1989

A Spectrophotometric Assay For Sanfilippo Syndrome, Type D (N-Acetylglucosamine 6-Sulfate Sulfatase Deficiency) With Prenatal Diagnostic Potential.

Rodney William Nowakowski
University of Alabama at Birmingham

Order Number 9003845

A spectrophotometric assay for Sanfilippo syndrome, type D (N-acetylglucosamine 6-sulfate sulfatase deficiency) with prenatal diagnostic potential

Nowakowski, Rodney William, Ph.D.

University of Alabama at Birmingham, 1989

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

A SPECTROPHOTOMETRIC ASSAY FOR SANFILIPPO SYNDROME, TYPE D (N-Acetylglucosamine 6-Sulfate Sulfatase Deficiency) WITH PRENATAL DIAGNOSTIC POTENTIAL

by

ROD W. NOWAKOWSKI

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Genetics in the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1989

ABSTRACT OF DISSERTATION

GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Doctor of Philosophy Major Subject Medical Genetics

Name of Candidate Rodney W. Nowakowski

Title A Spectrophotometric Assay for Sanfilippo Syndrome, Type D (N-Acetylglucosamine 6-Sulfate Sulfatase Deficiency), With Prenatal Diagnostic Potential

Sanfilippo syndrome, type D, is characterized by moderate physical abnormalities, progressive mental deterioration and deficient activity of N-acetylglucosamine 6-sulfate sulfatase, a lysosomal hydrolase involved in the degradation of heparin, keratan sulfate, and heparan sulfate. To date, demonstration of the enzyme deficiency typically relies on a radiolabeled trisaccharide substrate derived from heparan sulfate. The present study presents a spectrophotometric assay using the monosaccharide substrate, N-acetylglucosamine 6-sulfate. The reaction mixture was incubated for 6 h at 37 °C and, after Dowex chromatography, released N-acetylglucosamine was determined by a modification of the method of Reissig. Assay conditions were optimized for cultured skin fibroblasts and primary cultures of amniotic fluid cells. The pH optimum for each was 5.5. The assay was linear for 24 h and up to 0.1 absorbance unit. Specific activity for 11 normal cultured skin fibroblast cell lines was 7.7 (±4.1) μg N-acetylglucosamine released/h/mg protein. Specific activities for 3 cultured skin fibroblast cell lines from patients with Sanfilippo syndrome, type D, were 2.6%, 0%, and 7.8% of the normal mean and were therefore clearly distinguished from the normal controls. The specific

activity of N-acetylglucosamine 6-sulfate sulfatase in 122 chromosomally normal primary cultures of amniotic fluid cells was 6.2 (±2.6) μg N-acetylglucosamine released/h/mg. Selected characteristics of N-acetylglucosamine 6-sulfate sulfatase in primary cultures of amniotic fluid cells were determined. Sodium sulfate caused 95% inhibition and 100% inhibition when present in 10 mM and 30 mM concentrations, respectively, but a 40 mM sodium chloride concentration gave only 13% inhibition. N-acetylglucosamine 6-sulfate sulfatase was relatively stable at 55 °C after an initial reduction of approximately 35% during the first 60 min; however, at 70 °C, specific activity was reduced by 70% and 100% after 30 and 90 min, respectively. The Km and Vmax were 24.0 mM and 20.7 μg N-acetylglucosamine released/h/mg protein, respectively.

An enzyme deficiency in primary cultures of amniotic fluid cells, of the same magnitude as found in the cultured skin fibroblasts of known patients, would be detectable and, therefore, prenatal diagnosis by this new method is feasible.

Abstract Approved by: Committee Chairman Jerry L. Thompson

Program Director Wayne H. Finley

Date 5/12/89 Dean of Graduate School

DEDICATION

To my wife Deborrah, with love.

ACKNOWLEDGMENTS

I gratefully acknowledge the assistance and many contributions made by the members of my graduate committee, Dr. Larry DeLucas, Dr. Wayne Finley, Dr. Ken Taylor, Dr. Graeme Wilson, and especially Dr. Jerry Thompson, committee chairman, who gave freely of his time and energy. Thanks are due also to Dr. Gary Cutter for assistance in the statistical analysis of data, Dr. Rama Krishna for analysis of samples by nuclear magnetic resonance spectroscopy, Dr. Lennart Rodén for many helpful suggestions, and finally, to Dr. Henry B. Peters who made it all possible.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued) Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued) Page

LIST OF ABBREVIATIONS

AFC(s)
: amniotic fluid cell(s)

cDNA
: copy deoxyribonucleic acid

Gal(6S)
: galactose 6-sulfate

GalNAc(6S)
: N-acetylgalactosamine 6-sulfate

Glc(6S)
: glucose 6-sulfate

GlcNAc
: N-acetylglucosamine

GlcNAc(6S)
: N-acetylglucosamine 6-sulfate

GlcNAc 6-sulfatase
: N-acetylglucosamine 6-sulfate sulfatase

GlcNS(6S)-[6-^{3}H]Id
: O-(2-sulfamino-2-deoxy-α-D-glucopyranosyl 6-sulfate)-(1→4)-L-[6-^{3}H]idose

GlcNS(6S)-[6-^{3}H]Id(2S)
: O-(2-sulfamino-2-deoxy-α-D-glucopyranosyl 6-sulfate)-(1→4)-L-[6-^{3}H]idose 2-sulfate

GlcNAc(6S)-[6-^{3}H]Id(2S)
: O-(2-acetamido-2-deoxy-α-D-glucopyranosyl 6-sulfate)-(1→4)-L-[6-^{3}H]idose 2-sulfate

[1-^{14}C]GlcNAc(6S)
: N-acetylglucosamine[1-^{14}C] 6-sulfate

GlcNAc(6S)-UA-[1-^{3}H]aMan-ol
O-(α-D-6-sulfo-2-acetamido-2-deoxyglucosyl)-(1→4)-O-uronosyl(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol

GlcNAc(6S)-IdUA(2S)-[1-^{3}H]aMan-ol(6S)
O-(α-D-6-sulfo-2-acetamido-2-deoxyglucosyl)-(1→4)-(α-L-iduronic acid 2-sulfate)-2,5-anhydro-D-[1-^{3}H]mannitol

GlcNAc(6S)-[1-^{3}H]Gal-ol
O-(β-D-6-sulfo-2-acetamido-2-deoxyglucosyl)-(1→3)-D-[1-^{3}H]galactitol

GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-[1-^{3}H]Gal-ol
O-(β-D-6-sulfo-2-acetamido-2-deoxyglucosyl)-(1→3)-O-(β-D-6-sulfogalactosyl)-(1→4)-O-(β-D-6-sulfo-2-acetamido-2-deoxyglucosyl)-(1→3)-D-[1-^{3}H]galactitol

MPS III
mucopolysaccharidosis, type III (Sanfilippo Syndrome)

MPS IIID
mucopolysaccharidosis, type IIID (Sanfilippo Syndrome, Type D)

MSD
multiple sulfatase deficiency

CHAPTER I.

INTRODUCTION

Sanfilippo Syndrome

The Sanfilippo syndrome (R.C. Harris, 1961; Sanfilippo, Podosin, Langer, & Good, 1963) is a lysosomal storage disease and has been designated as mucopolysaccharidosis III (MPS III). The four types of the Sanfilippo syndrome (A, B, C, and D) have a clinical presentation characterized by physical changes similar to, but milder than, other mucopolysaccharidoses, severe mental deterioration that is progressive, and increased urinary excretion of partially degraded heparan sulfate (McKusick & Neufeld, 1983). Although the four types cannot be distinguished on clinical grounds alone (Haust & Gordon, 1986), each is biochemically distinct, having deficient activity of a different lysosomal enzyme involved in the degradation of heparan sulfate (Table 1). The reaction catalyzed by each of the enzymes in Table 1 is shown in Figure 1.

Table 1

Sanfilippo Syndrome Subtypes

Type	Deficient Enzyme Activity	Enzyme Classification
A	Heparin sulfamidase	EC 3.10.1.1
B	N-acetyl-α-D-glucosaminidase	EC 3.2.1.50
C	Acetyl CoA: α-glucosaminide N-acetyltranferase	EC 2.3.1.3
D	N-acetylglucosamine 6-sulfate sulfatase	EC 3.1.6.14

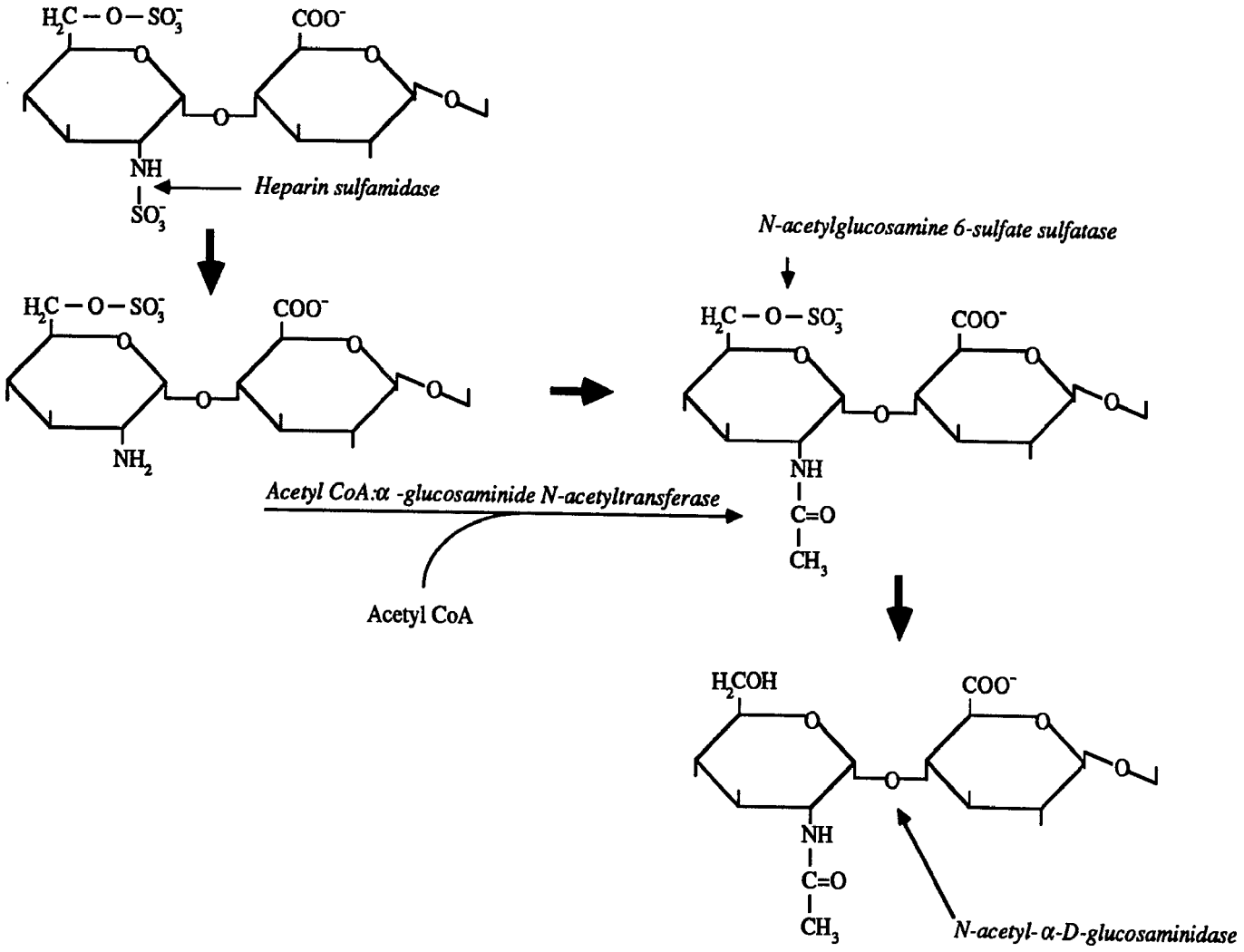


Figure 1. Reactions normally catalyzed by enzymes deficient in the Sanfilippo subtypes

Each type of the Sanfilippo syndrome is inherited as an autosomal recessive trait and considerable intratype and intertype variability have been found in the A, B, and C types (Van de Camp, Niermeijer, von Figura, & Giesberts, 1981). Van de Camp (1979) estimated the combined frequency of all types to be 1 in 24,000 in the Netherlands. Although this is considerably higher than estimates of other mucopolysacchridoses, it still may be an underestimate since the relatively mild physical changes could allow the condition to be diagnosed as simply mental retardation of nonspecific etiology (McKusick & Neufeld, 1983). Haust and Gordon (1986) compared several pooled series of MPS III patients classified on the basis of enzyme assays and found 68% type A, 22% type B, 9% type C, and only 1% type D. These frequencies may, however, reflect the finding that type A is more severe (Van de Camp et al., 1981) and that type D is less well known and may be recognized less often clinically.

The D Type of the Sanfilippo Syndrome

Sanfilippo syndrome, type D (MPS IIID), was first recognized when Kresse, Paschke, von Figura, Gilberg, and Fuchs (1980) reported two patients with clinical manifestations of the Sanfilippo syndrome who had heparan sulfaturia and who were unable to release sulfate from N-acetylglucosamine 6-sulfate (GlcNAc(6S)) residues in oligosaccharides derived from heparan sulfate. These patients did not exhibit keratansulfaturia even though GlcNAc(6S) residues in both heparan and keratan sulfate are desulfated by the same enzyme (Hopwood & Elliott, 1983a). The release of the β-linked GlcNAc(6S) residues from the nonreducing terminus of keratan sulfate, in spite of the deficient sulfatase, has been attributed to the action of β-N-acetylhexosaminidase A (Fuchs, Beck, & Kresse, 1985; Kresse, Fuchs, Glössl, Holtfrerich, & Gilberg, 1981). This, in part, accounts for the presence of sulfated N-acetylhexosamines found in the urine of MPS IIID patients (Hopwood & Elliott, 1983b). Fuchs et al. (1985) suggested that this finding alone might be sufficient for the diagnosis of MPS IIID but it was shown by

Hopwood and Elliott (1985) that patients with other MPS disorders also exhibit an elevated concentration of sulfated N-acetylhexosamines in their urine.

Coppa et al. (1983) reported two MPS IIID patients who showed clinical variability but were reluctant to attribute this to allelic mutations due to the small number of known patients and insufficient knowledge of the mutant enzyme. MPS IIID appears to be rare and, to date, only seven patients have been reported (Boustany, Openheimer, Johns, Horwitz, & Kolodny, 1988; Coppa et. al., 1983; Gatti et. al., 1982; Kaplan & Wolfe, 1987). The diagnosis was confirmed by enzyme assay for six of these patients and selected clinical and biochemical findings for these patients are summarized in Table 2. The diagnosis of MPS IIID is confirmed by the demonstration of a deficiency of N-acetylglucosamine 6-sulfate sulfatase (GlcNAc 6-sulfatase) in cultured skin fibroblasts or leukocytes. Enzyme characteristics in amniotic fluid cells and prenatal diagnosis have not been reported.

N-Acetylglucosamine 6-Sulfate Sulfatase

Early investigators (Dodgson & Lloyd, 1961) were unable to demonstrate 6-O-sulfatase activity in mammalian tissues toward the monosaccharide substrates GlcNAc(6S), GalNAc(6S), Glc(6S), and Gal(6S) and concluded that such enzymes were only present in relatively simple organisms. This conclusion was subsequently shown to be incorrect. The radiolabeled monosaccharide [1-^{14}C]GlcNAc(6S) has been used for the diagnosis of MPS IIID (Elliott & Hopwood, 1984) but has not found general usage perhaps because (1) the reported rate of desulfation was greater for a trisaccharide substrate (GlcNAc(6S)-IdUA(2S)-[1-^{3}H]aMan-ol(6S)) and because (2) free sulfate, an effective inhibitor of enzyme activity, was found to be difficult to remove after their preparation of GlcNAc(6S) by direct sulfation of N-acetylglucosamine (GlcNAc) with chlorosulfonic acid.

Table 2

Clinical Findings in Enzymatically Confirmed Sanfilippo D Patients

Patient #	1[a]	2[a]	3[b]	4[b]	5[c]	6[d]
Origin[e]	Italian	Italian	Italian	Italian	Italian	French-Irish
Consanguinity	No	Yes	No[f]	No[f]	No	Not reported
Sex	F	F	M	F	M	F
Age at report	$4^{6/12}$	$10^{6/12}$	$9^{8/12}$	$5^{4/12}$	9	5
First signs of neurological involvement	$3^{6/12}$	3	8	2	Not reported	3
Ocular involvement	No	Pigmentary retinal degeneration	No	No	No	Optic atrophy
Enzyme activity (% of normal mean)	6.1	6.1	4.6	4.4	0[g]	<3.4
Mild dysostosis multiplex	Yes	Yes	Yes	Yes	Yes	Yes
Mental retardation	Mild	Severe	Mild	Severe	Mild	Severe
Coarse facies/hirsutism and limitation of elbow extension	Yes	Yes	Yes	Yes	Yes	Yes
Hepatomegaly	Yes	Yes	Yes	No	No	Yes

[a] Gatti et al. (1982) [b] Coppa et al. (1983) [c] Kaplan & Wolfe (1987) [d] Boustany, Openheimer, Johns, Horwitz, & Kolodny, (1988)
[e] One patient in literature, not described clinically, is of East Indian origin [f] Reportedly non-consanguineous but from same small town [g] Affected brother with similar features at age $1^{3/12}$ but enzyme deficiency not confirmed

The substrate used typically for the diagnosis of deficient enzyme activity is the tritium-labeled trisaccharide, O-(α-D-6-sulfo-2-acetamido-2-deoxyglucosyl)-(1→4)-O-uronosyl-(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol, made from heparan sulfate through a lengthy sequence of steps (Kresse et al., 1980). Several disaccharide substrates also have been used to demonstrate GlcNAc 6-sulfatase activity (Table 3). These disaccharides and the other substrates mentioned above were all radiolabeled. There has been no report of a colorimetric or fluorometric assay to date.

Multiple sulfatase deficiency (MSD), another mucopolysaccharidosis, was first described by Austin, Armstrong, and Shearer (1965) as a variant of metachromatic leukodystrophy. As the name implies, MSD is characterized by deficient activity of multiple sulfatases. Patients with MSD have been shown to have deficient activity of GlcNAc 6-sulfatase (Basner et al., 1979; Horwitz, 1979), although one group (Yutaka, Okada, Kato, Inui, & Yabuuchi, 1981) noted normal levels in several cell lines from a single MSD patient. They attributed this finding to having grown the cells in medium at a higher than normal pH (7.4). This effect had been noted previously, in MSD cell lines cultured at high pH in MEM-HEPES buffered medium for another sulfatase, arylsulfatase A (Fluharty, Stevens, Davis, Shapiro, & Kihara, 1978). The cause of this pH effect is unknown. The differential diagnosis between MPS IIID and MSD requires the assay of additional sulfatases, and, as noted, cell lines should be cultured in a CO_2-HCO_3^- mediated buffer which keeps the pH at lower levels. GlcNAc 6-sulfatase has been purified from human urine (Basner, Kresse, & von Figura, 1979b) and human liver (Freeman & Hopwood, 1987). Enzyme properties found in these two studies are summarized in Table 4. The GlcNAc 6-sulfatase gene has been localized to chromosome 12q14 (Robertson, Callen, Baker, Morris, & Hopwood, 1988).

