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CAPRINE PLASMA α- AND β-MANNOSIDASE ACTIVITIES: ASSAY ANALYSIS AND EVALUATION OF THE EFFECTS OF AGE, SEX, AND REPRODUCTIVE STATUS AND POTENTIAL USE IN HETEROZYGOTE DETECTION OF β-MANNOSIDASE

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# CAPRINE PLASMA α- AND β-MANNOSIDASE ACTIVITIES: ASSAY ANALYSIS AND EVALUATION OF THE EFFECTS OF AGE, SEX, AND REPRODUCTIVE STATUS AND POTENTIAL USE IN HETEROZYGOTE DETECTION OF β-MANNOSIDOSIS

By

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ABSTRACT

CAPRINE PLASMA α- AND β-MANNOSIDASE ACTIVITIES: ASSAY ANALYSIS AND EVALUATION OF THE EFFECTS OF AGE, SEX, AND REPRODUCTIVE STATUS AND POTENTIAL USE IN HETEROZYGOTE DETECTION OF β-MANNOSIDOSIS

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The effects of age, sex, and reproductive status on caprine plasma α- and β-mannosidase activities as well as the potential use of plasma assays for heterozygote detection of caprine β-mannosidosis were investigated. Optimal conditions for the assay of caprine plasma α- and β-mannosidase activity were determined.

The pH optima were 4.0 and 5.0 for caprine plasma α- and β-mannosidase activity, respectively, and substrate hydrolysis was proportional to time beyond the incubation periods used in these studies. Age and sex affected caprine plasma α- and β-mannosidase activities, while plasma β-mannosidase activity was affected by reproductive status. Obligate heterozygotes for β-mannosidosis had plasma β-mannosidase values which were intermediate between those found in animals affected with β-mannosidosis and controls. Putative heterozygotes for β-mannosidosis could not be definitively identified, but likely candidates for future inbreeding experiments were discerned.

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# INTRODUCTION

Recently, a new inherited disorder of glycoprotein catabolism, β-mannosidosis, was defined in the caprine species. β-Mannosidosis is an autosomal recessive disorder characterized by profound neonatal neurological deficits and facial dysmorphia (Jones et al., 1979). Neurovisceral storage of the oligosaccharides Man(β1-4)GlcNAc(β1-4)GlcNAc and Man(β1-4)GlcNAc was associated with tissue deficiency of β-mannosidase (Jones and Laine, 1981; Jones and Dawson, 1981; Matsuura et al., 1981). Recent studies suggest that affected animals also have a complete deficiency of plasma β-mannosidase activity (Cavanagh et al., 1981). However, the determination of optimal conditions for the assay of caprine plasma α- and β-mannosidases, the influence of age, sex and reproductive status on the activities of plasma mannosidase in a clinically normal caprine population, and the use of plasma as a source of enzyme activity for heterozygote detection of β-mannosidosis have not been studied.

To determine optimal conditions for evaluating the activities of caprine plasma α- and β-mannosidase, the effects of reaction time and pH optimum on a colorimetric assay were investigated.

Because age, sex, and reproductive status significantly affect the activity of specific lysosomal hydrolases in man and cattle (Erickson et al., 1972; Griffiths et al., 1978; Annunziata and Di Matteo, 1978; Lombardo et al., 1981; Lowden, 1979; Jolly et al., 1974a; Jolly et al., 1973), these factors were examined in relation

to plasma α- and β-mannosidase activities in a population of clinically normal goats, and the results were compared with those from a herd of known and putative heterozygotes for β-mannosidosis. Plasma has been used successfully as a source of enzyme activity for heterozygote detection because it is easy to collect and has been successful in detecting carriers of other inborn errors of glycoprotein catabolism. In bovine α-mannosidosis, for example, heterozygotes were found to have enzyme values between those of normal and affected subjects (Jolly et al., 1974a; Jolly et al., 1973; Jolly and Desnick, 1979).

Plasma α-mannosidase activity was assayed in order to determine both its relationship to plasma β-mannosidase activity and its usefulness as a reference enzyme for plasma β-mannosidase activity in further characterizing heterozygotes (Jolly and Desnick, 1976; Winchester et al., 1976; Saiffer et al., 1975). α-Mannosidase seemed a logical choice for reference-enzyme studies, since its activity in tissue has been shown to be elevated in a goat with β-mannosidosis (Jones and Dawson, 1981).

This thesis is the first to study assay conditions for the caprine plasma mannosidases. It is also the first study to investigate the effects of age, sex, and reproductive status on plasma β-mannosidase activity in any species, to examine the effects of these factors on plasma α-mannosidase activity in the goat, and to use a readily available body fluid for detecting β-mannosidosis in caprine heterozygotes.

# REVIEW OF THE LITERATURE

## Overview of Lysosomal Storage Diseases

Inherited metabolic diseases have been studied extensively during the past two decades. As the pathogenesis of these diseases has been clarified, so has the understanding of normal biochemical pathways. This is particularly true of the lysosomal storage disorders. These diseases were first envisaged by Hers in 1963, when, from his study of Type II glycogen storage disease (an acid maltase deficiency), he proposed a category of diseases characterized by the absence of specific lysosomal hydrolases and concomitant accumulation of incompletely degraded macromolecules within lysosomes (Hers, 1963).

Hers also established four criteria for defining these diseases (Hers, 1965):

1. As seen by the electron microscope, the cellular inclusions are bound by single membranes and stain positively for acid phosphatase, i.e., they are in lysosomes.
2. The disorders are progressive.
3. Multiple organs are affected.
4. The stored material is heterogeneous.

Currently, there are more than 35 recognized recessive and X-linked inherited inborn errors of the lysosomal apparatus with a morphological hallmark of hypertrophy and hyperplasia of lysosomes (Desnick et al., 1978a; Hers, 1973). Although there is considerable overlap, these

diseases can be divided into five major categories based on the biochemical nature of the accumulated metabolites: 1) the glycogen storage diseases, 2) the lipidoses, 3) the mucolipidoses, 4) the mucopolysaccharidoses (errors of glycosaminoglycan catabolism), and 5) the oligosaccharidoses (disorders of glycoprotein catabolism). This report will concern itself primarily with disorders of glycoprotein catabolism.

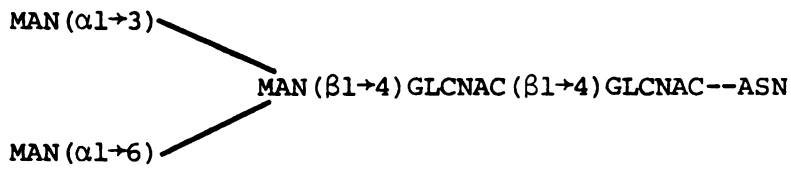
## Biochemistry of Glycoproteins

### Basic Structure

There are two basic classes of compounds which contain proteins covalently bound to sugars: the glycosaminoglycans and the glycoproteins. Glycosaminoglycans are high molecular weight linear carbohydrate polymers that are generally composed of disaccharide repeating units of a uronic acid (D-glucuronic acid or L-iduronic acid) and a hexosamine (N-acetylglucosamine) and, with the exception of hyaluronic acid, contain sulfate esters or sulfate amines. In contrast, glycoproteins do not contain uronic acid, lack a serially repeating unit, contain a relatively low number of sugar residues in the heterosaccharide portion (which is often branched), and contain several sugars which are not characteristic components of glycosaminoglycans (e.g., fucose, sialic acid, galactose) (Harper et al., 1979; Margolis and Margolis, 1979).

Oligosaccharide chains on glycoproteins are classified according to their amino acid linkages as well as their inner core structure. Although there are five major types of carbohydrate-peptide linkages, only the structure, synthesis, and catabolism of glycoproteins with N-glycosidic linkages are germane to this discussion.

The N-glycosidic linkage, which consists of N-acetylglucosamine β-linked to asparagine (GlcNAc-Asn), is the most ubiquitous carbohydrate-peptide linkage, being widely distributed in animals, plants, and microorganisms (Sharon and Lis, 1981). The oligosaccharides linked to asparagine typically have the following branched core structure:




Glycopeptides containing this structure fall into two broad categories: the neutral or oligomannosidic type, which contain only mannose and N-acetylglucosamine (as is found in chicken ovalbumin), and the acidic or complex type. The latter contain, in addition to mannose and N-acetylglucosamine, sialic acid, galactose, and fucose (Kornfeld and Kornfeld, 1976; Sharon and Lis, 1981). Examples of this type of compound are found in human or bovine immunoglobulin G.

## Metabolism of Glycoproteins

The biosynthesis of the asparagine-linked oligosaccharides is initiated by the transfer of UDP-GlcNAc to the polyisoprenoid lipid, dolichol phosphate. Sequential addition of sugars from their nucleotide derivatives to the N-acetylglucosaminylpyrophosphoryldolichol occurs to form the unit Man(β1-4)-GlcNAc(β1-4)-GlcNAc-P-P-dolichol. Additional mannose residues and three glucose residues are added to this structure to form the completed dolichol intermediate. The entire oligosaccharide moiety is then transferred to an asparagine residue on the nascent polypeptide chain. This asparagine-linked oligosaccharide is the precursor for formation of both oligomannosidic and complex glycoproteins (Figure 1).

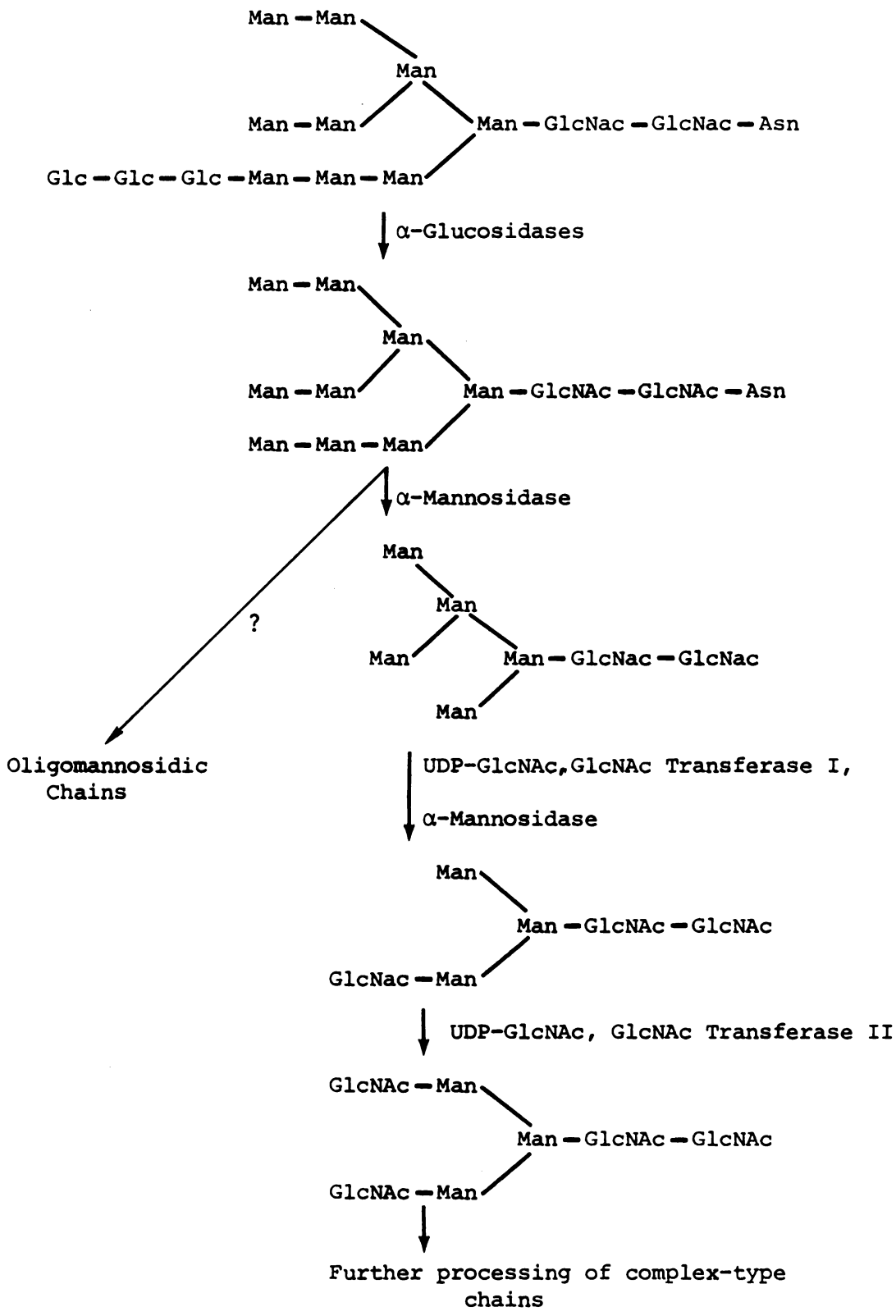



Figure 1. The synthesis of glycoproteins (Sharon and Lis, 1981).

