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City University of New York PH.D. 1982

BIOCHEMICAL GENETIC STUDIES OF THE FELINE MODEL OF HUMAN MUCOPOLYSACCHARIDOSIS VI, MAROTEAUX-LAMY DISEASE

by

MARGARET MARY MCGOVERN

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1982

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Chairman of Examining Committee

4/12/82
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BIOCHEMICAL GENETIC STUDIES OF THE FELINE MODEL OF HUMAN MUCOPOLYSACCHARIDOSIS VI, MAROTEAUX-LAMY DISEASE

by: Margaret Mary McGovern

Advisor: Robert J. Desnick

ABSTRACT

The enzymatic defect in feline mucopolysaccharidosis VI (MPS VI) was characterized and therapeutic strategies evaluated. A method was developed for the detection of heterozygotes for feline and human MPS VI. Arylsulfatase A (ASA) and B (ASB) activities were assayed in leukocyte extracts following separation of the enzymes by batch chromatography. ASB specific activities did not permit heterozygote identification, whereas the ASB to ASA activity ratio discriminated all 16 obligate heterozygotes for the feline and human disorders.

Normal feline and human hepatic ASB isozymes were purified to homogeneity with final specific activities of 1,100 and 800 umoles/h/mg protein, respectively. Both enzymes had the same pH optimum, however, the feline ASB was more electronegative, had a lower K_m and pI and was more thermostable. The molecular weight of the feline enzyme was twice that of human ASB by gel filtration, analytical polyacrylamide gel electrophoresis, and sucrose density-gradient centrifugation. SDS gel electrophoresis revealed a single protein band for each enzyme, and alkylation and cross linking studies were consistent with the feline

enzyme being a homodimer and the human isozyme a monomer.

Hepatic ASB from normal and MPS VI cats was purified over 2,800- and 1,800-fold, respectively. The MPS VI residual activity had a higher K_m, an altered electrophoretic mobility, half the native molecular weight, decreased stability, and was a monomer. When incubated with sulfhydryl reagents, the residual activity was enhanced, whereas the normal enzyme was unaffected. In the presence of dithiothreitol (DTT) or cysteamine, the residual activity had a molecular weight similar to that of the normal enzyme, suggesting that the monomeric residual enzyme was dimerized in the presence of thiol-reducing agents. When 2 mM DTT or cysteamine was incubated with whole blood from an MPS VI cat, the leukocyte residual ASB activity was increased 11- and 20-fold, respectively, and the accumulated dermatan sulfate degraded. Intravenous administration of DTT effected transient increase in leukocyte activity, but no decrease in substrate levels. In contrast, administration of cysteamine increased leukocyte residual activity more than 6-fold 30 min post-infusion, and decreased substrate to 35 percent of the initial level and remained at about 45% of pre-infusion values during the 120 min period studied.

FORWARD

Portions of this thesis have been presented in the following publications:

McGovern, M.M., Vine, D.T., Haskins, M.E., and Desnick, R.J.: An improved method for heterozygote identification in feline and human mucopolysaccharidosis VI, arylsulfatase B deficiency. Enzyme 26:206-12, 1981.

Vine, D.T., McGovern, M.M., Haskins, M.E., and Desnick, R.J.: Feline mucopolysaccharidosis type VI: Purification and characterization of the residual arylsulfatase B activity. Am. J. Hum. Genet. 33:916-927, 1981.

Vine, D.T., McGovern, M.M., Schuchman, E.H., Haskins, M.E., and Desnick, R.J.: Enhancement of the residual arylsulfatase B activity in feline mucopolysaccharidosis VI by thiol-induced subunit association. J. Clin. Invest. 69:294-302, 1982.

Desnick, R.J., McGovern, M.M., Haskins, M.E., Patterson, D.F., and Vine, D.T.: Characterization of the residual arylsulfatase B activity in feline mucopolysaccharidosis VI. Implications for enzyme manipulation therapy. In: Advances in the Treatment of Inborn Errors of Metabolism, R.W.E. Watts, R.A. Chalmers, M.d'A. Crawford, and D.A. Gibbs, eds., John Wiley and Sons, Ltd., Chichester, in press.

Desnick, R.J., McGovern, M.M., Schuchman, E.H., and Haskins, M.E.: Animal models of human lysosomal storage diseases. Molecular pathology and therapeutic studies. In: Animal Models of Inborn Errors of Metabolism, R.J. Desnick, D.F. Patterson, and D.F. Scarpelli, eds., Alan R. Liss, Inc., New York, in press.

Haskins, M.E., Jezyk, P.F., Desnick, R.J., McGovern, M.M., Vine, D.T., and Patterson, D.F.: Animal models of mucopolysaccharidosis. In: Animal Models of Inborn Errors of Metabolism, R.J. Desnick, D.F. Patterson, and D.F. Scarpelli, eds., Alan R. Liss, Inc., New York, in press.

McGovern, M.M., Vine, D.T., Haskins, M.E., and Desnick, R.J.: Arylsulfatase B. Physical and kinetic properties of the feline and human isozymes. Arch. Biochem. Biophys., in review.

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I. BACKGROUND AND RATIONALE

A. The Mucopolysaccharidoses.

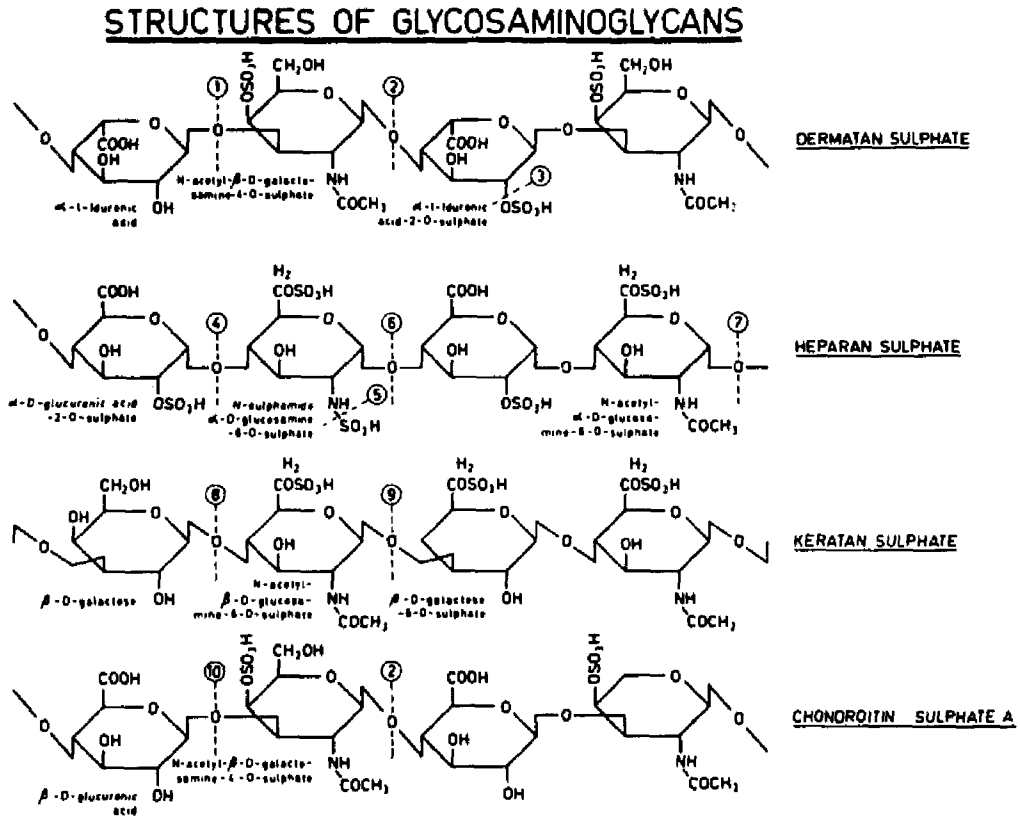
The mucopolysaccharidoses are a group of genetic disorders characterized by the accumulation of glycosaminoglycans (GAGs) in tissues and urine, particularly dermatan sulfate and heparan sulfate. These high molecular weight polymers, which are normal components of connective tissue, are made up of alternating residues of uronic acid (glucuronic or iduronic acid) and sulfated hexosamine (glucosamine or galactosamine) (Figure 1) (1).

Using cultured skin fibroblasts from patients with several phenotypically defined mucopolysaccharidoses, Neufeld and her coworkers have demonstrated that these syndromes result from defective GAG degradation (2). These studies were performed by the inclusion of ^{35}S-labeled sulfate in the media of the fibroblast cultures, allowing incorporation of labeled sulfate into newly synthesized GAG. In normal cells, ^{35}S incorporation reached a plateau by 2 days, whereas the radiolabel continued to be accumulated in cells from patients with the various mucopolysaccharidoses. When the cultures were transferred into fresh media, without ^{35}S sulfate, the clearance rate of radioactivity was significantly reduced in cells from patients as compared to normal controls. This indicated that the labeled GAGs in cells from mucopolysaccharidosis patients were not being properly degraded, whereas those in normal cells were.