Table 3

N-Acetylglucosamine 6-Sulfate Sulfatase Substrates

Substrate	Source	Reference
GlcNS(6S)-[6-^{3}H]Id	Heparin	Hopwood & Elliott (1981)
GlcNS(6S)-[6-^{3}H]Id(2S)	Heparin	Hopwood & Elliott (1981)
GlcNAc(6S)-[6-^{3}H]Id(2S)	Heparin	Hopwood & Elliott (1981)
[1-^{14}C]GlcNAc(6S)	-	Elliott & Hopwood (1984) Hopwood & Elliott (1981)
GlcNAc(6S)-UA-[1-^{3}H]aMan-ol	Heparan Sulfate	Basner, Kresse, & von Figura (1979b) Glössl, Truppe, & Kresse (1979) Kresse, Pachke, von Figura, Gilberg, & Fuchs (1980)
GlcNAc(6S)-IdUA(2S)-[1-^{3}H]aMan-ol(6S)	Heparin	Elliott & Hopwood (1984)
GlcNAc(6S)-[1-^{3}H]Gal-ol	Keratan Sulfate	Kresse, Pachke, von Figura, Gilberg, & Fuchs (1980) Matalon, Wappner, Deanching, Brandt, & Horwitz (1982) Toma, Di Ferrnate, Tenni, & Di Ferrante (1981)
GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-[1-^{3}H]Gal-ol	Keratan Sulfate	Kresse, Pachke, von Figura, Gilberg, & Fuchs (1980)

Table 4

Sumary of Properties for N-Acetylglucosamine 6-Sulfate Sulfatase Isolated From Human Liver and Urine

	Molecular Weight (kD) GEL Chromatography	SDS PAGE	pI	% Total	pH Optimum	Km	Vmax	Substrate
Urine [a]	97	-	7.7	-	5.5	150 µM	-	Heparan Sulfate Trisaccharide [b]
Liver [c]	Four forms (A, B, C & D) were isolated							
A	75	78	>9.5	30	5	11.7 µM	105	Heparin disaccharide [d]
B	75	48, 32	>9.5	60	5	14.2 µM	60	"
C	94	48, 45, 32	5.8	7	5	11.1 µM	53	"
D	95	-	5.4	3	-	-	-	"

[a] Basner, Kresse, & von Figura (1979b) [b] GlcNAc(6S)-UA-[1-^{3}H]aMan-ol [c] Freeman & Hopwood (1987) [d] GlcNAc(6S)-IdoA

Colorimetric Detection of N-Acetylhexosamines

The Morgan-Elson reaction (Morgan & Elson, 1934) is a colorimetric reaction specific for N-acetylated hexosamines. An alkaline solution containing N-acetylated hexosamine is heated and a colored product forms after the addition of p-dimethylaminobenzaldehyde. Morgan and Elson (1934) proposed that heating N-acetylglucosamine in an alkaline environment caused the formation of an oxazole derivative and that this intermediate compound condensed with p-dimethylaminobenzaldehyde, in acid solution, to form the colored end product. Improvements in the conditions under which this reaction occurs were reported by Aminoff, Morgan, and Watkins (1952). Their method was used by Jeanloz and Trémége (1956) to study the effect of substitution at different positions. They used methylated derivatives of GlcNAc and GalNAc and found that substitution at position 4 abolished the reaction, substitution in position 3 enhanced it, while substitution in position 6 did not affect color development. The method of Aminoff was improved by Reissig, Strominger, and Leloir (1955) who optimized conditions for color enhancement, speed of color development, specificity, and reduction in the effect of interfering substances. This spectrophotometric assay is sensitive and highly specific for N-acetylated hexosamines. A modification of the method of Reissig et al. (1955) was used for the detection of the N-acetylhexosamines, GlcNAc, and GlcNAc(6S), in this study (Appendix A).

Purpose of the Study

The present study was undertaken to develop an effective diagnostic assay for MPS IIID using an inexpensive and easily prepared monosaccharide substrate with a spectrophotometric procedure for the detection of free GlcNAc, the reaction product, after desulfation by GlcNAc 6-sulfatase (Appendix B). By eliminating the use of radiolabeled materials and the laborious preparation of the tritium labeled trisaccharide substrate customarily used, the assay would be within the capabilities of many laboratories,

potentially making the diagnosis more readily available. Additional aspects of this study were to determine selected characteristics of GlcNAc 6-sulfatase in primary cultures of amniotic fluid cells toward the monosaccharide substrate and, by establishing the range of activities for a large number of primary cultures of amniotic fluid cells, to determine the potential applicability of the spectrophotometric assay to prenatal diagnosis. Preliminary results of these investigations have been reported (Nowakowski, Thompson & Taylor, 1988a).

Through application of the techniques used in this study, it also was possible to develop a screening procedure for MPS IIID and several other mucopolysaccharidoses having elevated urinary sulfated N-acetylhexosamines. This related study is presented in Appendix C.

CHAPTER II.

METHODS AND MATERIALS

Chemical Reagents

Reagents were purchased as follows: Sephadex G-10, Pharmacia Fine Chemicals (Uppsala, Sweden); N-acetylglucosamine, N-acetylglucosamine 6-sulfate, 3,3-dimethylglutaric acid, p-dimethylaminobenzaldehyde, and bicinchoninic protein assay kit, Sigma Chemical Company (St. Louis, Mo.); Dowex AG 1-X8, 200-400 mesh, chloride form, Bio Rad (Richmond, CA); potassium tetraborate, Fisher Scientific (Fair Lawn, NJ); chloroform and chlorosulfonic acid, Aldrich Chemical Company (Milwaukee, WI); pyridine, Triton X-100, Brij 35, and Tween 20, Pierce Chemical Company (Rockford, IL). All other chemicals were of reagent grade or best available grade.

Optimization and Standardization of the Reissig Method

The method of Reissig et al. (1955) was modified as follows and applied to the detection of the substrate GlcNAc(6S) during its preparation and verification, and to the detection of released GlcNAc in the enzyme assay described below. Two reagents were used: potassium tetraborate ($K_2B_4O_7 \cdot 4H_2O$), 0.6 M with respect to B_4O_7, adjusted to pH 9.5 with concentrated HCl and 1 gm of dimethylaminobenzaldehyde (DMAB) dissolved in 10 ml of glacial acetic acid containing 12.5% (V/V) of 10 N HCl. The stock DMAB reagent was diluted immediately prior to use with 9 volumes of glacial acetic acid to 1 volume of the stock solution. A 500 µl sample was heated in boiling water for 3 min after the addition of 100 µl of the potassium tetraborate reagent. The sample was then cooled for 3 min in a room temperature water bath followed by the addition of 3 ml of the diluted

DMAB reagent and incubation in a 37 °C water bath for 20 min. It was again cooled for 3 min in a room temperature water bath and the absorbance was measured in 1.0 cm quartz cuvettes at 585 nm with a Bausch & Lomb Spectronic 2000 spectrophotometer. For larger or smaller samples the reagents were scaled up or down proportionately. The assay was optimized for detection of GlcNAc with respect to the pH of the potassium tetraborate reagent after which the following assay characteristics were determined for GlcNAc: absorption spectrum, sensitivity, change in absorbance with respect to time, effect of NaCl, and change in absorbance with respect to concentration.

Substrate Preparation

GlcNAc(6S) was prepared by direct sulfation of GlcNAc with chlorosulfonic acid by modification of the methods of Lloyd (1960, 1962) and Ishihara et al. (1976). One gram of GlcNAc was dissolved in 33 ml of pyridine. This solution was cooled to 0 °C followed by the dropwise addition of 680 µl of chlorosulfonic acid in 3.3 ml of chloroform with constant stirring. The mixture was stirred continuously for 30 min at 0 °C and then for 1 h at room temperature after which it was neutralized with 5 M NaOH (approximately 5.5 ml). Following neutralization, an oily layer separated to the bottom. This layer was extracted with a glass pipette and diluted in water. Initially, free SO_4^{-2} was precipitated with $Ba(OH)_2$ and Ba^{+2} was then removed by precipitation with dry ice or CO_2 gas after which the supernatant was diluted to reduce conductivity and applied to a column (1.5 x 10 cm) of Dowex AG1-X8, 200-400 mesh, chloride form. The precipitation steps were subsequently eliminated by desalting over a column (2.0 x 125 cm) of Sephadex G-10 eluted with 5% ethanol in water. The elution of SO_4^{-2} and Cl- were followed by $Ba(OH)_2$ and $AgNO_3$ precipitation, respectively. The elution of N-acetylhexosamine was followed by the modified Reissig reaction. The peak fractions having a positive reaction were pooled, concentrated by rotary evaporation in a Büchi Model RE-111A rotary

evaporator, diluted to reduce conductivity and applied to a column (1.5 x 10 cm) of Dowex AG1-X8, 200-400 mesh, chloride form, washed with 100 ml H_2O and eluted stepwise with 100 ml each of, 0.0015 M, 0.2 M, and 0.5 M NaCl. Fractions eluted with 0.2 M NaCl were combined, rotary evaporated, and desalted over a column (1.0 x 180 cm) of Sephadex G-10 eluted with 5% ethanol in water. Several preparations of GlcNAc(6S) were combined, desalted a second time over a column (2.0 x 125 cm) of Sephadex G-10, characterized for content, and used as substrate for assay of GlcNAc 6-sulfatase activity. During the course of study, GlcNAc(6S) became available as a commercial preparation; however, it was available only intermittently and varied in physical appearance. The commercial preparation and the GlcNAc(6S) made by direct sulfation were compared as described in the next section.

Substrate Characterization

Thin layer chromatography was performed with glass or polyester backed silica gel plates (Sigma). The 2 solvent systems used were n-butanol: acetic acid: H_2O (50: 25: 25) and n-propanol: H_2O (88: 12). Plates were developed for 15 h and dried after which each lane was marked in 1 cm fractions. Each fraction was then scraped into 500 µl of H_2O in a 13 x 100 glass tube and assayed by the modified method of Reissig. Molecular size exclusion gel chromatography was performed in a column (1.0 x 180 cm) of Sephadex G-10 eluted with 5% ethanol. Anion exchange chromatography was accomplished with 1 ml of Dowex AG 1- X8, 200-400 mesh in disposable polyethylene filter columns (Fisher). After sample application, columns were washed with 5 ml of H_2O and eluted stepwise with 5 ml each of 0.01 M, 0.02 M, 0.05 M, 0.1 M, 0.2 M, 0.5 M, and 1.0 M NaCl. Samples for nuclear magnetic resonance (NMR) spectroscopy were prepared as 5 mM solutions in D_2O (99.8%), and analyses were performed with a Bruker WH-400 spectrometer.

Cell Culture

Cultured skin fibroblasts from 5 normal individuals and an additional 5 cell lines of normal skin fibroblasts, recovered from liquid nitrogen storage (Laboratory of Medical Genetics, University of Alabama at Birmingham, Birmingham, Alabama), were used as controls along with cell line GM 302A (NIGMS Human Genetic Mutant Cell Repository, Camden, New Jersey). Cultured skin fibroblast cell lines from 3 patients with Sanfilippo syndrome, type D, were available for study: GM 5093 (NIGMS Human Genetic Mutant Cell Repository, Camden, New Jersey), WG 1004 (The Repository for Mutant Human Cell Strains, Montreal, Quebec), and MGE-VG (provided by Dr. E. Kolodny, Waltham, Massachusetts). Other sulfatase deficient cell lines were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, New Jersey) as follows: GM 0615 (Hunter syndrome), GM 2849 (Maroteaux-Lamy syndrome), GM 1881 (Sanfilippo syndrome, Type A), GM 4681 (multiple sulfatase deficiency), and GM 3245 (multiple sulfatase deficiency). One additional cultured skin fibroblast cell line from an MPS patient was recovered from liquid nitrogen (Laboratory of Medical Genetics, University of Alabama at Birmingham, Birmingham, Alabama): MGE-BN (multiple sulfatase deficiency).

Cell cultures were maintained at 37 °C with 5% CO_2 in closed flasks (Corning Glass Works, Corning, New York) with McCoy's 5a medium (Gibco, Grand Island, New York) supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic combination (Penicillin 10,000 U/ml, streptomycin 10,000 mcg/ml) and gentamicin sulfate (Elkins-Sinn, Inc., Cherry Hill, New Jersey) 40 µg/ml. Cells were subcultured at confluency following trypsinization. For enzyme studies, cells were harvested 10-14 days after subculture in 0.15 M NaCl with disposable plastic scrapers (Costar Corp., Cambridge, Massachusetts) and maintained in an ice bath while sonicated 5 times for 5 sec each time to disrupt cell membranes. The sonicate was centrifuged at 9,000 x g for 30 min at 5 °C and the supernatant retained for enzyme assay. Proteins were measured with the bicinchoninic

acid protein assay kit using bovine serum albumin as a standard. Smith et al. (1985) first reported the use of a bicinchioninic acid reagent for the measurement of proteins and showed that the procedure was simpler than the traditionally used method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951) and offered several advantages including less susceptibility to interference from nonionic detergents as well as some buffer salts.

Primary cultures of amniotic fluid cells with normal karyotypes (Laboratory of Medical Genetics, University of Alabama at Birmingham, Birmingham, Alabama) were maintained in 25 cm^2 flasks in the same manner described above for skin fibroblasts. Cells were harvested after 3-4 weeks in culture without having been subcultured. They were prepared for enzyme assay, in the same manner as the skin fibroblasts, in a total volume of 200 µl for each 25 cm^2 flask.

A conjunctival biopsy taken from a donor eye (Alabama Lion's Eye Bank, Birmingham, Alabama) was maintained in culture and prepared for enzyme assay in the same manner as described above for the skin fibroblasts.

Optimization of the Enzyme Assay

The incubation mixture, in a 1.5 ml Eppendorf tube (Bio Rad), consisted of 100 µl of 100 mM dimethylglutarate (DMG) buffer, pH 5.5, 50 µl of 31 mM GlcNAc(6S) containing 100 µg of sodium azide (NaN_3), and 50 µl of fibroblast sonicate (30-60 µg protein) in 0.15 M NaCl at 37 °C. After 6 h, the reaction mixture was diluted with 300 µl H_2O and added to 1 ml of Dowex AG 1-X8, 200-400 mesh, chloride form. The void volume and three 500 µl fractions, eluted with 1.5 mM NaCl, were analyzed for released GlcNAc by the modified method of Reissig (Appendix A) and compared to a standard consisting of 10 µg of GlcNAc in 500 µl total volume, assayed simultaneously in duplicate.

The following parameters of the enzyme assay for both skin fibroblasts and primary cultures of amniotic fluid cells were determined: the pH optimum, absorbance with respect

to protein concentration, and absorbance with respect to time. Two cultured skin fibroblast cell lines from the normal controls were used to determine the effect of selected nonionic detergents on enzyme activity. The detergents, Brij 35, Triton X-100, and Tween 20 (Sigma), were adjusted to their respective critical micellar concentrations in samples from both cell lines, after harvesting the cells from culture flasks and prior to sonication. The specific activities for each cell line were compared for samples prepared with and without detergent.

The degree of variability inherent in the overall assay procedure was assessed by the determining the following:

1. The consistency of the modified Reissig method for measurement of 30 standards of GlcNAc (10 μg/500 μl);
2. The consistency of the protein assay for 6 measurements of each of 4 standard solutions of bovine serum albumin (10, 20, 40, and 60 μg/100 μl);
3. The capacity of the Dowex columns to retain GlcNAc(6S);
4. The recovery of GlcNAc from the Dowex column within the 1 ml elution volume used in the assay;
5. The total substrate accounted for in an assay in terms of released GlcNAc and unreacted substrate; and
6. The repeatability of the procedure for duplicate assays of each of 12 samples of cultured amniotic fluid cells.

N-Acetylglucosamine 6-Sulfate Sulfatase Activity in Selected Tissues

Cultured Skin Fibroblasts

Specific activities in cultured skin fibroblasts were determined for the 11 control cell lines, 3 MPS IIID cell lines, and 7 other sulfatase deficient cell lines under identical and optimized assay conditions. The activities of the controls and the MPS IIID patients were compared to determine the specificity of the assay for detecting GlcNAc 6-sulfatase

deficiency. The specific activities for the multiple sulfatase deficient cell lines were compared to determine the degree, if any, of GlcNAc 6-sulfatase deficiency revealed by this assay.

Cultured Conjunctival Cells

Cultured conjunctival cells were assayed simply to establish whether or not GlcNAc 6-sulfatase activity was present in this tissue source. This was of interest because of the potential applicability of a positive result to diagnosis and management of GlcNAc 6-sulfatase deficiency (see discussion).

Primary Cultures of Amniotic Fluid Cells

The specific activity of GlcNAc 6-sulfatase from 122 primary cultures of amniotic fluid cells was determined and analyzed statistically. These data were compared to theresults for cultured skin fibroblasts to determine the degree of similarity, or difference, and hence allow inference about the theoretical possibility of prenatal diagnosis.

N-Acetylglucosamine 6-Sulfate Sulfatase Characteristics in Primary Cultures of Amniotic Fluid Cells

Enzyme characteristics of GlcNAc 6-sulfatase from primary amniotic fluid cell cultures were determined for the effect of sequential freezing in liquid nitrogen, repeated sonication, heat inactivation at 55 and 70 °C, and inhibition by sodium sulfate and sodium chloride. An exhaustive degradation was carried out with enzyme from a pool of 12 primary cultures of amniotic fluid cells over a 10-day period with a second aliquot of enzyme introduced on day 7. The Km and Vmax for GlcNAc 6-sulfatase were determined by the method of Wilkinson (1961) adapted to an Excel™ spreadsheet on a Macintosh™ SE computer.

CHAPTER III.

RESULTS

Optimization and Standardization of the Reissig Method

Maximum absorbance for 500 µl samples containing 10 µg of GlcNAc occurred with the potassium tetraborate solution at pH 9.5 (Fig. 2), and the method was modified to take advantage of this fact. Parameters for the detection of 10 µg GlcNAc in a total volume of 500 µl H_2O were determined, and maximum absorbance was found to occur at 585 nm (Fig. 3) and decreased only 2.0% over a 25-min period (Fig. 4). Absorbance for increasing concentrations of GlcNAc was linear from 0 to 20 µg (Fig. 5). The method was extremely sensitive, and the absorbance at low concentrations of GlcNAc is shown in Figure 6. The absorbance was not affected by salt for GlcNAc standard solutions prepared in 1 M and 2 M NaCl.

Substrate Preparation and Characterization

The direct sulfation of GlcNAc by chlorosulfonic acid resulted in Reissig-positive material that separated as 2 major peaks on Dowex chromatography (Fig. 7). The material eluting in the first major peak was desalted and used for comparison with the commercially prepared GlcNAc(6S). Samples from each source co-migrated on TLC in both solvent systems and co-eluted from Sephadex G-10 (data not shown). Both samples eluted from 1.0 ml columns of Dowex AG 1-X8 with 0.2 M NaCl, and the NMR absorption spectra were identical (Fig. 8).

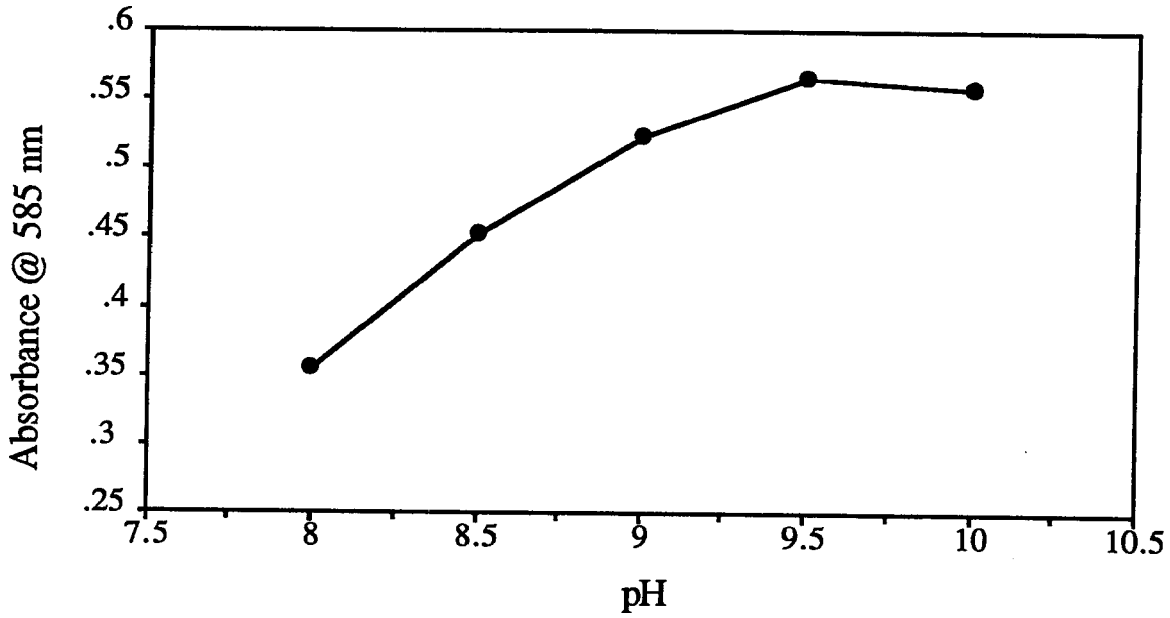


Figure 2. pH optimum for the potassium tetraborate reagent in the method of Reissig.