The three glucose units are now sequentially removed. After removal of the glucose units, four mannose units are excised followed by the addition of GlcNAc to the α(1-3)-mannose. The transfer of this GlcNAc residue then signals an α-mannosidase to cleave two mannose residues linked α-1-6 to the β-linked core mannose (Tabas and Kornfeld, 1978). Subsequently, one or two additional GlcNAc units are added to the α-1-6-linked mannose. Further processing may then occur in the endoplasmic reticulum or the Golgi apparatus where the glycoproteins assume their final structure (Sharon and Lis, 1981; Marvel, 1980).

Glycoproteins are catabolized within lysosomes by the sequential action of exoglycosidases (neuraminidase I, neuraminidase II, β-galactosidase, N-aspartyl-β-glucosaminidase B, α-mannosidase, β-mannosidase, and α-fucosidase), and an endoglycosidase (β-N-acetyl-glucosaminidase) (Dawson, 1979). Due to the sequential and primarily exoglycosidic nature of this catabolic scheme, a deficiency of any one enzyme precludes further processing by the more terminal catabolic glycosidases, and the oligosaccharide remnants remain stored within lysosomes. With the discovery of β-mannosidosis in goats, enzymatic deficiencies of all the exoglycosidases in glycoprotein catabolism have been discovered (Figure 2). Of interest is the fact that only deficiencies of β-mannosidase, α-mannosidase, and N-aspartyl-β-glucosaminidase result in the storage of products limited to glycoprotein catabolism. The remaining exoglycosidases act on glycolipid and/or glycosaminoglycan substrates in addition to glycoprotein substrates (Harper et al., 1979).

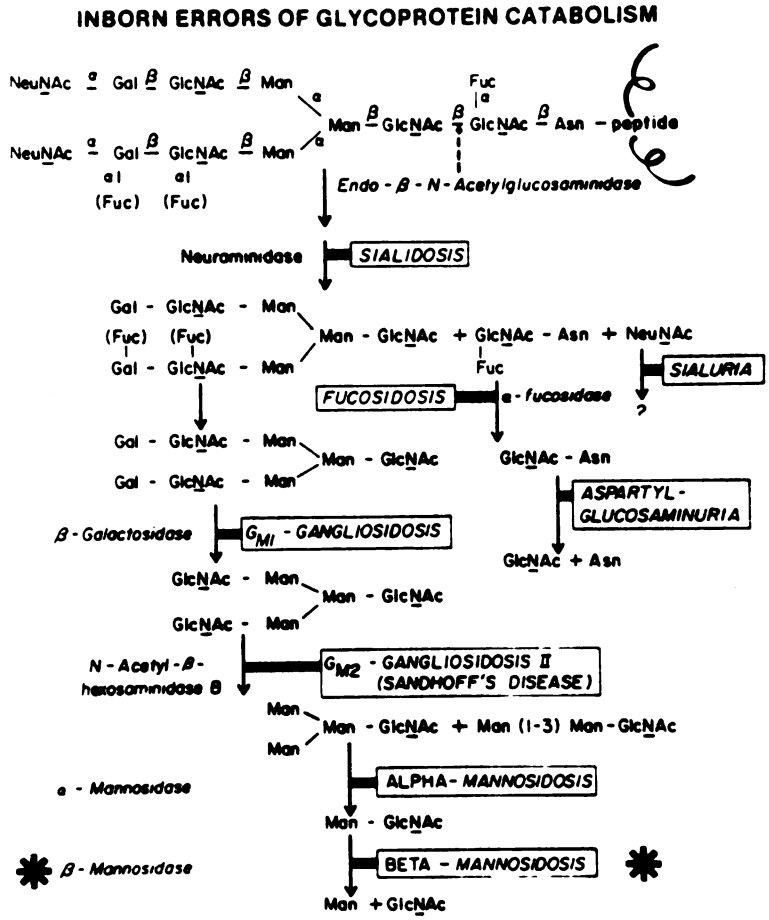



Figure 2. Inborn errors of glycoprotein catabolism (after Dawson, 1979).

## The Genetics of Lysosomal Storage Diseases

All glycoprotein storage diseases are inherited as autosomal recessive disorders characterized by the functional deficiency of a catabolic gene product leading to the accumulation of substrate or substrate precursors (Desnick et al., 1976a). Although the kinetic, physical, and antigenic properties for an increasing number of enzymes for glycoprotein catabolism can be assessed, characterization of the specific molecular defects has not been accomplished.

The major genetic defect for these disorders is assumed to be of a structural nature. Structural mutations include nucleic acid defects that alter binding sites required for the normal catalytic activity of lysosomal hydrolases. Thus, the enzyme in question is rendered partially or completely inactive. Most human enzymatic deficiencies result from point mutations due to changes affecting a single gene nucleotide (Stanbury et al., 1978). In approximately 70% of inborn genetic mutations, there will be a "missense" mutation leading to incorporation of a different amino acid in a protein chain (Jolly and Desnick, 1979; Stanbury et al., 1978).

Another type of genetic alteration affecting lysosomal hydrolases involved in glycoprotein catabolism concerns post-translational modifications of the enzyme protein. Since lysosomal hydrolases are glycoproteins, defective glycosylation or phosphorylation processes can be the cause of a partially inactive enzyme. For example, the basic defect proposed in a mucolipidosis known as I-cell disease is a deficiency of one of the enzymes catalyzing the formation of mannose-6-phosphate residues on lysosomal hydrolases. The lack of a phosphorylated mannosyl derivative attached to these enzymes prevents lysosomal targeting of these enzymes following Golgi synthesis and also interferes with

pinocytotic uptake following exocytosis (Sharon and Lis, 1981; Vladutiu and Rattazzi, 1975).

## The Genetics of Heterozygote Detection

Heterozygotes for lysosomal storage diseases are typically asymptomatic but exhibit codominant inheritance with levels of enzyme activity approximately half those of normal individuals (Jolly and Desnick, 1979). In most populations containing normal individuals and carriers of lysosomal enzymopathies, the carrier distribution curve will be bimodal with overlap between curves for the normal and heterozygous populations due to the wide range of values obtained in these control and carrier populations. The actual genotype of putative carriers in a proportion of cases, therefore, may be difficult to determine biochemically. In bovine α-mannosidosis, for example, the use of a plasma assay for detection of the deficient enzyme results in 15% of the putative heterozygotes having values intermediate between obligate carriers and controls (Jolly and Desnick, 1979; Jolly, 1974b; Jolly, 1978).

The genetic reason for the wide range of values obtained in both populations is due to the fact that at any locus there are likely to be variant alleles producing more or less normal gene products. These variants may code for isoenzymes with slightly different activity, kinetics, or stability, and the distribution of enzymatic activities in a population may reflect these variances. In studies of serum α-fucosidase levels, a low activity variant in some normal individuals is less than 10% of control values (Ramage and Cunningham, 1975). This variance in activity levels may be even more noticeable in the heterozygote population. Since heterozygotes carry two different alleles,

they typically exhibit a more complex pattern of isoenzymes than homozygotes (Stanbury et al., 1976; Desnick et al., 1976a).

## The Methods and Materials of Heterozygote Detection

All assays currently used for the detection of heterozygotes in glycoprotein storage diseases involve an analysis of enzyme activity. Although an alternative method for carrier detection might logically involve the examination of tissues or body fluids for abnormally stored or excreted oligosaccharides, the heterozygous state is not definitely associated with the accumulation or excretion of abnormal oligosaccharides, and this method will not be discussed.

Since glycohydrolases are present to some extent in essentially all body tissues and fluids, biological sample availability is a major consideration with respect to enzyme analysis for heterozygote detection. Heterozygote detection has been reported using both fluid and cellular elements of blood (Thompson et al., 1976a), fibroblasts (Milunsky et al., 1972), saliva (Den Tandt and Jaeken, 1979), urine (Saifer et al., 1976), tears (Carmody et al., 1973), and hair follicles (De Bruyn et al., 1979). However, glycohydrolases are generally measured in blood fluid or cells or from cultured fibroblasts.

### Heterozygote Detection Using Blood Plasma and Serum

The use of blood plasma and serum as enzyme sources for heterozygote detection has the major advantage of being readily obtainable from both man and animals. Thus, plasma and serum have been the most thoroughly evaluated of all tissues or fluids for use in heterozygote detection of glycoprotein storage diseases. These substances have been proposed as one of the preferred enzyme sources for carrier analysis

of mucolipidosis I and II, $GM_2$ gangliosidosis II, and α-mannosidosis in cattle (Jolly and Desnick, 1979).

The use of plasma is generally preferred over serum. The stability of lysosomal hydrolases is generally higher in plasma than in serum (Lombardo et al., 1980). Also, during serum preparation there is a release of significant amounts of lysosomal enzymes from the cellular elements of blood. One study reported that when serum is carefully prepared in order to prevent damage to platelets, the artificial alteration of serum lysosomal hydrolase activity does not occur, thus suggesting the actual increase in enzyme activity is due to the release of lysosomal hydrolases from platelets (Woolen and Walker, 1965). This study is somewhat contradicted by a report suggesting that even in plasma the release of acid hydrolases in platelets may account for up to one-third of the enzyme activity, suggesting that spontaneous release of enzymes by platelets occurs regardless of whether plasma or serum is used (Griffiths et al., 1978). The use of platelet poor plasma may avoid this lysosomal enzyme release.

If plasma is used as an enzyme source, the type of anticoagulant becomes a consideration. Although most plasma assays of lysosomal hydrolases use heparinized plasma, heparin has been reported to be an inhibitor of lysosomal acid phosphatase (Tietz, 1976). Chelating agents such as ethylenediaminetetraacetic acid (EDTA) may significantly reduce activity levels of metalloenzymes. For example, α-mannosidase, which requires zinc for maximal activity, is only 50% as active when EDTA is used as the anticoagulant compared to heparin (Jolly et al., 1973; Jolly and Desnick, 1979).

Inconclusive results are sometimes obtained when plasma or serum is used for heterozygote detection. In addition to the previously

discussed factors related to isoenzymes, a major reason is that the majority of the plasma acid hydrolases arise from the secretion or breakdown of organs in addition to the cells of blood (Kachmar and Moss, 1976; Griffiths et al., 1978). Thus, disease states, diet, age, sex, reproductive status, and environment all may be factors contributing to variance in lysosomal hydrolase activity. Also, plasma enzyme activity may not be representative of tissue activity. For example, Harzer and Sandhoff (1971) reported considerable variation in the N-acetyl-β-D-hexosaminidases A and B activities ratio in plasma compared to other body fluids and tissues.

## Heterozygote Detection Using Blood Cellular Elements

Since heterozygote and control populations cannot always be distinctly separated by plasma or serum assays, the lysosomal hydrolase activity in the cellular elements of blood has been examined. In the evaluation of these cellular elements, the assay of enzyme activity should be performed on populations of pure granulocytes, lymphocytes, or platelets (Nakagawa et al., 1980). One reason for using pure cellular elements is that each population has its own unique distribution of lysosomal hydrolases. Also, there may be considerable variation in the total leukocyte and platelet counts among healthy individuals as well as those suffering from disease. Therefore, the distribution of cell types and the enzyme activity can vary irrespective of the presence of lysosomal enzyme disease. The assay of enzyme activity from the specific cellular elements of blood tends to give a more definitive separation of heterozygotes from controls (Jolly and Desnick, 1979). However, because the isolation and assay of these cells are cumbersome and because of the difficulty in obtaining pure populations of cells,

blood cellular assays are typically used as a second enzyme source for inconclusive serum or plasma screening programs. Plasma or serum with supplemental leukocyte assays has been employed in the mass screening programs for carrier detection of Tay-Sach's disease in man and α-mannosidosis in cattle.

With respect to Tay-Sach's disease (a deficiency of β-hexosaminidase A), the serum assay is accurate for the genotype assignment of putative heterozygotes in 96% of the individuals examined. The remaining 4% fall into an inconclusive range. When the subjects with inconclusive serum test results are retested by the more accurate leukocyte assay (Kabak and Zeiger, 1972), a confidence limit of greater than 99% in genotype assignment is reached (Kabak et al., 1973; O'Brien, 1978).

Similarly, with bovine α-mannosidosis, a genotype assignment could be made in approximately 85% of the cases using a plasma α-mannosidase assay. The remaining 15% of the subjects were designated as equivocal. The latter group could then be differentiated by determination of the neutrophilic α-mannosidase to total hexosaminidase ratio (Jolly, 1974c; Jolly, 1978). Of interest is the fact that whereas heterozygotes for bovine α-mannosidosis can be relatively well differentiated, this does not appear to be the case for human α-mannosidosis. In man, it has been suggested that multiple determinations of α-mannosidase activity from several different sources may be required for heterozygote detection (Desnick et al., 1976b).

Heterozygote Detection Using Fibroblasts

The use of fibroblasts grown on tissue culture as an enzyme source for heterozygote detection has an advantage in that the skin biopsies from which they are grown are easily obtained from both man and animals.

