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Master Of Science degree in Biology

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Major professor

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Lysosomal Hydrolase Activities in Selected Organs and Central Nervous System Regions of Normal and β-Mannosidase-Deficient Goats

By

Robert James Kranich

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

Lysosomal Hydrolase Activities in Selected Organs and Central Nervous System Regions of Normal and β-Mannosidase-Deficient Goats

By

Robert James Kranich

Goats affected with β-mannosidosis have deficient tissue and plasma levels of the lysosomal enzyme β-mannosidase. Pathological characteristics include cytoplasmic vacuolation in the nervous system and viscera, and myelin deficits that demonstrate regional variation. This study was designed to investigate the distribution of enzyme activities in normal goats, to determine the correlation between β-mannosidase activity in normal animals and lesion severity in affected goats, and to assess regional enzyme activity changes in affected animals. Results indicate that β-mannosidase, α-mannosidase and α-fucosidase, enzymes of glycoprotein catabolism, show higher activity in spinal cord than in cerebral hemispheres, and higher activity in white matter compared to gray matter. Enzyme activity in normal tissue does not appear to be correlated with either the severity of vacuolation or the extent of myelin deficits in affected animals. The mechanism responsible for the organ-specific and CNS region-specific variation needs further investigation.

To my parents, Mary and Fred Kranich, and in memory of my grandmother Alvena Kranich, whose love and encouragement have made this possible.

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TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

	Abbreviation	Full Description
1.	CNS	Central Nervous System
2.	EDA	Ethylenediamine
3.	GlcNAc	N-acetylglucosamine
4.	Fuc	Fucose
5.	Man	Mannose
6.	Gal	Galactose
7.	NeuNAc	N-acetylneuraminidate
8.	Asn	Asparagine
9.	s	Seconds
10.	min	Minutes
11.	4-MU	4-Methylumbelliferone

INTRODUCTION

Caprine β-mannosidosis is a lysosomal storage disease involving N-linked glycoprotein catabolism. Affected goats have deficient tissue and plasma levels of β-mannosidase. Pathological characteristics include cytoplasmic vacuolation in the nervous system and viscera, and myelin deficits that exhibit regional variations. The current study was designed to (1) establish baseline enzyme activities in a statistically significant number of normal animals, and (2) investigate the possible correlation between enzyme activity in normal goats and the regional variation of lesions in β-mannosidase-deficient goats.

LITERATURE REVIEW

Lysosomes are organelles bound by a single lipoprotein membrane containing a characteristic complement of acid hydrolases that function optimally at acidic pH. The surrounding membrane is unique in that it contains transport proteins for the movement of digestive products out of the lysosome, and a H^+ pump that utilizes ATP to move H^+ into the organelle to maintain the low pH {Alberts et al., 1989}. Due to their broad complement of hydrolytic enzymes, these organelles collectively have the ability to digest each class of macromolecules. Although many cell types contain lysosomes, their enzyme complement will depend on the physiological demands of the cell, a concept often referred to as lysosomal heterogeneity {Pitt, 1975}.

In 1965 Hers {Hers, 1965} introduced the concept of lysosomal storage diseases when he described the condition of α-glucosidase deficiency. There are between 30 and 50 such diseases, each dealing with a particular aspect of enzyme production or processing {Durand, 1987}. In cases involving an enzyme deficiency such as β-mannosidosis, the organelle has lost the ability to degrade a particular macromolecule and this enzyme substrate accumulates. This accumulated product can cause cytoplasmic vacuolation and lead to the disruption of cellular function. Other possible causes of lysosomal diseases include the failure to synthesize activator proteins required by some lysosomal enzymes, the failure to synthesize the mannose-6-phosphate recognition marker which is

necessary for targeting many acid hydrolases to the lysosome, or the defective transport of metabolites across the lysosomal membrane {Durand, 1987}. The degree to which each cell type is afflicted can be dependent on the rate at which the uncatabolized material accumulates, the cell's excretory ability, and the lifespan of the cell {Hers, 1965}.

Lysosomal enzymes have been assayed in a variety of species, and their activities have been associated with various physiological functions. The data for enzyme activities are not always in agreement, which may be reflective of different methodologies. Certain lysosomal enzymes appear to have activities that vary with age {Alberghina and Giuffrida-Stella, 1988} or the developmental period of the animal {Verity et al., 1968; Shailubhai et al., 1990}, while in other cases activities may be correlated with a particular morphological event {Zanetta et al., 1980}. Studies have also shown that lysosomal enzyme activities in normal animals exhibit species variation {Freysz et al., 1979; Abe et al., 1979}, regional central nervous system (CNS) variations {Friede and Knoller, 1965}, and tissue-specific expression {Reiner and Horowitz, 1988}. Comparing lysosomal enzyme activities in neuronal, astroglial, and oligodendroglial cell fractions from different species {Abe et al., 1979; Freysz et al., 1979} has indicated that enzyme activities may differ between species when comparing similar cell fractions, and that differential lysosomal enzyme activity exists in different cell types. Yamanaka et al. {1981} have studied the relationship of enzyme activity and substrate turnover to the regional variation of CNS lesions as observed in globoid cell leukodystrophy. In this study, enzyme activity in specific regions of normal dogs could not be correlated with lesions in the same regions of affected animals;

however it was suggested that the metabolic activity of the enzyme substrate may be an important factor in determining the susceptibility or resistance of CNS regions to the lesions involved in globoid cell leukodystrophy.