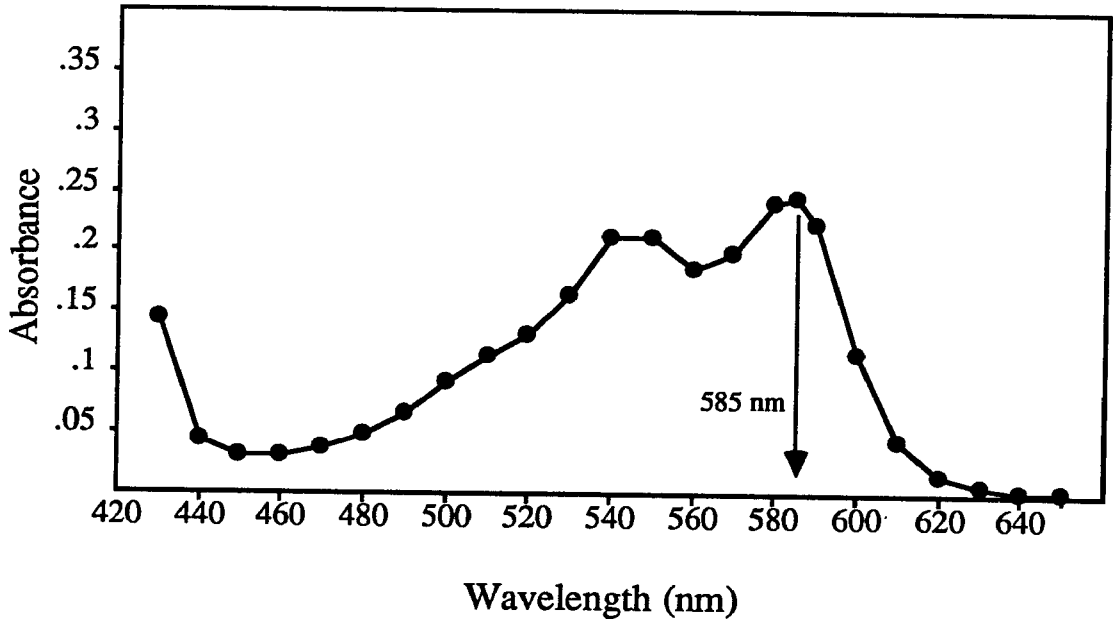


Figure 3. Absorption spectrum for GlcNAc assayed by the modified method of Reissig.

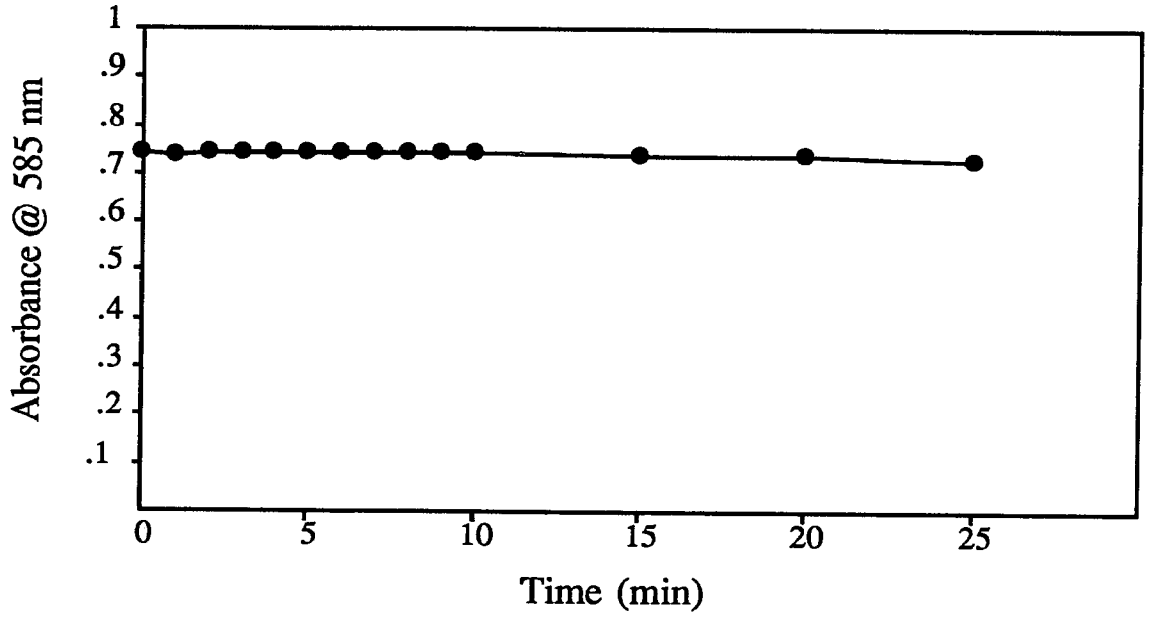


Figure 4. Change in color with respect to time for the modified method of Reissig.

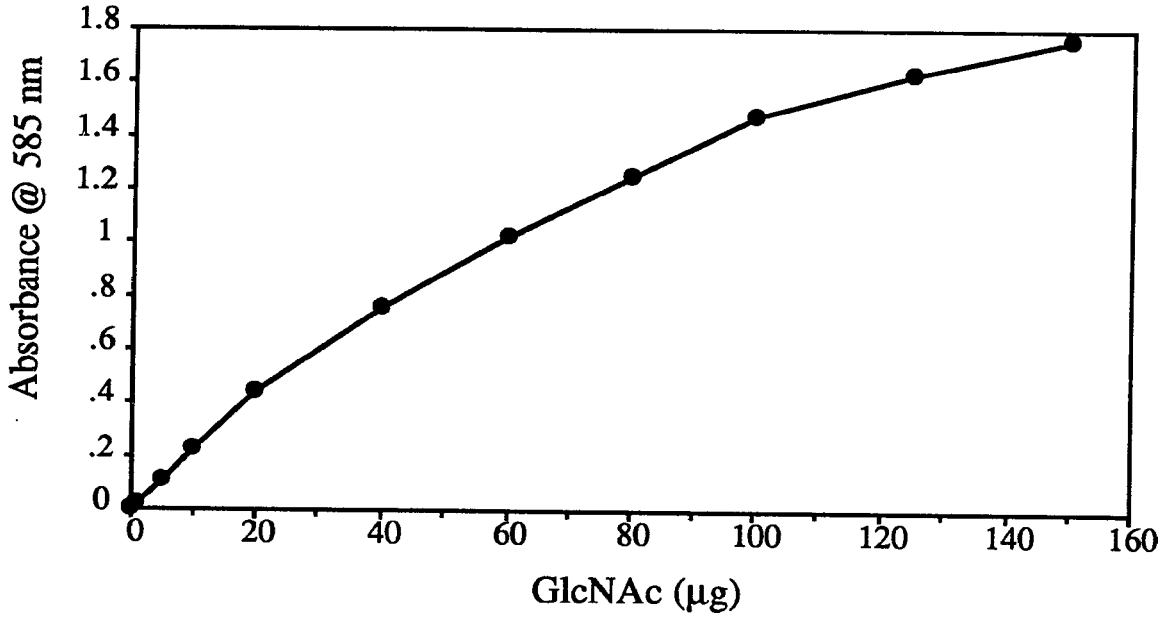


Figure 5. Change in absorbance with respect to the concentration of GlcNAc assayed by the modified method of Reissig.

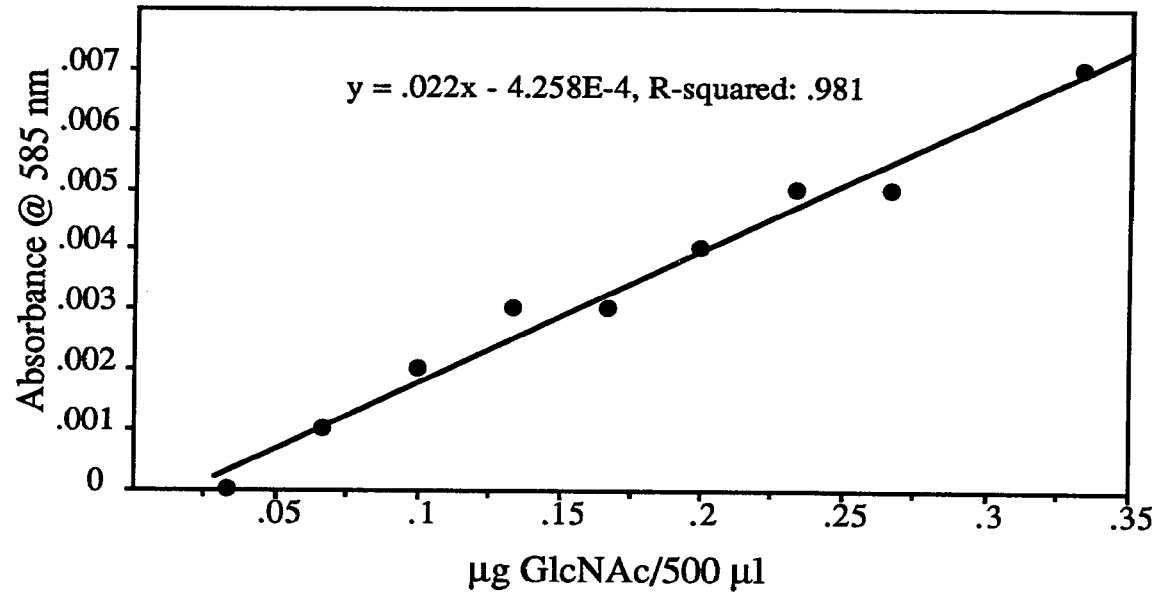


Figure 6. Sensitivity curve for low concentrations of GlcNAc assayed by the modified method of Reissig.

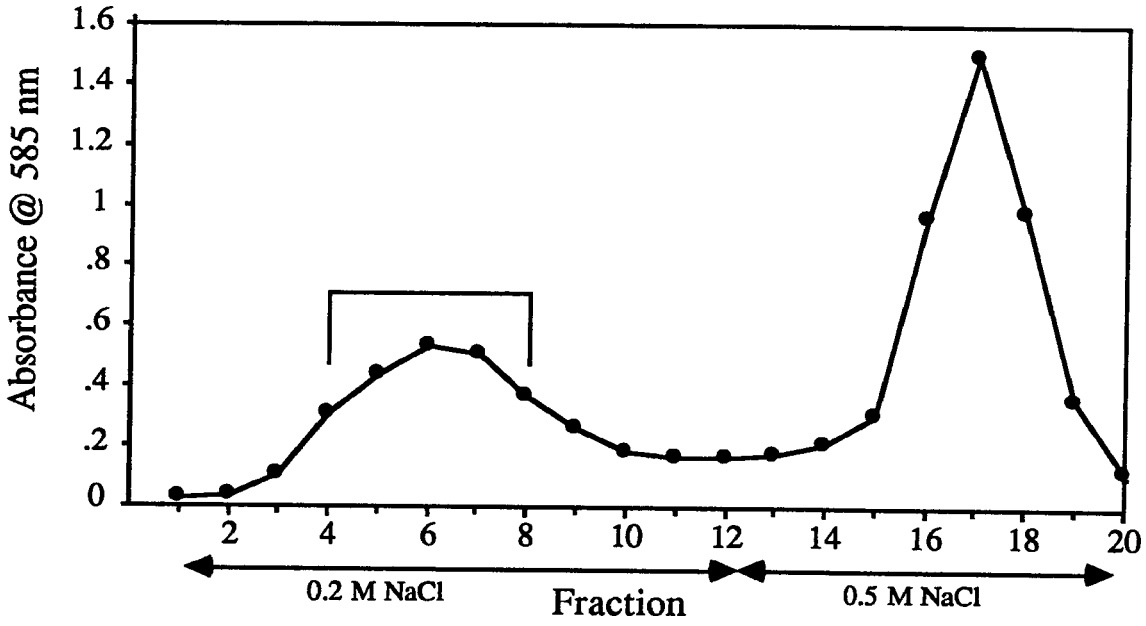
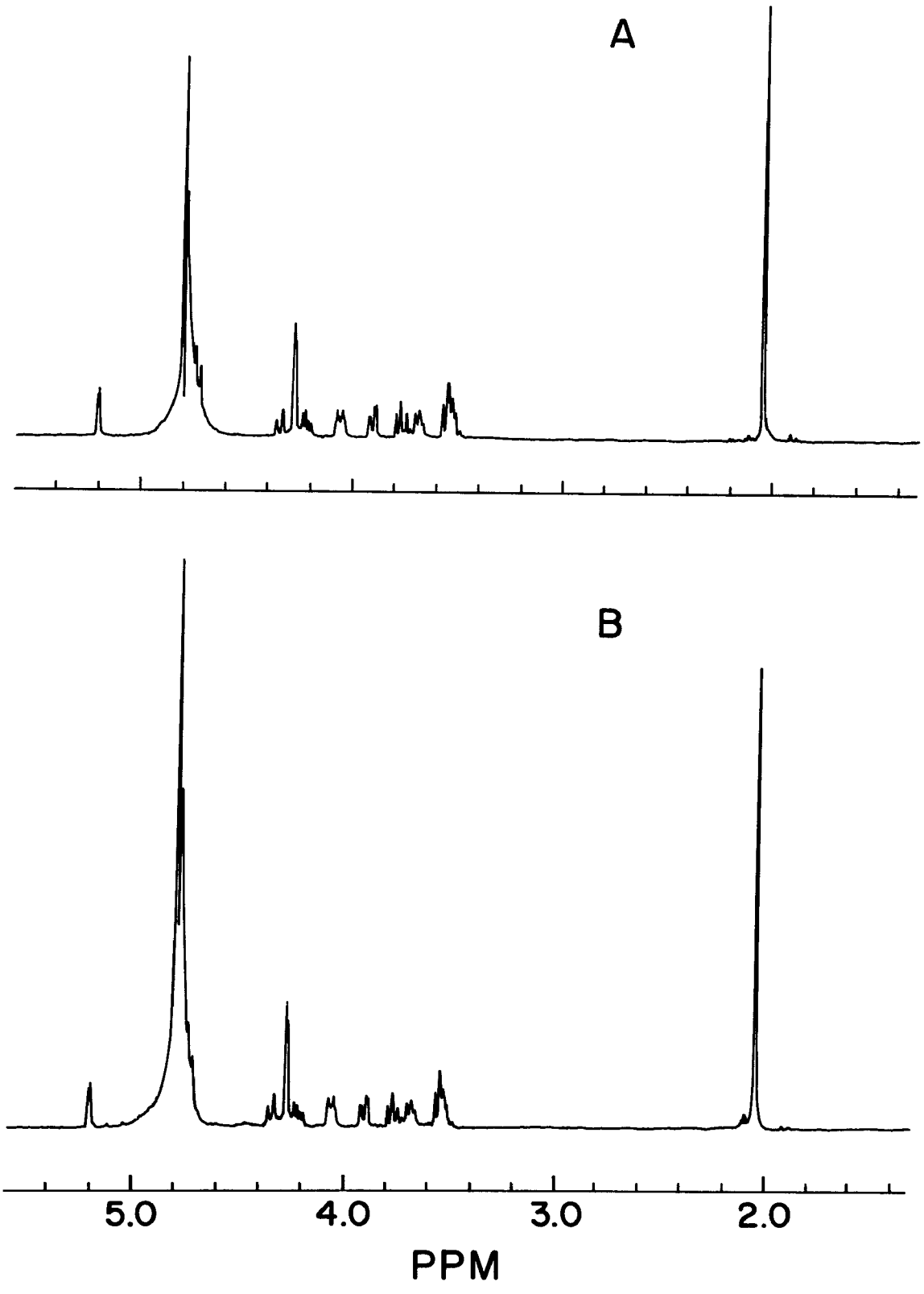


Figure 7. Dowex chromatography of the products after direct sulfation of GlcNAc. Fractions 4 through 8 retained for desalting and characterization.

Figure 8. Nuclear magnetic resonance spectroscopy of GlcNAc(6S) from a commercial preparation (A) and prepared by direct sulfation (B).


A
B
5.0
4.0
3.0
2.0
PPM

Free SO_4^{-2} and Cl^- were effectively removed by Sephadex G-10 chromatography (Fig. 9). When a single enzyme source (a pool of 24 primary cultures of amniotic fluid cells) was assayed with GlcNAc(6S) made by direct sulfation and GlcNAc(6S) from two separate commercial preparations, each was found to serve equally well as the substrate for GlcNAc 6-sulfatase activity (Table 5).

Table 5

A Comparison of N-Acetylglucosamine 6-Sulfate Sulfatase Activity With Substrate From Different Sources

	Specific Activity (μg GlcNAc released/h/mg protein)		
Source	Sample	Duplicate	Average
Commercial #1[a]	11.0	10.2	10.6
Commercial #2[a]	11.7	9.0	10.4
Direct Sulfation	16.2	11.0	13.6

[a] Both commercial preparations were from the same supplier but separate containers.

Optimization of the Assay For N-Acetylglucosamine 6-Sulfate Sulfatase

The pH optimum for skin fibroblasts and amniotic fluid cells from primary cultures was 5.5 (Fig. 10). Absorbance, for cultured skin fibroblasts, was linear with protein concentration to at least 0.1 absorbance unit and was also linear with respect to time for at least 24 h (Fig. 11). The protein concentration curve for primary cultures of amniotic fluid cells was linear to at least 0.05 absorbance units, and the absorbance with respect to time was linear for at least 24 h (Fig. 12). The assays in the present study were incubated for 6 h in order to have a relatively rapid procedure that still gave sufficient absorbance to separate normal from deficient GlcNAc 6-sulfatase activity. The detergents tested all gave lower specific activities for each of 2 cultured skin fibroblast cell lines compared to samples processed without detergent (Fig. 13).

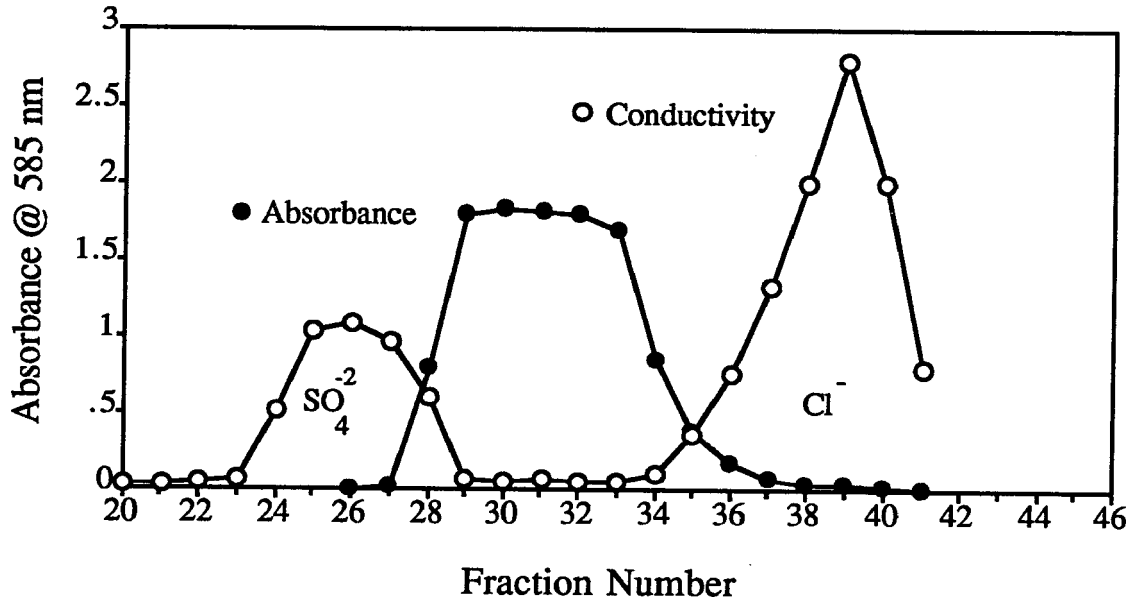


Figure 9. Chromatography on Sephadex G-10 of GlcNAc(6S) prepared by direct sulfation.

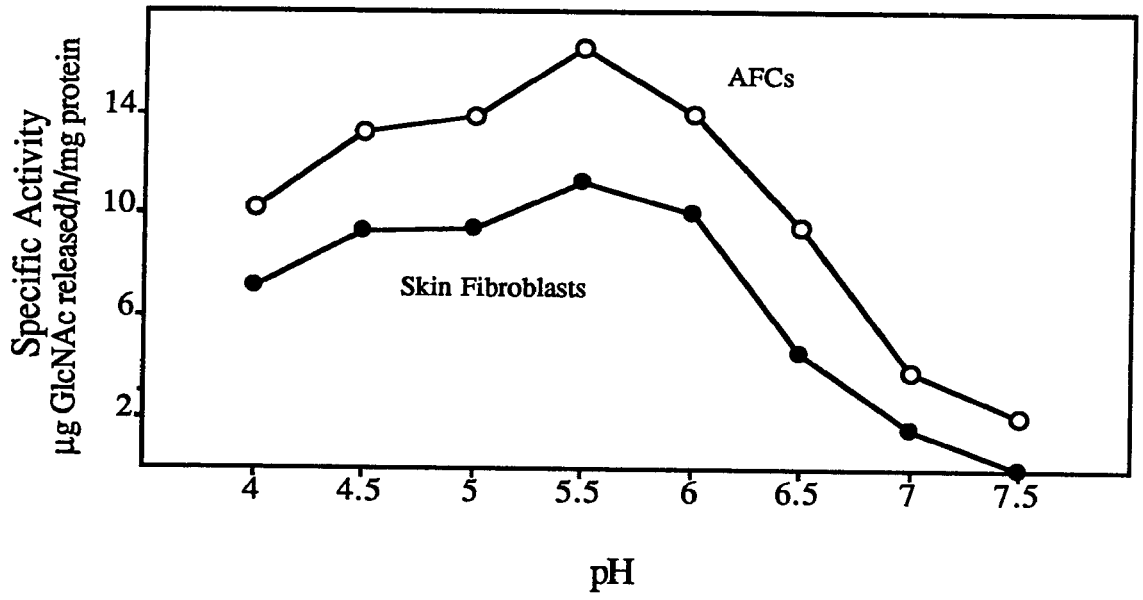


Figure 10. pH optimum for GlcNAc 6-sulfatase from cultured skin fibroblasts of a normal control and a pool (n=22) of primary cultures of amniotic fluid cells.