However, except for detecting the rare X-linked lysosomal storage disorders, the use of skin biopsies for heterozygote detection is generally impractical (Jolly and Desnick, 1979). Not only is tissue culture an expensive and time-consuming process, but the diagnostic precision of this method is reported to be seriously impaired by the marked variability of lysosomal enzyme activities measured in most types of cultured cells (Milunsky et al., 1972; Hultberg and Sjoblad, 1977). This variability appears to be considerably greater than in plasma (Carey and Pollard, 1977).

## The Measurement of Serum and Plasma Lysosomal Hydrolase Activity

The measurement of lysosomal hydrolases involves the use of either natural or artificial substrates. Natural substrates have the advantages of being both highly specific and facilitating the differentiation of unusual mutant genotypes. They have the distinct disadvantage of being difficult to obtain or synthesize in sufficient quantity or purity and require labeling with a radioactive marker (Hultberg and Öckerman, 1972). They will not be discussed in detail in this report.

Artificial substrates have the advantages of giving reproducible results, being easy to use, being commercially available, and being less expensive. Thus, they are generally used rather than natural substrates for measurement of lysosomal hydrolase activity (Jolly and Desnick, 1979). The major disadvantage of artificial substrates is that they may not accurately reflect the natural enzyme-substrate interactions, as various isoenzymes or forms of the same enzyme may have varying substrate specificities. For example, the isoenzymes β-hexosaminidase A and β-hexosaminidase B have different substrate specificities with only β-hexosaminidase A having the ability to cleave

$GM_2$ ganglioside (Sandhoff and Christmanou, 1979). Thus, Tay-Sachs disease, a genetic disorder due to a deficiency of β-hexosaminidase A, cannot be diagnosed using artificial substrates unless special techniques are employed to resolve the interference by β-hexosaminidase B (Johnson and Chutorian, 1979; Saiffer et al., 1975).

Two types of assays with artificial substrates are currently being used to measure lysosomal hydrolase activity: 1) a chromogenic assay using p-nitrophenyl glycosides in which enzyme activities are assayed by determination of the rate of formation of p-nitrophenol (p-NP) and 2) a fluorometric assay using 4-methylumbelliferyl (4MU) derivatives. With the 4MU assay, the enzymatic hydrolysis of these non-fluorescent substrates by the enzymes being assayed brings about the liberation of the highly fluorescent methylumbelliferone which can be measured in a standard fluorometer (Annunziata and Di Matteo, 1978). The fluorimetric assays are 7 to 10 times more sensitive and therefore facilitate enzyme analysis using minute biological samples (Annunziata and Di Matteo, 1978). A third and less frequently used assay involves phenyl-linked glycosides which, upon cleavage by a specific glycohydrolase, liberates phenol. A second determination, which must then be performed to quantify the amount of phenol which is liberated, makes phenol determinations rather cumbersome (Conchie et al., 1959).

## Factors Affecting the 4-Methylumbelliferyl- and p-Nitrophenol Assays

Intrinsic and extrinsic factors affect the plasma or serum 4-MU or p-NP assays of lysosomal hydrolases. The intrinsic factors are primarily the substrate concentration, the pH optimum, the effects of different buffer types on the assay, and stability with respect to the optimum storage and assay temperature. The extrinsic factors are those

related to the metabolic and physiologic differences inherent with the subjects. These are primarily age, sex, reproductive status, and environmental factors.

Intrinsic factors. Maximum reproducibility at the highest possible activity levels is the desired outcome for all enzyme assays. With respect to substrate concentration, this is most likely if all substrates are present in large excess, i.e., at concentrations at least 20 and preferably 100 times that of the Km value (zero order kinetics) and when not more than 20% of the substrate is transformed in the course of the enzyme assay (Kachmar and Moss, 1976).

Obtaining zero order kinetics may be a problem in specific lysosomal hydrolase assays because of the lack of substrate solubility. For example, 4-MU-α-mannopyrannoside has limited solubility in water (Öckerman, 1969), and p-NP-β-mannopyranoside has limited solubility even in special solvents as methylcellosolve (Cavanagh et al., in press). Another problem with these assays may be the low concentration of specific lysosomal hydrolases in plasma. The assay of such glycohydrolases as β-glucosidase (Öckerman, 1968) requires long incubation times which involve increased risk of non-enzymatic substrate decay, enzyme inactivation, and the risk of potentially exceeding the 20% optimum concentration of transformed substrate (Griffiths et al., 1978). Although the long incubation times are a greater problem with less sensitive p-NP assays, the dependency of activity on time should be evaluated for any assay for glycohydrolase activity.

The effects of pH on lysosomal hydrolase assays. The pH at which maximum activity occurs and the range over which the pH can change without affecting maximal activity vary from one enzyme to another and with the conditions of measurement. Enzyme assays should theoretically be carried out at the pH of optimal activity because the sensitivity of the measurement of the artificial substrates is maximal at this pH. Also, the pH activity curve has minimal slope at this pH so that a small variation of pH will cause a minimal change in enzyme activity (Kachmar and Moss, 1976). Therefore, an optimal pH must be determined for each lysosomal hydrolase.

In spite of the advantages of performing enzyme assays at an optimal pH, the diagnosis of affected or carrier subjects in certain disorders of glycoprotein catabolism may be aided by the use of a pH considerably different from optimum. This method is used in studies of human α-mannosidases to avoid interference by more neutral non-lysosomal enzymes (Kistler et al., 1977).

The effects of different buffer types on lysosomal hydrolase activities. Buffers are compounds which minimize the change of pH on the addition of a strong acid or base. To be most effective, the pKa of the buffer system should be 1 pH unit or less of the optimal pH of the enzyme system (Kachmar and Moss, 1976). The addition of various buffers controls the ionic environment in which the enzyme is functioning. This ionic environment has an effect on the reaction rate and the optimal pH for the specific enzyme measured. For example, tris-maleate and lactate both exhibit effective buffering capacities for the fluorimetric assay of plasma α-mannosidase activity (Öckerman, 1969); however,

neither buffer can be used when assaying β-glucosidase because these two buffers cause non-enzymatic hydrolysis of the 4-MU substrate (Öckerman, 1968).

The effects of temperature on lysosomal hydrolase activities. The rate of any chemical reaction is directly proportional to the temperature at which the reaction is taking place. Thus, it is essential to maintain a constant temperature during assays of lysosomal hydrolases in order to maintain maximum reproducibility. The standard temperature for assaying all lysosomal hydrolases is 37 C (Kachmar and Moss, 1976; Griffiths et al., 1978).

It is generally accepted that most lysosomal hydrolases are relatively stable upon storage at -20 C (Jolly and Desnick, 1979). In one report evaluating temperature stability of lysosomal hydrolases in tissue, it was concluded that the activity of these enzymes can remain relatively constant for several years in intact tissue stored at -20 C (Hultberg and Öckerman, 1972). α-Mannosidase in plasma from both man and cattle has also been reported to be highly stable at -20 C (Öckerman, 1969; Jolly et al., 1973). However, a recent study suggests that plasma enzyme activity decreases approximately 40% during storage of human α-mannosidase at -20 C for three days (Lombardo et al., 1980). This would suggest that the stability of glycohydrolases be evaluated at storage temperatures over time to insure assay validity.

Because a number of isoenzymes of specific lysosomal hydrolases exhibit considerable differences with respect to thermolability, heat inactivation has been used to provide better separation of both affected and heterozygote populations from controls. For example, the method generally employed for heterozygote detection in Tay-Sachs disease

involves measurement of total β-hexosaminidase activity in serum before and after thermal inactivation. Since β-hexosaminidase A is heat labile compared to the B isoenzyme, the loss of enzyme activity provides an indirect measurement of the A component (O'Brien, 1978; Nakagawa et al., 1978).

Extrinsic factors

The effects of age on plasma lysosomal hydrolase activities. In studies on man, most reports state that there is no change in plasma lysosomal hydrolase activity with age (Erickson et al., 1972; Griffith et al., 1978; Annunziata and Di Matteo, 1978). A recent report, however, suggests that age affects most plasma glycohydrolases in man, including α-mannosidase, as follows: there was an absolute maximum activity at birth, an absolute minimum activity at puberty, and a gradual rising then falling through early and middle adulthood, followed by a final elevation which remained constant through old age (Lombardo et al., 1981). In contrast to reports in man, Jolly (1974a) reported a twofold increase in acidic α-mannosidase activity from birth to maturity in cattle.

The effects of sex on lysosomal hydrolase activity. Reports in man indicated that mean plasma α-mannosidase activity and mean plasma glycohydrolase activities in general were not typically affected by sex (Erickson et al., 1972; Griffiths et al., 1978; Annunziata and Di Matteo, 1978). In cattle, however, mature females had significantly higher plasma activity than males (Jolly et al., 1973).

The effects of reproductive status on plasma glycohydrolase activities. The effects of reproductive status on plasma glycohydrolase activities have not been adequately studied in man, with the exception

of β-hexosaminidase A activity (Lowden,1979). In Tay-Sachs disease, identification of carriers during pregnancy is difficult because of the changes in the relative and absolute activities of the various molecular forms of the hexosaminidase isoenzymes with advancing pregnancy (Nakagawa et al., 1978). In cattle, no significant differences were noted in plasma α-mannosidase activity between gravid and non-gravid cows (Jolly et al., 1973). There has been no report on the effects of sexual neutering on lysosomal hydrolase activity in either man or animals.

The effects of environment on lysosomal hydrolase activities. No studies have been reported relating environmental factors to lysosomal hydrolase activity in man. In cattle, Jolly reported that environmental factors, including season of the year and diet, do have an effect on plasma α-mannosidase activity (Jolly et al., 1974a).

## The Use of Reference Enzymes

Quantitative measurements of enzyme activity require that activity be stated relative to some other reference parameter, for example volume, mg of protein, or some other variable factor as the activity of another enzyme. Use of a reference parameter is only valid if there is a positive correlation between it and the enzyme in question. With plasma or serum, volume is the most convenient, although not necessarily the best reference parameter (Kabak et al., 1973). In supernatants of tissue extracts, the amount of protein in the supernatant may be used, but this has the disadvantage that only a small proportion of the protein measured is the enzyme in question, and variable release of soluble proteins from different samples may contribute to assay

variability, thus potentially preventing definitive detection of heterozygotes (Jolly and Desnick, 1979).

Enzymatic activity may also be expressed relative to some other enzyme when activity levels of the two are highly correlated. Such reference enzymes have proved particularly useful in Tay-Sachs and other hexosaminidase deficiency diseases in which β-hexosaminidase A activity is usually measured relative to total β-hexosaminidase activity (Johnson and Chutorian, 1978); they have also proved useful in heterozygote detection of α-mannosidosis in cattle.

With respect to bovine α-mannosidosis, the ratio of neutrophilic α-mannosidase to total β-hexosaminidase activity (with which it is highly correlated) has been shown to be an alternative method of avoiding the aforementioned extrinsic factors affecting the assay. This ratio has also been shown to provide distinct separation between heterozygotes and controls (Thompson et al., 1976b; Winchester et al., 1976). Therefore, when developing an assay system for lysosomal hydrolases, it is desirable to measure a variety of prospective reference parameters in a normal population (from materials such as plasma and neutrophils) and to examine the correlation between the test enzyme and the prospective parameter. Those with the highest coefficients should provide the most satisfactory system for heterozygote detection (Jolly and Desnick, 1979).

## Current Knowledge of Acidic α-Mannosidases

Mammalian plasma and tissues have three distinct α-mannosidases (Phillips et al., 1974; Burditt et al., 1980a; Tabas and Kornfeld, 1978):

1. Acidic α-mannosidase, which has a pH optimum of 4.0-4.5, is found in lysosomes and is associated with the degradation of carbohydrate moieties of glycoproteins.
2. Intermediate α-mannosidase, which has a pH optimum of 5.5-6.0, is located in the Golgi apparatus and is believed to be associated with the trimming of the oligosaccharide precursors of N-glycosidically-linked chains in the synthesis of glycoproteins.
3. Neutral α-mannosidase, which has a pH optimum of 6.0-6.5, has a cytosolic distribution.

Although the pH activity of these isoenzymes overlaps, they can be distinguished by their kinetic and physicochemical properties.

Using ion exchange chromatography, acidic α-mannosidase can be resolved into two peaks identified as A and B. The B peak can be further broken down into two poorly defined subclasses, $B_1$ and $B_2$. In man, cattle, and cats, the A and B forms have similar kinetic properties, are activated by zinc and inhibited by cobalt, and are immunologically identical (Burditt et al.,1980b). The molecular differences between the A and B forms of acidic α-mannosidase are unknown, but because the activity of both forms is deficient in α-mannosidosis of humans (Carrol et al., 1972), cattle (Phillips et al., 1974), and cats (Burditt et al., 1980a), they are believed to be coded for by the same locus (Jolly, 1981).