Furthermore, it was noted that culture medium from the cells of one type of mucopolysaccharidosis could correct the defective GAG degradation in the cells of another. These findings suggested that specific "corrective factors" were present. Cross-correction experiments have

Figure 1. Structure of the glycosaminoglycans which accumulate in the mucopolysaccharidoses (from "Enzyme Therapy in Lysosomal Storage Diseases," 1974, p. 17) (1).

Figure 1


STRUCTURES OF GLYCOSAMINOGLYCANS
DERMATAN SULPHATE
HEPARAN SULPHATE
KERATAN SULPHATE
CHONDROITIN SULPHATE A

shown that most of the clinically defined disorders had distinct corrective factors (3). In some cases, cross-correction occurred among the cells of clinically indistinguishable patients, indicating that these disorders were due to mutations at different loci (i.e., Sanfilippo syndromes A and B). In contrast, other clinically distinct syndromes shared the same corrective factor, suggesting that these were due to different mutant alleles at the same locus (i.e., Hurler and Scheie diseases) (4,5).

The corrective factors have subsequently been isolated and identified; they are specific lysosomal enzymes (6-8). Thus, each mucopolysaccharidosis is now diagnosed by a combination of clinical features and urinary GAG excretion patterns (Table 1) as well as a particular enzyme deficiency (Figure 2) (9,10). The identification of the specific enzyme defect in each of the mucopolysaccharidoses 1) allows the development of heterozygote detection assays and prenatal diagnosis, 2) permits the study of the molecular pathology of disorders with residual activity, and 3) provides the rationale to develop therapeutic strategies for these diseases. In some of the mucopolysaccharidoses, advances have already been made in these areas (11).

Mucopolysaccharidosis VI (MPS VI), or Maroteaux-Lamy disease, was first described by Maroteaux et al. in 1963 (12). This disorder results from the deficient activity of arylsulfatase B (ASB) (EC 3.1.6.1) (13) and the lysosomal accumulation of dermatan sulfate. ASB has been shown to be an N-acetyl-galactosaminyl-4-sulfatase which cleaves 4-sulfate groups from the N-acetyl-galactosaminyl moieties of dermatan sulfate (14-16).

The clinical features of the disease include severe dysostosis

TABLE 1

Classification of the Mucopolysaccharidoses

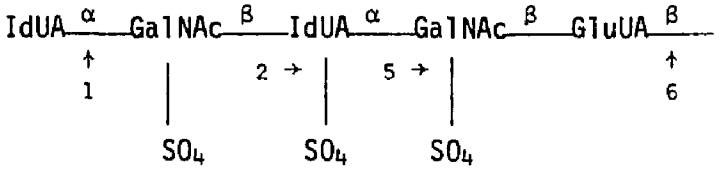
Designation			Deficient Enzyme	Urinary GAG	Clinical Features
MPS I	H	Hurler	α-L-iduronidase	Dermatan sulfate Heparan sulfate	Early corneal clouding, grave manifestations, death by age 10
MPS I	S	Scheie	α-L-iduronidase	"	Stiff joints, cloudy cornea, aortic valve disease, normal intelligence
MPS I	H/S	Hurler-Scheie	α-L-iduronidase	"	Phenotype intermediate between Hurler and Scheie
MPS II	A	Hunter, severe	Iduronate sulfatase	Dermatan sulfate Heparan sulfate	No corneal clouding, death before age 15
MPS II	B	Hunter, mild	Iduronate sulfatase	"	Survival to 30's to 50's, fair intelligence
MPS III	A	Sanfilippo A	Heparan sulfate-sulfatase	Heparan sulfate	Identical phenotypes, mild somatic and severe central nervous system effects
MPS III	B	Sanfilippo B	N-acetyl-α-D-glucosaminidase	"	
MPS III	C	Sanfilippo C	AcetylCoA:α-glucosaminide N-acetyl transferase	"	

Table 1. - Continued

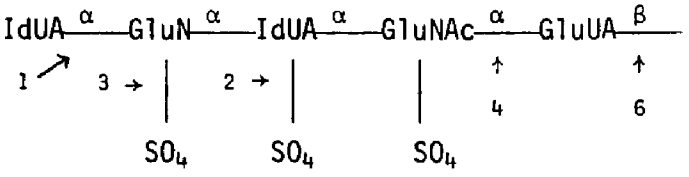
MPS III	D Sanfilippo D	N-acetylglucosamine-6-sulfate sulfatase	"	
MPS IV	A Morquio	Galactosamine-6-sulfate sulfatase	Keratan sulfate	Severe distinctive bone changes cloudy cornea, aortic regurgitation
MPS IV	B Morquio	β-Galactosidase	"	Mild bone changes, cloudy cornea
MPS V	Vacant			
MPS VI	Maroteaux-Lamy, severe	Arylsulfatase B	Dermatan sulfate	Severe osseous and corneal change, normal intellect
MPS VI	Maroteaux-Lamy, mild	Arylsulfatase B	"	Mild osseous and corneal change, normal intellect
MPS VII	β-Glucuronidase deficiency	β-Glucuronidase	Dermatan sulfate, Heparan sulfate	Hepatosplenomegaly, dysostosis multiplex, white cell inclusions, mental retardation

Figure 2. Degradation of dermatan sulfate and heparan sulfate by lysosomal enzymes which are deficient in the mucopolysaccharidoses. IdUA = iduronic acid; GalNAc = N-acetyl-galactosamine; GluUA = glucuronic acid; GluN = glucosamine; GluNAc = N-acetyl-glucosamine; SO_4 = sulfate.

Figure 2



Dermatan Sulfate



Heparan Sulfate

Number	Disease	Enzyme
1	MPS I	α-L-iduronidase
2	MPS II	α-L-iduronic acid sulfatase
3	MPS IIIA	heparan sulfate sulfatase
4	MPS IIIB	N-acetyl-α-D-glucosaminidase
5	MPS VI	arylsulfatase B
6	MPS VII	β-glucuronidase

multiplex, "Hurler-like" facial dysmorphia, short stature, corneal opacities, vacuolated lymphocytes and neutrophils, normal neurologic function and autosomal recessive inheritance (10,17,18). In addition, 6-10% of normal ASB activity is present in cultured fibroblasts and leukocytes (13,19-21) of individuals affected with this disease.

The residual ASB activity from fibroblasts of MPS VI patients has been characterized by several investigators (13,19,20) and found to be similar to the normal enzyme in its pH optimum, apparent Km, thermal stability at 60°C (19), and electrophoretic mobility (20). Immunologic studies, using antiserum prepared against homogeneous normal ASB, have revealed identical precipitin lines for the normal and residual ASB. Quantitative immunoassay showed that the enzymatic activity of the cross-reacting immunologic material (CRM) in human MPS VI was only 15% of normal (20).

B. The Feline Analogue of Human MPS VI.

MPS VI has also been reported in three families of Siamese cats (22-24). Affected animals can be identified within a week after birth by the presence of excessive urinary dermatan sulfate which can be demonstrated by cellulose acetate electrophoresis (23). As shown in Figure 3, affected cats have a characteristic appearance including a broad, flattened face, small ears and diffuse corneal clouding. In addition, affected animals are smaller than normal sex-matched relatives of the same age. Polymorphonuclear leukocytes in peripheral blood smears contain coarse granules which stain metachromatically with toluidine blue. Radiographic features of the disease include progressive epiphyseal dysplasia and bilateral hip subluxation with eventual degen-

Figure 3. The facies of a 6 month old Siamese cat with MPS VI. Note the flattened face and small ears.

Figure 3

eration of joints and fusion of cervical vertebrae. A few animals have had hind-limb paresis with depressed pain perception and increased extensor tone, presumably due to spinal cord compression secondary to progressive boney involvement. Animals with hind-limb paresis retained some voluntary control of hind-limb movement and were not incontinent. Although mentation is difficult to assess in animals, there have been no indications of deficits comparable to those defining mental retardation in man.

Significant pathologic changes are present in many systems. The liver and spleen are not grossly enlarged; however, membrane bound cytoplasmic vacuoles are present histologically and ultrastructurally in hepatocytes, Kupffer cells and splenic smooth muscle cells. Similar vacuoles are present in ocular fibroblasts and ciliary epithelium, cartilage, dermal atrioventricular heart valve fibroblasts, polymorphonuclear leukocytes, and aortic smooth muscle cells. Bone lesions consist of epiphyseal dysplasia with sclerosis and abnormal islands of cartilage, as well as fusions of cervical and lumbar vertebrae. Evidence of lysosomal storage within the central nervous system is limited to the perivascular cells of brain and cord and connective tissue cells of the meninges and choroid plexi. Spinal cord compression at T_{13} - L_2 was present in those animals with posterior paresis.

The abnormal accumulation of $^{35}SO_4$ by cultured fibroblasts was consistent with the defective degradation of GAG (23), and analysis of urinary mucopolysaccharides revealed markedly increased levels of dermatan sulfate. Analogous to the human disease, ASB activity in peripheral blood leukocytes, cultured fibroblasts and liver from homozygous affected cats was about 6% of that in normal cats. Obligate heterozy-

gotes (parents of affected cats) have ASB activities intermediate between those of normal and affected cats (23,24). Pedigree information from independently ascertained families was consistent with autosomal recessive inheritance and affected cats of both sexes have been bred successfully (Figure 4).