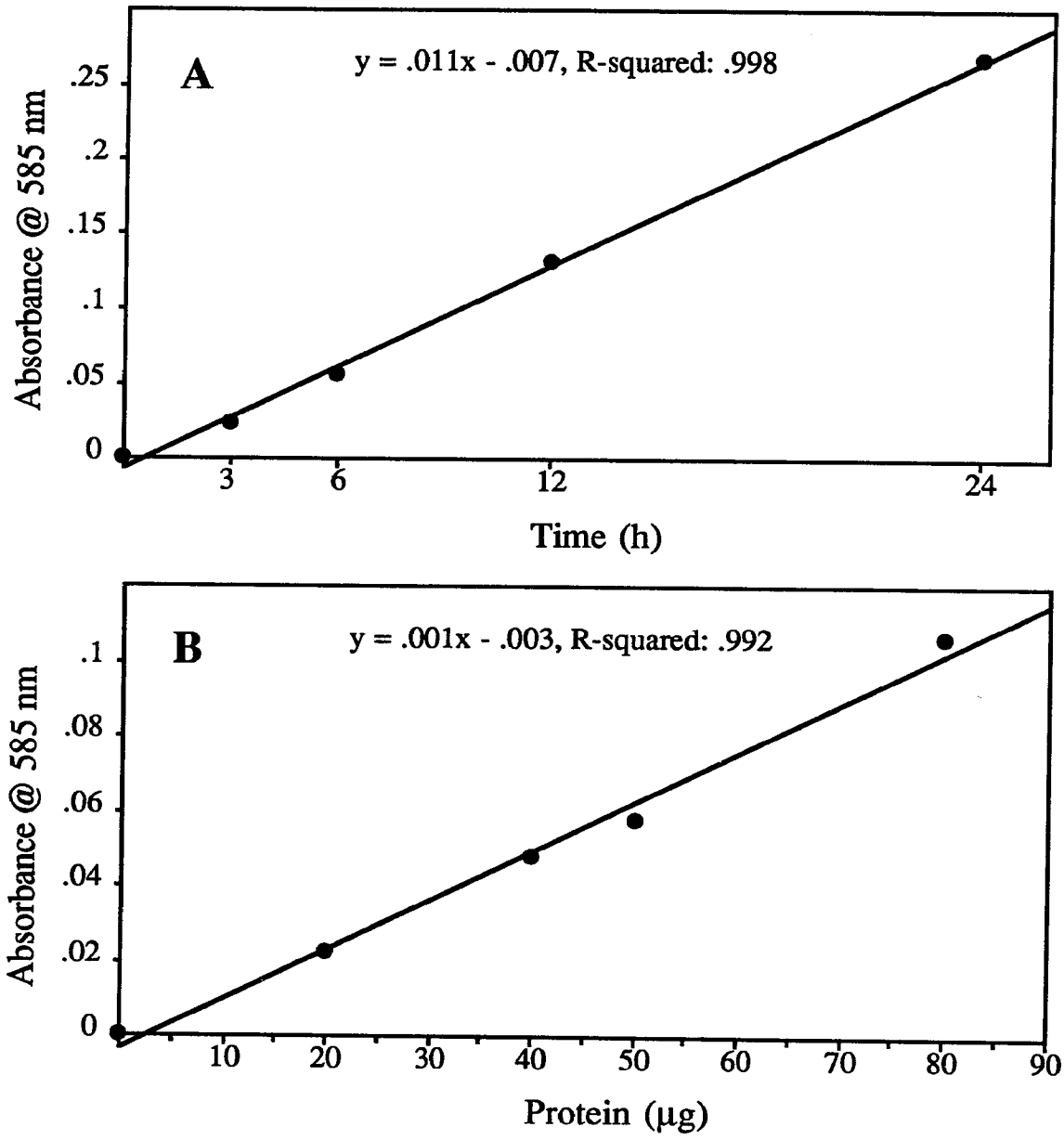


Figure 11. Time curve (A) and 6 h protein concentration curve (B) for GlcNAc 6-sulfatase from cultured skin fibroblasts.

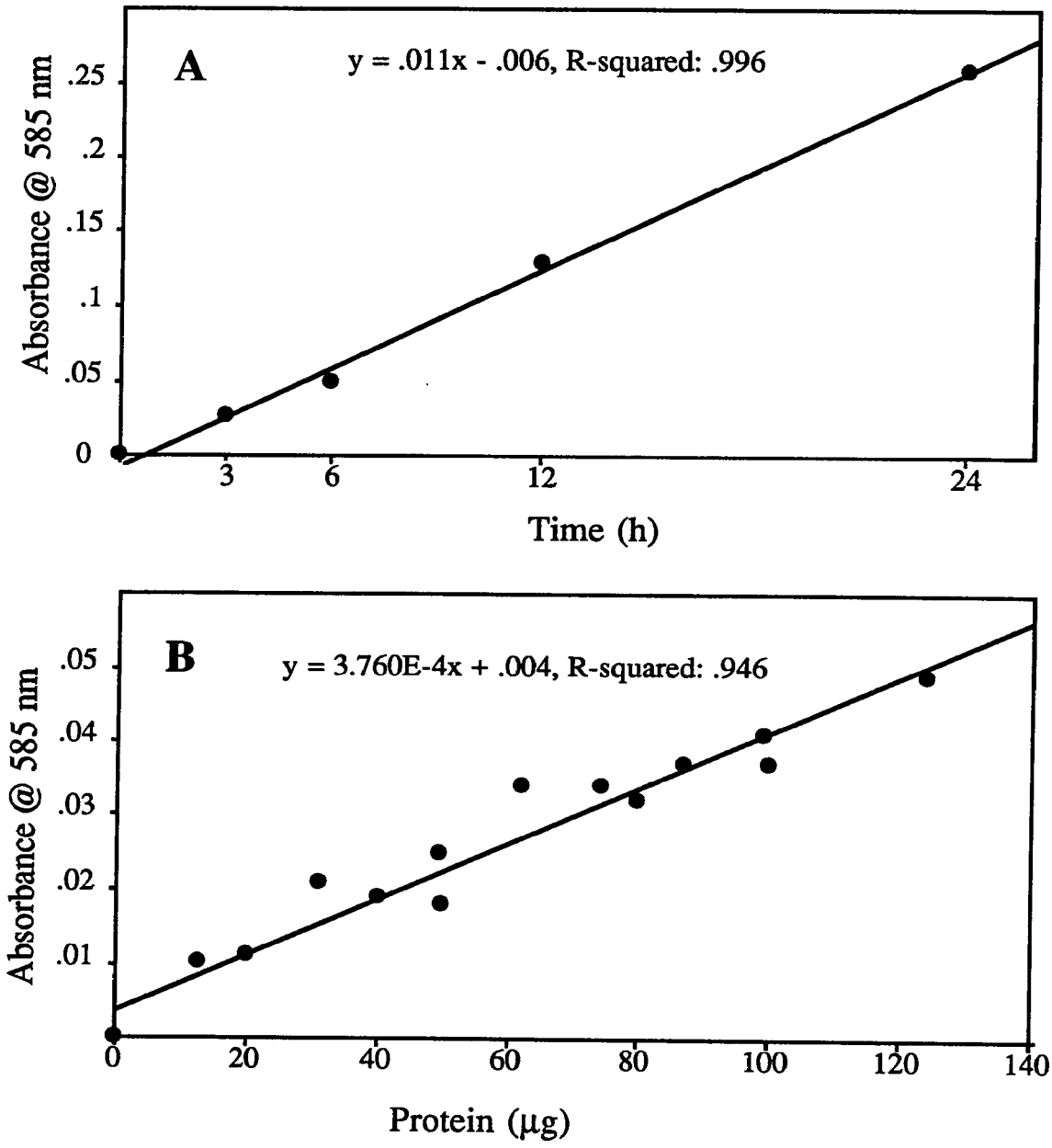


Figure 12. Time curve (A) and 6 h protein concentration curve (B) for GlcNAc 6-sulfatase from a pool (n=22) of primary cultures of amniotic fluid cells.

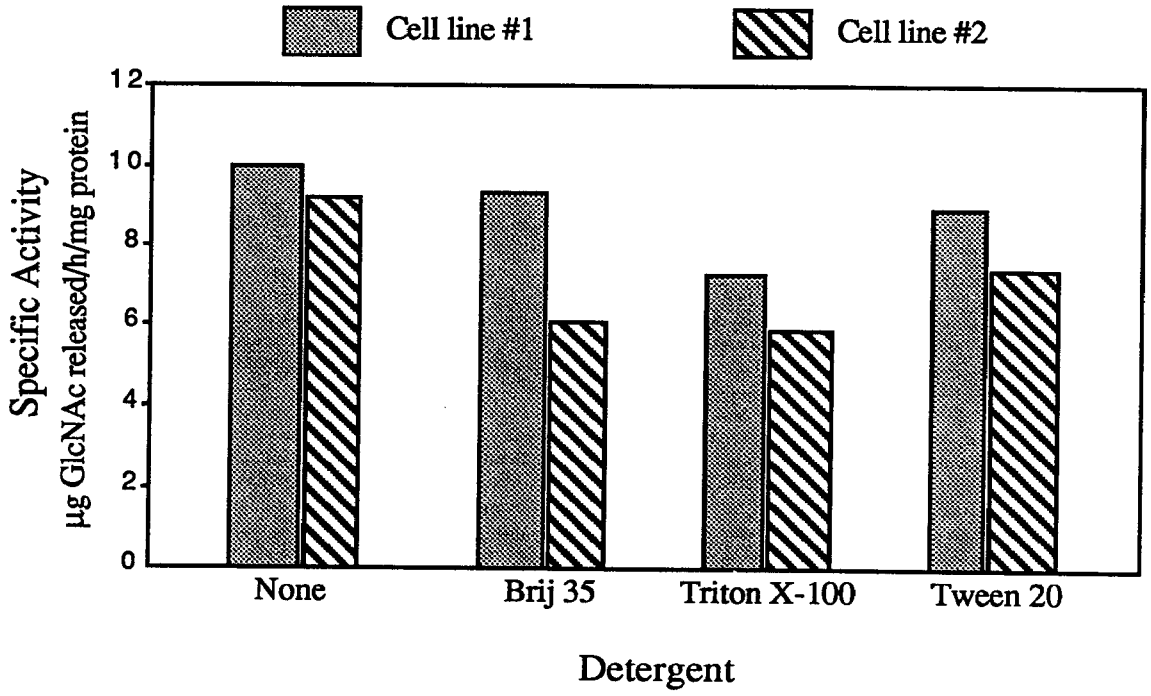


Figure13. Specific activity of GlcNAc 6-sulfatase from skin fibroblasts processed in selected nonionic detergents each present at its critical micellar concentration.

The bicinchoninic acid protein assay gave consistent results for repeated measurements of each of 4 standards (Fig. 14). The modified method of Reissig was also consistent for repeated measurements of 10 μg standards (Fig. 15). A column (1.0 ml) of Dowex could retain at least 2,000 μg of GlcNAc(6S). A 10 μg sample of GlcNAc in 500 μl of water was applied to a column (1.0 ml) of Dowex, and 100% was recovered within four 500 μl fractions when the column was washed with water. Substrate recovery was 92% in terms of released GlcNAc and unreacted substrate eluted from the column with 1.0 M NaCl. Duplicate assays of 12 separate samples of cultured amniotic fluid cells (Table 6) were analyzed statistically by the paired comparisons test (Daniel, 1983) for consistency. The null hypothesis of a mean difference of zero could not be rejected at the 0.05 significance level.

N-Acetylglucosamine 6-Sulfate Sulfatase Activity in Selected Tissues

Cultured Skin Fibroblasts

The specific activity of GlcNAc 6-sulfatase from cultured skin fibroblasts of normal controls ranged from 3.8 to 17.0 μg GlcNAc released/h/mg protein with a mean of 7.7 (± 4.1). The specific activities of GlcNAc 6-sulfatase in cultured skin fibroblasts from the 3 known patients with Sanfilippo syndrome, type D, were 2.6%, 0%, and 7.8% of the normal mean (Table 7). Specific activity of GlcNAc 6-sulfatase in cultured skin fibroblasts from mucopolysaccharidoses patients with other sulfatase deficiencies were in the normal range (Table 8).

Cultured Conjunctival Cells

The average specific activity of cultured conjunctival cells assayed in duplicate was 5.7 μg GlcNAc released/h/mg protein.

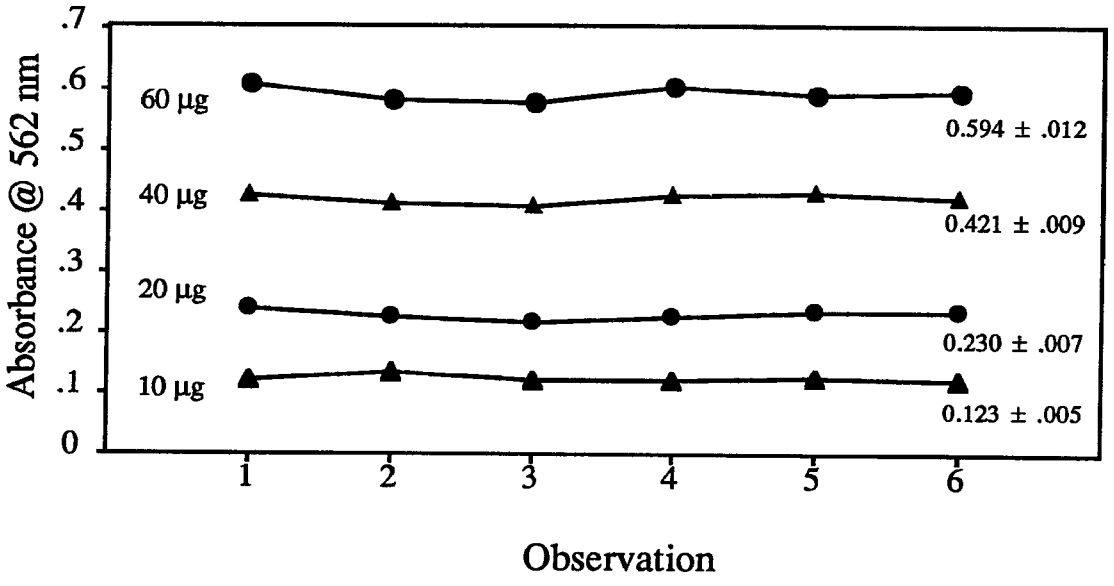


Figure 14. Six consecutive determinations of 4 BSA standards. Each observation was made on a separate day with new reagents.

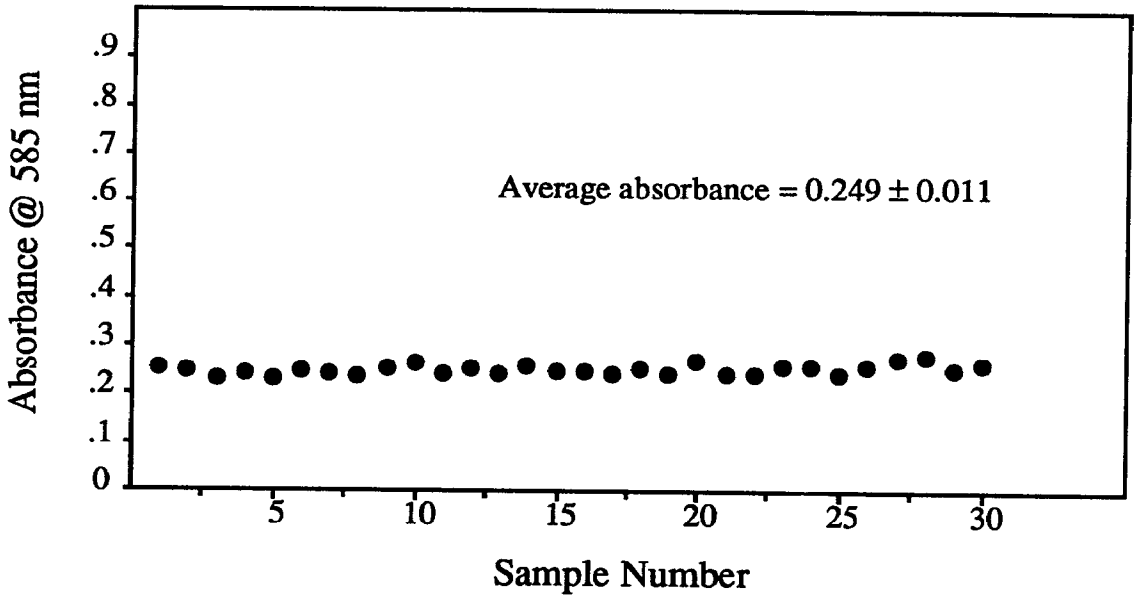


Figure 15. Absorbances of 10 µg GlcNAc standards from 30 consecutive Reissig assays.

Table 6

Repeat Determinations of N-Acetylglucosamine 6-Sulfate Sulfatase Activity in Twelve Separate Samples of Cultured Amniotic Fluid Cells

Sample #	1	2	3	4	5	6	7	8	9	10	11	12
Assay	Specific Activity[a]											
1st	5.0	9.0	7.0	6.9	4.4	4.7	5.6	8.4	5.7	4.5	6.6	8.3
2nd	5.8	9.0	6.1	8.8	5.4	7.4	6.5	9.0	6.9	5.5	4.0	5.7

a (μg GlcNAc released/h/mg protein)

Amniotic Fluid Cells

The specific activity of GlcNAc 6-sulfatase from 122 primary amniotic fluid cell cultures ranged from 1.9 to 13 μg GlcNAc released/h/mg protein with a mean of 6.2 (± 2.6) (Fig. 16). The distribution of specific activities for primary AFC cultures was skewed and kurtotic and did not fit a normal distribution by the Shapiro-Wilk test (Shapiro & Wilk, 1965); however, after a $\log_e$ transformation (Fig. 17), a normal distribution could not be rejected statistically. Analysis of variance for the distributions of AFCs and cultured skin fibroblasts after a $\log_e$ transformation did not reject the hypothesis that they were equal ($F = 2.20$, $p = 0.1406$).

The AFC data were examined by cluster analysis to determine if evidence existed for more than one distribution as might be expected if there were isozymes present as found by Vance et al. (1980) for N-acetyl-α-D-glucosaminidase. Four, three and two clusters did not reveal separate, statistically supportable, distributions among either the raw data or the $\log_e$ transformed data.

Table 7

N-Acetylglucosamine 6-Sulfate Sulfatase Activity in Cultured Skin Fibroblasts From Normals and Sanfilippo D Patients

Cell Line		Specific Activity (μg GlcNAc released/h/mg protein)[a]
Normal	TPN 4911	3.8
Normal	TPN 4922	4.8
Normal	GM 302A	4.9
Normal	MGE-DSE	5.1
Normal	TPN 5023	5.4
Normal	MGE-RWN	5.9
Normal	TPN 5011	6.6
Normal	TPN 5041	7.2
Normal	MGE-RKM	11.3
Normal	MGE-TS	12.6
Normal	MGE-JNT	17.0
Sanfilippo D	GM 5093	0.2
Sanfilippo D	WG 1004	0.0
Sanfilippo D	MGE-VG	0.6

[a] Control Mean ($\pm\sigma$) = 7.7 ($\pm$ 4.1)

Table 8

N-Acetylglucosamine 6-Sulfate Sulfatase Activity in Cultured Skin Fibroblasts From Patients With Other Sulfatase Deficiencies

Cell Line		Enzyme Deficiency	Specific Activity (μg GlcNAc released/h/mg protein)
Hunter	GM 0615	Iduronate sulfatase	3.0
Maroteaux-Lamy	GM 2849	N-acetylgalactosamine 4-sulfate sulfatase	4.1
Sanfilippo A	GM 1881	Heparan N-sulfatase	4.8
Multiple sulfatase	GM 4681	Several sulfatases	0.0
Multiple sulfatase	GM 3245	Several sulfatases	0.6
Multiple sulfatase	MGE-BN	Several sulfatases	0.3

Enzyme Characteristics in Primary Cultures of Amniotic Fluid Cells

Repeated freezing, up to 5 times, had little effect on enzyme activity. There was a decrease of only 16% following the first 3 freeze cycles (Fig. 18). Sonication had no adverse effect on enzyme activity up to 45 sec in 5-sec intervals (Fig. 19).