Glycoproteins consist of an amino acid sequence with O-linked or N-linked oligosaccharides. The pathway of interest for the current study involves the catabolism of carbohydrate moieties of N-linked glycoproteins. Figure 1 summarizes the biochemical pathway for glycoprotein catabolism. Approximately 85-90% of brain glycoproteins have N-glycosidic bonds, and the remaining 10-15% have O-glycosidic linkages {Margolis and Margolis, 1989}. The carbohydrate portion of the molecule has been implicated in a variety of roles including conformational stability, molecular charge, and protease resistance {Paulson, 1989}. Glycoproteins can be found in myelin and myelin-forming cells where they function in the process of myelinogenesis {Quarles, 1989} as well as many other processes.

Oligodendrocytes and astrocytes are neuroglial cells found in CNS gray and white matter regions. In white matter, oligodendrocytes provide myelin sheaths for axons which aid the conduction of nerve impulses. The corresponding cell type in the peripheral nervous system is the Schwann cell. Astrocytes have a broader range of functions compared to oligodendrocytes. It is believed that the cytoplasmic processes characteristic of astrocytes are involved in the exchange of fluid, gases, and metabolites between the nervous tissue and the blood and cerebrospinal fluid {Rhodin, 1974}. It has also been suggested that astrocytes have an important degradative function based on studies involving assays of lysosomal hydrolases, and that astrocytes are involved in the removal of cellular

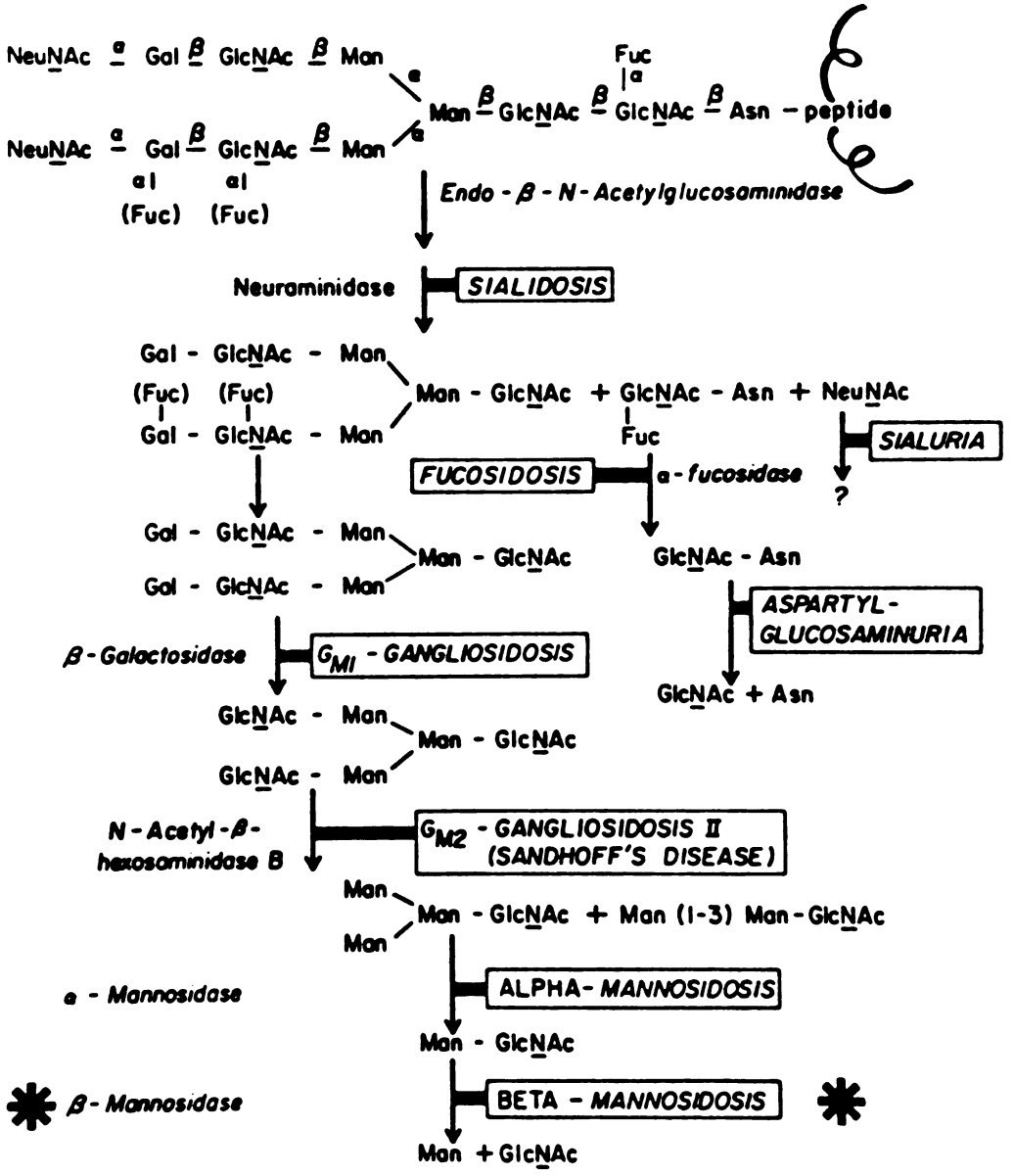


Figure 1. Diagram summarizing the degradative pathway for the oligosaccharide portion of glycoprotein catabolism.

debris in the brain {Hof and Kimelberg, 1985}.