Although all subjects affected with α-mannosidosis have deficient activity levels when compared to species-matched controls, residual acidic α-mannosidase activity in α-mannosidosis has been reported in man (Burditt et al., 1980b) and cattle (Winchester et al., 1976; Hocking et al., 1972). In affected calves, the proportion of residual activity

varies from one organ, cell type, or body fluid to another but is generally 2% to 15% of the activity in the corresponding normal tissue, cell, or fluid (Jolly, 1981). Characterization suggests that a mutant enzyme, the product of a defect in the structural gene for acidic α-mannosidase, accounts for the residual activity (Burditt et al., 1980b). In general, the mutant enzyme has the following characteristics: it is less stable than the normal enzyme (Burditt et al., 1980b); it has a higher kM value for synthetic substrate than the normal enzyme (Desnick et al., 1976b); the mutant enzyme may or may not cross react with anti-α-mannosidase serum (Burditt et al., 1980b); cobalt tends to activate rather than inhibit the mutant enzyme (Desnick et al., 1976b); and zinc does appear to lower the kM but to a lesser degree than in controls (Desnick et al., 1976b).

## Non-Caprine β-Mannosidase

Although β-mannosidase has been predicted to be a key enzyme in sequential exoglycosidase degradation of glycoprotein oligosaccharide units, very little information regarding the mammalian enzyme has been reported apart from studies on human serum (Chester and Öckerman, 1981) and rat liver (LaBadie and Aronson, 1973), and that the ratio of α-mannosidase to β-mannosidase is very high (Jones and Dawson, 1981). β-Mannosidase has been purified and characterized from fungi (Elbein et al., 1977; Bouquelet et al., 1978; Wan et al., 1976; Sone and Misake, 1978), pineapple bromelain (Li and Lee, 1972), gastropods (Sugahura et al., 1972; Murmatsu and Egani, 1967), and hen oviduct (Sukeno et al., 1972). A summary of these findings is presented in Table 1.

From the data, β-mannosidase from both human serum and rat liver has pH optimum intermediate or pxoimate to values of other organisms.

Table 1. Biochemical characteristics of non-caprine β-mannosidases

| Source | Reference | Km (mM) | Molecular Weight | Optimal pH | Thermolability (% Activity Lost) | Comments |
|---|---|---|---|---|---|---|
| **FUNGI** | | | | | | |
| *Aspergillus niger* | Elbein 1977 | 2.0 pNP* 1.0 Trisaccharide | 120,000 | 3.5-4.0 | N.R.** | --- |
| *Aspergillus niger* | Bouquelet 1978 | 0.5 pNP 8.4 Disaccharide | 130,000 | 3.5 | 55 C--2 hrs no activity lost | Contains 20% carbohydrate |
| *Polyporus sulfurous* | Wan 1976 | 1.6 pNP | 64,000 | 2.8 | N.R. | EDTA has no effect; 2 fractions are obtained from chromatography |
| *Tremella fuciformis* | Sone 1978 | 2.1 pNP | 140,000 | 5.0 | N.R. | EDTA has no effect; 2 mM zinc added to reaction causes 14% reduction in enzyme activity. Unstable below pH 3.5 |
| **PLANT** | | | | | | |
| Pineapple bromelain | Li 1972 | N.R. | N.R. | 3.0 | 55 C--1 hr 50% | Stable at low pH |

Table 1 (continued)

| Source | Reference | Km (mM) | Molecular Weight | Optimal pH | Thermolability (% Activity Lost) | Comments |
|---|---|---|---|---|---|---|
| ANIMAL | | | | | | |
| *Achatina fulica* | Sugahara 1972 | 6.5 (phenyl) | N.R. | 4.5 | 55 C--5 min at pH 5-7 15% inactivated, at pH 3-4 80% inactivated, stable at -20 C | --- |
| *Turbo cortunus* | Muramatsu 1967 | 7.1 (phenyl) | N.R. | 4.0 | 60 C--3 min 50% inactivated stable at -20 C | EDTA has no effect, unstable below pH 3.5 |
| Hen oviduct | Sukeno 1972 | 2.9 pNP | 100,000 | 4.6 | 65 C--60 min 100% inactivated | EDTA and zinc have no effect on the assay; stable at high pH; 2 fractions obtained from chromatography |
| Rat liver | LaBadie 1973 | 6.0 pNP | N.R. | 4.6 | 65 C--60 min at pH 6.5 39% inactivated | $Zn^{+2}$ has no effect on assay; stable between pH 4.5-7.5 |
| Human serum | Chester 1981 | 2.8 pNP | N.R. | 4.25 | 50 C--15 min 100% inactivation; stable at -20 C or +4 C for 7 days | $Co^{+2}$, $Zn^{+2}$, EDTA have no effect on assay |

*pNP = p-nitrophenol assay **N.R. = not reported

The kM using pNP substrate is considerably higher for rat liver than other species evaluated, including man. In all cases where examined, β-mannosidase appears to be unstable at low pH, stable upon freezing and, in contrast to α-mannosidase, does not appear to be a metalloenzyme.

## Caprine β-Mannosidosis

### Introduction

β-Mannosidosis is a rapidly fatal neurovisceral storage disease due to a deficiency of the terminal enzyme in glycoprotein catabolism, β-mannosidase. The disease was originally observed in New South Wales (Hartley and Blakemore, 1973) and arose independently in a herd of goats in Michigan (Jones et al., 1979). The disease was first defined by Jones et al. when oligosaccharide (Jones et al, 1980; Jones and Laine, 1981; Matsuura et al., 1981) and enzyme (Jones and Dawson, 1981; Cavanagh et al., 1981) studies were performed on tissue, urine and plasma obtained from neonatal Nubian goats exhibiting severe neurologic defects and facial dysmorphia (Jones et al.,1979).

### Mode of Inheritance

β-Mannosidosis appears to be a Mendelian disorder which exhibits autosomal recessive inheritance. This is suggested by results of both inbreeding and outbreeding experiments on a herd of goats in which the gene for β-mannosidosis is known to exist. The experiments exhibit the following features typical of an autosomal recessive disorder:

1. The β-mannosidosis phenotype has only been expressed experimentally by inbreeding animals which are either known heterozygotes or putative heterozygotes.
2. No first generation breedings of known heterozygotes to randomly selected controls has produced affected animals.

3. Siblings of an affected goat appear to have a one in four chance of being affected (of 24 offspring currently produced by inbreeding, 6 have been affected).
4. Both males and females have been produced by inbreeding animals in which the phenotypic expression was not present.

Gross Changes and Clinical Features

The primary gross abnormalities in β-mannosidosis are present at birth and are characterized by facial dysmorphia and joint abnormalities (Jones et al., 1979; Jones et al., 1981). A depressed nasal bridge, frontal bossing, narrowed palpebral fissures, and an elongate, narrow muzzle comprise the facial dysmorphia. The joint abnormalities are progressive and are characterized by carpal contractures and hyperextension defects of the pastern joints. On necropsy, in addition to the above changes, cardiac hypertrophy, pectus carinatum, enlargement of spleen and kidney, and cerebral ventricular dilatation were noted. Lack of myelin is evident in the cerebrum and cerebellum, and proximal musculature appears atrophic. With computerized axial tomography, affected animals exhibit obvious cerebral ventricular dilatation but no other consistent radiographic lesions.

On neurologic examination, goats affected with β-mannosidosis exhibit a prominent intention tremor, pendular nystagmus, spasticity, and inability to walk. Patellar reflexes are typically not present at birth but were accentuated in an animal surviving into the second month. Electromyographic abnormalities include spontaneous potentials resembling positive sharp waves and fibrillation potentials suggestive of denervation. No significant alterations are present in electroencephalographic or funduscopic examination.

Clinicopathologic data, including calcium, phosphorus, BUN, creatinine, SGOT, CPK, alkaline phosphatase, and complete blood count, are not consistently altered. In recent studies, using serum electrophoresis, there was a prominent decrease in $\alpha_2$ and $\gamma$-globulins in neonatal affected animals. This difference was explained by the fact that these animals did not obtain a significant amount of colostrum postpartum.

Morphologic Alterations

An evaluation of the morphologic alterations of β-mannosidosis is currently in press (Jones et al., in press). They will be only briefly discussed here.

Light microscopy. The hallmark of β-mannosidosis under the light microscope is diffuse PAS-negative cytoplasmic vacuolation. This alteration is prominent on toluidine blue-stained semithin sections but may be difficult to discern on routine H&E sections. The vacuolar alterations are consistently present in fibroblasts, macrophages, endothelial cells, and perithelial cells. All parenchymal cells, with the exception of epidermis, are also affected to some degree. Prominent examples of the pathologic alterations of β-mannosidosis are observed in the brain and the proximal tubules of the kidney.

The lesions in the brain are most extensive in, but not limited to, the cerebral cortex and the cerebellum. These lesions could be grouped into three major types of pathologic alterations (Hartley and Blakemore, 1973; Jones et al., 1979; Jones et al., 1981; Jones et al., in press):

1. Neuronal lesions. There is a fine to coarse, typically cell-specific vacuolation of neuronal cell bodies. The greatest percentage of neuronal cell bodies exhibiting vacuolation is

in the cerebral cortex, but the most severely affected cells are the cerebellar Golgi type 2 cells and neurons of the dentate nucleus.

2. Axonal and myelin lesions. There is a severe lack of myelin of the cerebrum and cerebellum and optic nerves. This myelin paucity is not present in peripheral nerves. The central nervous system axonal lesions include "spheroids" which consist of aggregates of dense bodies and mitochondria.
3. Glial alterations. In addition to gliosis, the changes in the glial cells are characterized by primarily cytoplasmic vacuolation. Oligodendroglial cells were sparse and appeared to be extensively vacuolated.

In the kidney, the epithelium of the proximal convoluted tubules is characterized by numerous large vacuoles which appear to entirely replace the normal cytosolic parenchyma. In contrast, the distal tubules, the collecting ducts, and parietal and visceral glomerular epithelium exhibit only a fine vacuolation typical of that observed in most other organs (Jones et al., 1981; Hartley and Blakemore, 1973).

Ultrastructural Alterations

All tissues exhibited essentially similar ultrastructural alterations. These are characterized by cytoplasmic vacuoles .2-.8 μm in diameter. The vacuoles are lined by a single membrane, often containing membranous and floccular material, and are related to the forming face of the Golgi complex (Jones et al., 1981).

## Nature of the Storage Product

Recent studies on the nature of the storage product in β-mannosidosis have shown a pathologic storage of the oligosaccharides Man(β1-4)GlcNAc-(β1-4)GlcNAc and Man(β1-4)GlcNAc in the brain (Jones and Laine, 1981) and kidney (Matsuura et al., 1981), as well as elevated levels of mannose and GlcNAc residues in the urine (Jones and Dawson, 1981). The accumulation and excretion of these compounds presumably arises from the turnover of glycoproteins containing N-linked glycosyl chains.

The storage of the trisaccharide Man(β1-4)GlcNAc(β1-4)GlcNAc is of interest because of the chitobiosyl linkage, which is apparently not broken down by endo-β-glucosaminidase. The reason for this lack of cleavage is not yet known; however, its presence brings to question the function and activation factors required for endo-β-glucosaminidase action (Jones and Laine, 1981; Matsuura et al., 1981).

Phospholipid, neutral lipid, glycolipid and gangliosides all are apparently not specifically altered in animals affected with β-mannosidosis. In β-mannosidosis the storage product is limited to oligosaccharide products of perturbed glycoprotein catabolism.

## Nature of the Enzyme Defect

A deficiency of both tissue (Jones and Dawson, 1981) and plasma (Cavanagh et al., 1981) β-mannosidase activity has been demonstrated in goats affected with β-mannosidosis using the synthetic substrate p-nitrophenol-β-mannoside. With respect to tissue activity, no enzyme activity was reported in the brain or kidney. In the liver, β-mannosidase activity was markedly deficient but not completely absent as in other organs. The presence of this significant activity was due to an overlap from a β-mannosidase with a more neutral pH (Dawson,

1982). On evaluation of a number of other glycohydrolases involved in glycoprotein catabolism in an affected goat, tissue α-mannosidase and α-fucosidase activity varied from 2 to 20 times normal depending on the tissues analyzed. Interestingly, an obligate heterozygote which was examined had tissue β-mannosidase, α-mannosidase, and α-fucosidase activity levels all intermediate between levels in the affected animal and control levels, suggesting the possibility of using these assays for heterozygote detection.

Similarly, plasma assays have shown affected animals to have essentially no β-mannosidase activity. However, unlike tissue studies, the plasma α-mannosidase activity in affected goats is similar to that of control goats (Cavanagh et al., in press).

Complete characterization of caprine β-mannosidase is currently in progress. However, the hepatic enzyme reportedly has an optimum tissue pH of 5.0 with a second activity peak at a more neutral pH (Dawson, 1982).