C. The Use of Animal Models for Molecular Pathology and Therapeutic Studies.

During the past decade, efforts have focused on the discovery and characterization of naturally occurring animal models of human disease (25-27). Animal analogues of a variety of human "inborn errors of metabolism" have been identified (28). These models provide the unique opportunity to investigate the molecular pathology of their human counterparts. Specifically, characterization of the underlying genetic defect and its resultant pathophysiologic consequences permits the rational design and evaluation of various therapeutic strategies which could not be assessed adequately in clinical trials due to the limitations of human experimentation. Therefore, it is the purpose of this section to discuss selected animal models of human lysosomal storage diseases, emphasizing the study of their molecular pathology and their usefulness as prototype systems for the evaluation of novel therapeutic strategies.

Table 2 lists the animal models of human lysosomal storage diseases; the disorders are classified as glycogenoses, glycoproteinoses, glycosphingolipidoses or mucopolysaccharidoses on the basis of the primary accumulated substrate. Although other models of human lysosomal storage diseases have been described (25,26), the criteria for inclusion

Figure 4. Pedigree of several families of Siamese cats affected with MPS VI.

Figure 4

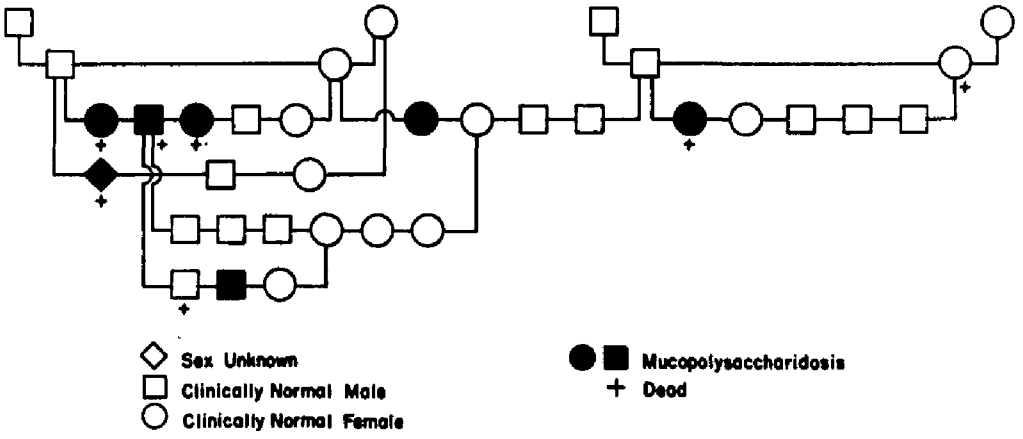

Sex Unknown
Clinically Normal Male
Clinically Normal Female
Mucopolysaccharidosis
Dead

TABLE 2. Animal Models of Human Lysosomal Storage Diseases*

Disease	Enzymatic Defect	Species	Reference
Glycogenosis:			
Glycogenosis Type II (Pompe)	Acid α-Glucosidase	Shorthorn Cow	29
Glycoproteinoses:			
α-Mannosidosis	Acid α-Mannosidase	Angus Cow	30
		Domestic Cat	39
β-Mannosidosis	Acid β-Mannosidase	Nubian Goat	31
Glycosphingolipidoses:			
G_{M1}-Gangliosidosis			
Type 2	Acid β-Galacto-sidase	Siamese Cat	32
		Friesian Calf	40
		Mixed Breed Dog	41
G_{M2}-Gangliosidosis			
Type 2	β-Hexosaminidase A&B	Domestic Cat	33
Type 3	β-Hexosaminidase A, Partial Activity	Yorkshire Swine	42
Gaucher Type 2	Acid β-Glucosidase	Silky Hair Terrier Dog	43
Krabbe	Galactosyl ceramide: β-Galactosidase	West Highland/ Cairn Terriers	37
		Twitcher Mouse	38
Niemann-Pick Type A	Sphingomyelinase	Poodles	36
		Siamese Cat	44
Mucopolysaccharidoses:			
MPS I-H (Hurler)	α-L-Iduronidase	Domestic Cat	35
MPS VI (Maroteaux-Lamy)	Arylsulfatase B	Siamese Cat	23

*Includes only enzymatically confirmed models.

in Table 2 was the actual demonstration of the same enzyme deficiency as in the analogous human disorder. Each of these models is inherited as an autosomal recessive trait.

Recognition of the affected proband and/or identification of the proband's parents or heterozygous relatives has permitted the establishment of active breeding colonies for cows with Pompe disease (29) and α-mannosidosis (30); goats with β-mannosidosis (31); cats with G_{M1}-gangliosidosis type 2 (32), G_{M2}-gangliosidosis type 2 (33,34), mucopolysaccharidosis Type I-H (35) and mucopolysaccharidosis Type VI (23,24); dogs with Niemann-Pick disease (38), as well as dogs (37) and mice (38) with Krabbe disease. These models are the best characterized with respect to the clinical manifestations, natural history of the disease, morphologic pathology, and the nature of the metabolic defect (i.e., accumulated substrate(s) and deficient enzyme).

1. Molecular pathology. Requisite to the use of animal models for the study of human disease is the necessity to establish the degree of homology between the human and animal counterparts. Obviously, an animal disease which has an identical molecular etiology to that of the corresponding human disorder will provide the ideal model for investigation of disease pathogenesis and treatment. For inborn errors of metabolism, an animal homologue would be one with the identical molecular defect at the genic level (e.g., the same or equivalent base substitution in the mutant gene). However, it is unlikely that any given animal model will share the precise gene mutation which occurs in the human disease. In fact, it is known that multiple mutations in the structural gene for any given protein can cause defective catalysis or function, as

has been so well-demonstrated for the over 140 and 250 different mutations in the human molecules, glucose 6-phosphate dehydrogenase (45-47) and hemoglobin (48), respectively.

To date, studies of the molecular pathology in animal models of human lysosomal storage diseases have focused at the level of the metabolic defect. In each animal model disease listed in Table 2, the homology of the metabolic defect has been documented by the demonstration of the same enzymatic deficiency as in the corresponding human disorder. Several of these animal models have enzymatic defects with residual activities which permit further comparison of their mutations with those in the human diseases. Interestingly, residual activities occur in both the human and animal counterparts of mannosidosis and G_{M1}-gangliosidosis. Comparison of the kinetic, physical and immunologic properties of these defective enzymes revealed that neither of these animal models were homologues, but rather, were analogues of their human disease counterparts. These studies are summarized in Table 3 and are discussed below.

a) <u>α-Mannosidosis</u>. The residual α-mannosidase activities in both the bovine and human disorders had increased K_m values, decreased thermostabilities and cross-reacted with antibodies to the respective normal enzymes, consistent with a structural gene mutation (49). The only difference between the human and bovine residual enzymes was the amount of activity detected ($\sim$ 2-8% vs. $\sim$ 15% of normal, respectively). In contrast, the residual activity in feline mannosidosis was only 2% of normal levels and had similar physical and kinetic properties to the normal feline enzyme (39). Although the immunologic properties of the

TABLE 3. Comparison of the Properties of Residual Activities in the Liver from Human and Animal Models of Lysosomal Storage Diseases

Disease	Residual Activity	pH	Kinetics	Thermo-stability	Mol. Wt.	Cations, Cofactors Effectors	IEF*/ Electrophor-etic Migration	Cross Reacting Material	Refer-ences**
Mannosidosis:									
Human	2-8%	N*	Km	NS	N	N	N	+	52
Bovine	15%	N	Km	NS	N	N	N	+	49
Feline	2%	N	N	N	NS	N	NS	NS	39
G_{M1}-Gangliosidosis Type2:									
Human	2-18%	N	Km	NS	N	NS	Electro-positive	+	53
Feline	10%	N	Km	NS	NS	NS	Electro-positive	-	50
Canine	1%	N	N	N	N	NS	N	NS	51

* N = normal; IEF = isoelectric focusing; NS = not studied.

residual feline enzyme were not investigated, it was suggested that the residual activity was the result of a structural gene mutation that led to enhanced susceptibility of the enzyme to proteolysis or an alteration in some property which decreased catalytic activity; a regulatory gene mutation, although less likely, was also considered (39). Further purification and characterization, including CRM studies, are required to discriminate among these possibilities.

b) G_{M1}-Gangliosidosis. The feline model of G_{M1}-gangliosidosis has 10% residual hepatic activity which has an altered K_m toward both the natural and artificial substrates, is thermolabile, migrates less anodally and is not recognized (CRM-negative) by the antibody raised against normal feline β-galactosidase (50). In contrast, the canine enzyme has 1% of normal hepatic activity, normal kinetic and physical properties and an amount of CRM directly corresponding to the amount of activity (51). Thus, the feline mutation is consistent with a structural gene mutation, but the nature of the canine mutation is unclear; possible mutations include mRNA processing, regulatory gene as well as structural gene defects. Discrimination of the different possibilities may require isolation of the gene and characterization of its transcriptional and translational integrity.