Caprine β-mannosidosis is an autosomal recessive defect {Fisher et al., 1986} of glycoprotein catabolism. Goats affected with the disease have deficient tissue {Jones and Dawson, 1981; Healy et al., 1981} and plasma {Cavanagh et al., 1982; Jones et al., 1984; Healy et al., 1981} activities of the lysosomal enzyme β-mannosidase (EC 3.2.1.25). As a result there is accumulation of the trisaccharide Man(β1-4)GlcNAc(β1-4)GlcNAc and the disaccharide Man(β1-4)GlcNAc along with other more complex oligosaccharides {Jones and Laine, 1981; Matsuura et al., 1981; Matsuura and Jones, 1985}. Clinical characteristics include the inability to rise, marked intention tremor, and deafness {Jones et al., 1983; Kumar et al., 1986}. Pathological characteristics include cytoplasmic vacuolation in the nervous system and viscera with variation among cell types, and dysmyelination in the CNS but not in peripheral nerves {Jones et al., 1983}.

There is evidence {Lovell and Jones, 1983; 1985} suggesting that the myelin deficit associated with β-mannosidosis is primarily due to oligodendrocyte defects during or prior to myelination: (1) A reduction in the number of oligodendrocytes observed postnatally in areas of severe and moderate myelin deficiency suggests impaired proliferation or cell death of this cell type. (2) Vacuolation in many of the remaining oligodendrocytes suggests the possibility of impaired function, including myelination capabilities. (3) A lack of evidence indicating myelin degeneration suggests a defect in myelinogenesis rather than the destruction of myelin sheaths after formation. (4) In affected animals up to 4 weeks of age, normal myelin sheaths have been observed, which suggests that once myelin sheaths are formed by functional oligodendrocytes they can be

maintained. (5) The presence of internodes without myelin adjacent to internodes with myelin, and the normal myelination of the peripheral portion of cranial nerves adjacent to hypomyelination of the central portion, suggest that axonal abnormalities are not primarily responsible for myelin deficits.

The myelin deficits associated with this disease demonstrate regional variations {Lovell and Jones, 1983; 1985} that appear to correlate with the time of myelination. For example, the spinal cord, myelinating early in development shows a mild myelin deficiency, and glial cells that for the most part appear normal. In contrast, the corpus callosum, myelinating late in development, shows almost complete absence of myelin, and a large decrease in the number of glial cells. In general, the brain regions that myelinate late in development are more severely affected. In the white matter, astrocytes appear relatively normal except for mild vacuolation, while some oligodendrocytes are severely affected. Many of the oligodendrocytes that persist postnatally show dark cytoplasm and vacuolation.

Cytoplasmic vacuolation occurs to different degrees in various organs and cell types of β-mannosidase-deficient goats {Jones et al., 1983}. For example, cell types of specific organs that show especially severe cytoplasmic vacuolation include the proximal convoluted tubular epithelium of the kidney, and the follicular epithelium of the thyroid. Within the CNS, there is also extensive variation among cell types. Neurons of the cerebral cortex are in general more severely affected than neurons of the brainstem and spinal cord. One hypothesis proposed to explain these differences is that the activity of β-mannosidase in specific cell types is correlated with the extent of vacuolation.

In a previous study {Boyer et al., 1990}, β-mannosidase activity was

measured in the gray matter and white matter of cerebral hemispheres and spinal cord of two normal newborn goats. This was part of an experiment designed to compare the accumulation of oligosaccharides, the enzyme activity, and the extent of myelin deficits or vacuolation in different regions. Previous studies {Jones and Dawson, 1981; Jones and Laine, 1981; Matsuura et al., 1981; Jones et al., 1984; Pearce et al., 1987} had suggested that the level of β-mannosidase activity in normal animals correlates with the accumulation of oligosaccharides in the tissues of affected animals. Since cerebral hemispheres have a greater severity of lesions in affected animals and there is normally a high concentration of neurons in cerebral cortex, it was expected that in normal animals higher enzyme levels would occur in cerebral hemispheres compared to spinal cord. The results showed that β-mannosidase activity and the concentration of the accumulated trisaccharide were higher in spinal cord compared to cerebral hemispheres. Although the results were not statistically significant as only two animals were used, it appeared that β-mannosidase activity correlated with accumulation of the trisaccharide, but not with the regional pattern of lesion severity.

In addition to β-mannosidase-deficient goats {Hartley and Blakemore, 1973; Jones et al., 1983; Healy et al., 1981}, the disease has been described in humans {Cooper et al., 1986; Wenger et al., 1986} and Salers calves {Abbitt et al., 1991; Orr, 1990; Bryan, 1990}. Although each account reports a deficiency of β-mannosidase, the clinical and pathological characteristics are not identical for each species. In both the caprine and bovine species the disease involves extensive dysmyelination and is fatal within days to months after birth, while most human patients are able to live into adulthood with mild retardation. This

species difference may be related to the accumulated oligosaccharides resulting from the disease. In all three species the disaccharide accumulates and is the major storage product in the human; however, in both the caprine and bovine species the predominant accumulated substrate is a trisaccharide. The differences in the accumulated substrates may be suggestive of differences in glycoprotein catabolic pathways for ruminant and non-ruminant species {Hancock et al., 1986}. Other examples of species variation in lysosomal storage diseases is summarized by Aronson and Kuranda {1989}; different species showed differences in the accumulated material even though they were deficient in the same lysosomal hydrolase.

Lysosomal enzyme activities have been reported in various organs from normal and β-mannosidase-deficient caprine {Healy et al., 1981; Jones and Dawson, 1981; Pearce et al., 1987}, bovine {Jolly et al., 1991}, and human studies {Wenger et al., 1986; Dorland et al., 1988; Cooper et al., 1988; Kleijer et al., 1990}. A common characteristic among lysosomal storage diseases is an increased activity of lysosomal hydrolases other than the defective enzyme {Hers, 1973}. Increased activities of most hydrolases assayed has also been reported in β-mannosidase-deficient animals (Table 1), including caprine {Healy et al., 1981; Jones and Dawson, 1981} and bovine examples {Jolly et al., 1991}.