Sodium sulfate and sodium chloride were both inhibitory toward GlcNAc 6-sulfatase activity, the latter significantly moreso. Sodium sulfate caused 95% inhibition and 100% inhibition when present in 10 mM and 30 mM concentrations, respectively (Fig. 20). Sodium chloride was considerably less inhibitory with a 40 mM concentration giving only a 13% reduction in specific activity compared to the control which was 8.0 mM due to the NaCl present in the enzyme preparation.

GlcNAc 6-sulfatase was relatively stable at 55 °C after an initial reduction of approximately 35% during the first 60 min; however, at 70 °C., specific activity was

reduced by 70% and 100% after 30 and 90 min, respectively (Fig. 21). The Km and Vmax for GlcNAc(6S) sulfatase in primary cultures of AFCs were determined from the averaged values of 3 separate experiments (Fig. 22). The Km was 24.0 mM and the Vmax was 20.7 μg GlcNAc released/h/mg protein. After exhaustive degradation, 12.4% of the substrate was cleaved by GlcNAc 6-sulfatase from a pool of primary cultures of amniotic fluid cells.

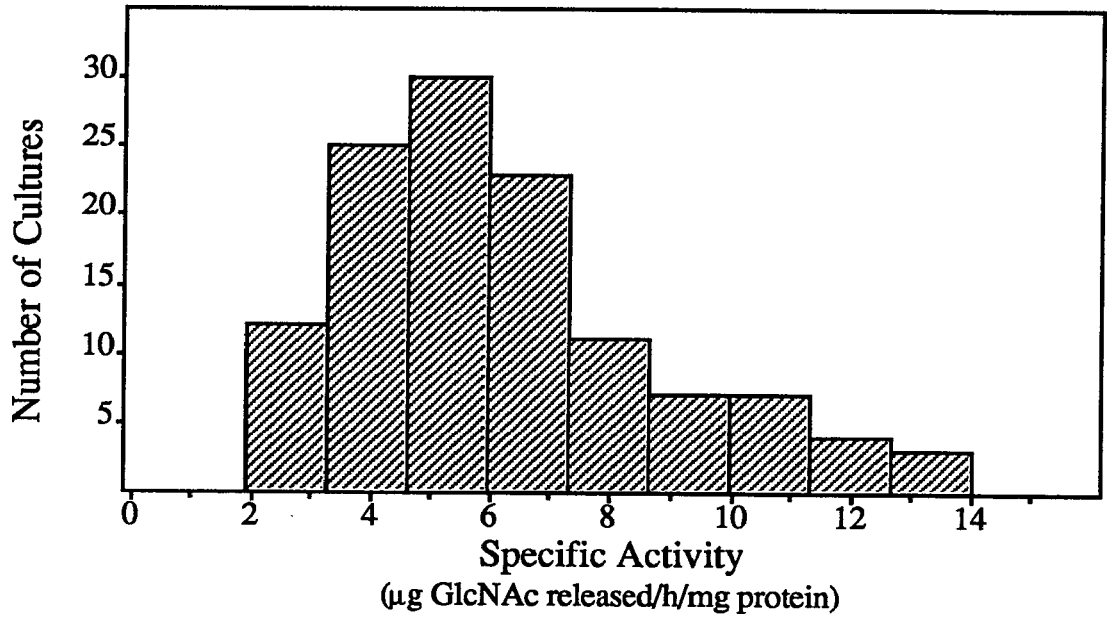


Figure 16. The distribution of specific activities for GlcNAc 6-sulfatase from 122 primary cultures of amniotic fluid cells.

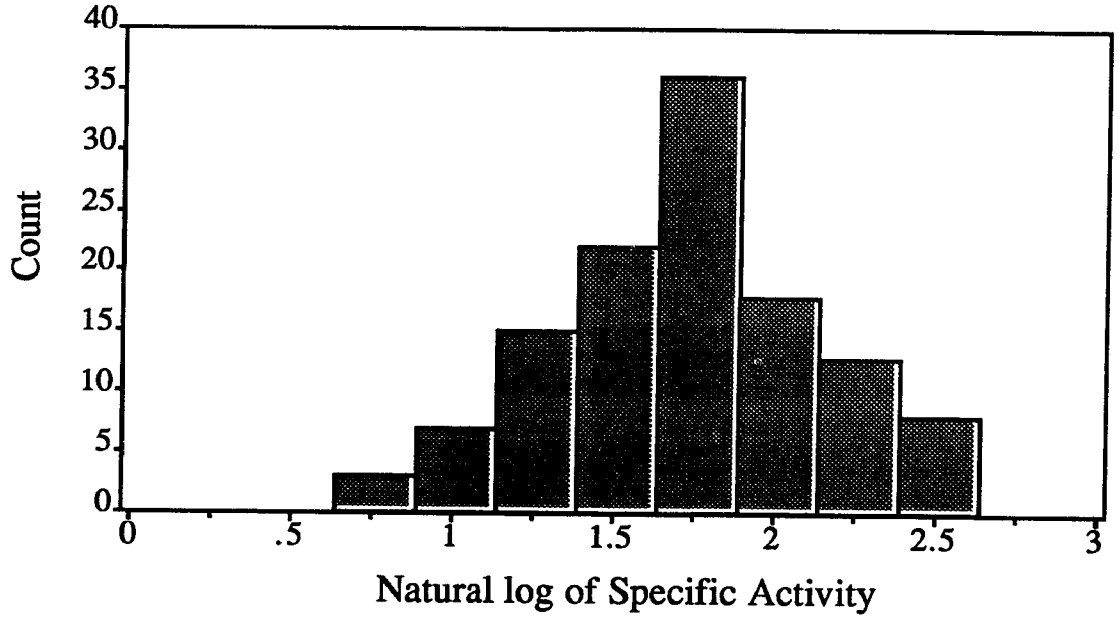


Figure 17. The distribution of the log normal transformed specific activities for GlcNAc 6-sulfatase from 122 primary cultures of amniotic fluid cells.

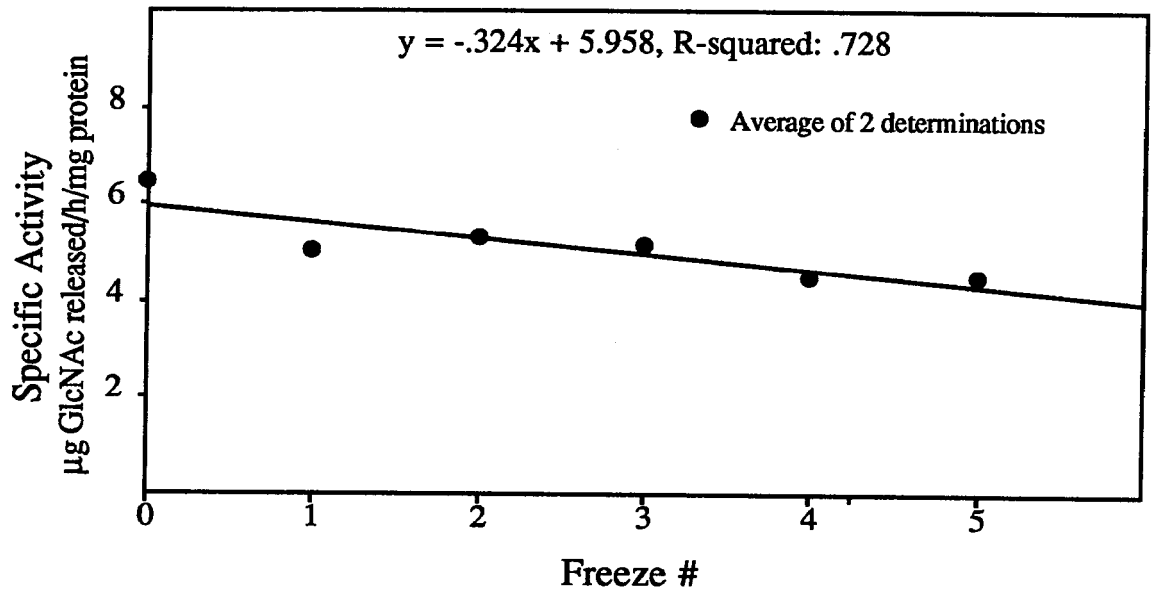


Figure 18. Effect of sequential freeze-thaws on GlcNAc 6-sulfatase activity in amniotic fluid cells. Enzyme source was a pool of 12 primary cultures.

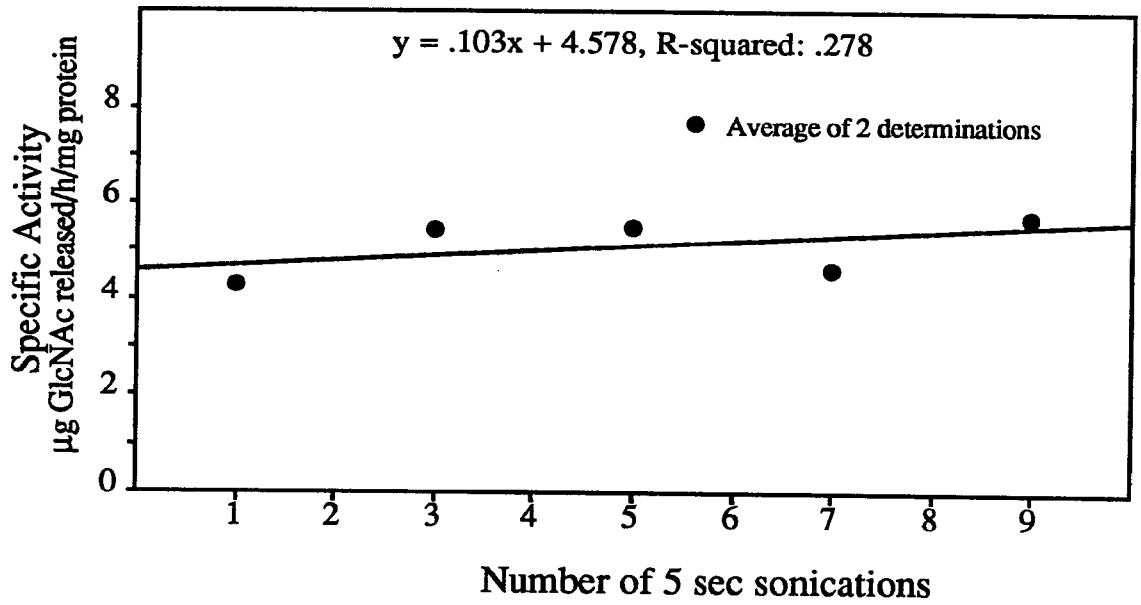


Figure 19. Effect of multiple sonications on GlcNAc 6-sulfatase activity in amniotic fluid cells. Enzyme source was a pool of 13 primary cultures.

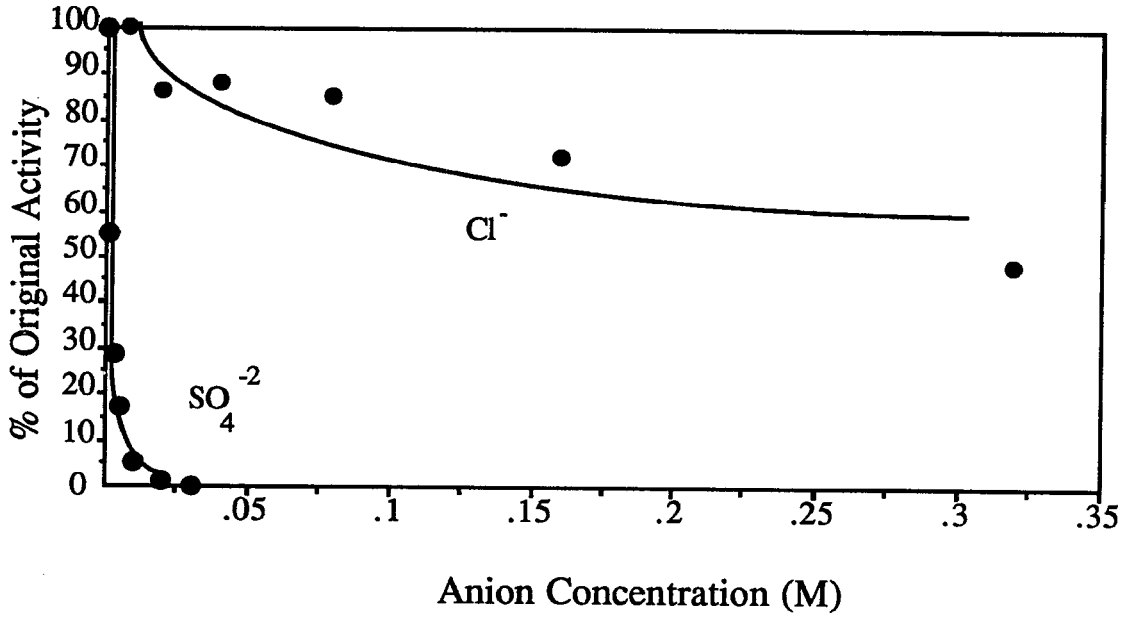


Figure 20. Effect of sodium chloride and sodium sulfate on GlcNAc 6-sulfatase activity in primary cultures of amniotic fluid cells.

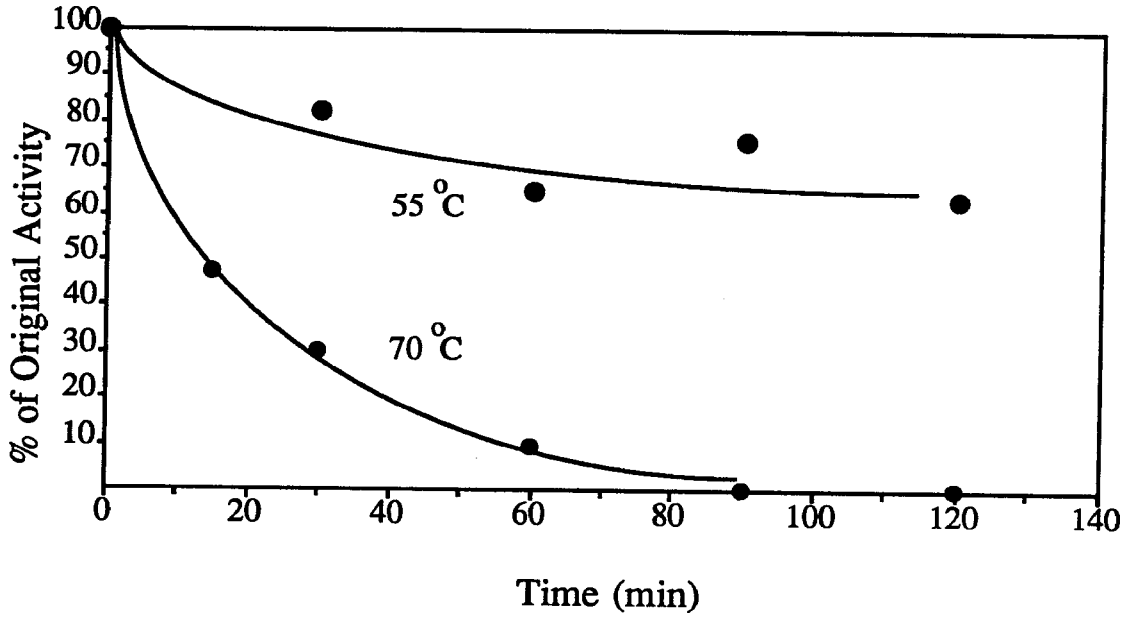


Figure 21. Heat inactivation of GlcNAc 6-sulfatase at 55 °C and 70 °C. Enzyme source was a pool of primary amniotic fluid cell cultures.

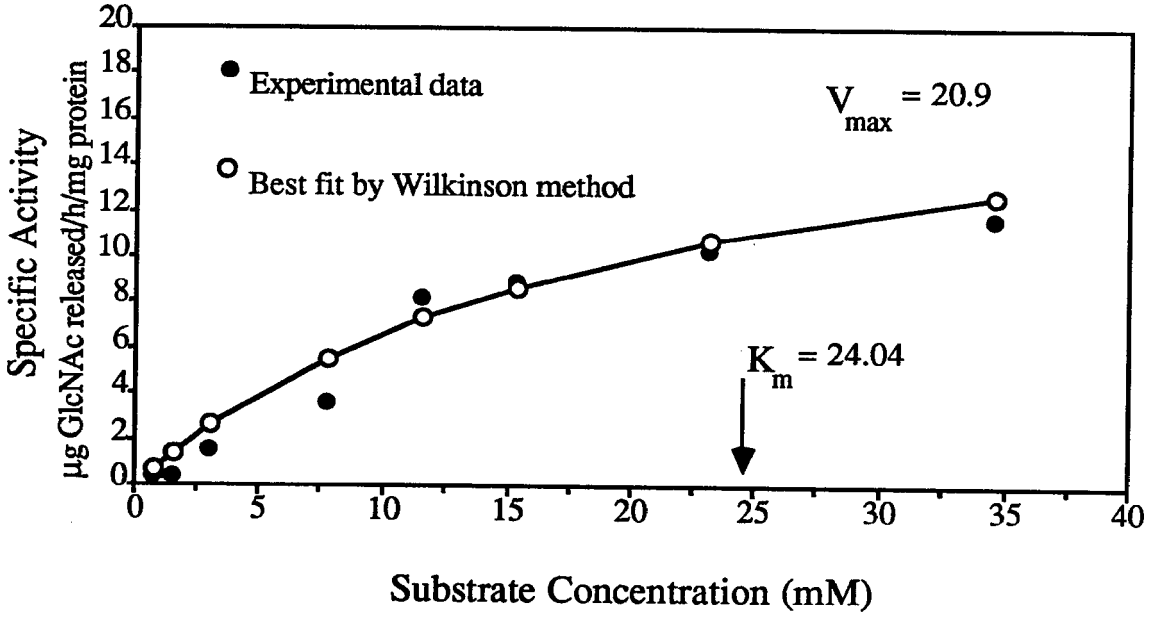


Figure 22. GlcNAc 6-sulfatase activity as a function of substrate concentration. Enzyme source was a pool of primary cultures of amniotic fluid cells.

CHAPTER IV.

DISCUSSION

The monosaccharide substrate and spectrophotometric assay were effective in measuring GlcNAc 6-sulfatase activity and clearly distinguished MPS IIID patients from normal controls. The specific activities for GlcNAc 6-sulfatase from cultured skin fibroblast cell lines of the 3 known patients with Sanfilippo syndrome, type D, expressed as a percentage of the normal mean (0% to 7.8%), compared closely to those previously reported for the 6 enzymatically confirmed patients assayed by different procedures (0 to 6.1%, Table 1). The deficiency of GlcNAc 6-sulfatase activity found in multiple sulfatase deficient cell lines was not consistent with the findings of Yutaka et al. (1981). They used a disaccharide substrate and made multiple determinations of the specific activity of GlcNAc 6-sulfatase in several cultures from a single multiple sulfatase deficient fibroblast cell line. The activity reported was usually normal but in some samples a reduction of between 30-50% of the control values was found. They proposed that the normal activity levels might have resulted from the fact that cells were cultured in medium that was maintained at a pH of 7.4. Similar results were obtained by Fluharty et al. (1978) who reported a rise in arylsulfatase A activity when multiple sulfatase deficient cell lines were maintained at that same pH. The cell lines in the present study were maintained in a CO_2-HCO_3^- buffered medium which gives lower pH levels.

GlcNAc 6-sulfatase from primary cultures of amniotic fluid cells was found to be a relatively stable enzyme with respect to heat inactivation, repeated freezing, and repeated

sonication. This is advantageous for both enzyme isolation and sample preparation, which necessitate these and related procedures that are potentially damaging to enzyme activity. Basner et al. (1979b) found greater heat sensitivity for GlcNAc 6-sulfatase isolated from human urine, with more than 90% of enzyme activity toward a trisaccharide substrate destroyed after only 3 min at 70 °C. In the present study, 90% of the enzyme activity was lost after 60 min. This difference may, however, simply reflect the different enzyme preparation and assay procedures. Inhibition by $Na_2SO_4^{-2}$ was potent. The 95% inhibition with 10 mM $Na_2SO_4^{-2}$ found in the present study was similar in magnitude to that found by Basner et al. (1979b) who reported that a 17 mM concentration gave ≥95% inhibition. This emphasizes the importance of using a substrate preparation free of SO_4^{-2}. The effect of NaCl was less than that suggested by Kresse et al. (1982) who recommended that cell pellets not be homogenized in 0.15 M NaCl since this concentration was considered highly inhibitory toward GlcNAc 6-sulfatase activity. The AFC cultures used in this study were prepared in 0.15 M NaCl but the concentration in the incubation mixture was significantly less than that (0.037 M). Even when the concentration was increased more than fourfold, to 0.16 M, there was only a 28% reduction in specific activity.