2. Therapeutic Trials in Animal Models. Considerable attention has been focused on the development of strategies to treat patients with inherited metabolic diseases. Theoretically, the ideal cure for these diseases would be the insertion of the segment of DNA coding for the normal gene product. Therapeutic intervention at the level of the

primary genetic defect, or gene therapy, is presently precluded by our inability to insert a gene and effect its normal expression. However, recent developments in recombinant DNA methodology and their rapid application to in vitro gene product production have signaled that we are on the threshhold of future technical accomplishments which may lead to "gene transfer" as a means to replace defective human genes.

To date, therapeutic endeavors to ameliorate the molecular pathology of selected inherited metabolic diseases have primarily involved gene product therapy and manipulations at the level of the metabolic or cellular defect. These efforts have included cofactor supplementation, allotransplantation, enzyme replacement, plasmapheresis and surgical bypass procedures. Several of these strategies have been evaluated in the animal models of the lysosomal storage diseases. These studies illustrate the value of animal model systems to obtain critical information regarding the ability of selected therapeutic strategies to alter the biochemical abnormalities and clinical course of these diseases.

a) Enzyme Replacement Therapy. During the past decade, investigators have been intrigued by the possibility of enzyme therapy for various inborn errors of metabolism, particularly the lysosomal storage diseases. The rationale for enzyme therapy in these diseases evolved from two fundamental observations: a) the identification of lysosomes as the subcellular site of pathology, and b) the elucidation of the basic role of the lysosome in cellular catabolism. Thus, it was reasoned that, after endocytosis, exogenously supplied enzyme would be brought into contact with accumulated substrate by fusion of the various components of the lysosomal apparatus.

Human trials of enzyme replacement therapy have met with several difficulties, including the inability to serially evaluate the physiologic, immunologic and biochemical factors affecting the fate of the administered enzyme. Suitable animal models, such as β-glucuronidase deficient mice and G_{M2}-gangliosidosis cats, have provided the means to evaluate and manipulate the factors that maximize enzyme stability and tissue distribution prior to human trials. These studies also provided data regarding potential toxic and immunologic complications.

For example, the tissue and subcellular fate as well as immunologic safety of intravenously administered bovine β-glucuronidase have been studied extensively in C3H/HeJ Gus^h β-glucuronidase deficient mice (54-56). A selective inactivation assay provided the means to conveniently differentiate the bovine enzyme from residual murine activity (54). Following intravenous injection, bovine β-glucuronidase was rapidly cleared from the murine circulation ($T_{1/2} \sim 3$ min) with almost exclusive enzyme uptake by the hepatic lysosomes. These observations, and similar findings in early human trials, identified the need to develop entrapment strategies to deliver the enzyme to non-hepatic sites of pathology.

Intravenous administrations of bovine β-glucuronidase entrapped in positively- and negatively-charged liposomes and in autologous murine erythrocytes were evaluated to determine the ability of these carrier vehicles to target-deliver and protect the entrapped enzyme (55,56). These studies have shown that enzyme entrapped in negatively-charged liposomes prolonged the hepatic retention of enzymatic activity compared to that of unentrapped enzyme (8 days vs. 1 day) and allowed delivery of enzyme to the kidney. Although positively-charged liposomes were cap-

able of delivering enzyme to liver with a longer retention time ($\sim$11 days), they were noted to cause temporary labilization of the lysosomal membrane resulting in the intracellular release of endogenous lysosomal hydrolases (57). Furthermore, immunologic studies demonstrated that administration of the enzyme-loaded liposomes elicited an immune response to both the liposome carrier and the entrapped enzyme (58). Although liposomes have been purported to be ideal vehicles for the delivery of enzyme to specific tissues (59), these animal model studies illustrated the potential physiologic complications of these enzyme carriers prior to clinical use.

Compared to unentrapped enzyme, intravenously administered erythrocyte-entrapped β-glucuronidase was retained in the murine circulation four times longer with more efficient delivery of enzyme to renal and splenic tissues. Ultrastructural examination revealed that the major site of erythrocyte-entrapped enzyme uptake was in Kupffer cells, not hepatocytes, indicating that this method of entrapment may be useful for diseases with substrate accumulation in the reticuloendothelial system (e.g., Type 1 Gaucher disease). In marked contrast to enzyme administered unentrapped or in liposomes, erythrocytes loaded with the heterologous bovine β-glucuronidase did not elicit an immune response following repeated intravenous administrations (60).

These studies also demonstrated that intravenously administered enzyme, whether entrapped in liposomes or erythrocytes, or unentrapped, was unable to cross the blood-brain barrier and gain access to the central nervous system. Thus, it was recognized that efforts to treat diseases with neurologic involvement required the development of strategies to reversibly open the blood-brain barrier for neuronal enzyme

uptake. Using the feline model of G_{M2}-gangliosidosis, Kattazzi et al. (34,61) evaluated the effectiveness of hyperbaric oxygen and intracarotid air micro-embolism as methods to reversibly open the blood-brain barrier. Following exposure to hyperbaric oxygen, β-hexosaminidase A was injected into the femoral or carotid vein and neural uptake was determined. In order to prolong the half-life of exogenous enzyme in the circulation and maximize its central nervous system exposure, hepatic clearance of the enzyme was partially inhibited by the infusion of receptor-blocking mannosaccharides (i.e., mannans). This inhibition resulted in a 3 to 5-fold increase in neural enzyme uptake over that observed without blockage of the hepatic uptake receptors. However, hyperbaric treatment followed by the intracarotid administration of large doses of enzyme resulted in the neural uptake of only about 1% of injected enzyme.

More encouraging results were obtained in preliminary experiments of intracarotid air micro-embolization. Following the injection of small volumes of air, mannans and β-hexosaminidase A were administered intravenously into normal or G_{M2}-gangliosidosis cats (34,61). One hour after enzyme injection, the exogenous β-hexosaminidase A activity reached 20-30% of the endogenous level in normal feline brain, suggesting that air micro-embolization or similar mechanical methods to open the blood-brain barrier will permit access to neuronal sites of substrate deposition by nonselective extravasation of plasma containing the administered enzyme. More recently, these investigators have improved their techniques, permitting increased amounts of injected enzyme to reach the central nervous system (62,63). Clearly, animal models are essential for the development and evaluation of novel therapeutic strat-

egies for human neurologic disorders.

b) Allotransplantation. An intriguing means for transferring normal genetic information into individuals with certain structural and metabolic gene defects is allotransplantation (64-67). This approach exploits the grafting of cells, tissues or organs containing the normal DNA for the production of functional gene products in the recipient.

Experimental transplantation designed to correct inborn errors of metabolism has been undertaken in only a few animal disorders. Mukherjee et al. (68) reported that orthotopic liver grafts resulted in increased UDP-glucuronyl transferase activity and markedly decreased the hyperbilirubinemia in Gunn rats. Subsequently, Sutherland and co-workers (69) administered isolated liver cells into the portal vein of Gunn rats and demonstrated decreased levels of plasma bilirubin.

Animal model studies have provided the rationale for allogenic bone marrow transplantation for disorders in which the primary disease pathology results from defects in lymphocytes, phagocytes, erythrocytes or platelets. For example, cyclic neutropenia, which occurs in both man and dogs, is thought to be due to a regulatory defect which affects the pluripotential stem cells. Marrow transplantation in dogs with this disease resulted in normal granulocytopoiesis and corrected the neutrophil defect (70). It should also be noted that bone marrow stem cells are the progenitors of other mesodermal cell types, including Kupffer cells and osteoclasts. Thus, successful transplantation of normal stem cells will provide a continuous source of differentiated cells for the correction of disorders in which the differentiated cell is the target

site of disease pathology. For example, congenital severe osteopetrosis is a recessively inherited disorder of humans characterized by the progressive deposition of bone matrix leading to blindness, deafness, anemia, frequent fractures, increased susceptibility to infection and progressive hepatosplenomegaly. Studies of the molecular pathology of the murine analogue for this disease revealed that the abnormal resorption was the result of an intrinsic osteoclast defect (71). Moreover, allogenic bone marrow transplantation in affected mice lead to the amelioration of the disease (71,72). Based on the murine model studies, bone marrow transplantation was undertaken in several patients with the human disease with therapeutic benefit (73,74).

Successful bone marrow transplantation and correction of the enzymatic defect have been accomplished in murine acatalasemia and murine β-glucuronidase deficiency. In acatalasemia, catalase deficiency in peripheral leukocytes leads to an inability to kill certain types of bacteria resulting in recurrent infections. Acatalasemic mice, which are abnormally sensitive to injections of exogenous hydrogen peroxide, were marrow transplanted and the defect corrected as demonstrated by increased blood catalase levels and increased survival after hydrogen peroxide challenges (75). Marrow transplantation in murine β-glucuronidase deficient mice, using total body irradiation to make space for the graft, resulted in successful grafting and increased levels of β-glucuronidase activity in liver and plasma (76).

