine while the other prong was replaced by a short acetyl group. The optimum taurocholate concentration was much sharper for the latter substrate, and this optimum fell approximately on the critical micellar concentration of taurocholate. When the hydrophobic tail was eliminated altogether by ozonolysis, detergent not only was not necessary for catalytic activity, but in fact it inhibited the hydrolysis of the Forssman hapten pentasaccharide. The comparison of V_{max} and K_M values indicated that this enzyme preparation had a higher affinity for the lipids than the oligosaccharides when taurocholate was present (Table 9). The results imply that the detergnet plays an important role in forming a mixed micelle with the Forssman hapten so that the substrate is in a dispersed state that is suitable for formation of an enzyme-substrate complex. At a concentration of 4 mg/ml taurocholate and 150 nmoles of substrate, the ratio of substrate to number of micelles (micellar weight = 2200 (222)) is approximately 1:2. There is an excess of pure micelles and thus at substrate concentrations below 1 mM

Table 9. Comparison of the Kinetic Parameters of the Hydrolysis of Different Substrates by α-N-Acetylgalactosaminidase.

Substrate	K_M	V_{max}	pH Optimum	Detergent
	(M)	(μmoles/min/mg)		(mg/ml)
GL-5	2.6×10^{-4}	4.2	3.9	4
GL-5 Pentasaccharide	1.0×10^{-2}	1.7	--	--
Acetyl-sphingosyl-GL-5-pentasaccharide	2.5×10^{-4}	0.81	--	4
p-Nitrophenyl-α-GalNAc	2.9×10^{-3}	14.1	4.5	--
GalNAc-(α1→6)-1,2:3,4-di-O-IP-Gal	4.8×10^{-3}	1.1	--	--
Porcine Submaxillary Mucin	2.3×10^{-6}	0.42	4.2	--

the enzyme activity obeys Michaelis-Menten kinetics if the enzyme activity is proportional to the mixed-micellar concentration (the micelles that contain both Forssman glycolipid and taurocholate molecule are mixed micelles) which in turn approximates the substrate concentration if the equilibrium between monomeric and micellar Forssman hapten favours the formation of the latter form. This is indeed the case for most lipids and detergents (222). The stimulation of taurocholate on Forssman hapten hydrolysis can hardly be explained by this simple micelle picture. The micellar weight is too small to be able to provide a surface on which the enzyme can adhere unless the mixed micelles form large globular micelle clusters of secondary micelles (223). The relatively sharp taurocholate optimum at 4-5 mg/ml for N-acetyl-sphingosyl-Forssman pentasaccharide is not understood. The possibility of appreciable activation of the enzyme by its direct interaction with the detergent can be ruled out by the inhibition of the hydrolysis of Forssman hapten oligosaccharide in the presence of taurocholate at 4 mg/ml. The gradual decrease in activity to hydrolyse Forssman hapten at taurocholate concentrations beyond 7 mg/ml probably represents increasing inactivation of the enzyme at the higher detergent concentrations. Substrate inhibition (224) similar to the action of sphingomyelinase on sphingomyelin in Triton X-100 has not been observed.

The action of this enzyme seems to be better for glycolipids in the presence of detergent than for hydrophilic substrates because it hydrolyses Forssman with a much smaller K_M.

The K_M for phenyl-α-N-acetylgalactosaminide (6.4 mM) compares favorably with the K_M of p-nitrophenyl-α-N-acetylgalactosaminide found

in this work (2.9 mM). Both artificial substrates are aromatic glycosides and their K_M values are in the same order of magnitude. The pH optimum for the hydrolysis of the former substrate was found to be at pH 4.3 as compared to pH 4.5 for the latter. This difference may have arisen from the charge present in the nitrophenyl group in the nitrophenyl glycoside.