A variety of lysosomal enzymes have been studied in association with α-mannosidosis, another lysosomal storage disease of glycoprotein catabolism. Similar to β-mannosidosis, species variation exists for α-mannosidosis in feline {Cummings et al., 1988}, bovine {Embury and Jerrett, 1985}, and human cases {Mitchell et al., 1981}. Table 2 outlines that, with few exceptions, most enzymes

Table 1. Summary of published research for lysosomal enzymes in ß-mannosidase-deficient species.

Enzyme	Kidney	Liver	Brain
Acid Phosphatase	(I)1	(I)1	
Arylsulfatase	(I)1	(I)1	
α-Fucosidase	(I)1, (I)2, (I)4	(I)1, (I)2, (I)4	(I)2, (I)4
α-Galactosidase	(I)1	(I)1	
ß-Galactosidase	(I)1, (I)4	(I)1, (D)4	(I)4
α-Glucosidase	(I)1, (I)4	(I)1, (I)4	(I)4
ß-Glucosidase	(I)1	(I*)1	
ß-Glucuronidase	(I)1	(I)1	
Hexosaminidase*	(I)1, (I)4	(I)1, (I)4	(I)4
α-Mannosidase	(I)1, (I)2, (I)4	(I)1, (I)2, (I)4	(I)2, (I)4
Phosphodiesterase	(I)1	(I*)1	

I Increased enzyme activity
I* Increased activity for one of two affected animals
D Decreased enzyme activity

1. Healy et al., 1981
2. Jones and Dawson, 1981
3. Pearce et al., 1987
4. Jolly et al., 1991

* Hexosaminidase represents the activities of ß-N-acetylgalactosaminidase, ß-N-acetylglucosaminidase, and hexosaminidase as outlined in publications 1 and 4 above.

Table 2. Summary of published research for tissue enzyme activities in α-mannosidase-deficient species.

Enzyme	Persian Cats[1]	Domestic Long-haired Cats[2]	Galloway Calves[3]	Human[4]
ß-N-Acetylgalactosaminidase	I			
ß-N-Acetylglucosaminidase			I	
Arylsulfatase A	I			
Arylsulfatase B	I			
α-Fucosidase	I	I		I*
ß-Galactosidase	I	I	I	I*
α-Glucosidase			I	I
ß-Glucosidase	I	D	I	
ß-Glucuronidase	I	I	I	I*
Hexosaminidase		D		
α-Iduronidase		I		
Sphingomyelinase		D		

I - Increased enzyme activity
D - Decreased enzyme activity

1. Vandevelde et al., 1982. Enzyme activities were determined in brain tissue.
2. Cummings et al., 1988. Enzyme activities were determined in brain tissue - cerebral cortex.
3. Embury and Jerrett, 1985. Enzyme activities were determined in kidney, liver, and brain.
4. Mitchell et al., 1981. Enzyme activities were determined in liver and brain. * In fibroblasts cultures these enzyme activities were normal compared to the controls, while α-mannosidase activity was markedly decreased.

show an increase in activity in α-mannosidase-deficient species, a pattern similar to β-mannosidase-deficient species and other lysosomal storage diseases.

The current study was initiated to investigate the possible relationship between the enzyme activity of normal animals and the regional variation of lesions involved in β-mannosidase-deficient goats. A sufficient number of normal goats were used to allow for statistically significant determinations of baseline enzyme activities in selected organs along with gray and white matter of cerebral hemispheres and spinal cord. Three lysosomal hydrolases involved in the glycoprotein catabolic pathway (Figure 1) including α-mannosidase (EC 3.2.1.24), β-mannosidase, and α-fucosidase (EC 3.2.1.51) were assayed. In addition, acid phosphatase (EC 3.1.3.2) was assayed as an enzyme not involved in this pathway. Enzyme activities in affected animals were assayed to establish the change in activity of selected enzymes in the glycoprotein catabolic pathway.

MATERIALS AND METHODS

Materials

4-Methylumbelliferone (4-MU), free acid (M 1381), ethylenediamine (EDA), free base (E 4379), and Coomassie Brilliant Blue G-250 (B 1131) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Methoxy ethanol was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Leupeptin and pepstatin A were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Each artificial substrate was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.): 4-methylumbelliferyl-α-D-mannopyranoside (M 4383), 4-methylumbelliferyl-β-D-mannopyranoside (M 9134), 4-methylumbelliferyl phosphate (M 8883), and 4-methylumbelliferyl-α-L-fucoside (M 4633).

Methods

Animals

Animals used in this study included six normal goats (two females, four males) and two goats affected with β-mannosidosis (one female, one male) ranging in age from 3 to 8 days old. Affected animals were identified by the absence of plasma β-mannosidase activity and the presence of typical phenotypic and histopathological characteristics {Jones et al., 1983; Kumar et al., 1986}. Tissue samples from selected organs and regions of the CNS were removed after euthanasia and stored in a freezer at -20°C.