More recently, bone marrow transplantation has been proposed as a therapeutic strategy for the treatment of a variety of human inherited enzymatic deficiency diseases (77). The proponents of this strategy have transplanted patients with several lysosomal storage diseases; the

early results in one recipient with MPS I-H (Hurler disease) have been reported (77). The rationale for bone marrow transplantation in this and other lysosomal disorders is based on the concept that a continuous supply of normal circulating phagocytes will be capable of correcting the defective lysosomal catabolism in these storage diseases. It is presumed that monocytes will penetrate a variety of tissues and short-lived neutrophils will exocytose their lysosomal hydrolases, thus providing a continuous supply of normal enzyme. Furthermore, in disorders with primary liver involvement, the disease course may be altered by the replacement of Kupffer cells containing the active enzyme. This hypothesis seems reasonable for disorders in which reticuloendothelial cells and other bone marrow cells are the primary sites of pathology, such as Type 1 Gaucher disease and MPS Types IV and VI. However, it is doubtful that inborn errors with primary neuronal substrate accumulation (e.g., Tay-Sachs disease, metachromatic leukodystrophy or MPS Types I-H, II, IIIA, IIIB, IIIC, IIID or VII) would benefit from marrow transplantation, since it is unlikely that adequate numbers of marrow-derived cells can gain access to the neural cellular sites of pathology. Therefore, studies in appropriate animal models should be conducted to evaluate the capability of marrow engraftment to alter the course of lysosomal storage diseases with primary neurologic involvement. In this regard, one important animal model experience should be noted.

Jolly et al. (78) have described the biochemical and clinical findings in a freemartin with mannosidosis. In this "experiment of nature" a calf with mannosidosis received an in utero marrow graft from a normal littermate. The level of α-mannosidase activity in leukocytes of the chimeric calf was in the heterozygote range. In addition, the

calf had a marked decrease in the number of vacuolated lymphocytes, and a reduced level of accumulated mannose-rich oligosaccharides in visceral tissues. However, the level of substrate accumulation was not significantly reduced in the brain and the calf exhibited the typical neurologic manifestations and course of the disease. These results are instructive since they indicate that the marrow chimera did not alter the neurologic course of this lysosomal storage disease. Clearly, animal models provide the opportunity to fully assess the effectiveness and limitations of marrow transplantation. Such studies should provide essential data for subsequent human trials.

c) Enzyme Manipulation. As noted above, several of the human and animal lysosomal storage diseases have defective enzymes with residual activity. Thus, efforts to purify and characterize the residual activities in these diseases may lead to the development of novel therapeutic strategies designed to manipulate and enhance the function and/or stability of the defective enzyme. For example, characterization of the residual enzymatic activity in bovine mannosidosis has led to clinical trials of enzyme manipulation therapy as discussed below.

The finding that zinc cations stimulated normal plant, mammalian and human acid α-mannosidase activity (79,80), as well as residual α-mannosidase in tissues and fluids from bovine and human mannosidosis (52,61), stimulated the trial of cofactor supplementaton in bovine mannosidosis. Following oral zinc supplementation, a modest increase in the activity of the residual acid α-mannosidase was observed in bovine liver, kidney and pancreas (organs in which zinc accumulates). A concomitant decrease in the levels of mannosyl-oligosaccharides also was

observed in these tissues. However, in the brain of the treated calf, the residual enzymatic activity and oligosaccharide content were not changed. These findings indicated that the effect of zinc supplementation may be confined to tissues that accumulate zinc and that inadequate zinc uptake by tissues of the nervous system may have precluded a therapeutic effect.

It is anticipated that the study of animal models of inborn errors of metabolism will have a dramatic impact on the future development and evaluation of effective therapies for a variety of human enzyme deficiency diseases. Thus, the feline analogue of human MPS VI represents a valuable tool in understanding the molecular pathology of this disease and for the development of therapeutic strategies.

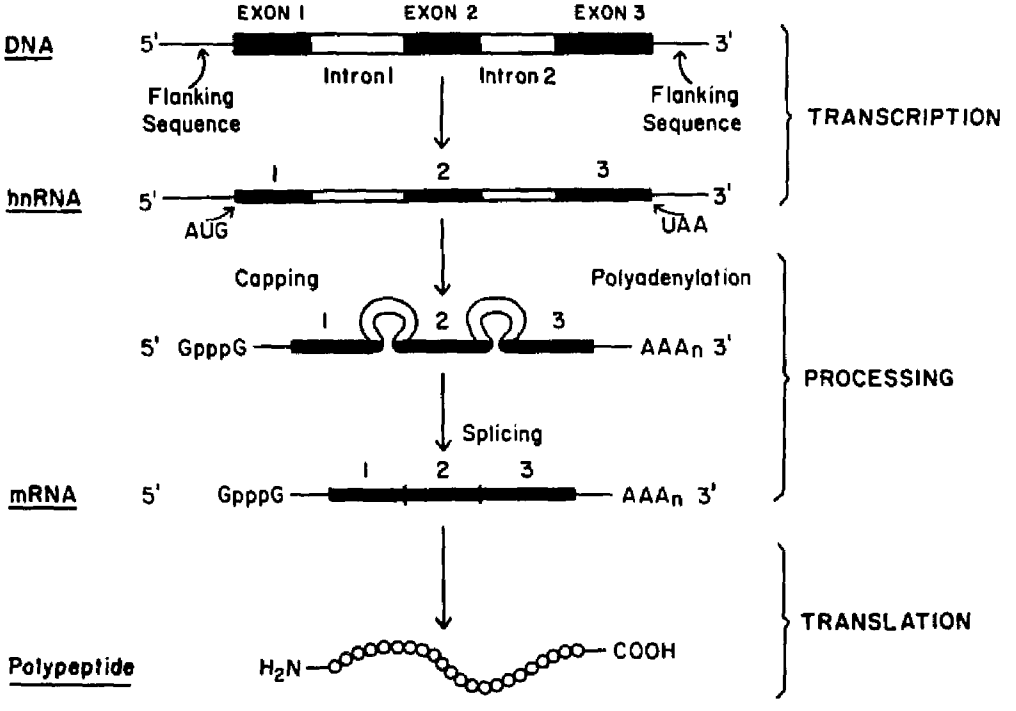
D. The Nature of Inherited Enzymatic Defects.

Recent advances in molecular biology have revolutionized our concept of eukaryotic gene structure. Previously, it was thought that a structural gene consisted of only the deoxynucleotide sequence which was transcribed into mRNA and then directly translated into the amino acid sequence of the gene product. This concept has been revised by the finding of deoxynucleotide sequences within and flanking the structural gene which do not encode for the amino acid sequence of the polypeptide product. As shown in Figure 5, the intervening sequences (introns) separate the nucleotide sequences (exons) which are ultimately translated into the gene product. The flanking regions at the 5' and 3' ends presumably contain the signals for the initiation of transcription and for RNA processing. The entire gene (both introns and exons) is transcribed into a precursor RNA or hnRNA (heterogeneous nuclear RNA) which

Figure 5. Current concept of eukaryotic gene structure, transcription and translation.

Figure 5

CURRENT CONCEPT OF THE EUKARYOTIC STRUCTURAL GENE



then undergoes a series of processing events to produce the mature mRNA, which contains only the exon sequences. These processing events include capping and polyadenylation at the 5' and 3' ends, respectively, and excision (splicing) of the intron sequences.

Various types of heritable mutations could result in a defective enzymatic activity. These include mutations in 1) either the exon, intron or flanking regions of the structural gene, 2) other genes controlling posttranslational modifications of the gene product, and 3) regulatory genes controlling the synthesis of the active enzyme.

1. Structural Gene Defects. Missense (base substitutions), nonsense (chain terminating base substitutions) and frameshift (insertions or deletions) mutations can occur in any intron, exon or flanking region of the structural gene. A particular mutation can alter the fidelity of transcription, RNA processing or translation, depending on the specific site and type of mutation. Certain mutations (e.g., in the intron, intervening sequence junction or flanking region) may result in the quantitative deficiency, or absence, of normal gene product.

Qualitative mutations that alter the structure and function of the normal gene product result from single base substitutions in the exon portion of the gene. From the genetic code, it can be calculated that approximately 70% of single base substitutions in a DNA triplet located in an exon will change that codon to incorporate a different amino acid (missense mutations), about 25% of the substitutions will insert the same amino acid (degenerate mutations), and about 5% of substitutions will code for chain termination of the nascent polypeptide sequence (nonsense mutations). Although the rate of gene product synthesis

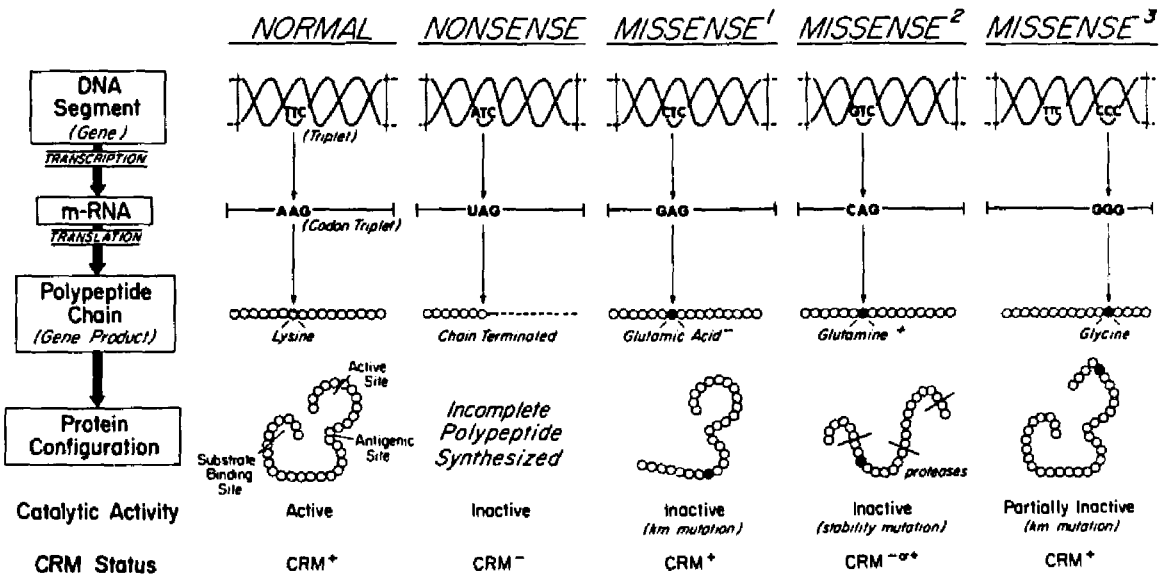
remains relatively normal, missense and nonsense mutations in an exon can alter the kinetic, stability, or other properties of the enzyme, rendering it catalytically inactive or partially active. In the latter case, a mutation which results in a partially active enzyme permits purification of the residual activity and comparison of its properties with the normal enzyme. In this way, insight into the nature of the enzymatic defect can be obtained.