It is interesting to note that α-N-acetylgalactosaminidase hydrolysed GL-5 pentasaccharide with a very high K_M, about 50-fold higher than the hydrolysis of GL-5 (Table 9). Both the pNP-α-GalNAc and GalNAc-(α1→6)-1,2:3,4-di-O-isopropylidene-galactose, which have more nonpolar groups adjacent to the N-acetylgalactosamine residue than the GL-5 pentasaccharide, were hydrolysed with lower K_M values. The hydrolyses of GL-5 and acetyl-sphingosyl-GL-5-pentasaccharide required the presence of detergents that would also be able to reduce the polarity of the environment near the active site. The high V_{max} of the hydrolysis of pNP-α-GalNAc may be due to the nature of the linkage of the carbohydrate to an aromatic ring, which is capable of stabilizing both a carbonium ion and a carbanion intermediate, thus perhaps reducing the activation energy of the reaction. The fact that p-nitrophenyl glycoside substrates degrade on storage, even at 0°, is evidence for the easier degradation of p-nitrophenyl-glycosides. In contrast, spontaneous hydrolysis of oligosaccharides is not usually observed unless the solutions are contaminated by bacteria or fungi.

The kinetic parameters for the hydrolysis of porcine submaxillary mucin are more difficult to evaluate. There are about 38 blood group A^+ pentasaccharide groups on each molecule of blood group A^+ mucin (73). There are also α-linked N-acetylgalactosamine residues linked

directly to the protein molecule that can also be hydrolysed by α-N-acetylgalactosaminidase. The K_M of the mucin must be increased at least 38-fold if all the pentasaccharide groups are accessible to the enzyme. It will be further decreased by the N-acetylgalactosamine residues directly linked to the protein. The pH optimum in the hydrolysis of the mucin showed a biphasic curve with increased hydrolysis activity below pH 3.7 (not shown in the pH optimum curve). This may be reflective of the hydrolysis of two types of N-acetylgalactosamine residues on the mucin molecule.

The competitive inhibition by N-acetyl-talosamine-(α1→6)-1,2:3,4-di-O-isopropylidene-galactose is unique in that N-acetyl-talosamine differs from N-acetylgalactosamine in the configuration at C-2 of the monosaccharide. It implies that the catalytic site of the enzyme cannot distinguish between the R or S configurations at C-2. It is surprising though, that galactose and galactosamine hydrochloride at 10 mM did not inhibit the hydrolysis of Forssman hapten. The presence of a bulky N-acetylgroup on the terminal sugar is probably a requirement for recognition by the enzyme.

The high yield of TalNAc-(α1→6)-di-O-isopropylidene-galactose in its synthesis and its inhibition properties make it potentially important as a ligand for affinity chromatography in the purification of α-N-acetylgalactosaminidase.

The presence of two enzymatically active protein peaks on native gel electrophoresis of the porcine α-N-acetylgalactosaminidase is probably due to aggregation of the enzyme, which chromatographed on Sephadex G-150 as a single symmetrical peak. The identity of the isoelectric focussing forms in migration on native gel electrophoresis

probably indicates that the difference in these two forms arises from minute differences in the protein molecule. It has been suggested that neuraminidase treatment changes the mobilities of α-galactosidases (132, 136). No sialic acid residue was found in this enzyme. The change in charge may be due to deamidation of glutamine, asparagine, 4-L-aspartylglycosylamine, or 5-L-glutamylglycosylamine, resulting in the loss of an amino group or an oligosaccharide and an amino group. This would shift the pI of the enzyme to a more acidic form. Indeed a glycosyl asparaginase has been purified from hog kidney (225).

Porcine α-N-acetylgalactosaminidase has been shown to be an exoglycosidase. Endoglycosidases are not uncommon. Endo-α-N-acetylgalactosaminidases were found in *Streptomyces griseus* (226), *Diplococcus pneumoniae* (227, 228) and *Clostridium perfrigens* (229). Endo-β-N-acetylglucosaminidase were demonstrated to be present in *Streptomyces griseus* (230-232), *Diplococcus pneumoniae* (232), and hen oviduct (233). Endo-β-galactosidases were purified from *Escherichia freundri* (234) and *Diplococcus pneumoniae* (235). These enzymes are very useful tools in liberating oligosaccharides from glycoproteins and glycopeptides (215, 236, 237). No mammalian endoglycosidase has yet been characterized.

The natural substrates of the pig liver α-N-acetylgalactosaminidase are probably the blood group A active glycolipids, which have been shown to be present in porcine gastric mucosa and porcine mucins (Table 1). The kinetic parameters of hydrolysis of the blood group A substances were not examined because of the lack of material. Under the conditions used for the hydrolysis of Forssman hapten, the enzyme did hydrolyse the blood group A-active glycosphingolipids to give

glycolipids of higher mobilities.