## Summary of Literature Review

Lysosomal storage diseases are a diverse class of enzymopathies characterized by the functional deficiency of a catabolic gene product leading to the intralysosomal accumulation of substrate or substrate precursors. The oligosaccharidoses are a category of lysosomal storage diseases which involve errors of glycoprotein catabolism. All glycoprotein storage diseases are autosomal recessive with heterozygotes exhibiting approximately one-half the enzyme activity of normal individuals. Attempts to detect carriers of these disorders, therefore, typically involve measuring the activity of the partially deficient enzyme and comparing this to putative carrier and control values. The

analysis of enzyme activity most frequently used for heterozygote detection involves the use of either chromogenic or fluorimetric substrates on blood plasma, serum, or cellular elements. Although most carriers can be separated from genotypically normal subjects using these methodologies, definitive diagnosis of all putative carriers may not be possible due to an overlap in activity levels between normal subjects and heterozygotes. There are three major causes for this overlap:

1. Genetic factors. These refer to the fact that at any locus there may be variant alleles producing active gene products with slightly different activity, stability, or kinetics. These different gene products may lead to considerable activity variance in both controls and heterozygotes.
2. Variability inherent within the assay itself. These factors include the tissue used as an enzyme source, the choice of substrate and the substrate concentration, the pH at which the enzyme is assayed, the type of buffer used, the temperature at which the enzyme is assayed, and the stability of the enzyme to freezing.
3. Extrinsic factors related to the metabolic and physiologic differences inherent within the subjects examined. These factors include age, sex, reproductive status, and environment.

β-Mannosidosis, the most recently described disorder of glycoprotein catabolism, is a rapidly fatal neurovisceral storage disease due to a deficiency of the terminal enzyme in glycoprotein catabolism, β-mannosidase. This disorder has currently only been defined in the caprine species, where it exhibits autosomal recessive inheritance. The major features of goats affected with this disease are:

1. Grossly, there are facial dysmorphia, joint abnormalities, and profound neurologic deficits.
2. Histologically, there are diffuse cytoplasmic vacuolation and demyelination of the central nervous system.
3. Biochemically, there are neurovisceral storage of the oligosaccharides, Man(β1-4)GlcNAc(β1-4)GlcNAc and Man(β1-4)GlcNAc, and negligible β-mannosidase activity in plasma or tissue.

## OBJECTIVES

The objectives of this research were:

1. To establish the pH optimum for the assay of caprine plasma α- and β-mannosidases.
2. To determine the effects of assay incubation time on caprine plasma α- and β-mannosidase activities.
3. To determine the range and mean plasma α- and β-mannosidase activities in a control population of Michigan goats with respect to age, sex, and reproductive status.
4. To determine whether the plasma β-mannosidase assay and/or the use of the plasma α-mannosidase to β-mannosidase ratio can serve as diagnostic tests for detection of heterozygotes for β-mannosidosis.

# MATERIALS AND METHODS

## Processing Plasma from Control and Breeding Herd Populations

Specimens were randomly selected from 12 goat herds in 3 Michigan counties during April and May, 1981. Blood was collected from 27 males and 25 females aged 1 to 7 days (mean age 4 days), defined as neonatal goats; from 27 males and 25 females, aged 22 to 28 days (mean age 24 days), defined as young goats; and from 24 males and 26 females, aged 6 to 96 months (mean age 36 months), defined as adult goats. All animals were clinically normal and their reproductive status was noted at the time of obtaining blood.

Blood was also collected in a similar manner from a breeding herd of known and putative heterozygotes for β-mannosidosis at Michigan State University. This herd included 8 males and 7 females, all putative carriers, whose blood was drawn twice, first as neonatal goats and then again as young goats (except for 1 male) when they had reached the age of 22 to 28 days. The adult population from this herd consisted of 4 males and 10 females aged 3 to 40 months, of which 1 male and 2 females are known through inbreeding to be heterozygotes, with the remainder being putative carriers.

The blood was collected from the jugular vein in sterile heparinized vacutainers and stored at 4 C during transportation to the laboratory. The plasma was separated by centrifugation (500 g, 15 minutes, 4 C),

usually within 1 to 2 hours of collection. Specimens were then aliquoted in 0.5 ml volumes and stored at -20 C until enzyme analysis. All analyses were performed within 4 months of the collection date.

## Determination of Plasma α- and β-Mannosidase Activities

The assays for plasma α- and β-mannosidases were performed using the appropriate pNP derivative (Dawson and Tsay, 1977). Each assay mixture contained the following: 20 µl of 10 mmol substrate, 100 µl of 0.1 M sodium citrate-phosphate buffer (pH 4.4 for the α-mannosidase assay and pH 5.0 for the β-mannosidase assay, except where noted), and 50 µl of diluted plasma 1:2 (v/v) for both α- and β-mannosidase assays of goats 28 days or less and 1:1 (v/v) for both assays of adults. The reaction mixture in the α-mannosidase assay was incubated for 1 hour at 37 C. The reaction mixture in the β-mannosidase assay was incubated for 20 hours at 37 C. Following incubation, the reaction was stopped by the addition of 0.5 ml of 3.5% trichloroacetic acid (w/v) and subsequent addition of 0.8 ml of 0.25 M glycine $NaHCO_3$ buffer, pH 10.0. The mixture was then centrifuged in a Clay-Adams Serofuge and the supernatant was used for obtaining spectrophotometric readings at 410 nm. Plasma enzyme activities represent the average of 3 replications and are expressed as nanomoles of p-nitrophenol formed per hour per milliliter of plasma (nmol/ml/hr). As a means of minimizing experimental error, no data were accepted unless the range of the 3 replications was less than 0.020 spectrophotometric absorbance units. All statistical comparisons were performed with the improved Bonforoni t-test (Games, 1977) and, unless otherwise stated, data were considered significant for $p<0.05$.

## Determination of the Effects of Assay Incubation Time on Enzyme Activity

The effects of assay incubation time on plasma α-mannosidase activity were determined by performing the assay at 15 minute intervals through the range of 15 to 120 minutes. The effects of incubation time on β-mannosidase activity were determined by performing the assay using 2, 4, 8, 10, 24, and 25 hour incubation times.

## Determination of the pH Optima

The pH optima for the α- and β-mannosidase assays were established using 0.5 M HCl-KCl (pH 2.0), 0.5 M glycine-HCl (pH 2.5), 0.1 M citrate-phosphate buffer (pH 3.0 to 7.0) at 0.5 intervals,and 0.1 M acetate at 0.2 pH intervals through the range of 3.8 to 5.6.

# RESULTS

## The Effect of Incubation Time on Plasma α- and β-Mannosidase Activity

Figure 3[A] illustrates that the hydrolysis of pNP-β-mannopyranoside by plasma diluted 1:1 with saline was proportional to time up to 24 hours. Short reaction times with undiluted plasma were not routinely used because of high sample blanks. Similarly, hydrolysis of pNP-α-mannopyranoside by plasma diluted 1:1 was proportional to time up to 2 hours (Figure 3[B]).

## pH Activity Curves

The dependency of plasma α- and β-mannosidase activity on pH was evaluated through the range of 2.0 to 8.0 using HCl and KCl, glycine HCl and citrate-phosphate buffers (Figure 4[A,B]). From the pH activity curves, plasma β-mannosidase exhibits activity between pH 4.0 and 7.0 with optimal activity at pH 5.0. In contrast, plasma α-mannosidase exhibits activity between pH 3.0 and 7.0 with an optimal activity at pH 4.0.

## Analysis of Control Animals

### Effects of Age on Plasma α- and β-Mannosidase Activity

The mean plasma α- and β-mannosidase activities in both sexes decreased with maturity. Male and female adult goats had significantly lower mean plasma α- and β-mannosidase activities than either neonatal

Figure 3A. Effect of assay incubation time at 37 C on caprine plasma β-mannosidase.

Figure 3B. Effect of assay incubation time at 37 C on caprine plasma α-mannosidase.

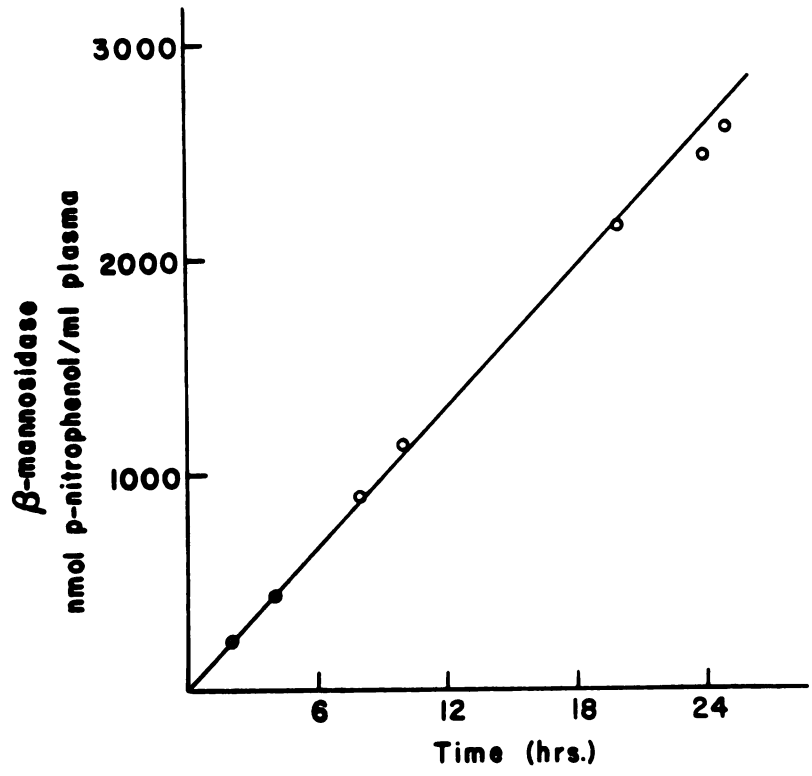



Figure 3A

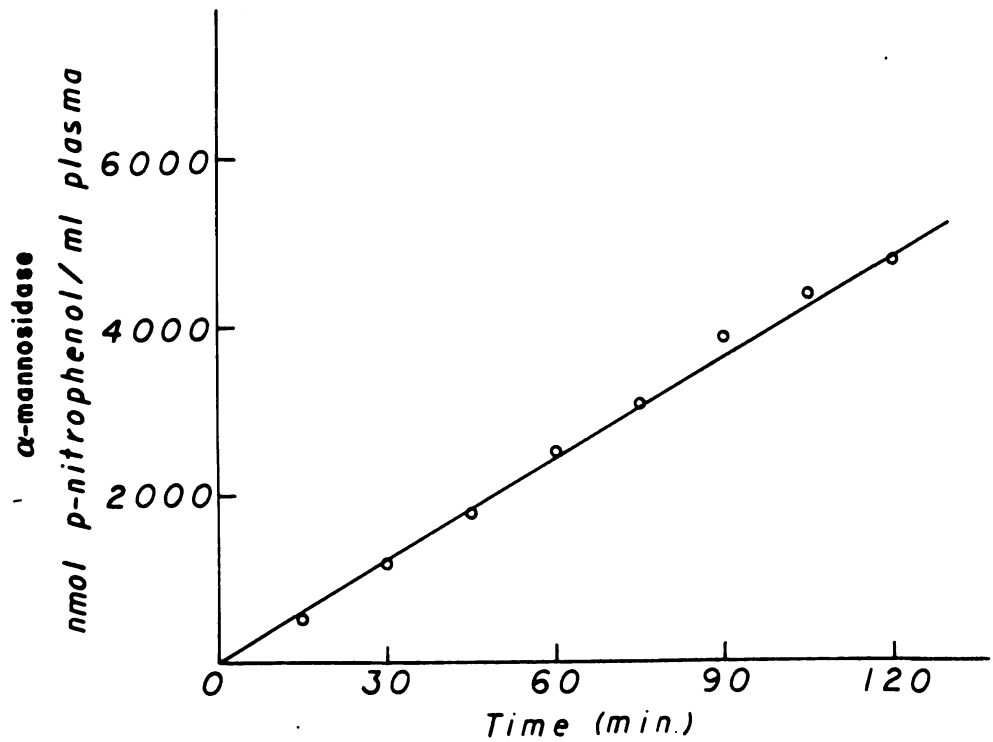



Figure 3B

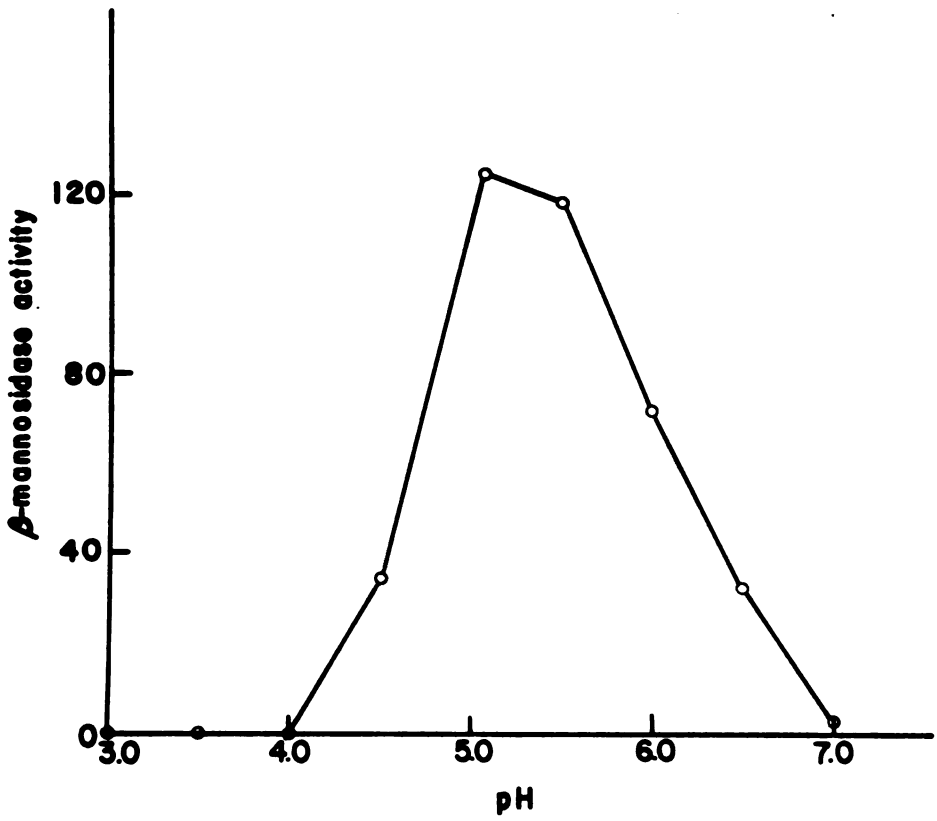



Figure 4A. Effect of pH on caprine plasma β-mannosidase.