As illustrated in Figure 6, a normal structural gene will be transcribed and translated into a normal gene product with a specific sequence of amino acids that specifies a unique three-dimensional configuration. This configuration establishes at least three functional sites on the active enzyme molecule: the substrate binding site, the catalytic or active site, and at least one major antigenic site. There may also be sites for allosteric, coenzyme, and subunit interactions, etc. A single base substitution in an exon portion of the gene may alter an enzyme's structure in such a manner as to deleteriously affect one or more of these functional properties.

Nonsense or chain-terminating mutations in an exon result in an incompletely synthesized enzyme. If the nonsense mutation occurs early in the enzyme's amino acid sequence, the resultant polypeptide will not have sufficient structure for catalytic activity or immunologic recognition of its antigenic site(s) [i.e., cross-reacting material (CRM)-negative, Figure 6]. Missense mutations can modify the catalytic, substrate binding, cofactor binding or allosteric sites, resulting in kinetic mutations which may partially or totally impair catalytic function. These mutations can be CRM-positive or negative depending on whether they alter the antigenic sites. Missense mutations also can

Figure 6. Effect of various single base substitutions on the structure, function and stability of an enzyme protein.

Figure 6


NORMAL
NONSENSE
MISSENSE 1
MISSENSE 2
MISSENSE 3
DNA Segment (Gene)
TRANSCRIPTION
m-RNA
TRANSLATION
Polypeptide Chain (Gene Product)
Protein Configuration
(Triplet)
AAG
(Codon Triplet)
UAG
GAG
CAG
GGG
Lysine
Chain Terminated
Glutamic Acid
Glutamine
Glycine
Active Site
Substrate Binding Site
Antigenic Site
Incomplete Polypeptide Synthesized
proteases
Catalytic Activity
Active
Inactive
Inactive (km mutation)
Inactive (stability mutation)
Partially Inactive (km mutation)
CRM Status
CRM+
CRM−
CRM+
CRM−α+
CRM+

affect the physical stability of the enzyme by altering its conformation or subunit interactions. Unstable proteins may be rapidly degraded by cellular proteases resulting in non-catalytic, CRM-negative mutations.

In the future, it is likely that these mutations will be characterized at the genic level using sophisticated nucleic acid and recombinant DNA techniques. The precise molecular defects (e.g., specific base substitutions, deletions, etc.) in each of the lysosomal storage diseases (and their subtypes and variants) will be identified, analogous to the recent accomplishments in the dissection of the molecular pathology of the human hemoglobinopathies and thalassemias (82-85). At present, such studies are not possible for the lysosomal storage diseases since none of the genes have been isolated and cloned.

Recently, a variety of strategies have evolved for the isolation of eukaryotic genes which are expressed at extremely low levels. One approach involves the use of synthetic oligonucleotides for priming the synthesis of a cDNA specific for the gene in question or, alternatively, to directly screen cloned cDNA libraries for a segment corresponding to the gene's mRNA (86). The first step toward this goal is the determination of the amino acid sequence of each enzyme and the construction of a DNA oligonucleotide probe. The availability of an oligonucleotide probe also would permit the analysis of CRM-negative mutations by establishing the absence, presence or quantity of mRNA and DNA present in cells of the mutant genotype (87). Such studies would identify gene deletions or mRNA processing defects.

Since the exact molecular defects in the human lysososmal storage diseases cannot be determined at the genic level as yet, efforts have focused on the characterization of the enzymatic defects, particularly

in those disorders with detectable residual activity. Information concerning the nature of a mutation which results in a partially active enzyme can be obtained by comparison of the kinetic and physical properties of the normal and residual activities. An increased K_m (decreased substrate binding) or a decreased V_{max} (reduced product formation due to defective product release or enzyme instability) would lead to abnormal substrate accumulation. In addition, the incorporation of an inappropriate amino acid, particularly if charged differently, may significantly alter the enzyme's configuration and render it unstable and susceptible to degradation by endogenous proteases; it is anticipated that most stability mutations would be catalytically inactive and CRM-negative. Of the enzymatic defects characterized to date, most residual activities have had markedly increased K_m values or both increased K_m and decreased V_{max} values. The latter presumably reflects mutations which alter substrate binding as well as render the enzyme less stable.

Missense or nonsense mutations in the structural gene exons may also alter the enzyme's ability to interact normally with critical small molecules, such as allosteric effectors and cofactors. A number of human inborn errors have been identified that involve enzymes requiring the binding of a specific vitamin cofactor for the normal expression of enzymatic activity (88). These mutations often fall into two groups - those that respond to cofactor supplementation therapy and those that do not. Presumably, the former mutations represent defects in the coenzyme binding site of the enzyme that increase the K_a for the coenzyme, whereas the latter group represents defects that severely deform the coenzyme binding or other crucial sites. Similarly, multimeric enzyme proteins may be inactive if subunit assembly cannot occur due to a mutation in

the structural gene coding for one polypeptide of a heteromultimeric enzyme or for the common subunits of a homomultimer.

2. Posttranslational Defects. Missense or nonsense mutations in genes controlling the posttranslational modifications of the enzyme protein also may cause catalytic deficiencies. For example, one type of posttranslational modification involves processes which control the subcellular compartmentalization of an enzyme, including the cleavage of a peptide leader sequence (89,90), the synthesis of a specific membrane binding or transport protein, or the addition of specific oligonucleotide moieties. Such abnormalities have been elegantly demonstrated for the β-glucuronidase isozymes in mice (91), and more recently, for the defective lysosomal localization of many hydrolases in Mucolipidoses II and III (92,93). In the former, a defect in the protein responsible for the microsomal localization of β-glucuronidase has been described (94). In Mucolipidoses II and III, the deficient activity of an enzyme, UDP-N-acetyl-glucosaminylphosphotransferase (92,93), results in the failure to form the mannose-6-phosphate residues on the oligosaccharide moieties of most lysosomal hydrolases; the mannose-6-phosphate recognition signal appears to mediate the intracellular trafficking of lysosomal enzymes to the lysosomal apparatus (95-101). The absence of this signal results in the multiple deficiency of lysosomal hydrolases intracellularly and the extracellular accumulation of these enzymes.

3. Regulatory Gene Defects. Finally, mutations in regulatory genes controlling initiation, temporal modulation, or rates of enzyme synthesis could occur. These mutations may be in genes distant from the

structural gene locus. Alternatively, such mutations could be in the 5' flanking region of the structural gene which presumably contains "promoter sequences" required for initiation of transcription (RNA polymerase recognition and/or binding). Although there are no well-documented examples of human enzymatic defects resulting from regulatory gene mutations, it is likely that these occur in nature, but presumably represent lethal mutations. However, a partial defect of a regulatory gene might be viable and result in an enzymatic deficiency.

E. Purification and Characterization of Mammalian ASB.

ASB has been characterized from several mammalian sources including human placenta (102), liver (103-106), brain (107), and lung (108), bovine brain (109) and liver (110-112), chicken liver (113), ovine brain (114), rabbit kidney (115) and murine liver and kidney (116). The physical and kinetic properties of the reported homogeneous enzyme preparations are summarized in Table 4.

It is notable that the pH optimum, pI's, K_m values and molecular weights of these preparations are similar, whereas the specific activities vary greatly (7,200 to 7,800,000 nmoles/h/mg protein). Despite the large number of studies on mammalian ASB, there are large discrepancies concerning the homogeneity of the described preparations. Thus, careful purification and quantitation of ASB activity is necessary in order to reliably and reproducibly purify the enzyme to homogeneity. This would allow further, careful characterization of the enzyme, including amino acid composition analysis, subunit structure determinations and immunologic studies.