The Role of Carbohydrates on α-N-Acetylgalactosaminidase

The carbohydrate analysis of the enzyme indicated that the only sugars present were mannose and N-acetylglucosamine. This is different from hexosaminidase which also has sialic acid and presumably galactose (104).

As shown in Table 10, most glycopeptides have an inner core composed of mannose and N-acetylglucosamine while the periperal carbohydrates can be N-acetyl-neuraminic acid, galactose and fucose. The structure of the oligosaccharide residues in this α-N-acetylgalactosaminidase is probably similar to the inner core of the oligosaccharide of ovalbumin, which contains only mannose and N-acetylglucosamine. The role of carbohydrates on glycoproteins is not well understood except perhaps for suspected roles as cell surface receptors, freezing point depression, change in viscosity of solutions or hepatic recognition of non-N-acetylneuraminic acid-containing glycoproteins (238).

Carbohydrates play an important role in the metabolism of glycosidases in cell cultures as well as in animals. Bovine testicular β-galactosidase, which contains only mannose and N-acetylglucosamine, is rapidly assimilated by generalized gangliosidosis skin fibroblasts. Pretreatment of the enzyme with mannosidase reduced the rate of assimilation by 97% (239). Similarly, treatment of rat liver lysosomal β-glucuronidase, N-acetyl-β-glucosaminidase, α-L-fucosidase and α-D-mannosidase, as well as rat preputial gland β-glucuronidase, with sodium periodate resulted in a near abolition of rapid clearance of the enzymes from the circulation when injected into rats (240). The isolation of

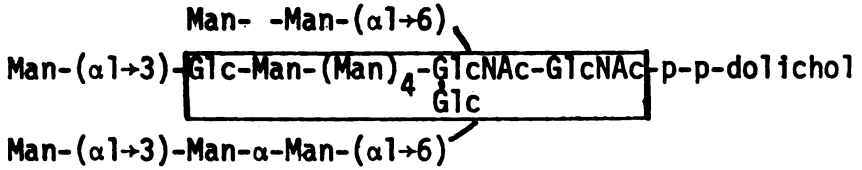
Table 10. Structures of Oligosaccharides in Glycoproteins

Glycoprotein	Source	Structure	Ref.
Serotransferrin	human	NeuAc-(α2→6)-Gal-(β1→4)-GlcNAc-(β1→2)-Man-(α1→3) NeuAc-(α2→6)-Gal-(β1→4)-GlcNAc-(β1→2)-Man-(α1→6)	216
Prothrombin	bovine	NeuAc-Gal-GlcNAc-β-Man NeuAc-Gal-GlcNAc-β-Man	242
Immunoglobulin M Glycopeptide V_1	human	NeuAc-(α2→6)-Gal-(β1→6)-GlcNAc-(β1→2)-Man-(α1→6) NeuAc-(α2→6)-Gal-(β1→6)-GlcNAc-(β1→2)-Man-(α1→3)	238
Ovalbumin Glycopeptide V	chicken	Mam-(α1→6) Man-(α1→3)	215
Glycopeptide IV		Man-(α1→6) Man-(α1→3) Man-(α1→2)-Man-(α1→3)	

Table 10. (cont'd).