Tissue Preparation

Activities of α-mannosidase, β-mannosidase, acid phosphatase, and α-fucosidase were determined in extracts of liver, kidney, thyroid, muscle, cerebral hemisphere gray and white matter, spinal cord gray and white matter, optic nerve, and corpus callosum. α-Fucosidase activity was not measured in muscle. For optic nerve and corpus callosum, samples from more than one animal were combined to provide sufficient tissue for each assay. Liver samples were not available from two normal animals. Cerebral hemisphere gray matter (dorsal and lateral cortex) and white matter (centrum semiovale) samples were dissected just prior to assay from a frozen coronal section taken at the level of the optic chiasm. Spinal cord gray matter and white matter were dissected from cervical cord after removal of the meninges. Tissues were removed from the freezer individually and weighed to minimize thawing. After weighing, the tissue was minced using a razor blade and suspended in extraction buffer containing protease inhibitors giving a final concentration of 0.2 g/ml. Extraction buffer (pH 5.5) contained 0.01 M citrate, 0.05 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.02% NaN_3, 10% glycerol, 0.2 mg/L leupeptin, and 0.7 mg/L pepstatin A. Each tissue was held on ice until all samples were prepared for cell disruption.

Cells and organelles were ruptured by sonication (Heat Systems Ultrasonics, Inc. sonicator, Model W185-F) at 4°C for 30-180 s at setting #3. The total time was divided into 10 s bouts of sonication separated by 50 s of cooling time; samples were on ice throughout disruption. Sonication was continued until a homogenous solution was attained, or 3 min total time, whichever came first. Tissues were centrifuged at 15000 g (Eppendorf, Model 5414) for 10-20 min, and

the supernatants removed and stored at 4°C for enzyme assay the same day.

Substrate Preparation

Artificial substrates were used for each of the enzyme assays using established methods {Healy et al., 1981; Jones et al., 1984}. The substrate for α-mannosidase was 2 mM 4-methylumbelliferyl-α-D-mannopyranoside in 31 mM citrate-37 mM phosphate buffer, pH 4.0. The substrate for β-mannosidase was 2 mM 4-methylumbelliferyl-β-D-mannopyranoside in 24 mM citrate-51 mM phosphate buffer, pH 5.0. The substrates for α- and β-mannosidase were frozen in bulk preparations (25 ml), thawed for each assay and returned to -20°C for long-term storage. The substrate for acid phosphatase was 2 mM 4-methylumbelliferyl phosphate in 0.1 M sodium acetate buffer (Walpoles Buffer), pH 4.8. This substrate was stored at -80°C in 1 ml aliquots; only sufficient quantities were removed as necessary for each assay. The substrate for α-fucosidase was 2 mM 4-methylumbelliferyl-α-L-fucoside in 24 mM citrate-51 mM phosphate buffer, pH 5.0. This substrate was prepared fresh for each assay by dissolving the solid in a small quantity of dimethyl sulfoxide at 37°C. The desired volume was then obtained by diluting with 37°C 24 mM citrate-51 mM phosphate buffer, pH 5.0.

Standard Curve

Once in solution, 4-MU free acid is stable for 30 days. During the course of this study, fresh 4-MU free acid was prepared monthly and a standard curve was generated with each new preparation. Six dilutions (10, 2, 1.33, 0.66, 0.22, 0.11 nmoles) of 2 mM 4-MU free acid was prepared in 2-methoxy ethanol; 95% ethanol can be substituted. Each of the six points for the standard curve was

prepared by serial dilutions of the 2 mM 4-MU free acid. The fluorimeter high voltage was set before running the test samples using a portion of the 10 nmol dilution. Each time an enzyme assay was run, two of the dilutions (10 and 1.33 nmol) were repeated as a check against the standard curve.

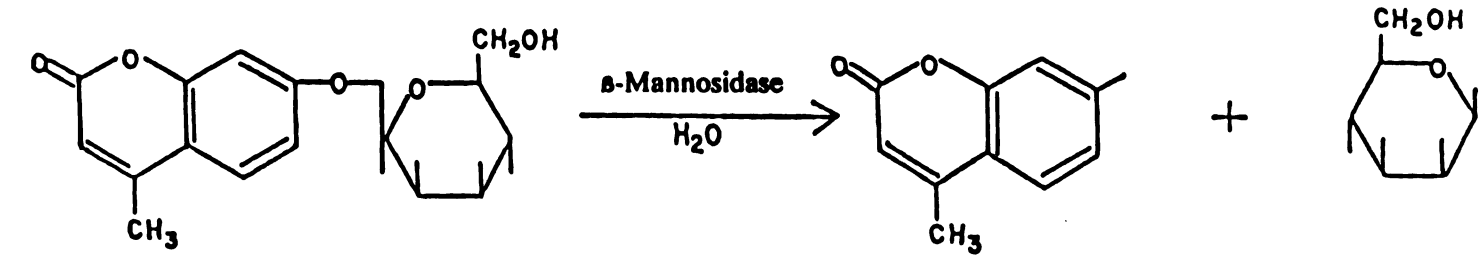
Enzyme Assay

The overall reaction is illustrated in Figure 2 using 4-methylumbelliferyl-β-D-mannopyranoside as an example. The 4-methylumbelliferone is a fluorescent product which can be measured using a fluorimeter (Gilford, Model Fluoro IV) with an excitation setting of 365 nm and an emission of 450 nm.

Tissue samples were diluted using the buffer appropriate for the enzyme being assayed. Dilutions were made (Table 3) to provide readings within range of the standard curve. All samples were run in duplicate. For enzyme assays, 100 ul of substrate was added to 50 ul of the tissue extract, and incubated at 37°C for 15 min (organ samples) or 30 min (CNS samples). The reaction was quenched with 1.7 ml 0.1 M EDA and the fluorescence was quantified. The following controls were run for each assay:

(1) Sample Blank - 50 ul tissue extract, 100 ul substrate, and quenched immediately with 1.7 ml 0.1 M EDA. This blank was incubated for the duration of the assay, and its level of fluorescence was subtracted from each of the tissue samples. This control enabled the detection of any inherent fluorescence within the tissue sample.