Table 4. Purification and Characterization of ASB from Mammalian Tissues

Source	Specific Activity	Fold Purification	Yield (%)	pH Optimum	pI	Km (mM)	Molecular Weight*	Forms**	Reference
Human:									
Placenta	220,000	610	7	5.8	7.6	1.04	71,500	3	102
					7.9	1.96	60,000		
					8.1	0.65	48,000		
Liver	245,000	107	29	5.6	-	2.9	51,000	2	105
	5,600,000	970	36	6.1	-	0.8	47,000-54,000	1	103
	7,200	290	4.3	6.0-6.6	8.5	0.67	40,500	2	104
Bovine:									
Brain	5,820,000	-	-	-	8.3	-	60,000	4	109
Liver	7,800,000	10,000	10	5.7	8.3	-	47,000	2	110
Rabbit:									
Kidney	2,520,000	620	3.7	6.0	-	2.0	-	1	115

*Determined by gel filtration.
**Number of isozymes distinguishable by ion exchange chromatography.

F. Rationale.

The feline analogue of human MPS VI provides the unique opportunity to characterize the molecular pathology in this disease and to develop various therapeutic strategies. Physical and kinetic studies of the normal human, normal feline, and feline MPS VI ASB may provide insight into the defect in this disease, and provide a basis for the development of therapeutic strategies to be tested *in vivo* using the animal model.

II. OBJECTIVES

It was the purpose of these studies to investigate the molecular pathology of the feline analogue of human MPS VI, and based on these studies, to develop therapeutic strategies which can be evaluated in the animal model. This research included:

1) Development of a rapid and reliable method to identify heterozygotes within the feline MPS VI colony for breeding purposes.
2) Purification and characterization of normal human and feline hepatic ASB.
3) Purification and characterization of the residual ASB activity in feline MPS VI.
4) Production of antibodies to purified human and feline hepatic ASB and determination of CRM in feline MPS VI.
5) Attempts to correct the enzymatic defect by manipulation of the residual activity in vitro.
6) Attempts to correct the enzymatic defect in feline MPS VI in vivo.

III. MATERIALS

Normal human, normal feline, and feline MPS VI livers were obtained within 30 min of death and stored at -55°C until used.

Sephadex G-200 and G-25, concanavalin A-Sepharose, Blue Sepharose, and molecular weight standards (aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome C) were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. DEAE-cellulose and CM-cellulose were purchased from Whatman, Inc., Clifton, NJ. Coommassie brilliant blue G-250, p-nitrocatechol sulfate, 1-O-methyl-α-D-glucopyranoside, diaminobenzidine tetrahydrochloride, dimethyl suberimidate, cysteamine, cystamine, iodoacetate, iodoacetamide, and p-chloromercuribenzoate were from Sigma Chemical Co., St. Louis, MO. Ovine anti-rabbit peroxidase conjugated IgG was from Cappel Laboratories, Cochranville, PA. Mercaptoethanol was from Eastman Kodak Co., Rochester, NY. The Bio-Rad protein assay and materials for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories, Richmond, CA. Pronase from Streptomyces griseus was purchased from Boehringer Mannheim, Indianapolis, IN. Fluorescamine was obtained from Hoffman-LaRoche, Inc., Nutley, NJ. EDTA tetrasodium salt was from Fisher Scientific Co., Fairlawn, NJ, and LiCl was from Mallinckrodt, Inc., Paris, KT. Alcian Blue 8GN was obtained from MCB Manufacturing Chemists, Inc., Cinncinatti, OH. Cellulose acetate gels (14.5 X 5.5 cm; 250 μ thick) were from Kalex, Inc., Manhasset, NY. Acrodisc filters were purchased from Gelman Scientific, Ann Arbor, MI, and Amicon ultrafiltration apparatus and membranes were from Amicon Corp., Lexington, MA. The microzone electrophoretic cell (Model R-101) and Duostat power supply for cellulose acetate electrophoresis were obtained from Beckman Instruments, Inc., Anaheim, CA. Ampholytes were

purchased from LKB Instruments, Hicksville, NY. 4-Methylumbelliferyl sulfate was purchased from Research Products International, Inc., Elk Grove Village, IL. Agarose for immunoelectrophoresis was from Seakem Marine Colloids Div. FMC Corp., Rockland, ME. All other reagents were of the highest grade available.

IV. METHODS

A. Enzyme Assays.

ASA and ASB activities were assayed essentially by the method of Roy (117) with the following modifications. Assays were performed by incubation of 0.2 ml of enzyme source with 0.2 ml of 10 mM p-nitrocatechol sulfate (40 mM for MPS VI ASB) in 0.5 M sodium acetate buffer, pH 5.6, for 1 h at 37°C. The reaction was stopped by the addition of 0.1 ml of 2 N NaOH and the samples were read at 515 nm in a Gilford 2400 spectrophotometer. All assays were performed in duplicate.

Natural substrate assays were performed by Dr. Reuben Matalon (University of Illinois) (118) and Dr. Hayato Kihara (Pacific State Hospital, CA) (119).

B. Protein Assays.

Protein concentrations were determined by the Bio-Rad assay according to the manufacturer's instructions (120) with the exception of samples used for dermatan sulfate quantitation which were assayed by the fluorescamine method (121).

C. Specimen Preparation for Heterozygote Detection.

Heparinized blood (10-20 ml) was obtained from five homozygous affected, fourteen obligate heterozygotes, seventy-one related cats from the MPS VI breeding colony, and thirteen unrelated normal cats. In addition, heparinized blood was obtained from an affected individual with human MPS VI, her two siblings, parents, maternal aunt, paternal grandmother, and thirty unrelated normal individuals.

Leukocytes were isolated as previously described (122). The leuko-

cyte pellet was washed 3 times with isotonic saline and frozen at -20°C until used. Cell pellets were suspended in 1.0 ml of distilled water and then subjected to 3 cycles of freezing and thawing. The suspension was centrifuged at 10,000 X g for 10 min and the supernatant used for analysis.

D. Separation of ASA and ASB from Peripheral Leukocytes.

DEAE-cellulose was suspended in distilled water and the fines removed; equilibration was performed in an equal volume of 0.1 M Tris/ HCl buffer, pH 7.5. An aliquot (1.0 ml) of this suspenson was mixed with 0.5 ml of the leukocyte supernatant for 1 h at 25°C, then centrifuged at 2,000 X g for 10 min. The supernatant containing the ASB activity was removed and assayed for arylsulfatase activity and protein. To elute the ASA activity bound to the DEAE-cellulose, the resin was washed twice with 3.0 ml of buffer and then mixed with 0.5 ml of 0.3 M NaCl in Tris buffer, pH 7.5, for 30 min. The suspension was centrifuged at 2,000 X g for 10 min and the supernatant was assayed for arylsulfatase activity and protein.

E. Purification of Human and Feline ASB.

All purification procedures were carried out at 4°C. Human and feline liver were homogenized in 3 volumes of 10 mM Tris/HCl buffer, pH 7.5, containing 0.05% Triton X-100 in a Waring blender (30 sec X 3), followed by further homogenization with a Brinkman Polytron for 30 sec. Each homogenate was centrifuged at 27,000 X g for 1 h and the residue was discarded.

1. Preparative DEAE-Cellulose Chromatography. The ASB and ASA in the supernatants were completely separated by batch DEAE-cellulose chromatography. Each supernatant was mixed overnight with 2 volumes of 0.1 M Tris/HCl buffer, pH 7.5, containing DEAE-cellulose (6 ml DEAE-cellulose/mg protein) which had been previously equilibrated with the same buffer. Under these conditions, the ASA bound to the resin, whereas the ASB did not. The DEAE-cellulose suspensions were suction-filtered over a Buchner funnel, and the filtrate containing the ASB activity was used for further purification.

2. Concanavalin A-Sepharose (Con A) Chromatography. The filtrates obtained above were diluted 3-fold with 25 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The diluted filtrates were mixed for 3 h with Con A (1 ml Con A/mg protein). The Con A was removed by suction filtration over a coarse-porosity fritted glass funnel and washed extensively with the above buffer until the absorbance (OD_{280}) of the eluate was less than 0.03. The bound glycoprotein was eluted by mixing the washed Con A with three 50 ml aliquots of the above buffer containing 1 M 1-O-methyl-α-D-glucopyranoside. The aliquots were combined, concentrated to 10 ml by ultrafiltration using a PM-10 membrane, and dialyzed against 4 liters of 25 mM Tris/HCl buffer, pH 7.5, with three changes of buffer over 12 h.

3. Blue Sepharose Chromatography. The dialyzed concentrates were adjusted to pH 6.0 with 1 M acetic acid and applied to columns (1 X 16 cm for the feline; 0.3 X 7 cm for the human) containing Blue Sepharose equilibrated with 10 mM Tris/acetate buffer, pH 6.0. The columns were

washed with 5 bed volumes of the above buffer before elution of the enzyme with a linear NaCl gradient (0 to 0.3 M) in 10 mM Tris/HCl buffer, pH 7.4. Fractions containing ASB activity were pooled, concentrated to 5 ml and dialyzed against 25 mM Tris/HCl buffer, pH 7.4

4. CM-Cellulose Chromatography. The concentrated feline and human enzyme preparations were adjusted to pH 5.0 with 1 M acetic acid and chromatographed over CM-cellulose columns (2 X 8 cm for the feline; 0.5 X 8 cm for the human) which previously were equilibrated in 25 mM sodium acetate/acetic acid buffer, pH 5.0. The columns were washed with 5 bed volumes of buffer and eluted with a linear NaCl gradient (0 to 0.5 M). Fractions containing ASB activity were pooled and concentrated as described above to 3 ml.