The use of detergents did not give a greater specific activity of GlcNAc 6-sulfatase as might be expected if significant quantities were membrane-bound and not freed by sonication. It has been suggested, at least indirectly, that detergents might be useful; for example, Elliott and Hopwood (1984) homogenized fibroblasts in Triton X-100 (1 g/L) in preparation for the assay of GlcNAc 6-sulfatase activity. The present study, however, indicated that the detergents tested, including Triton X-100, were moderately inhibitory.

The overall assay procedure was found to be relatively free of the potential for variation. Evidence for this, with respect to cell preparation, was the stability of the enzyme when subjected to repeated freezing, repeated sonication, and heat inactivation. The modified Reissig method was very consistent for repeated measures of the GlcNAc

standard and implies that the measures of released GlcNAc during the assay of GlcNAc 6-sulfatase activity also were consistent. The bicinchoninic acid assay, used for the measurement of proteins, gave consistent results for repeat measurements of standard BSA solutions and it is therefore unlikely that specific activity determinations were affected by inconsistent measures of protein values in the cell sonicates. The amount of Dowex (1 ml) used to separate the reaction products could hold at least a fourfold excess of substrate without spillover, and virtually all of the substrate in an assay could be accounted for in terms of the reaction product, released GlcNAc, plus unreacted substrate recovered from the column after elution of the reaction products. These facts and the fact that 100% of GlcNAc was recovered from the same column size within the normal elution volume of the assay indicates that greater release of GlcNAc was not due to substrate eluting prematurely from the column and that lower specific activities were not due to retention of the released GlcNAc.

A monosaccharide substrate for GlcNac 6-sulfatase was used in only two previous studies (Basner et al., 1979b; Elliott & Hopwood, 1984). Neither group used a spectrophotometric procedure, and only the latter used the monosaccharide substrate to study an affected patient. The kinetic values reported by the previous investigators and those of the present study, converted to the same units, are compared in Table 9. Although the spectrophotometric procedure gave substantially higher specific activities, the Km and Vmax for GlcNac 6-sulfatase also were higher. Neither of the previous investigators indicated the manner in which these kinetic parameters were determined so it is difficult to make a meaningful comparison. It seems reasonable, however, to conclude that the assay developed in the present study offers an effective alternative to those used previously.

Table 9

A Comparison With Previously Reported Kinetic Properties of N-Acetylglucosamine 6-Sulfate Sulfatase Toward the Monosaccharide Substrate GlcNAc(6S)

Reference	Enzyme Source	Specific Activity	Km	Vmax
Elliott & Hopwood (1984)	Skin Fibroblasts	0.11-0.53	0.33 mM	2
Basner et al. (1979)	Urine	63	1.5 mM	-
Present Study	Skin Fibroblasts [b]	581	16.6 mM	1,629
	Amniotic Fluid Cells [b]	468	24.0 mM	1,561

[a] pmol GlcNAc released/min/mg protein [b] mean value

The specific activities for the primary cultures of amniotic fluid cells were not necessarily expected to be normally distributed. Many biological functions fail to follow a truly normal distribution, but the transformed data are often shown to follow a log normal distribution (Elveback, Guillier, & Keating, 1970; Flynn, Piper, Garcia-Webb, McPherson, & Healy, 1974); for example, a $\log_e$ distribution also was found by Vance et al. (1980) for N-acetyl-α-D-glucosaminidase, another heparan sulfate degrading enzyme, the deficiency of which results in Sanfilippo syndrome, type B. The enzyme sources for their study were serum and plasma. The advantage gained when data can be transformed to a normal approximation is that it allows the use of more powerful statistical analyses than the nonparametric tests required otherwise (E.K. Harris & DeMets, 1972; Strike, 1981). This formed the basis in the present study for comparing the log normal transformed data of cultured skin fibroblasts and primary cultures of AFCs to assess the similarity of their distributions and consequently the potential for prenatal diagnosis. The similarity of the distribution of GlcNAc 6-sulfatase activity in cultured skin fibroblasts and primary cultures of amniotic fluid cells from normals indicates that an enzyme deficiency in an affected fetus, similar in magnitude to the affected patients, would be easily distinguished.

Histological study of conjunctival biopsies has been used traditionally in the diagnosis of MPS disorders (Libert, 1980; Kenyon, 1982) and more recently in monitoring the results of bone marrow transplantation in MPS patients (Summers et al., 1988). The presence of GlcNAc 6-sulfatase activity in cultured conjunctival cells makes this an alternate tissue source for enzyme analysis to be used in diagnosis and management of patients with deficient activity of this enzyme. It has been shown that conjunctival tissue grows well in culture and offers the potential for enzymatic diagnosis of lysosomal storage diseases (Nowakowski, Thompson & Baker, 1988b, Appendix D). Conjunctival biopsies are safe, easily performed with topical anesthetic (Kenyon, 1982), and, unlike skin biopsies, are nondisfiguring. It would be advantageous to combine these conjunctival attributes and correlate enzyme activity with histological change in the same tissue for future monitoring of therapeutic trials.

CHAPTER V.

CONCLUSIONS

The Sanfilippo D patients were distinguished clearly from normal controls by the spectrophotometric assay using the monosaccharide substrate. Multiple sulfatase patients also were deficient in GlcNAc 6-sulfatase activity, and the differential diagnosis requires assays of additional sulfatases. The assay is effective, relatively fast and simple to perform. The sulfated monosaccharide substrate is considerably less difficult to prepare than oligosaccharide substrates and also is available from a commercial source. The use of a spectrophotometric procedure eliminates the need to work with radioactive materials. These features make the diagnostic assay potentially more accessible since it could be performed in additional laboratories.

GlcNAc 6-sulfatase was active in primary cultures of amniotic fluid cells and showed low susceptibility to inactivation by heat, NaCl, freezing, and sonication, all of which can be encountered in the preparation of cell pellets for enzyme assay. This shows that prenatal monitoring of enzyme activity can be accomplished and does not require any unusual precautionary measures. There has been no report of prenatal diagnosis for MPS IIID, but it would be feasible to use this new procedure if it is assumed that an affected fetus would have a deficiency of GlcNAc 6-sulfatase activity of the same magnitude as the affected patients in this study. Final proof of this assumption will, of course, await the study of an at-risk pregnancy.

APPENDIX A

Modified Reissig Method for Estimation of N-Acetylamino Sugars

Reissig, J.L., Strominger, J.L. & Leloir, L.F. (1955). A modified colorimetric method for the estimation of N-acetylamino sugars. The Journal of Biological Chemistry, 217, 959-966.

REAGENTS

1. $K_2B_4O_7 \cdot 4H_2O$ (MW 305.51) - 25 ml, 0.6 M with respect to borate (B_4O_7), pH 9.5

   Add 4.66 g to 22 ml of H_2O and heat gently to dissolve. Adjust pH with concentrated HCl (about 300-500 µl) and QS to 25 ml total volume with H_2O.

2. p-Dimethylaminobenzaldehyde (DMAB, MW 149.2) stock - 32 ml. Can be stored refrigerated for about 1 month.

Concentrated HCl	3.42 ml
H_2O	0.58 ml
Glacial acetic acid	28.00 ml
DMAB	3.2 g

3. GlcNAc (MW 221) standard: 50 mg GlcNAc in 50 ml H_2O gives a 1 µg/µl solution.

PROCEDURE

Add 100 µl of $K_2B_4O_7 \cdot 4H_2O$ reagent to 500 µl sample and heat in boiling water bath for 3 min. Cool in a room temperature water bath for 3 min, add 3 ml of DMAB reagent, diluted 1:9 with glacial acetic acid, and incubate at 37 °C for 20 min. Cool in a room temperature water bath for 3 min and read absorbance at 585 nm against a blank of water.

	BLANK	STANDARD
H_2O	500 µl	490 µl
GlcNAc (10 µg)	-	10 µl of stock solution #3 above
TOTAL	500 µl	500 µl

APPENDIX B

Spectrophotometric Assay for N-Acetylglucosamine 6-Sulfate Sulfatase

REAGENTS

1. Buffer: 100 mM dimethylglutarate (DMG, MW 160.2), pH 5.5

   Add 0.4 g DMG to 20 ml of H_2O and adjust pH to 5.5 with 1 M NaOH (about 4.4 ml). Q.S. to 25 ml final volume with water.

2. 1 M NaOH (MW 40): 4 g/100 ml

3. NaN_3 (MW 65): 20 mg/ml

4. Substrate: GlcNAc(6S) (MW 323) stock solution of 250 mg/5 ml. Freeze in 1 ml aliquots.

5. Dowex AG1-X8, 200-400 mesh, chloride form. Charge initially with 3 M HCl for approximately 30 min, wash with deionized water to pH 5 or greater for use. Recharge weekly with 1 M HCl for 15-30 min and wash until pH is 5.0 or greater before use.

6. NaCl (MW 58.44): 0.88 g/100 ml gives a 0.15 M solution. Dilute 1 : 99 for a 1.5 mM solution.

INCUBATION MIXTURE

	Volume (µl)		Final composition
Buffer	100		50.0 mM
Substrate	10	(500 µg)	7.7 mM
H_2O	35		-
NaN_3	5		0.05 %
Enzyme	0-50		-
NaCl	0-50		-
TOTAL	200		-

PROCEDURE

Incubate at 37 °C for 6 h. Dilute with 300 µl H_2O and add to 1 ml volume of Dowex AG 1-X8, 200-400 mesh, in Quik-Sep column. Collect void and three 500 µl fractions eluted with 1.5 mM NaCl. Assay by modified method of Reissig for released GlcNAc and compare sum of absorbances from the 4 fractions to a 10 µg standard (10 µl of standard solution in 490 µl H_2O) which reads approximately 0.250.

NORMAL REFERENCE VALUES

Cell type	Specific Activity (µg GlcNAc released/h/mg protein)
Primary amniotic fluid cell cultures	6.2 ± 2.6 (n = 122)
Cultured skin fibroblasts	7.7 ± 4.1 (n = 11)
Cultured conjunctival cells	5.7 (n = 2)

APPENDIX C

A New Screening Method for Mucopolysaccharidoses With Urinary Excess of Sulfated N-Acetylhexosamines

Note

This appendix is presented in the format of a manuscript prepared for publication. The references, however, are contained within the reference section of the dissertation itself.

A NEW SCREENING METHOD FOR MUCOPOLYSACCHARIDOSES WITH URINARY EXCESS OF SULFATED N-ACETYLHEXOSAMINES

Rod W. Nowakowski,[†*] Jerry N. Thompson,[†Δ] Deborah S. Edge[†]

Laboratory of Medical Genetics,[†] Department of Optometry,[*]Departments of Biochemistry and Pediatrics,[Δ] The Medical Center, University of Alabama at Birmingham.

ABSTRACT

Patients with Sanfilippo syndrome, type D, multiple sulfatase deficiency, and other mucopolysaccharidoses previously have been reported to excrete elevated amounts of sulfated N-acetylhexosamines in their urine. Based on this finding, a new and simple colorimetric screening test for these disorders is presented. An aliquot of urine from each of 23 normal controls and 6 patients with different mucopolysaccharidoses was analyzed after anion-exchange chromatography by a modification of the method of Reissig. This method is both specific and sensitive for N-acetylated hexosamines. Patients with Sanfilippo syndromes, type A and D, Maroteaux-Lamy syndrome, Morquio syndrome, type A, and multiple sulfatase deficiency were clearly distinguished from normal controls. The screening procedure was altered slightly to allow quantitation and revealed levels of N-acetylhexosamines similar to those reported previously using other more laborious techniques. The procedures for both screening and quantitation are presented.

INTRODUCTION

Sanfilippo syndrome, type D (MPS IIID) is characterized by moderate physical abnormalities, progressive mental deterioration, and deficient activity of N-acetylglucosamine 6-sulfate sulfatase, a lysosomal enzyme involved in the degradation of heparin, keratan sulfate, and heparan sulfate (McKusick & Neufeld, 1983). Patients excrete partially degraded heparan sulfate in their urine. The fact that they do not store or excrete keratan sulfate, in spite of the deficient sulfatase, has been attributed to the action of β-N-acetylhexosaminidase A which can cleave the terminal, nonreducing, β-linked GlcNAc(6S) residues in keratan sulfate but not the α-linked GlcNAc(6S) residues in heparan sulfate (Kresse et al., 1981). This accounts, in part, for the increased urinary content of GlcNAc(6S) in MPS IIID as was reported by Hopwood and Elliott (1983b), and Fuchs et al. (1985). The latter group suggested that this observation could allow a specific diagnosis to be made without an enzyme assay; however, Hopwood and Elliott (1985) showed that MPS patients in general had some elevation of urinary sulfated N-acetylhexosamines and that those with Morquio syndrome, type A (N-acetylgalactosamine 6-sulfate sulfatase deficiency), and Maroteaux-Lamy syndrome (N-acetylgalactosamine 4-sulfate sulfatase deficiency), as well as those with multiple sulfatase deficiency had significantly elevated levels. They quantified urinary content of GlcNAc(6S), GalNAc(6S), GalNAc(4S) and GalNAc(4,6 diS) in 3 normal controls, 14 patients with mucopolysaccharide (MPS) disorders, and 1 each with multiple sulfatase deficiency, mucolipidosis type II, and α-mannosidosis. The presence of at least some of the sulfated

N-acetylhexosamines found in the urine of these patients, as in MPS IIID, was proposed to be the result of the action of β-N-acetylhexosaminidase A on keratan sulfate, chondroitin sulfate, and/or dermatan sulfate.

The Morgan-Elson reaction (1934) is a colorimetric reaction specific for N-acetylated hexosamines. Improvements in the conditions under which this reaction occurs were reported by Aminoff et al. (1952) and their method was used by Jeanloz and Trémége (1956) to study the effect of substitution at different positions. This latter group studied methylated derivatives of GlcNAc and GlcNAc found that substitution at position 4 prevented color formation, substitution at position 6 did not affect it, and substitution at position 3 enhanced color production by approximately 50%. The method of Aminoff was improved by Reissig et al. (1955) who optimized conditions for color enhancement, speed of color development, specificity, and reduction in the effect of interfering substances. They found that the color formation for GalNAc was approximately one-third that of GlcNAc and that they were additive when combined. A modification of the method of Reissig was used for the detection of N-acetylhexosamines in this study.

The present study was undertaken to develop a new screening procedure, based on the method of Reissig, for MPS IIID and other mucopolysaccharidoses with increased sulfated N-acetylhexosamines in the urine. The intent was to develop a procedure that would give a visual result relatively quickly and that could be altered slightly to provide quantitation if desired.

METHODS AND MATERIALS

Chemical Reagents

Reagents were purchased as follows: N-acetylglucosamine 6-sulfate and p-dimethylaminobenzaldehyde, Sigma Chemical Company (St. Louis, Missouri.); Dowex AG 1-X8, 200-400 mesh, chloride form, Bio Rad (Richmond, California); and potassium tetraborate, Fisher Scientific (Fair Lawn, New Jersey). All other chemicals were of reagent grade.

The Method of Reissig

The method of Reissig et al. (1955) was modified as follows. Two reagents were used: a potassium tetraborate solution, 0.6 M with respect to borate (B_4O_7), adjusted to pH 9.5 with concentrated HCl and a solution consisting of 1 g of dimethylaminobenzaldehyde (DMAB) dissolved in 10 ml of glacial acetic acid containing 12.5% (V/V) of 10 N HCl. The stock DMAB reagent was diluted immediately prior to use with 9 volumes of glacial acetic acid to one volume of the stock solution. A 500 µl sample was heated in boiling water for 3 min after the addition of 100 µl of the potassium tetraborate reagent. The sample was then cooled for 3 min in a room temperature water bath followed by the addition of 3 ml of the diluted DMAB reagent and incubation in a 37 °C water bath for 20 min. It was again cooled for 3 min in a room temperature water bath, and the absorbance was measured 1.0 cm quartz cuvettes at 585 nm in a Bausch & Lomb Spectronic 2000 spectrophotometer. The reagent volumes were scaled up proportionately for assay of the 1 ml samples in the screening procedure. The following characteristics of the modified Reissig assay were determined: pH optimum of the tetraborate buffer, change in

absorbance with time, absorbance with respect to concentration of GlcNAc(6S), repeatability for consecutive assays of 10 μg standards, and effect of NaCl.

Sample preparation

The urine sample from an MPS IIID patient was provided by Drs. Edwin Kolodny and Marvin Natowicz (Waltham, Massachusetts). All others were recovered from frozen storage (UAB Laboratory of Medical Genetics, Birmingham, Alabama). Urine samples were prepared by bench-top centrifugation for 1 min at 1,950 rpm. Aliquots of the supernatant were used for determination of creatinine values and for the screening and quantitation techniques described below.

Development of the Screening Procedure

Consideration was first given to direct assay of whole urine. The absorbance of whole urine from 5 normal controls was measured at 585 nm to determine background interference. Urine from normal controls was used to simulate affected patients by adding GlcNAc(6S). Treated and untreated samples were assayed by the modified method of Reissig. A natural background absorbance at 585 nm and a strong Reissig-positive reaction even in untreated samples eliminated the use of whole urine as the screening sample. Since the N-acetylhexosamine of interest was sulfated, anion-exchange chromatography was employed as an isolation technique. A sample size equivalent to 2 μmol of creatinine was calculated, based on the predicted absorbance by the modified method of Reissig, for patients having urinary levels of sulfated N-acetylhexosamines equivalent to those found by Hopwood and Elliott (1985). The levels of GlcNAc(6S) and GalNAc(6S) reported for normals and MPS patients were added and ordered numerically (Table 1). The GalNAc(6S) component was reduced by two-thirds, in accordance with its reduced color formation in this reaction. The reported levels of GalNAc(4S) and GalNAc(4,6 diS) were not considered since substitution at position 4 eliminates color production. Samples were eluted with NaCl from 0.5 ml of Dowex AG1 - X8, 200-400

mesh, chloride form, in disposable polyethylene filter columns (Fisher). The elution profile was reduced systematically by increasing the NaCl concentration to obtain the maximum N-acetylhexosamine content in a single fraction. Control urines, treated with GlcNAc(6S) to simulate affected patients, were used to determine the recovery of GlcNAc(6S) from the anion-exchange columns used in both the screening and quantitation procedures described below.

Screening procedure

A urine sample equivalent to 2 μmol of creatinine was applied to 0.5 ml of Dowex AG 1-X8, 200-400 mesh, chloride form. The void, three 0.5 ml fractions eluted with 0.015 M NaCl and one 0.5 ml fraction eluted with 2 M NaCl were discarded. Next, a 1.0 ml fraction eluted with 2 M NaCl was collected in a 13 x 100 mm test tube and assayed by the modified method of Reissig for N-acetylhexosamine content along with a standard of 20 μg of GlcNAc(6S) in 1.0 ml of water. The color and absorbance of this single fraction were recorded.

Quantitation procedure

A sample equivalent to 2 μmol creatinine, in a minimum volume of 500 μl, was applied to 0.5 ml of Dowex as in the screening procedure above. A 0.5 ml fraction of the void, three 0.5 ml fractions eluted with 0.15 M NaCl, and eight 0.5 ml fractions eluted with 2 M NaCl were analyzed for N-acetylhexosamine content along with a standard of 10 μg GlcNAc(6S) in 500 μl of water. Fractions 4 through 11 represented charged material, and the absorbances of these fractions were added to calculate the total charged Reissig-positive material.