(2) Substrate Blank - 50 ul of buffer (appropriate type and pH) and 100 ul substrate. This tube was incubated for the duration of the assay and quenched along with the tissue samples. This blank controlled for any



4-Methylumbelliferyl-ß-D-mannopyranoside | 4-Methylumbelliferone | ß-Mannose

Figure 2. Enzyme assay reaction using an artificial substrate. The artificial substrate for ß-mannosidase is used here for illustration.

Table 3. Tissue Dilutions For Enzyme Assays and Protein Determinations

Tissue	α-Mannosidase Dilution: Normal\Affected	β-Mannosidase Dilution: Normal\Affected	α-Fucosidase Dilution: Normal\Affected	Acid Phosphatase Dilution: Normal\Affected	Protein Assay Dilution: Normal\Affected
Liver	1:10/1:20	None/None	1:5/1:40	1:200/1:300	1:40/1:40
Kidney	1:60/1:120	None/None	1:40/1:80	1:200/1:300	1:40/1:40
Thyroid	1:30/1:60	1:5/None	1:40/1:80	1:100/1:100	1:40/1:40
Muscle	None/ND	None/ND	None/ND	1:30/ND	1:20/ND
Cerebral Hemisphere Gray Matter	1:10/1:100	None/None	1:5/1:10	1:60/1:200	1:30/1:10
Cerebral Hemisphere White Matter	1:10/1:50	None/None	1:5/1:10	1:60/1:100	1:10/1:10
Spinal Cord Gray Matter	1:10/1:50	None/None	1:5/1:10	1:60/1:100	1:10/1:10
Spinal Cord White Matter	1:10/1:50	None/None	1:5/1:10	1:60/1:50	1:10/1:10
Optic Nerve	1:10/ND	None/ND	1:5/ND	1:60/ND	1:5/ND
Corpus Callosum	1:10/ND	None/ND	1:5/ND	1:60/ND	1:20/ND

ND - Not Determined

inherent fluorescence from the artificial substrate, and was used to set the fluorimeter autoblank before reading the tissue samples.

(3) Plasma Control - Plasma samples were prepared from a normal goat outside of the test population, and stored at -80°C in 0.5 ml aliquots. Each time an assay was conducted for the activity of α-mannosidase or β-mannosidase, duplicate tubes of plasma were prepared for each substrate and run with the tissue samples. Plasma controls provided (1) a comparison between independent assays for each of the above two enzymes, and (2) a means to analyze independent preparations of each artificial substrate. An acceptable enzyme assay was defined to be one in which the plasma control value was within a given range as determined by independent assays conducted prior to the start of the current study. If the plasma values were outside of this acceptable range the enzyme assay was repeated. Substrates were prepared on multiple occasions during the course of this study, and the plasma control was used as a means to judge if the preparation gave values consistent with previous assays. An acceptable substrate preparation was defined to be one in which the plasma control value was within a given range as determined by independent assays conducted prior to the start of the current study. If the plasma values were outside of this acceptable range the preparation was discarded.

(4) Kidney Control - Using a normal goat outside of the test population, a large quantity of supernatant was prepared following the procedures and concentration as outlined previously. This was stored at -80°C in 1.0 ml

aliquots. Each time an assay was conducted for the activity of acid phosphatase or α-L-fucosidase, duplicate tubes were diluted for each substrate and incubated with the tissue samples. This control provided the same system checks as outlined above for the Plasma Control, including (1) a comparison between independent assays for each of the above two enzymes, and (2) a means to analyze independent preparations of each artificial substrate. The same guidelines for an acceptable enzyme assay and substrate preparation apply for this control as for the above Plasma Control.

Protein Assay

Protein concentration for each tissue supernatant was determined using the Bradford method {Bradford, 1976} and bovine serum albumin as the standard. This method utilizes the ability of Coomassie Brilliant Blue G-250 to bind protein and shift the absorption maximum of the dye from 465 to 595 nm as determined by spectrophotometric analysis (Beckmann, Model T 64).

For the reaction, 90 ul extraction buffer, 10 ul diluted (with extraction buffer) supernatant, and 1000 ul Coomassie Brilliant Blue reagent were combined, vortexed, and read immediately using the spectrophotometer. See Table 3 for a summary of the tissue dilution for each organ/CNS region. Before each assay, a standard curve was run with four data points: 0 mg/ml, 2 mg/ml, 4 mg/ml, and 6 mg/ml, using bovine serum albumin as the standard. The 0 mg/ml sample was used to set the Blank for the spectrophotometer. The sample tubes were then run (in triplicate) for each of the tissue supernatants.

Statistical Analysis

Statistical comparisons were performed using the Sign Test {Sokal and Rohlf, 1969}.

RESULTS

Normal Goats

Lysosomal enzyme activities of crude tissue preparations from organs: In normal goats, enzyme levels for kidney and thyroid are similar for three of the enzymes measured (α-mannosidase, α-fucosidase, and acid phosphatase); however, the activity of β-mannosidase in the thyroid was 5.7 times higher than that of kidney (Table 4). Muscle contained the lowest activity for all enzymes measured. Enzyme activities in the liver varied in comparison to kidney, with α-mannosidase and α-fucosidase activities lower in the liver than kidney, and β-mannosidase and acid phosphatase activities similar in liver and kidney. Thus the activities of enzymes in the glycoprotein catabolic pathway did not necessarily show the same relationship in the different organs.