Glycoprotein	Source	Structure	Ref.
Liver G_{M1} Gangliosidosis type I oligosaccharide	human	Gal-(β1→4)-GlcNAc-(β1→2)-Man-(α1→6) Man-(β1→4)-GlcNAc Gal-(β1→4)-GlcNAc-(β1→2)-Man-(α1→3)	213
Urine Fucosidosis oligosaccharide	human	Fuc-(α1→2)-Gal-(β1→4)-GlcNAc-(β1→2)-Man-(α1→3) Man-(β1→4)-GlcNAc Fuc-(α1→2)-Gal-(β1→4)-GlcNAc-(β1→2)-Man-(α1→6)	214
IgG	porcine	NeuAc-(α2→6)-Gal-(β1→4)-GlcNAc-(β1→2)-Man-(α1→3) Man-(β1→4)-Glc- GlcNAc-(β1→2)-Man-(α1→6) NAc[-(3←1α)-Fuc]- (β1→4)-GlcNAc-β-Asn	243
Hepatic Binding Protein Glycopeptide I	rabbit	NeuAc-(α2→?)-Gal-(β1→?)-GlcNAc-(β1→2) NeuAc-(α2→?)-Gal-(β1→?)-GlcNAc-(β1→?)-Man-(α1→3) Man-(β1→4)-GlcNAc- NeuAc-(α2→3)-Gal-(β1→?)-GlcNAc-(β1→4) (β1→?)-GlcNAc-Asn	214
Glycopeptide II		$(Man)_{6-7}$-α-Man-(β1→?)-GlcNAc-(β1→?)-GlcNAc-Asn	
Glycoasparagines Aspartylglycosylaminuria urine I	human	NeuAc-(α2→3)-Gal-(β1→4)-GlcNAc-β-Asn	245
II		NeuAc-(α2→?)-Gal-(β1→?)-GlcNAc-(β1→?)-Gal-(β1→4)-GlcNAc-β-Asn	

Table 10. (cont'd).

Glycoprotein	Source	Structure	Ref.
Thyroid Oligosaccharide lipid	calf		246

carbohydrate-binding proteins from specific organs such as the β-galactoside binding lectin of calf heart and lung (241) seems to imply that the carbohydrates on proteins play an important role in being recognized by specific organs in animals.

SUMMARY

The structure of a glycosphingolipid with Forssman reactivity was characterized by the use of GLC analyses of the carbohydrate composition, permethylation to determine the linkages and stereospecific enzymes to determine the sequence of the carbohydrate residues and their anomeric configurations.

In the course of the purification of α-N-acetylgalactosaminidase from porcine liver, methods like Con A-Sepharose, ampholine elution of enzyme activities from DEAE-cellulose, and hydroxylapatite were found to be very useful. The enzyme is difficult to purify because it is very unstable when present in dilute concentrations. A large-scale purification has to be performed so that even when highly purified, the enzyme can still be dissolved in a volume that can be handled and yet is not inactivate due to high dilution.

When the enzyme has been purified, it appears to consist of two subunits of 52,000 daltons each. Only mannose and glucosamine are present in the oligosaccharide residues. The enzyme is not heat-stable and is rapidly inactivated at temperatures above 60°.

The substrate concentrations in many of the assays were not saturating because of the availability of substrate. Even pNP-α-N-acetylgalactosaminide was in short supply because the manufacturer discontinued its production for a long period. One of the reasons was the difficulty in synthesizing α-linked amino sugar derivatives. The

α-anomerity was hindered because of the hinderance of the bulky N-acetyl group present on the molecule. The failure in synthesis of an affinity adsorbant was also related to the difficulty in synthesing the disaccharide. The difficulty was increased because of the relatively high cost of N-acetylgalactosamine.

Many of the assays in this work are tedious and time consuming and thus some of the kinetic aspects of the enzyme toward certain substrates were not exhaustive evaluations. The work did shed some light on the efficiencies of the enzyme to hydrolyse different substrates by finding the K_M and V_{max} of the enzyme with these substrates. In general, the enzyme seemed to hydrolyse the lipid substrates with a higher affinity. The pNP substrate was hydrolysed with the highest V_{max}.

This enzyme should be a useful tool in studying the lipid storage diseases. The presence of the antibody against this enzyme or the inhibition N-acetyltalosamine-(α1→6)-Gal in cell culture should lead to induction of the accumulation of blood group A^+ glycosphingolipids. This would facilitate the study of the metabolism of blood group glycolipids by making them more readily available.

Previous preparations of this same enzyme were always contaminated with β-N-acetylgalactosaminidase. The purification procedure in this work eliminated all the detectible β-N-acetylgalactosaminidase activities. This will be very useful for the unambiguous assignment of anomerities in oligosaccharides.

This enzyme can also be used in the treatment of cell surfaces and thus altering the antigenicity of the cells (e.g. A^+ RBC can be treated to become an A^- RBC). This may be one of the ways to elucidate the function of blood group substances.

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