Table 1

N-acetylhexosamine values from Hopwood & Elliott (1985) adjusted to predict expected absorbances for screening and quantitation procedures

	A*	B+	C†	D**	E++	F††
SAMPLE	(μmol/mmol Creatinine)			(μg)	(Absorbance @ 585 nm)	
Normal	0.03	0.00	0.03	0.02	0.000	0.000
Mucolipidosis II	-	0.12	0.12	0.08	0.000	0.001
a-Mannosidosis	0.09	0.04	0.13	0.08	0.000	0.001
Normal	0.13	0.03	0.16	0.10	0.000	0.001
Normal	0.19	0.06	0.25	0.16	0.001	0.002
MPS VIB	0.38	0.28	0.66	0.42	0.001	0.005
MPS IS	0.82	0.08	0.90	0.58	0.002	0.006
MPS IIIB)	0.95	0.06	1.01	0.65	0.002	0.007
MPS IIB	0.92	0.27	1.19	0.77	0.002	0.008
MPS VIA #3	0.95	0.64	1.59	1.03	0.003	0.011
MPS IIIA	1.27	0.42	1.69	1.09	0.003	0.012
MPS VIA #2	1.86	0.51	2.37	1.53	0.005	0.017
MPS VIA #1	2.12	0.67	2.79	1.80	0.006	0.020
MPS IIA	3.80	0.50	4.30	2.78	0.009	0.031
___PREDICTED CUT-OFF (ABSORBANCE = 0.01) FOR SCREENING PROCEDURE____						
MPS IVA #1	0.32	5.80	6.12	3.96	0.012	0.044
Multiple Sulfatase	8.86	3.16	12.02	7.77	0.024	0.086
MPS IH	12.66	0.00	12.66	8.18	0.025	0.090
MPS IVA #2	6.33	11.29	17.62	11.38	0.035	0.126
MPS IIID #2	34.81	0.47	35.28	22.79	0.071	0.252
MPS IIID #1	53.80	0.00	53.80	34.75	0.108	0.384

*A. GlcNAc(6S) +B. 1/3 GalNAc(6S) †C. Sum of columns A & B giving GlcNAc(6S) equivalent **D. μg of GlcNAc(6S) equivalent in 2 μmol creatinine ++E. Expected absorbance in screening procedure based on 27% recovery ††F. Expected total absorbance in quantitation procedure based on 96% recovery

RESULTS

The pH optimum for the potassium tetraborate reagent was 9.5. Absorbance was linear to at least 10 μg GlcNAc(6S) in 500 μl total volume. Color was stable with only a 2% decrease in absorbance at 25 min. Twenty consecutive 10 μg GlcNAc(6S) standards had a mean absorbance of 0.115 (± .005). The standard produced a light pink color, and the lowest absorbance that consistently gave an easily recognizable faint pink color was 0.01. Color development was not affected for NaCl concentrations up to at least 2 M.

The mean background absorbance at 585 nm for whole urine from 5 normal individuals was 0.054 (± 0.033). The absorbances ranged from 0.031 to 0.111 and were not related to creatinine value. Direct assay of untreated whole urine samples showed strong yellow-brown color development, and even though samples treated with GlcNAc(6S) had greater color formation with a pink cast, the difference was not considered sufficient for visual separation as would be required for a useful screening test.

Mean recovery of GlcNAc(6S) from the Dowex elution of treated controls (i.e., simulated patients) was 96% in the quantitation procedure and 27% in the screening procedure. Results of the screening and quantitation procedures, performed as outlined in Methods and Materials, on an aliquot of urine equivalent to 2 μmol of creatinine, for 23 normal controls and 6 patients with mucopolysaccharidoses are shown in Table 2. The elution profiles for the quantitation of these same patients and controls are shown in Figure 1.

Table 2

Screening and Quantitation Results For Normal Controls and MPS Patients

	----------SCREENING----------		QUANTITATION
Sample	Color	Absorbance	Total Absorbance Fractions 4-11[a]
Blank	Pale Yellow-Green	0.0	-
Control #1	Same as blank	0.001	0.005
Control #2	Same as blank	0.002	0.0
Control #3	Same as blank	0.0	0.001
Control #4	Same as blank	0.0	0.002
Control #5	Same as blank	0.002	0.002
Control #6	Same as blank	0.001	0.0
Control #7	Same as blank	0.0	0.002
Control #8	Same as blank	0.002	0.002
Control #9	Same as blank	0.0	0.001
Control #10	Very faint orange[b]	0.004	0.001
Control #11	Same as blank	0.0	0.0
Control #12	Same as blank	0.0	0.0
Control #13	Same as blank	0.0	0.008
Control #14	Same as blank	0.0	0.004
Control #15	Same as blank	0.0	0.0
Control #16	Same as blank	0.0	0.001
Control #17	Same as blank	0.001	0.003
Control #18	Same as blank	0.0	0.0
Control #19	Same as blank	0.001	0.007
Control #20	Same as blank	0.0	0.0
Control #21	Same as blank	0.0	0.0
Control #22	Same as blank	0.0	0.004
Control #23	Same as blank	0.0	0.0
Sanfilippo B	Same as blank	0.008	0.0
----------	----------	---------- [c]	
Sanfilippo A	Faint pink	0.012	0.022
Maroteaux-Lamy	Faint pink	0.012	0.036
Morquio A	Faint pink	0.012	0.037
Sanfilippo D	Pink/yellow[b]	0.028	0.100
Multiple sulfatase	Light pink	0.027	0.127

[a] Represents charged Reissig-positive material, presumably sulfated [b] The source of the yellow/orange coloration has not yet been identified [c] Visible cut-off for screening procedure (Absorbance = 0.01)

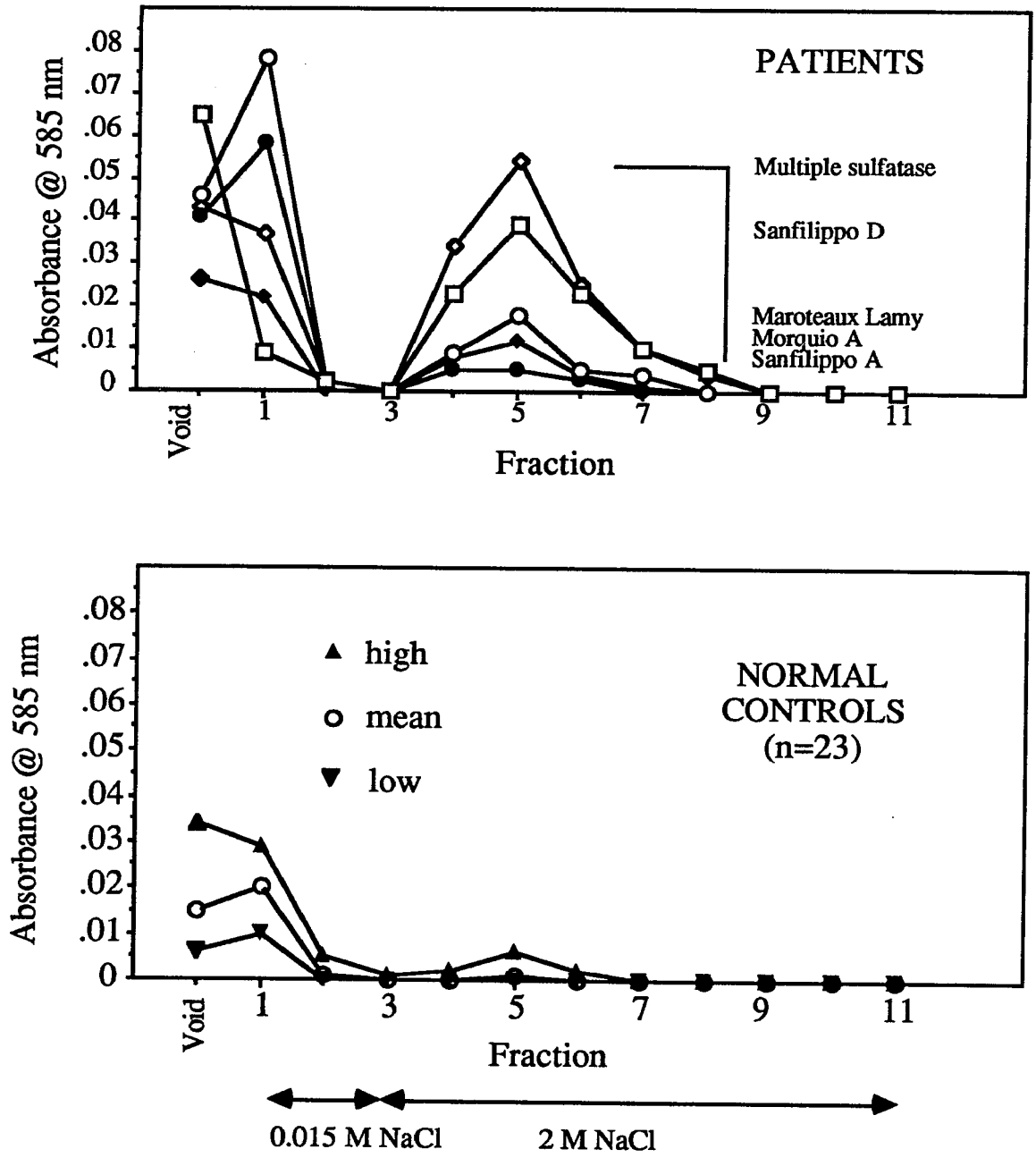


Figure 1. Elution profile of the quantitation procedure for normal controls and patients with mucopolysaccharidoses. Quantitation procedure described under Methods and Materials

DISCUSSION

The natural absorption of urine at 585 nm as well as the background color development in the direct assay of whole urine was effectively reduced by limiting the urine sample size to the equivalent of 2 μmol of creatinine in a minimum volume of 500 μl and by employing anion exchange chromatography to remove nonanionic but Reissig-positive material.

The screening procedure clearly distinguished MPS IIID, MPS IIIA, Maroteaux-Lamy, Morquio syndrome, type A, and multiple sulfatase deficient patients from the normal controls. The values found for known patients were quite close to the anticipated results predicted from the data of Hopwood and Elliott (1985); for example, the screening test was predicted (Table 1) to distinguish patients with MPS IIID, Morquio syndrome, type A, and multiple sulfatase deficiency from normal controls. The patient with Maroteaux-Lamy syndrome also was distinguished from normals, and the multiple sulfatase deficient patient had values of sulfated N-acetylhexosamine closer to those of the MPS IIID patient than reported previously. No particular significance is placed on these results since the total number of patients in the two studies is still relatively small and intertype variability is not uncommon in the MPS disorders. Additionally, the patients in this study with MPS IIID and multiple sulfatase deficiency had similar levels of GlcNAc 6-sulfatase activity in cultured skin fibroblasts (4% and 8% of normal, respectively), which implies that the levels of GlcNAc(6S) in their urines also might be similar.

The potential for false positives appears low. Only one of the 23 controls differed from the blank on the screening procedure. The aberrant-appearing control had a faint

orange background, but the absorbance at 585 nm was only 0.004 and this was substantially different from the affected patients whose absorbances ranged from 0.008 to 0.028. The additional step of measuring the absorbance on the screening procedure when a questionable color results is easily accomplished and keeps the overall screening procedure simple.

The DMAB reagent is stable up to one month if refrigerated (Reissig et al. 1955), and we have found the potassium tetraborate reagent to be stable for at least two weeks at room temperature. The screening procedure gives a visual result which can be extended for spectrophotometric quantitation if desired. Such quantitation, however, does not distinguish between the contribution of GlcNAc(6S) and GalNAc(6S) which would be desirable to separate patients with Morquio syndrome, type A, from those with Sanfilippo syndrome, type D, although a good clinical summary should accomplish the same goal. The screening test presented is relatively rapid, sensitive, and inexpensive to perform. Used in conjunction with others, it offers the advantage of additional specificity in that a strong positive result, in our experience to date, implies one of two disorders and gives direction to the subsequent assays for enzyme deficiencies.

APPENDIX D

Diagnosis of Feline G_{M1} Gangliosidosis by Enzyme Assay of Cultured Conjunctival Cells

Investigative Ophthalmology & Visual Science, Vol. 29, No. 3, March 1988

Diagnosis of Feline G_{M1} Gangliosidosis by Enzyme Assay of Cultured Conjunctival Cells

Rod W. Nowakowski,* Jerry N. Thompson,† and Henry J. Baker‡

G_{M1} gangliosidosis is characterized by a deficiency in the lysosomal hydrolase β-galactosidase, progressive nervous system disease and ocular lesions. Diagnosis of G_{M1} gangliosidosis in humans and cats with the analogous disease has been made by measurement of the enzyme activity in various tissues including brain, liver and cultured skin fibroblasts. The authors report the use of cultured conjunctival cells for this purpose derived from cats with feline G_{M1} gangliosidosis, a model of the human disease (juvenile G_{M1} gangliosidosis, Derry's disease). Full thickness conjunctival biopsies from three cats with G_{M1} gangliosidosis and two normal controls were used to initiate cell cultures. Optimal conditions for β-galactosidase activity were established with an uncultured conjunctival biopsy from a normal cat. The fluorgen, 4-methylumbelliferyl-β-D-galactopyranoside was used as substrate. After 2 months in culture, and 2 weeks after subculture, cells from cats affected with G_{M1} gangliosidosis exhibited specific activities for β-galactosidase of 10, 9 and 12 nmoles 4MU/hr/mg protein, whereas specific activities for normals were 630 and 469 nmoles 4MU/hr/mg. Enzymatic analysis of cultured conjunctival cells may offer an effective alternative for the diagnosis of G_{M1} gangliosidosis. Invest Ophthalmol Vis Sci 29:487–490, 1988

G_{M1} gangliosidosis, a genetic disorder of man and other animals, is characterized by a deficiency in a lysosomal enzyme, acid β-galactosidase. In humans, three phenotypes exist, infantile, juvenile and adult.[1] They can be distinguished by their age of onset and clinical findings. In the infantile form, the most severe, a cherry-red spot may be seen in the macula in approximately 50% of the cases. Diagnosis of all types is confirmed by the demonstration of a deficiency in the acid β-galactosidase in various tissues and body fluids. Leukocytes, tears, urine and cultured fibroblasts have been used for enzyme assay. Heterozygote detection and prenatal diagnosis are possible.

Defective catabolism of G_{M1} ganglioside has also been documented in cats, dogs and cattle and has been reviewed previously.[2] Biochemical and ultrastructural studies in cats with G_{M1} gangliosidosis have shown remarkable similarity to the juvenile (Type II or Derry's) disease in man.[3] The deficiency of acid β-galactosidase in affected cats has been demonstrated for various tissues including brain, liver, skin and cultured fibroblasts.[4]

The demonstration of morphological changes in the ultrastructure of conjunctiva has been used in the diagnosis of metabolic diseases.[5–9] The present study extends the use of conjunctiva through enzymatic analysis of cells cultured from conjunctival biopsies.

An additional aspect of this investigation was to determine acid β-galactosidase activity of conjunctival cells from a normal cat at initial biopsy and after successive passages in culture. Previous investigators[10,11] have shown that enzyme activities in cell lines from different tissues vary with time in culture. This has not been reported for β-galactosidase in feline conjunctival cells. Any variation would be important to know since normal controls are used for comparison when an assay is performed to diagnose an enzyme deficiency.

Materials and Methods

Optimal conditions for β-galactosidase activity using 4-methylumbelliferyl-β-D-galactopyranoside (Sigma Chemicals, St. Louis, MO) as substrate were established with an uncultured conjunctival biopsy taken from a normal cat under general anesthesia.

From the *Department of Optometry, the †Laboratory of Medical Genetics and Departments of Pediatrics and Biochemistry, and the ‡Department of Comparative Medicine, University of Alabama at Birmingham, Birmingham, Alabama.

Supported in part by BRSG S07 RR05807 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, NS 10967 awarded by the National Institute of Neurological, Communicative Disorders and Stroke, National Institutes of Health, the Department of Health and Human Services (Maternal and Child Health Project 905) and the Clinical Service grant (C426) from the March of Dimes Birth Defects Foundation.

Submitted for publication: January 23, 1987; accepted October 8, 1987.

Reprint requests: Rod W. Nowakowski, OD, UAB School of Optometry, 1716 University Boulevard, Birmingham, AL 35294.

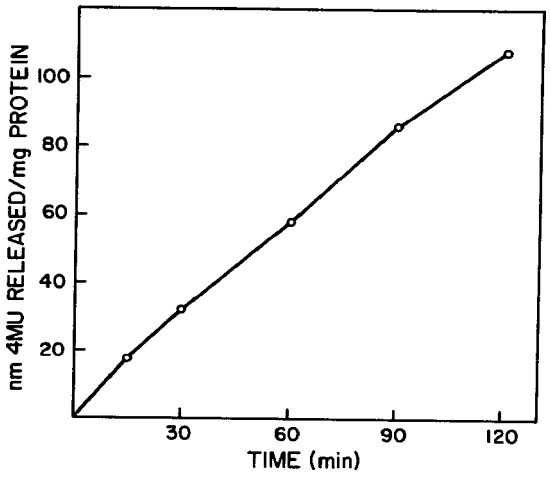


Fig. 1. Activity of acid β-galactosidase as a function of time. Enzyme source was homogenate of uncultured conjunctival biopsy from a normal cat. Assay conditions are described under *Materials and Methods.*

The tissue was placed in 1.5 ml of 0.5% n-octyl-β-D-glucoside and homogenized by 20 strokes in a Dounce homogenizer. The homogenizer was rinsed four times with 100 μl/rinse of 0.5% octylglucoside (Sigma). The total, homogenate and washings, was then transferred to a centrifuge tube and centrifuged at 5°C for 30 min at 10,000 *g*. The supernatant was retained for assay. Determination of enzyme activity was carried out in a total volume of 0.5 ml by modification of a previously reported procedure.[12] The optimized reaction mixture consisted of 0.1 M sodium acetate, pH 4.5, 0.05 M NaCl, 2.5 mM 4-methylumbelliferyl-β-D-galactopyranoside and conjunctival supernatant. After 2 hr at 37°C, the reaction was stopped by the addition of 2.5 ml glycine-NaOH buffer, pH 11. Fluorescence was determined at 365 (excitation) and 450 (emission) nm. Specific activity was expressed as nmoles 4-methylumbelliferone released/hr/mg protein. Protein was determined by the method of Lowry et al.[13]

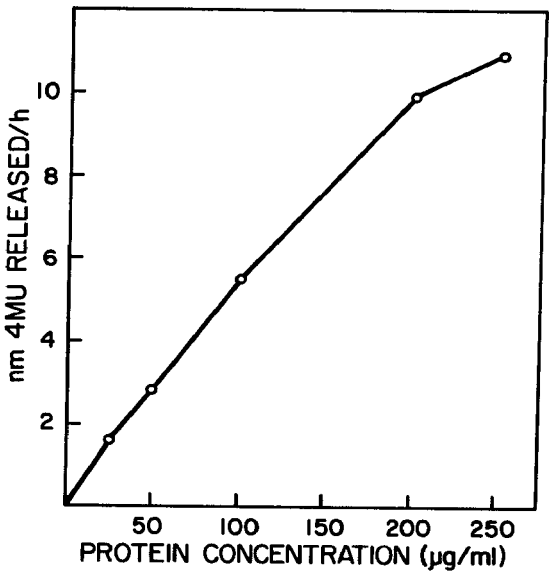


Fig. 2. Release of 4-methylumbelliferone as a function of enzyme concentration. Enzyme source was homogenate of uncultured conjunctival biopsy from a normal cat. Assay conditions are described under *Materials and Methods.*

Full thickness conjunctival biopsies taken under sterile conditions from anesthetized cats with G_{M1} gangliosidosis and from normal controls were used to initiate cell cultures. Several tissue pieces approximately 1 mm^2 in size were placed in 25 cm^2 Corning tissue culture flasks and incubated at 37°C in McCoy's 5A medium supplemented with 10% fetal bovine serum and 1% antibiotics. Medium was changed twice weekly and cells were subcultured after reaching confluency. Cultures were maintained for 2 months. Two weeks after the last passage, the cells were harvested, sonicated in 0.15 M NaCl, centrifuged at 10,000 *g* for 30 min at 5°C and the supernatant used for the determination of β-galactosidase activity. Cultured cells from three mutant and two normal cats were studied.

A separate conjunctival biopsy from the normal cat used in the optimization study was placed in culture, in the manner indicated above, in order to determine the specific activity at successive passages in culture. At 2 week intervals, some cultures were harvested, sonicated in 0.5% octylglucoside, centrifuged and the supernatant assayed for β-galactosidase activity as indicated above while the remaining cells were subcultured and maintained for the next 2 week interval with twice weekly changes of medium.