Lysosomal enzyme activities of crude tissue preparations from CNS regions: In both the cerebral hemispheres and spinal cord of normal goats, each of the three enzymes involved in the glycoprotein catabolic pathway, α-mannosidase, β-mannosidase, and α-fucosidase, had significantly ($p < 0.05$) higher levels of activity in white matter compared to gray matter (Table 4). For these three enzymes, gray and white matter regions of the spinal cord were significantly ($p < 0.05$) higher than the corresponding region in the cerebral hemispheres. For acid phosphatase, the only significant difference among CNS regions was the increase in activity in spinal cord gray matter compared to cerebral hemisphere

Table 4. Specific activities of lysosomal enzymes from crude tissue preparations of normal newborn goats

Tissue	α-Mannosidase	β-Mannosidase	α-Fucosidase	Acid Phosphatase
Liver**	139 (85)	12 (1.2)	59 (4.5)	1408 (321)
Kidney	977 (271)	11 (4.2)	1024 (324)	1854 (627)
Thyroid	883 (218)	63 (16)	822 (147)	1968 (484)
Muscle	21 (7.8)	1.9 (0.6)	57 (32)	462 (301)
Cerebral Hemisphere Gray Matter	61 (16)	1.7 (0.2)	65 (31)	699 (132)
Cerebral Hemisphere White Matter	110 (24)	4.5 (0.5)	98 (31)	673 (80)
Spinal Cord Gray Matter	109 (21)	4.7 (0.8)	120 (38)	923 (130)
Spinal Cord White Matter	200 (58)	11 (2.3)	172 (42)	875 (198)
Optic Nerve*	105 (25)	5.4 (0.2)	149 (47)	786 (174)
Corpus Callosum**	79 (19)	3.2 (1.0)	82 (42)	731 (114)

The values (nmol/mg-hr) represent the means ± (SD) of six normal animals, unless otherwise indicated (* n=3, ** n=4)

gray matter. As expected, optic nerve and corpus callosum showed activities similar to that of cerebral hemisphere white matter.

Affected Goats

Lysosomal enzyme activities of crude tissue preparations from organs: As expected in this disease, β-mannosidase activity was not measurable in organs except for the liver (2.7 and 4.4 nmol/mg-hr for A1 and A2 respectively). The residual activity in the liver is consistent with previous reports of a non-lysosomal form of the enzyme {Dawson, 1982; Cavanagh et al., 1985}. The other enzyme activities measured showed a modest increase or little change in the organs studied (Table 5).

Lysosomal enzyme activities of crude tissue preparations from CNS regions: All enzymes with the exception of β-mannosidase, (Table 5) showed increased activities to various extents in all regions studied. For both α-mannosidase and α-fucosidase, spinal cord activities in both gray and white matter were 5-7 times higher than in normal tissue; cerebral hemisphere gray matter activities showed an 4-10 fold elevation over control activities, and cerebral hemisphere white matter showed a 3-4 fold elevation also. Acid phosphatase activities consistently showed a smaller elevation (2-3 fold) in all regions compared to other enzymes.

Table 5. Changes in lysosomal enzyme activities in crude tissue preparations of goats affected with β-mannosidosis

	α-Mannosidase		α-Fucosidase		Acid Phosphatase	
Tissue	A1	A2	A1	A2	A1	A2
Liver	1.0x	7.2x	10.2x	3.1x	1.5x	2.4x
Kidney	1.5x	1.3x	2.4x	1.8x	1.7x	1.0x
Thyroid	1.2x	1.0x	1.5x	1.2x	0.8x	0.9x
Cerebral Hemisphere Gray Matter	4.7x	10.6x	4.9x	7.7x	2.1x	1.6x
Cerebral Hemisphere White Matter	3.4x	4.1x	3.5x	4.4x	2.5x	2.3x
Spinal Cord Gray Matter	7.3x	6.3x	7.1x	5.4x	3.2x	1.9x
Spinal Cord White Matter	5.5x	5.6x	6.5x	5.6x	3.3x	2.0x

Each value represents the ratio of the specific activity in tissue from an affected animal (A1 or A2) to the mean for normal goat tissue (shown in Table 4)

DISCUSSION

To our knowledge, this study represents the first data for the specific activities of these glycoprotein catabolic enzymes in defined regions of the CNS. In normal goat CNS, α-mannosidase, α-fucosidase, and β-mannosidase all have higher specific activities in white matter than in gray matter, suggesting differential regulation (for example, changes among multiple enzyme forms, changes in mRNA levels, or altered rates of degradation). The results also raise the possibility of an enhanced role for these enzymes in white matter, perhaps related to turnover of glycoproteins in myelin or axonal membranes. In addition, specific activities of the three glycoprotein catabolic enzymes were consistently higher in spinal cord than in cerebral hemispheres, again suggesting differential regulation of activity in different regions of the CNS. CNS regional differences in hydrolase activity including acid phosphatase have been previously reported in several species including monkey {Friede and Knoller, 1965}, generally with higher activity in gray matter correlating with higher concentration of lysosomes in neurons. In contrast, the current study showed no difference in acid phosphatase activity between gray matter and white matter. It is not clear whether the lack of regional difference in acid phosphatase activity found in the current study reflects technical differences compared to other reports or reflects species differences. However, the finding of a different distribution of activity for acid phosphatase compared to the glycosidases measured suggests the hypothesis

of a separate pattern of distribution of enzyme activity in CNS regions for some glycosidases, i.e. a pattern that does not correlate with the distribution of lysosomes. Differences among cells types may contribute to the regional differences measured in hydrolase activities. Previous reports {Raghavan et al., 1972; Abe et al., 1979; Freysz et al., 1979} comparing activity of acid hydrolases in bulk isolated neurons, astrocytes and oligodendrocytes of several species yielded somewhat variable results, but indicated that, in most cases, hydrolase activities were about the same or higher in neurons compared to glial cells. Thus substantially higher glycosidase activity in white matter glial cells probably is not responsible for the higher glycosidase activity in goat white matter; however, species variability and other factors still need to be assessed.