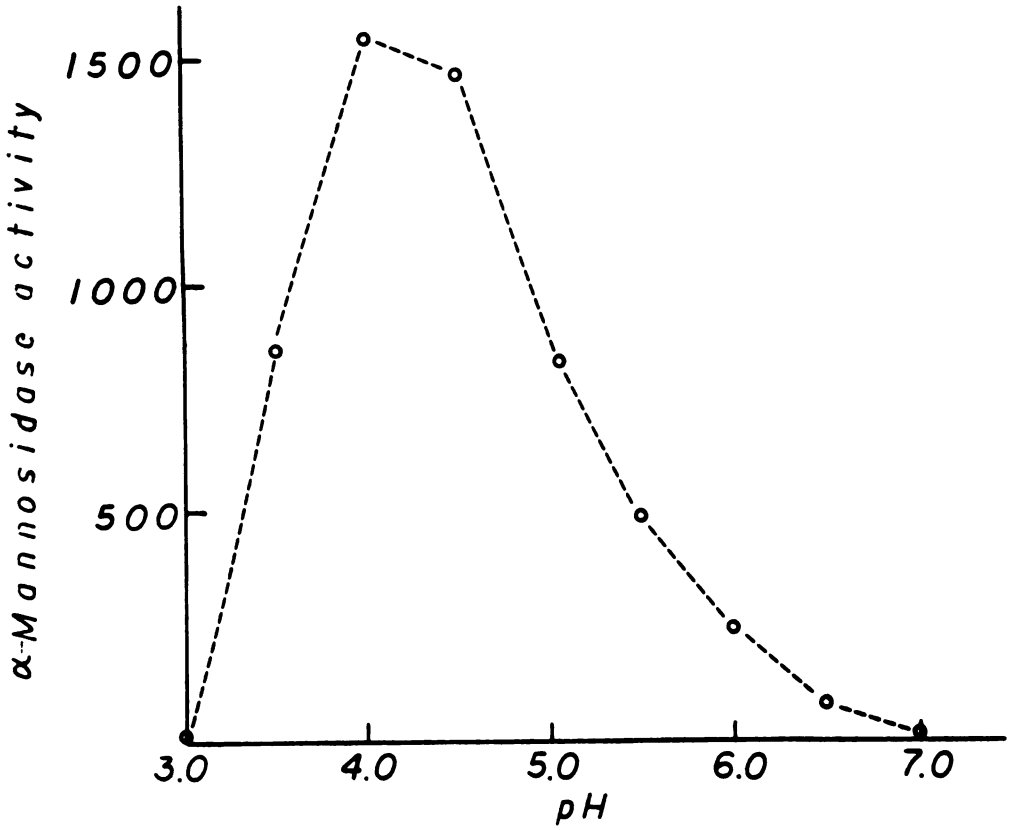



Figure 4B. Effect of pH on caprine plasma α-mannosidase.

or young goats ($p<0.10$ for the mean β-mannosidase values for young male and adult male goats) (Figure 5[A,B]). Except for significantly higher β-mannosidase values in neonatal male goats when compared to young male goats, mean plasma mannosidase levels did not differ in these age groups. The lack of any correlation between age and either plasma α- or β-mannosidase activity in adult goats (Table 2) suggests that there is no progressive increase or decrease in these two enzymes beyond sexual maturity.

## Effects of Sex and Reproductive Status on Plasma α- and β-Mannosidase Activity

The effect of sex on mean plasma α- and β-mannosidase activity seems related to sexual maturity (Table 3, Figure 5[A,B]). In adult goats, males had significantly higher mean plasma α- and β-mannosidase activities than females, but in young goats only the mean plasma α-mannosidase level was higher. Sex appears to have no significant effect on mean plasma α- and β-mannosidase activities in neonatal goats.

Reproductive status affects mean plasma β-mannosidase levels more than mean plasma α-mannosidase levels (Table 3). Gravid goats had significantly higher mean plasma β-mannosidase activity than lactating goats, and intact young male goats had higher levels than their neutered counterparts. Of the 2 neutered adult male goats and the 2 adult female goats not pregnant, none had mean plasma values appreciably different from its respective sex-matched population as a whole. However, the small sample size of each subpopulation precluded meaningful statistical evaluation.

Figure 5. The effects of age and sex on plasma (A) α-mannosidase and (B) β-mannosidase activity in the control subpopulations. Each vertical bar is the mean ± std. error. Neonates (0-7 days); Young (22-28 days); Adult (6-96 months).

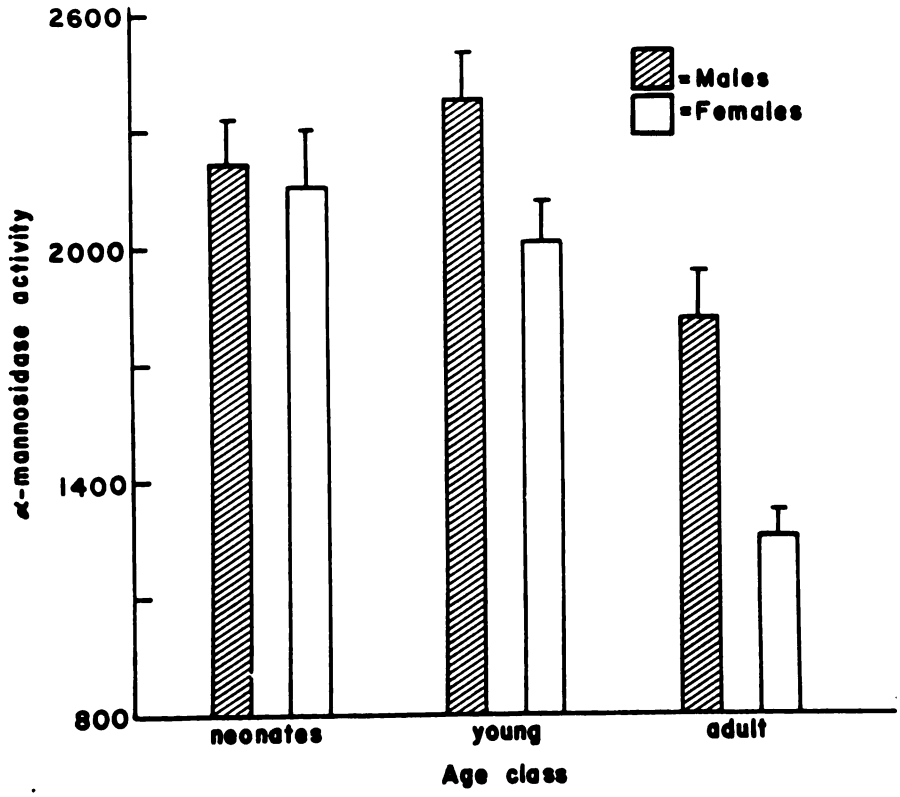



Figure 5A

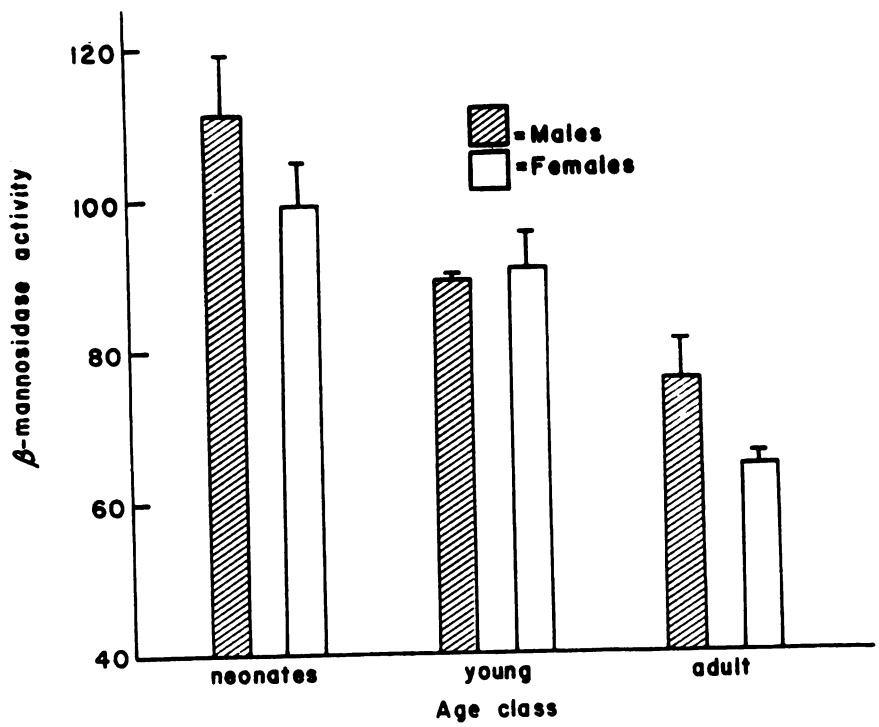



Figure 5B

Table 2. Correlation coefficients (r) relating age in months to (1) plasma α- and (2) β-mannosidase activity in adult male and female goats

| Class | N | Age to Plasma α-Mannosidase Activity (1) | Age to Plasma β-Mannosidase Activity (2) |
|---|---|---|---|
| Adult males | 24 | 0.001 | 0.025 |
| Adult females | 26 | -0.248 | -0.561 |

The lack of any correlation between age and either plasma α- or β-mannosidase activity in adult goats suggests that there is no progressive increase or decrease beyond sexual maturity.

Table 3. Mean plasma α- and β-mannosidase activity in neonatal, young, and adult control goats according to age, sex, and reproductive status

| Class | N | Mean Plasma Activity (nmol/ml/hr) ± Std. Error α-mannosidase | β-mannosidase |
|---|---|---|---|
| Neonatal Goats (1-7 days old) | | | |
| Male | 27 | 2,206±117 | 113.1±6.4 |
| Female | 25 | 2,158±136 | 98.7±6.7 |
| Young Goats (22-28 days old) | | | |
| Male | 27 | 2,387±108 | 88.8±2.2 |
| Intact | 16 | 2,347±133 | 93.4±2.8 |
| Neutered | 11 | 2,445±188 | 82.2±2.7 |
| Female | 25 | 2,019±104 | 91.7±4.3 |
| Adult Goats (6-96 months old) | | | |
| Male | 24 | 1,814±116 | 77.2±3.7 |
| Female | 26 | 1,262± 63 | 64.1±3.1 |
| Gravid | 14 | 1,327± 95 | 70.5±3.2 |
| Lactating | 10 | 1,186± 97 | 55.7±5.6 |

## Analysis of Breeding Herd Goats

### Use of Plasma β-Mannosidase Activity as a Means for Detecting Heterozygotes for β-Mannosidosis

The 3 adult obligate heterozygotes in the β-mannosidosis breeding herd had values approximately one-half of those of the sex-matched adult control goats. The lone male carrier had a plasma β-mannosidase value of 39.7 nmol/ml/hr, compared to the mean value of 77.2 nmol/ml/hr for the adult male controls (Figure 6[A]). The 2 carrier females had plasma β-mannosidase values of 31.7 nmol/ml/hr and 35.0 nmol/ml/hr, compared to 64.1 nmol/ml/hr for the adult female controls (Figure 6[B]). Although the large overlap of plasma β-mannosidase values in relation to age, sex, and reproductive status (data not shown) between the breeding herd and the control populations prevented a distinct separation of heterozygotes from genotypically normal goats, 2 adult male and 1 adult female putative heterozygotes had values lower than the sex-matched obligate heterozygotes and distinct from their respective adult control populations (Figure 6[A,B]).