All animals used in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Results

The activity of β-galactosidase in uncultured conjunctival tissue from a normal cat, as a function of time exposed to substrate, is shown in Figure 1. The activity did not show a substantial deviation from linearity over 2 hr of incubation. Activity was linear with enzyme concentration to 100 μg/ml (Fig. 2). The optimum pH for substrate hydrolysis was 4.5 (Fig. 3). Stimulation of β-galactosidase hydrolytic activity by NaCl, shown in Figure 4, demonstrates peak activity

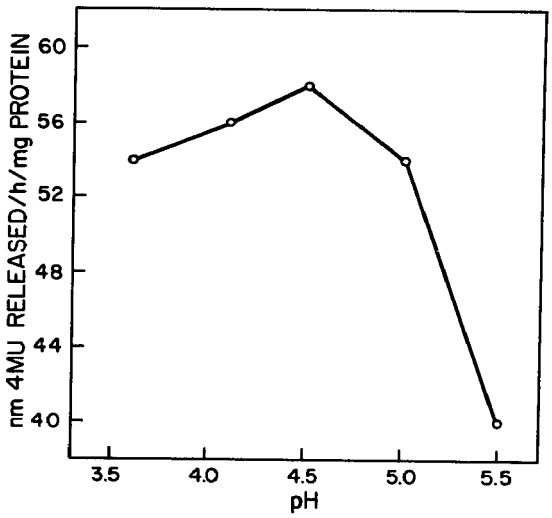


Fig. 3. Specific activity of acid β-galactosidase as a function of reaction mixture pH. Enzyme source was homogenate of uncultured conjunctival biopsy from a normal cat. Assay conditions are described under *Materials and Methods.*

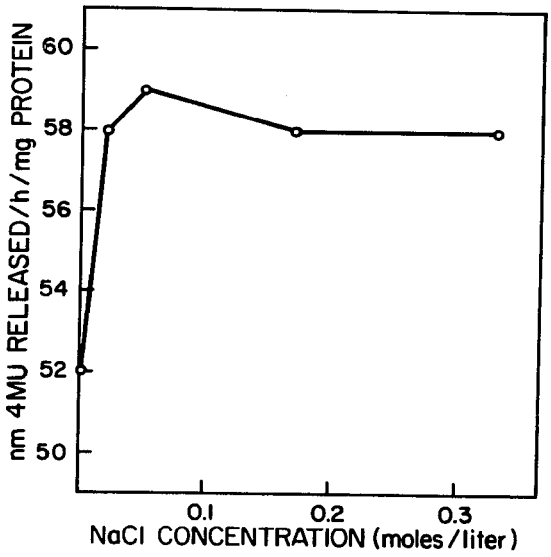


Fig. 4. Specific activity of acid β-galactosidase as a function of sodium chloride concentration in reaction mixture. Enzyme source was homogenate of uncultured conjunctival biopsy from a normal cat. Assay conditions are described under *Materials and Methods.*

at 0.05 M. Under the above conditions, the specific activity in the uncultured conjunctival biopsy from a normal cat was 59 nmoles 4MU/hr/mg protein.

The specific activity of β-galactosidase of the cultured conjunctival cells from a normal cat was determined biweekly. Table 1 shows that the specific activity increased with each successive passage. The cells in the initial outgrowth from the biopsy were epithelial-like in appearance. After the first passage, and subsequently, the predominant cell type noted in culture was fibroblast-like, as shown in Figure 5.

The specific activities for the three mutants using the optimized conditions above were 10, 9 and 12 nmoles 4MU/hr/mg protein and for the two normals were 630 and 469 nmoles 4MU/hr/mg protein after 8 weeks in culture. The average value of the specific activities for the mutants was reduced to approximately 2% of that for the normals. Within each group the relative range was virtually identical with the lowest value being 75% of the highest for the mutant cats and 74.4% for the normal cats.

A mixture of supernatants from a normal and an affected cat containing equal amounts of protein had a specific activity higher than the expected average indicating that an inhibitor was not present (data not shown).

By comparing specific activities of reaction mixtures containing octylglucoside versus water for both a normal and an affected cat it was also demonstrated that the presence of octylglucoside did not adversely affect the specific activity of acid β-galactosidase. In fact, the specific activity was slightly higher in the mixture containing octylglucoside for both the normal and the affected cat (data not shown).

Discussion

Cats with G_{M1} gangliosidosis were distinguished clearly from normals on the basis of the specific activity of acid β-galactosidase in cultured conjunctival cells. The increasing specific activity with time in culture implies that a comparison of acid β-galactosidase activity between affected and normal cats could be substantially altered if any change in specific activity dependent on time in culture were not taken into consideration. Enzymatic analysis of cultured cells from a conjunctival biopsy may be an effective alternative means of diagnosis when G_{M1} gangliosidosis is suspected in humans. The procedure is easily per-

Table 1. Specific activity of acid β-galactosidase in normal cat conjunctival cells with successive passages at 2 week intervals

Cells	*Passage*	*Weeks*	*Specific activity (nm 4MU released/ hr/mg protein)*
Uncultured	0	0	59
Cultured	1	2	96
Cultured	2	4	290
Cultured	3	6	441

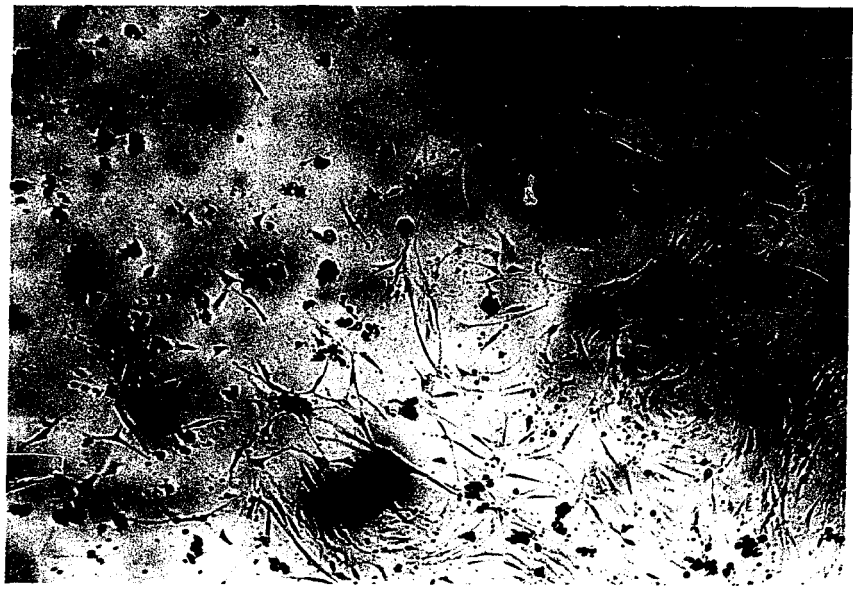


Fig. 5. Feline conjunctival cells in culture. Fibroblast-like cells are attached and epithelial-like cells from the initial biopsy outgrowth are seen as free-floating dark clumps. Magnification ×41.

formed with a topical anesthetic.[5-7] It is safe and, unlike skin biopsies, is nondisfiguring.

Key words: β-galactosidase, cat, G_{M1} gangliosidosis, tissue culture, conjunctiva

References

1. O'Brien JS: The gangliosidoses. *In* The Metabolic Basis of Inherited Disease, 5th Ed, Stanbury JB, Wyngaarden JB, Frederickson DS, Goldstein, JL, and Brown MS, editors. New York, McGraw-Hill Book Company, 1983, pp. 945–969.
2. Baker HJ, Reynolds GD, Walkley SU, Cox NR, and Baker GH: The gangliosidoses: Comparative features and research application. Vet Pathol 16:635, 1979.
3. Farrell DF, Baker HJ, Herndon RM, Lindsey JR, and McKhann GM: Feline GM_1 gangliosidosis: Biochemical and ultrastructural comparisons with the disease in man. J Neuropathol Exp Neurol 32:1, 1973.
4. Baker HJ and Lindsey JR: Animal model of human disease: Feline GM_1 gangliosidosis. Am J Pathol 74:650, 1974.
5. Arsenio-Nunes ML, Goutières F, and Aicardi J: An ultramicroscopic study of skin and conjunctival biopsies in chronic neurological disorders of childhood. Ann Neurol 9:163, 1980.
6. Kenyon KR: Conjunctival biopsy for diagnosis of lysosomal disorders. *In* Clinical, Structural, and Biochemical Advances in Hereditary Eye Disorders. New York, Alan R. Liss, Inc., 1982, pp. 104–122.
7. Libert J: Diagnosis of lysosomal storage diseases by the ultrastructural study of conjunctival biopsies. Pathol Annu 15(Pt I):37, 1980.
8. Quigley HA and Goldberg MF: Conjunctival ultrastructure in mucolipidosis III (pseudo-Hurler polydystrophy). Invest Ophthalmol 10:568, 1971.
9. Van Hoof F, Libert J, Aubert-Tulkens G, and Serra MV: The assay of lacrymal enzymes and the ultrastructural analysis of conjunctival biopsies: New techniques for the study of inborn lysosomal diseases. Metab Ophthalmol 1:165, 1977.
10. Cristofalo VJ, Parris N, and Kritchevsky D: Enzyme activity during the growth and aging of human cells in vitro. J Cell Physiol 69:263, 1967.
11. Wang KM, Rose NR, Bartholomew EA, Balzer M, Berde K, and Foldvary M: Changes of enzymic activities in human diploid cell line WI-38 at various passages. Exp Cell Res 61:357, 1970.
12. Holmes EW and O'Brien JS: Feline GM_1 gangliosidosis: Characterization of the residual liver acid β-galactosidase. Am J Hum Genet 30:505, 1978.
13. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 193:265, 1951.

REFERENCES

Aminoff D., Morgan, W. J. T. & Watkins, W. M. (1952). Studies in immunochemistry. II. The action of dilute alkali on the N-acetylhexosamines and the specific blood-group mucoids. The Journal of Biological Chemistry, 51,379-389.

Austin, E., Armstrong, D. & Shearer, L. (1965). Metachromatic form of diffuse cerebral sclerosis. V. The nature and significance of low sulfatase activity: A controlled study of brain, liver, and kidney in four patients with metachromatic leukodystrophy. Archives of Neurology, 13, 593-614.

Basner, R., von Figura, K, Glössl, J., Klein, U., Kresse, H., & Mlekusch, W. (1979a). Multiple deficiency of mucopolysaccharide sulfatases in mucosulfatidosis. Pediatric Research, 13, 1316-1318.

Basner, R., Kresse, H., & von Figura, K. (1979b). N-acetylglucosamine 6-sulfate sulfatase from human urine. The Journal of Biological Chemistry, 254, 1151-1158.

Boustany, R-M N., Openheimer E., Johns, D.R., Horwitz, A.L. & Kolodny, E.H., (1988). Poster presentation at the First International Congress on Mucopolysaccharidosis and Related Diseases, Minneapolis, Minnesota, May 20-22.

Coppa, G.V., Giorgi, P.L., Felici, L., Gabrielli, O., Donti, E., Bernasconi, S., Kresse, H., Paschke, E., & Mastropaolo, C. (1983). Clinical heterogeneity in Sanfilippo disease (mucopolysaccharidosis III) type D: presentation of two new cases. European Journal of Pediatrics, 140, 130-133.

Daniel, W.W. (1983). Biostatistics: A foundation for analysis in the health sciences (3rd ed.). (pp. 86-89). New York: John Wiley & Sons.

Dodgson, K.S., & Loyd, A.G. (1961). Potassium glucose 6-O-sulphate as a substrate for glycosulphatase. The Journal of Biological Chemistry, 78, 319-324.

Elliott, H. & Hopwood, J.J. (1984). Detection of the Sanfilippo D syndrome by the use of a radiolabeled monosaccharide sulfate as the substrate for the estimation of N-acetylglucosamine 6-sulfate sulfatase. Analytical Biochemistry, 138, 205-209.

Elveback, L.R., Guillier, C.L., & Keating, Jr., F.R. (1970). Health, normality, and the ghost of Gauss. The Journal of the American Medical Association, 211, 69-75.

Fluharty, A.L., Stevens, R.L., Davis, L.L., Shapiro, L.J. & Kihara, H. (1978). Presence of arylsulfatase A (ARS A) in multiple sulfatase deficiency disorder fibroblasts. The American Journal of Human Genetics, 30, 249-255.

Flynn, F.V., Piper, K.A.J., Garcia-Webb, P., McPherson, K., & Healy, M.J.R. (1974). The frequency distributions of commonly determined blood constituents in healthy blood donors. Clinica Chimica Acta, 52, 163-171.

Freeman C. & Hopwood, J.J. (1987). Human liver N-acetylglucosamine-6-sulphate sulphatase. Biochemical Journal, 246, 355-365.

Fuchs, W., Beck, M., & Kresse, H. (1985). Intralysosomal formation and metabolic fate of N-acetylglucosamine 6-sulfate from keratan sulfate. European Journal of Biochemistry, 151, 551-556.

Gatti, R., Borrone, C., Durand, P., De Virgilis, S., Sanna, G., Cao, K., von Figura, K., Kresse, H., & Paschke, E. (1982). Sanfilippo type D disease: Clinical findings in two patients with a new variant of mucopolysaccharidosis III. European Journal of Pediatrics, 138, 168-171.

Glössl, J., Truppe, W., & Kresse, H. (1979). Purification and properties of N-acetyl galactosamine 6-sulphate sulphatase from human placenta. The Journal of Biological Chemistry, 181, 37-46.

Harris, E.K., & DeMets, D.L. (1972). Estimation of normal ranges and cumulative proportions by transforming observed distributions to Gaussian form. Clinical Chemistry, 18, 605-612.

Harris, R.C. (1961). Mucopolysaccharide disorder: A possible new genotype of Hurler's syndrome. American Journal of Diseases of Children, 102, 741.

Haust, M.D., & Gordon, B.A. (1986). Ultrastructural and biochemical aspects of the Sanfilippo syndrome,-Type III genetic mucopolysaccharidosis. Connective Tissue Research, 15, 57-64.

Hopwood, J.J., & Elliott, H. (1981). Selective depolymerization of heparin to produce radiolabelled substrates for sulfamidase, 2-acetamido-2-deoxy-α-D-glucosidase, acetyl-CoA:2-amino-2-deoxy-α-D-glucoside N-acetyl transferase, and 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase. Carbohydrate Research, 91, 165-190.

Hopwood, J.J. & Elliott, H. (1983a). N-Acetylglucosamine 6-sulfate residues in keratan sulfate and heparan sulfate are desulfated by the same enzyme. Biochemistry International, 6, 141-148.

Hopwood, J.J. & Elliott, H. (1983b). Isolation and characterization of N-acetylglucosamine 6-sulfate from the urine of a patient with Sanfilippo type D 836.

Hopwood J. J. & Elliott, H. (1985). Urinary excretion of sulphated N-acetylhexosamines in patients with various mucopolysaccharidoses. Biochemical Journal, 229. 579-586.

Horwitz, A.L. (1979). Genetic complementation studies of multiple sulfatase deficiency. Proceedings of the National Academy of Sciences of the United States of America, 76, 6496-6499.

Ishihara, K., Sobue, M., Uemura, D., Tsuji, M., Nakanishi, Y., & Suzuki, S. (1976). N-acetylglucosamine 4,6-bisulfate in rat urine: isolation, identification and chemical synthesis. Biochimica et Biophysica Acta, 437, 416-430.

Jeanloz, T. & Trémége, M. (1956). Color reaction of N-acetylamino sugar derivatives with p-dimethylaminobenzaldehyde. Federation Proceedings, 15, 282.

Kaplan, P. & Wolfe, L.S. (1987). Sanfilippo syndrome type D. Journal of Pediatrics, 110, 267-271.

Kenyon. K.R. (1982). Conjunctival biopsy for diagnosis of lysosomal disorders. In D.L. Daentl, (Ed). Clinical, Structural, and Biochemical Advances in Hereditary Eye Disorders (pp. 103-122). New York, NY: Alan R. Liss, Inc.

Kresse, H., Paschke, E., von Figura, K., Gilberg, W., & Fuchs, W. (1980). Sanfilippo disease type D: Deficiency of N-acetylglucosamine 6-sulfate sulfatase required for heparan sulfate degradation. Proceedings of the National Academy of Sciences of the United States of America, 77, 6822-6826.

Kresse, H., Fuchs, W., Glössl, J., Holtfrerich, D., & Gilberg, W. (1981). Liberation of N-acetylglucosamine-6-sulfate by human β-N-acetylhexosaminidase A. The Journal of Biological Chemistry, 256, 12926-12932.

Kresse, H., von Figura, K., Klein, U., Glössl, J., Paschke, E., & Pohlmann, R. (1982). Enzymic diagnosis of the genetic mucopolysaccharide storage disorders. Methods in Enzymology, 83, 559-572.

Libert, J. (1980). Diagnosis of lysosomal storage diseases by the ultrastructural study of conjunctival biopsies. Pathology Annals, 15(Pt I), 37-66.

Lloyd, A.G. (1960). Studies on sulphatases. Preparation of substrates for the assay of glycosulphatase. The Journal of Biological Chemistry, 75, 478-482.

Lloyd, A.G. (1962). Fractionation of the products of the direct sulphation of monosaccharides on anion-exchange resin. Biochemical Journal, 83, 455-460.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry, 193, 265-275.

Matalon, R., Wappner, R., Deanching, M., Brandt, I.K., & Horwitz, A. (1982). Keratan and heparan sulfaturia: Glucosamine 6-sulfate sulfatase deficiency. Annals of Clinical and Laboratory Science, 12, 234-238.

McKusick, V.A. & Neufeld, E.F. (1983). The mucopolysaccharide storage diseases. In J.B. Stanbury, Wyngaarden, J.B., Frederickson, D.S., Goldstein, J.L., and Brown, M.S. (Eds). The Metabolic Basis of Inherited Disease (5th ed), (pp. 751-777). New York, NY: McGraw-Hill Book Company.

Morgan, W. T. J., & Elson, L. A. (1934). A colorimetric method for the determination of N-acetylglucosamine and N-acetylchondrosamine. Biochemical Journal, 28, 988-995.

Nelson, J. C., Haynes, E., Willard, R., & Kuzma, J. (1971). The distribution of euthyroid serum protein-bound iodine levels. The Journal of the American Medical Association, 216, 1639-1641.

Nowakowski, R.W., Thompson, J.N. & Taylor, K.B. (1988a). A spectrophotometric assay for Sanfilippo D syndrome with prenatal diagnostic potential. The American Journal of Human Genetics (Supplement), 43(3), A 242.

Nowakowski, R.W., Thompson, J.N. & Baker, H.J. (1988b). Diagnosis of feline G_{M1} gangliosidosis by enzyme assay of cultured conjunctival cells. Investigative Ophthalmology and Visual Science, 29, 487-490.

Reissig, J.L., Strominger, J.L. & Leloir, L.F. (1955). A modified colorimetric method for the estimation of N-acetylamino sugars. The Journal of Biological Chemistry, 217, 959-966.

Robertson, D.A., Callen, D.F., Baker, E.G., Morris, C.P. & Hopwood, J.J. (1988). Chromosomal localization of the gene for human glucosamine-6-sulphatase to 12q14. Human Genetics, 79, 175-178.

Sanfilippo, S.J., Podosin, R., Langer, L., & Good, R.A. (1963). Mental retardation associated with acid mucopolysaccharidurìa (heparitin sulfate type). Journal of Pediatrics, 63, 837-838.

Shapiro, S.S. & Wilk, M.B. 1965. An analysis of variance test for normality of complete samples. Biometrika, 52, 591-611.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., & Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Analytical Biochemistry, 150, 76-85.

Strike, P.W. (1981) Clinical reference values. In Medical laboratory statistics (pp 51-65). Boston: WRIGHT-PSG.

Summers, C.G., Whitley, C.B., Pineda II, R., Ramsay, N.K.C., Kersey, J.H., & Krivit, W. (1988). Paper presented at the First International Congress on Mucopolysaccharidosis and Related Diseases, Minneapolis, Minnesota, May 20-22.

Toma, S., Di Ferrante, D.T., Tenni, R., & Di Ferrante, N. (1981). Preparation from keratan sulfate of substrates for the measurement of 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase and (1→3)-N-acetyl-β-D-glucosaminidase. Carbohydrate Research, 88, 93-105.

Vance J.M., Pericak-Vance, M.A., Elston, R.C., Conneally, P.M., Namboodiri, K.K.,Wappner, R.S., & Yu, P.L. (1980). Evidence of genetic variation for α-N-acetyl-D-glucosaminidase in Black and White populations: A new polymorphism. The American Journal of Medical Genetics, 7, 131-140.

Van de Camp, J.J.P. (1979). The Sanfilippo Syndrome. A clinical and genetical study of 75 patients in the Netherlands. Unpublished master's thesis, University Hospital Leiden, Leiden, The Netherlands.

Van de Camp, J.J.P., Niermeijer, M.F., Von Figura, K., & Giesberts, M.A.H. (1981). Genetic heterogeneity and clinical variability in the Sanfilippo syndrome (types A, B, and C). Clinical Genetics, 20, 152-160.

Wilkinson, G.N. (1961). Statistical estimations in enzyme kinetics. Biochemical Journal, 80, 324-332.

Yutaka, T., Okada, S., Kato, T., Inui, K., & Yabuuchi, H. (1981). Properties of sulfatases in cultured skin fibroblasts on multiple sulfatase deficient patients. Clinical Genetics, 20, 296-303.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Rodney W. Nowakowski

Major Subject Medical Genetics

Title of Dissertation A Spectrophotometric Assay for Sanfilippo Syndrome, Type D (N-Acetylglucosamine 6-Sulfate Sulfatase Deficiency), With Prenatal Diagnostic Potential

Dissertation Committee:

, Chairman Wayne V. Finley

Director of Graduate Program Wayne V. Finley

Dean, UAB Graduate School

Date 3/24/89

PS-1428