In normal animals, specific activities of the three enzymes in the glycoprotein catabolic pathway did not exhibit a consistent pattern across all of the organs studied. β-Mannosidase activity shows a substantially higher activity in the normal thyroid than in other organs, as previously demonstrated {Pearce et al., 1987}. High activity of the glycoprotein catabolic pathway in thyroid follicular cells is expected due to the demands of thyroglobulin catabolism. However, since α-mannosidase activity is similar in kidney compared to thyroid, the current study suggests that either β-mannosidase is selectively higher in thyroid, or selectively lower in kidney, compared to other enzymes in the pathway. In the interpretation of data from all tissues, the correlation of activity based on artificial and natural substrates must be considered. Since this correlation has not been established for β-mannosidase, the possibility (although unlikely) does exist that the regional distribution of β-mannosidase activity with a native substrate is different than

observed here.

One objective of this study was to investigate the correlation between the severity of lesions in affected animals and the level of enzyme activity in normal tissue. In affected goats, neuronal cytoplasmic vacuolation is more severe and widespread in cerebral cortex than in spinal cord gray matter and it was hypothesized that the extent of lysosomal vacuole accumulation may be related to the level of β-mannosidase activity in those cells. However, normal cerebral hemisphere gray matter showed lower β-mannosidase activity than spinal cord gray matter. In addition, myelin deficits are much more severe in cerebral hemispheres than in spinal cord, but normal cerebral hemisphere white matter showed lower activity than spinal cord white matter. Accumulation of trisaccharides in affected goats correlates with normal enzyme activity, since the oligosaccharide accumulation due to β-mannosidase deficiency is higher in the spinal cord of affected goats than in either cerebral hemisphere gray or white matter {Boyer et al., 1990}. Thus β-mannosidase activity in normal goat CNS does not correlate with the severity of lesions in affected goats, but does correlate with the extent of oligosaccharide accumulation. The lack of correlation between enzyme activity and severity of white matter lesions in specific regions has also been reported for other genetic deficiencies {Yamanaka et al., 1981} and the involvement of other metabolic factors such as turnover was suggested.

In storage diseases involving a lysosomal enzyme defect, increased activity of other lysosomal enzymes is a common characteristic {Hers, 1973}. For example, in feline, bovine, and human α-mannosidosis {Cummings et al., 1988; Vandevelde et al., 1982; Borland et al., 1984; Embury and Jerrett, 1985; Mitchell

et al., 1981; Van Hoof, 1973; Ockerman, 1973} and in caprine and bovine β-mannosidosis {Healy et al., 1981; Pearce et al., 1987; Jones and Dawson, 1981; Jolly et al., 1991}, increased activities of most hydrolases measured have been reported in brain, liver, and/or kidney. In the present study, kidney demonstrated a moderate increase in glycosidase activity, consistent with previous results {Jones and Dawson, 1981}. In liver, occasional large increases were seen in affected goats, but the activity levels in both normal and β-mannosidase-deficient liver were extremely variable. In thyroid, no significant increase was measured; the reason for the differences among organs needs further investigation. In general, glycosidase activities in CNS regions of β-mannosidase-deficient goats increased 3-fold to 10-fold , a larger and more consistent increase than seen in organs. In contrast acid phosphatase activity increased about 2-fold to 3-fold in the CNS. These results suggest that specific regulatory mechanisms may exist for the glycosidases measured, with activity regulated differently in CNS than in organs.

The occurrence of different levels of activity in different organs was expected, since variations in enzyme populations within lysosomes of different tissues is well established and the levels of expression for lysosomal enzymes are known to vary among tissues and during development. It has been proposed that the level of expression for various enzymes in a given tissue is developmentally regulated and under normal conditions remains constant after development is complete {Lusis and Paigen, 1975}. However, changes in the activity of given lysosomal enzymes can occur in response to hormones and transformation {Dong et al., 1989; Pfister et al., 1984; Perrild et al., 1989}, as well as in lysosomal storage diseases. The genes for lysosomal enzymes have been considered to be

housekeeping genes, but some have recently been shown to contain TATA sequences {Quinn et al., 1987; Bishop et al., 1988}, which provides a basis for the regulation of expression at the mRNA level. However, little is known about the control of mRNA synthesis or other regulatory mechanisms for lysosomal enzymes. Genetic enzyme deficiencies such as β-mannosidosis provide good systems for investigating the regulation of lysosomal enzyme activity in the presence of metabolic perturbations. In this study, substantially increased activity of glycosidases occurred, especially in the CNS. Further investigation is necessary to assess the contribution of specific cell types to the increased activity and to determine the mechanism of regulation.

SUMMARY

The current study was initiated to investigate the possible relationship between enzyme activity of normal animals and the regional variation of lesions involved in β-mannosidase-deficient goats. Results indicate that β-mannosidase, α-mannosidase and α-fucosidase, enzymes of glycoprotein catabolism, show higher activity in spinal cord than in cerebral hemispheres, and higher activity in white matter than in gray matter. Enzyme activity in normal tissue does not appear to be correlated with either the severity of vacuolation or the extent of myelin deficits in affected animals. The mechanism responsible for the organ-specific and CNS region-specific variation needs further investigation.

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