Attempts to detect heterozygotes in neonatal and young goats were hindered because of the absence of known heterozygotes in these age groups and because of the large differences in the individual plasma β-mannosidase values obtained from the breeding-herd animals at 1 week and at 4 weeks of age. Only 2 males and 1 female from these populations had low activity levels as both neonates and young goats (Figure 6[C,D,E,F]).

Figure 6. Comparison of plasma β-mannosidase activities of obligate and putative heterozygotes with age- and sex-matched controls. A - adult males (6-96 months); B - adult females (6-96 months); C - young males (22-28 days); D - young females (22-28 days); E - neonatal males (1-7 days); F - neonatal females (1-7 days).

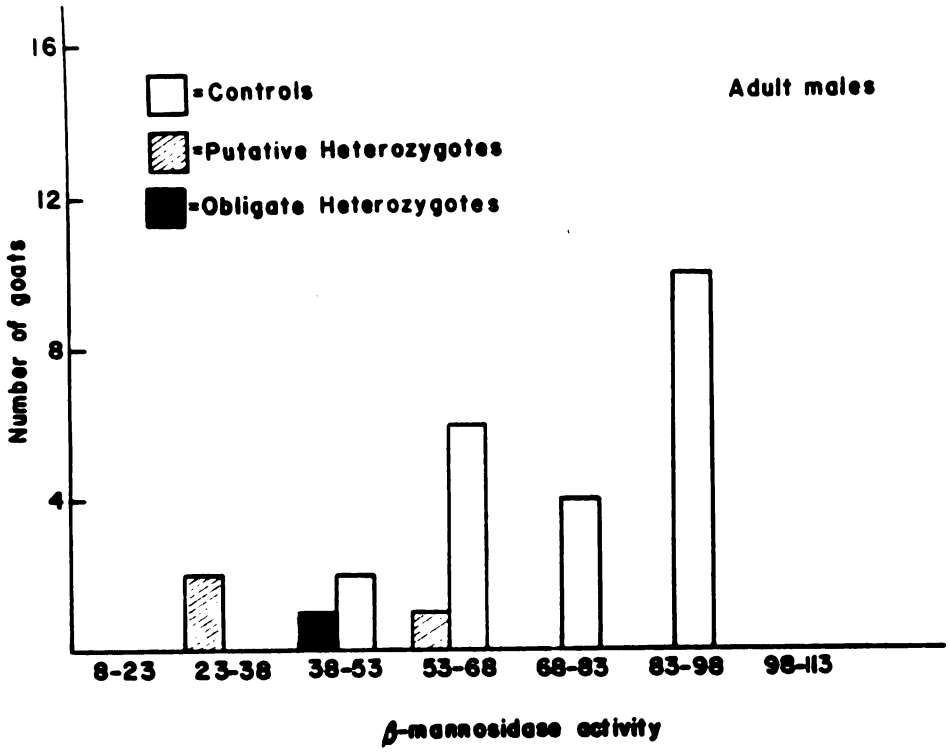



Figure 6A

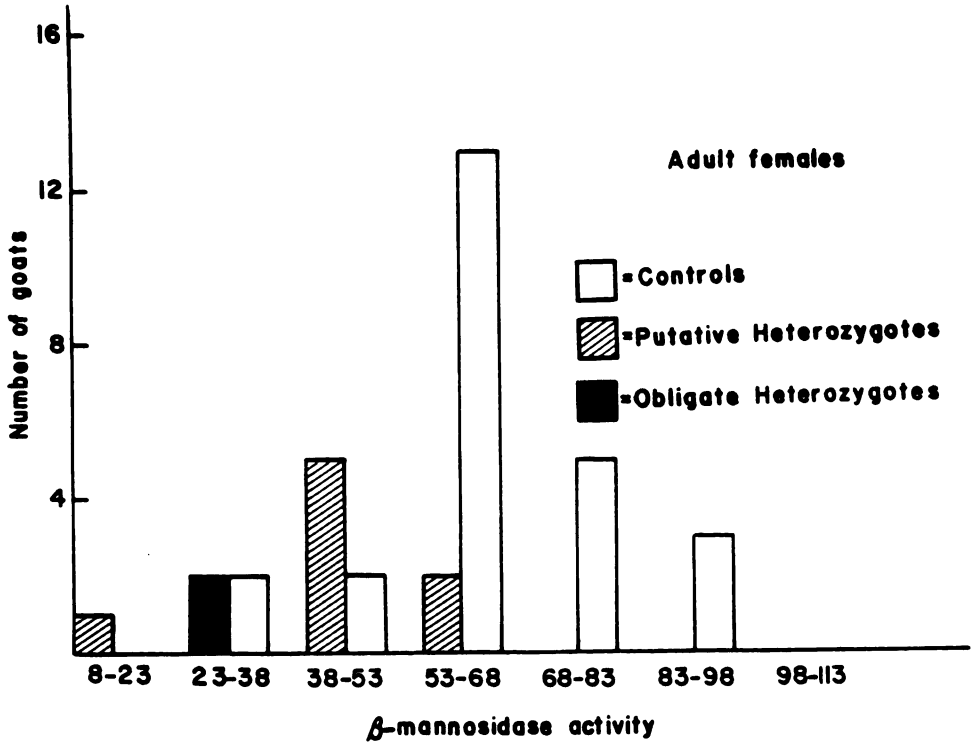



Figure 6B

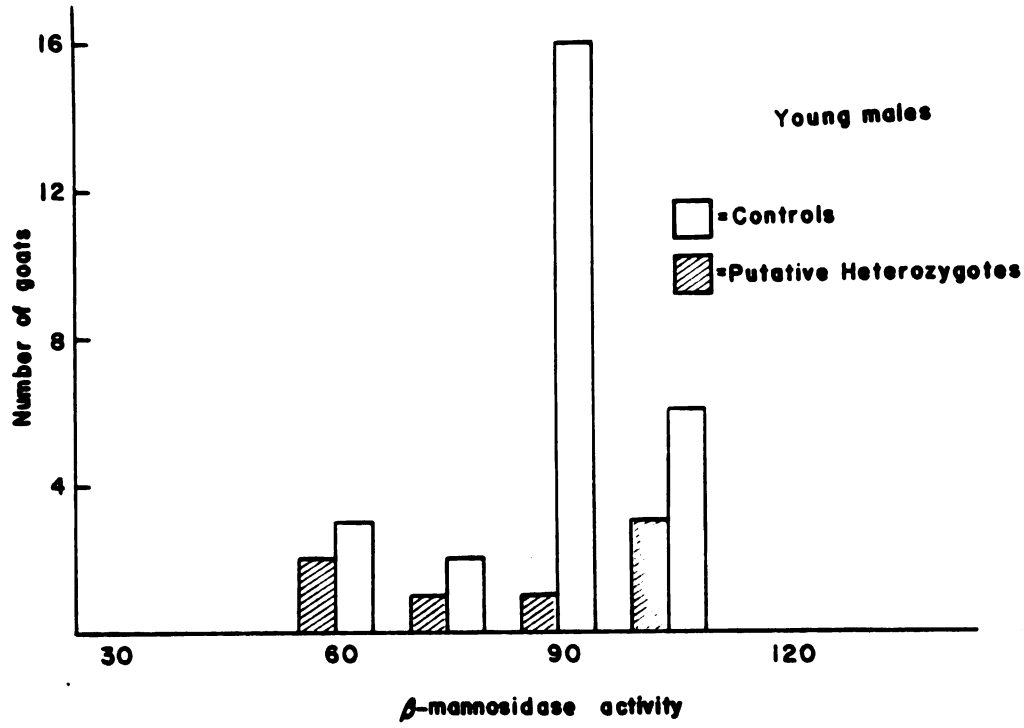



Figure 6C

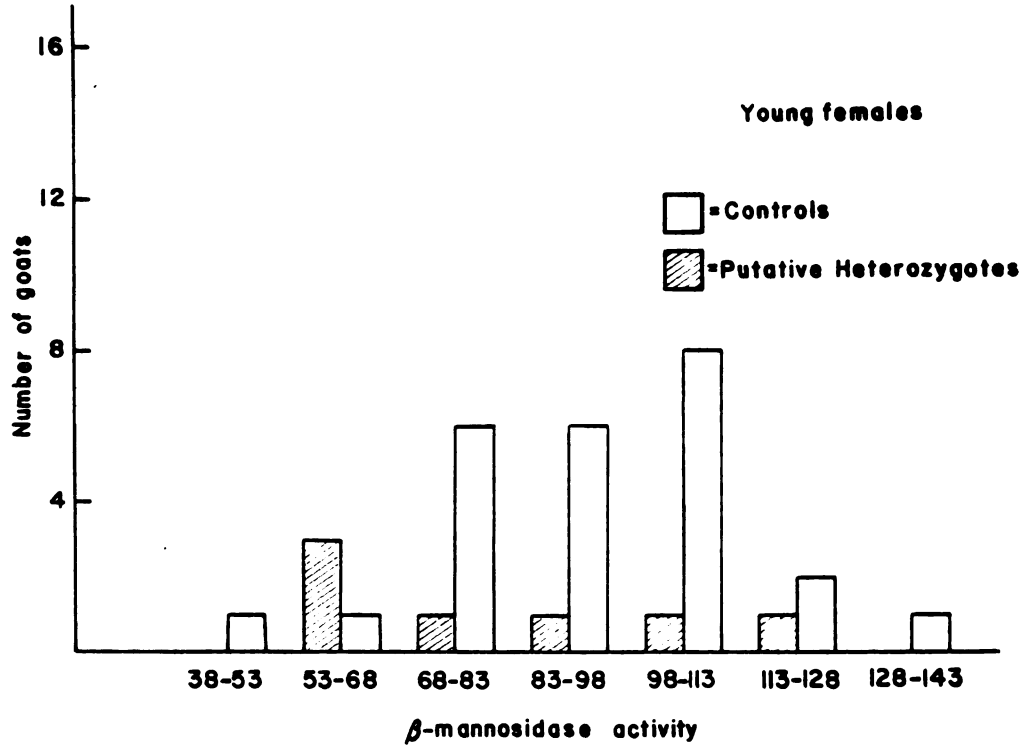



Figure 6D

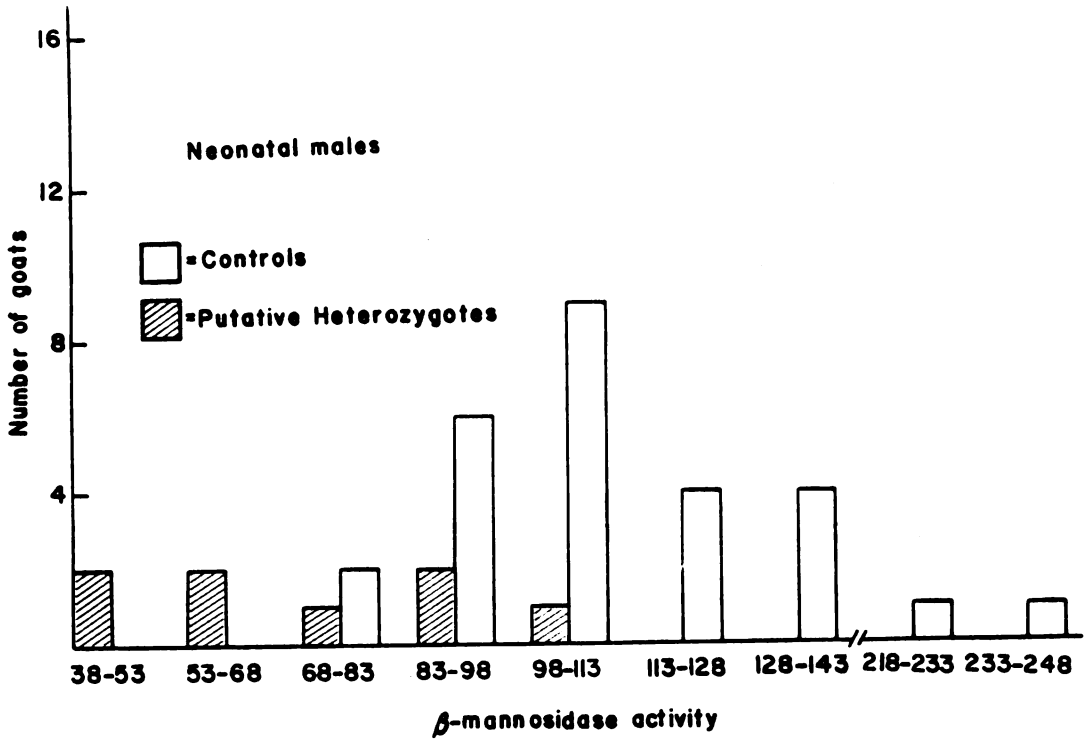



Figure 6E

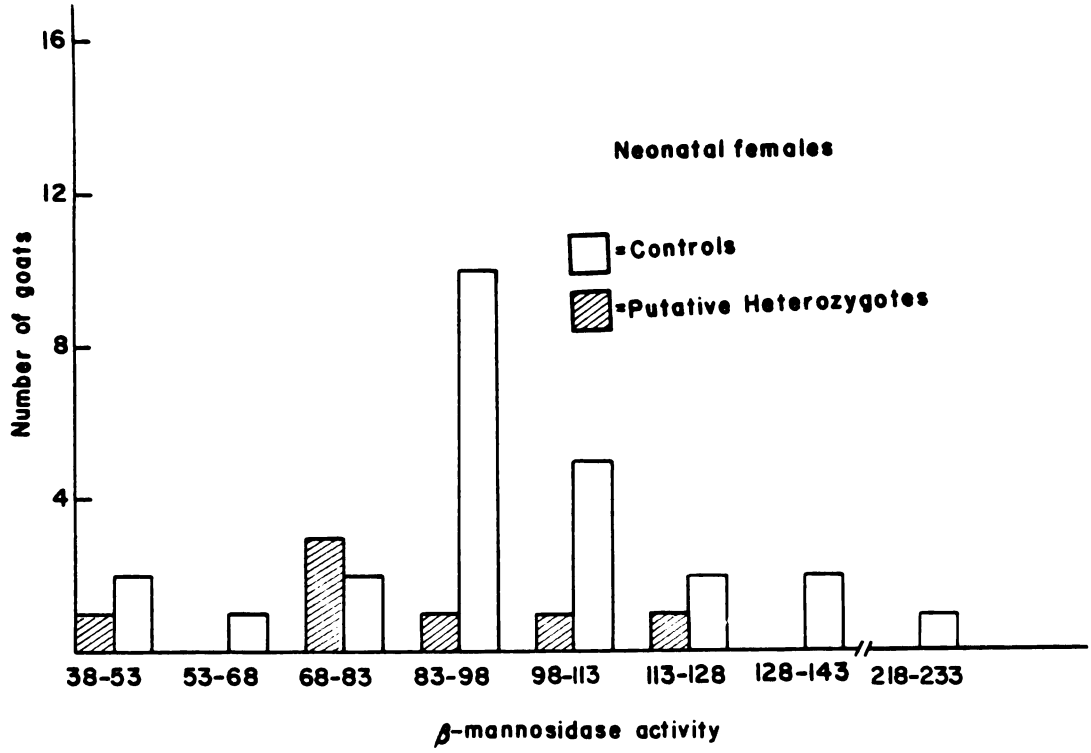



Figure 6F

## The Relationship Between Plasma α-Mannosidase and β-Mannosidase Activities

The correlation coefficient relating plasma α-mannosidase to plasma β-mannosidase for all the control goats is 0.284. Correlation coefficients were also low when the relationship between the 2 plasma mannosidases was evaluated according to age, sex, and reproductive status, and this suggests that there is no relationship between the activities of the 2 plasma mannosidases. Despite these low correlations, the ratio of α- to β-mannosidase activities was determined for controls and compared to the ratios obtained from obligate and putative heterozygotes. Although these values varied considerably and offered no better separation on an individual basis than that obtained from plasma β-mannosidase alone, the mean α- and β-mannosidase ratio for the control population (22.4) varied considerably from the mean ratio for the known heterozygotes (50.4).

# DISCUSSION

## The Effect of Incubation Time on Plasma α- and β-Mannosidase Activities

Substrate hydrolysis by the plasma mannosidases was proportional to time well beyond the respective incubation periods used in this study to evaluate control and β-mannosidosis breeding herd goats. This implies that the long incubation times used, primarily with respect to β-mannosidase assay, should not detract from the validity of the assay. Long incubation times are common when colorimetric assays are used to measure lysosomal hydrolase activity. A more sensitive plasma β-mannosidase assay with a shorter incubation time may be possible now that the 4-MU derivative of β-mannopyranoside is commercially available.

## pH Activity Curves

The pH optimum of 5.0 for caprine plasma β-mannosidase was the same as has been reported in caprine tissue using both pNP and 4-MU substrates (Jones and Dawson, 1981). This value is somewhat higher than values reported in human serum (pH 4.25) and in rat liver (pH 4.6) (Chester and Öckerman, 1981; LaBadie and Aronson, 1973). Other workers have shown the pH optimum of β-mannosidase from diverse, non-mammalian sources to be in the range of 2.8 to 5.0 (Sone and Misake,1978; Bouquelet et al., 1978; Elbein et al., 1977; Wen et al, 1976;Li and Lee, 1972; Sugahura et al., 1972; Sukeno et al., 1972; Muramatsu and Egani, 1967). An additional pH optimum for caprine β-mannosidase reported from crude

liver preparations (Jones and Dawson, 1981) was not observed. The instability of caprine β-mannosidase at low pH corresponds to reports in rat liver, *Tremella fuciformis*, and *Turbo cortunus*, where partially purified β-mannosidase activity was shown to fall rapidly below pH 3.5 (LaBadie and Aronson, 1973; Sone and Misake, 1978; Muramatsu and Egani, 1967), as well as for human serum β-mannosidase, which was stable between pH 4 and 6 for at least 24 hours at 37 C (Chester and Ockerman, 1981). These results differ from pineapple bromelain β-mannosidase, which appears to be relatively stable at low pH (Li and Lee, 1972).

The pH optimum of caprine plasma α-mannosidase was 4.0. This corresponds with the pH optimum of plasma α-mannosidase of 4.0 to 4.5 in other mammalian species (Phillips et al., 1974; Opheim and Touster, 1978; Burditt et al., 1980b). The lack of an additional pH optimum in the plasma α-mannosidase activity is not surprising. Separation of acidic and neutral forms of α-mannosidase generally requires the use of DEAE-cellulose chromatography (Winchester et al., 1976).

The α-mannosidase assays performed on controls and β-mannosidosis breeding herd goats were carried out at pH 4.4 because this value corresponded to the pH optimum for plasma α-mannosidase in the bovine (Jolly et al., 1973). The difference between caprine plasma α-mannosidase activity at 4.4 and the pH optimum of 4.0 is less than 5%; therefore, the results of this study are valid.

## Analysis of Control Animals

The effects of age, sex, and reproductive status on plasma α-mannosidase in cattle have been reported previously (Jolly et al., 1974a; Jolly et al., 1973), as have the effects of age and sex on this

same glycohydrolase in man (Erickson et al., 1972; Griffiths et al., 1978; Annunziata and Di Matteo, 1978; Lombardo et al., 1981). Earlier values of caprine serum α-mannosidase activity with p-nitrophenyl-α-D-mannopyranoside as substrate were higher than the levels reported here (Garcia et al., 1979). This may be due to the lower substrate concentration in our study.

Our data suggest that the mean plasma α- and β-mannosidase levels in the control population tend to remain constant in goats through 4 weeks of age and then decrease sometime during sexual maturity (Figure 6[A,B]). The mean male plasma β-mannosidase values are the only exception in that they begin to decrease at an earlier age. The absence of any relationship between the age of the adult goats and plasma α- and β-mannosidase values suggests that the mean plasma levels of the mannosidases do not change significantly after sexual maturity.

The age dependence of α-mannosidase levels in goats through maturity is exactly opposite from that observed in cattle, which exhibit more than twofold increase in acidic α-mannosidase activity from birth to maturity (Jolly et al., 1973; Jolly and Desnick, 1979). For man, most reports state that there is no change in plasma α-mannosidase activity with age (Erickson et al., 1972; Griffiths et al., 1978; Annunziata and Di Matteo, 1978). However, the findings in a recent study suggest that age does affect most plasma lysosomal hydrolases, including α-mannosidase, as follows: there is an absolute maximum activity at birth, an absolute minimum activity at puberty, a gradual rising and then falling through early and middle adulthood, and a final increase that remains constant through old age (Lombardo et al., 1981).

This report is of interest because our values for goats appear to follow this course, at least initially.

This study indicates that the effects of sex on mean plasma α- and β-mannosidase activities in goats are most pronounced in mature animals. Adult males have significantly higher mean plasma mannosidase levels than females (Table 2, Figure 6[A,B]). This distinction is less clear in the non-adult control populations. The results for plasma α-mannosidase values in goats also differ from those in cattle, i.e., mature bovine females have higher α-mannosidase values than mature males (Jolly et al., 1974). Reports on man indicate that sex has no effect on plasma α-mannosidase or plasma glycohydrolase values in general (Erickson et al., 1972; Griffiths et al., 1978; Annunziata and Di Matteo, 1978; Lombardo et al., 1981).

The reproductive status of the goats affected plasma β-mannosidase activity more than plasma α-mannosidase activity. Gravid females had significantly higher mean plasma β-mannosidase values than did lactating females, and intact young males had significantly higher values than young neutered goats. In contrast, the mean caprine α-mannosidase values are not apparently affected by reproductive status. The only study on reproductive status in cattle reported no discernible differences between mean plasma α-mannosidase levels of gravid and non-gravid cows (Jolly et al., 1974). There are no reports on plasma α-mannosidase values in steers or on the effects of reproductive status on plasma α-mannosidase activity in man.

## Use of Plasma β-Mannosidases as a Means for Detecting Heterozygotes for β-Mannosidosis

Heterozygotes for β-mannosidosis have plasma β-mannosidase activities between the mean values for sex- and age-matched control populations and those for animals affected with β-mannosidosis (Cavanagh et al., 1981). These results are consistent with an earlier report that an obligate heterozygote for β-mannosidosis showed a partial deficiency of tissue β-mannosidase activity (Jones and Dawson, 1981).

In this study, a definitive diagnosis of putative carriers could not be made because of the small number of obligate heterozygotes in the β-mannosidosis breeding herd and the wide range of values obtained in the control population. However, 2 adult male and 1 adult female putative carriers that have values lower than their sex-matched heterozygotes appear promising for future inbreeding studies, as do 2 male and 1 female non-adult putative carriers that had low plasma β-mannosidase levels as both neonatal and young goats.

The use of plasma lysosomal hydrolase activity in detecting heterozygotes for glycoprotein storage diseases has previously been reported, and the results compare quite favorably with our own observations (Jolly and Desnick, 1979). These studies typically show that plasma as a source of enzyme activity is highly successful in diagnosing individuals affected with glycoprotein storage disease (Jolly and Desnick, 1979; Winchester et al., 1976; Stanbury et al., 1978). However, detection of the carrier state may be rendered difficult by the range of values from normal subjects, which overlap and obscure the values of putative heterozygotes (Jolly and Desnick, 1979; Stanbury et al., 1978; Desnick et al., 1976B; Johnson and Chutorian, 1978).

Because of the low correlation coefficient between plasma α- and β-mannosidase activities, there is an indistinct separation between putative heterozygotes and controls with the α- to β-mannosidase ratio as the principal criterion, and thus α-mannosidase should not be used as a reference enzyme in detecting β-mannosidosis heterozygotes. This is unfortunate, since relating the activity of β-mannosidase to an enzyme with which it is highly correlated would negate the variation of this enzyme's activity introduced by age, sex, and reproductive status (Winchester et al., 1976). In spite of the lack of correlation, the mean α- to β-mannosidase ratio for the obligate heterozygotes does differ considerably from that of the control population as a whole. This suggests that even though individual obligate heterozygotes cannot be identified from the α- to β-mannosidase ratio, population differences may still exist.

It is now clear that future evaluation of plasma α- and β-mannosidase levels in goats should consider age, sex, and reproductive status. The marked contrast between age and sex effects on plasma α-mannosidase values in goats and cattle suggests that factors affecting plasma α-mannosidase activity are species-specific and that results obtained from cattle cannot be assumed valid for other species as well. Future studies on the regulation of mannosidase activity in different species are important to the understanding of cell regulation of mannosidase activity in glycoprotein catabolism. Finally, although neither plasma β-mannosidase activity alone nor plasma α- to β-mannosidase ratios distinguish β-mannosidosis heterozygotes from the control populations, the 3 obligate heterozygotes did have plasma β-mannosidase values intermediate between those of affected and control animals and had plasma α- to β-mannosidase ratios higher than controls. Thus, plasma

mannosidase assays may be used to select the most likely heterozygous candidates for the β-mannosidosis breeding program. Future results from the breeding program that establish whether goats with low β-mannosidase values are heterozygotes will be important in determining the validity of this approach.

## SUMMARY

The effects of age, sex, and reproductive status on caprine plasma α- and β-mannosidase activities as well as the potential use of plasma assays for heterozygote detection of caprine β-mannosidosis were investigated. Optimal conditions for the assay of caprine plasma α- and β-mannosidase activity were determined.

The pH optima were 4.0 and 5.0 for caprine plasma α- and β-mannosidase activities, respectively, and substrate hydrolysis was proportional to time beyond the incubation periods used in these studies. Age and sex affected caprine plasma α- and β-mannosidase activities, while plasma β-mannosidase activity was affected by reproductive status. Obligate heterozygotes for β-mannosidosis had plasma β-mannosidase values which were intermediate between those found in animals affected with β-mannosidosis and controls. Putative heterozygotes for β-mannosidosis could not be definitively identified, but likely candidates for future inbreeding experiments were discerned.

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