5. Sephadex G-200. The concentrated enzymes were applied separately to a Sephadex G-200 column (1.5 X 100 cm) which previously had been equilibrated with 10 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl. Slow flow rates (0.4 ml/min) were used to allow optimal separation of the enzyme from other proteins. Fractions with enzymatic activity were pooled and concentrated as described above. The human ASB was homogeneous and used for characterization studies. The feline ASB sample was further purified as described below.

6. DEAE-Cellulose Chromatography. The feline ASB sample was dialyzed against 10 mM Tris/HCl buffer, pH 7.5, and the dialysate applied to a column (0.5 X 8 cm) containing DEAE-cellulose which previously was equilibrated with 10 mM Tris/HCl buffer, pH 7.4. The enzyme was

bound to the anion exchange resin under these conditions, and was eluted with a linear NaCl gradient (0 to 0.3 M). Fractions containing ASB activity were pooled, concentrated and rechromatographed on Sephadex G-200 as described above.

F. Purification of Normal and MPS VI Residual ASB Activity.

Hepatic tissue from a MPS VI (30 g) and a normal Siamese cat (80 g) was homogenized in 3 vol of 0.01 M Tris/HCl buffer, pH 7.5, in a Virtis homogenizer. Following three cycles of freeze-thaw, each suspension was further homogenized with a Brinkman Polytron for 20 sec and then centrifuged at 27,000 X g for 45 min. The ASB and ASA in the supernatants were separated by batch anion exchange chromatography. The supernatants were added to 2 volumes of 0.1 M Tris/HCl buffer, pH 7.5, containing DEAE-cellulose (6 ml DEAE/mg protein; DE 52), which was previously equilibrated with the same buffer. The suspension was stirred overnight at 4°C. Under these conditions, the ASA was bound to the resin, but the ASB was not. The DEAE-cellulose suspension was suction-filtered over a Buchner funnel and the filtrate containing the ASB activity was diluted 6-fold with 25 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The diluted filtrate was mixed with Con A (3 ml Con A/mg protein), and stirred for 3 h at room temperature. The Con A was removed by suction-filtration over a coarse-porosity fritted glass funnel and then was poured into a 10 ml disposable syringe fitted with a glass wool plug. The column was washed extensively with the above buffer until the absorbance (OD_{280}) of the eluted fractions was less than 0.03 OD. Then, the bound glycoprotein was eluted with 1 M 1-O-methyl-α-D-glucopyranoside at a flow rate of 60 ml/h. The fractions

containing the ASB activity were pooled, concentrated to 14 ml by ultrafiltration using a PM-10 membrane and dialyzed against 4 liters of 25 mM Tris/HCl buffer, pH 7.5, with three changes of buffer over 12 h. The dialyzed concentrate was chromatographed over DEAE-cellulose (6 X 1.5 cm and 16 X 1.5 cm columns for the MPS VI and normal preparations, respectively), which had been equilibrated previously with 25 mM Tris/HCl buffer, pH 7.5. The column was eluted with 60 ml of the same buffer, before a linear NaCl gradient (0 to 0.3 M) was applied. Fractions (2.5 ml) were collected at a flow rate of 0.3 ml/min. Fractions containing the ASB activity were pooled, concentrated to about 3 ml by ultrafiltration and then chromatographed over Sephadex G-100 (0.5 X 100 cm column). The column was eluted with 0.05 M Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl, at a flow rate of 5 ml/h. The fractions (2.5 ml each) containing the ASB activity were pooled and concentrated as above. These partially purified preparations were used to characterize their physical and kinetic properties as described below.

G. Analytical Polyacrylamide Gel Electrophoresis.

Analytical polyacrylamide gel electrophoresis using 7 percent disc gels (0.5 X 7 cm) was performed in β-alanine/acetate buffer, pH 4.0, as described by Reisfeld et al. (123). A constant current of 4 mA/gel was applied for 3 h or until the tracking dye reached the bottom of the tube. Detection of ASB activity was accomplished by incubating the gels with 20 mM pNCS for 1 h at 37°C. For visualization of enzyme activity, the gels were transferred into 2 N NaOH for 2-3 min. The gels were also stained for protein with Coomassie brilliant blue G-250 as previously described (124).

H. Cellulose Acetate Gel Electrophoresis.

Cellulose acetate gel (cellogel) electrophoresis was performed by a modification of the method of Rattazzi et al. (125). Cellogel sheets (17 cm X 17 cm X 0.35 mm) were rinsed in distilled water followed by equilibration for 1 h in 0.036 M sodium barbitol, 0.037 M sodium acetate/ acetic acid buffer, pH 6.5, containing 1 mM β-mercaptoethanol and 0.01% sodium azide. The gels were blotted and placed into an electrophoresis chamber containing the same buffer used for equilibration. Samples (75 to 100 µl) were applied to the center of the gel and electrophoresed at room temperature for 3 h at a constant current of 15 mAmp. The ASA and ASB activities were visualized by incubation of the gel for 1 h at 37°C with a filter paper overlay saturated in a solution of 4-methylumbelliferyl sulfate (4-MUS) (10 mg/ml) in 0.5 M sodium acetate/ acetic acid buffer, pH 6.0. For visualization of bands, the gel was exposed to ammonium hydroxide for 2-3 min in a closed chromatography tank.

I. Isoelectric Focusing.

Flat bed isoelectric focusing was performed on an LKB Multiphor apparatus using a pH 6 to 8.5 gradient and polyacrylamide gels prepared according to the manufacturer's instructions (126). The gel was maintained at 4°C and a constant voltage (800 V) was applied for 3 h. The pH was determined with a surface electrode and the activity bands were visualized by incubating the gel for 1 h at 37°C with a filter paper overlay saturated with 4-MUS as described above.

J. Molecular Weight Determinations.

The apparent molecular weights of the purified human and feline ASB

activities were determined by gel filtration on Sephadex G-200. The following proteins were used as standards: aldolase (M_r = 158,000), bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 45,000) and chymotrypsinogen (M_r = 24,000). Molecular weights were also determined using the method of Hendrick and Smith (127) by electrophoresis in gels of different polyacrylamide concentrations. The protein standards used were: aldolase (M_r = 158,000), bovine serum albumin (M_r = 68,000), chymotrypsinogen (M_r = 24,000) and cytochrome C (M_r = 12,400). Determination of the molecular weight of denatured ASB was performed on SDS gels according to the method of Weber and Osborn (128).

Sucrose density-gradient centrifugation was performed by the method of Martin and Ames (129). Linear sucrose gradients of 5 to 20% sucrose in 0.1 M Tris/HCl buffer, pH 7.5, were prepared. Samples (50 µl) containing purified human or feline enzyme and marker proteins were layered onto the top of the gradient. Marker proteins included lactic dehydrogenase (M_r = 135,000), hexokinase (M_r = 98,000), and ovalbumin (M_r = 47,000). Centrifugation was at 40,000 rpm for 18 h at 4°C in a Beckman L5-75 Ultracentrifuge with a SW57 rotor. After centrifugation, 0.25 ml fractions were collected and assayed for protein and ASB activity.

K. Subunit Structure Determinations.

The subunit structure of the normal feline enzyme was determined by treatment with thiol reagents, as described by Fisher et al. (130). Two electrophoretically different forms of ASB were prepared by treating 3,000 U of purified normal feline enzyme with 14 mM iodoacetate and 3,000 U with 14 mM iodoacetamide. The alkylated enzymes were dissociated into subunits by dialysis against 0.1 M Tris/HCl buffer, pH 7.0,

containing 0.4 M sucrose, 1 M NaCl, and 2 M urea. An equal amount of the iodoacetate-treated enzyme was mixed with the iodoacetamide-treated ASB and the mixtures were frozen at -55°C for 2 h, thawed, and then dialyzed against 10 mM Tris/HCl buffer, pH 7.4, to recombine the subunits. In addition, a control mixture was made which contained equal amounts of the iodoacetamide- and iodoacetate-treated enzymes, after each was dissociated and reassociated. Each preparation was subjected to analytical polyacrylamide gel electrophoresis as described above. As a further control, aliquots of normal feline ASB were also treated with varying concentrations of iodoacetate (0.14-14 mM), dialyzed against 10 mM Tris/HCl buffer, pH 7.4, and analyzed on polyacrylamide gel electrophoresis without subjecting the preparations to the dissociation-reassociation procedure.

The subunit structure of the human and feline isozymes was further investigated by treatment with the crosslinking reagent, dimethyl suberimidate, as described by Davies and Stark (131). Aliquots (3,000 U) of the homogeneous human and feline enzymes were treated with a final concentration of 2 mg/ml of dimethyl suberimidate in 0.2 M triethanolamine hydrochloride, pH 8.5, and the reaction mixture left at room temperature for 3 h. The enzymes were denatured for 2 h at 37°C in 1% SDS and 1% β-mercaptoethanol; each preparation was then subjected to SDS gel electrophoresis according to the method of Weber and Osborn (128) using 5% gels.

L. Physiokinetic Studies.

Enzymatic activity versus pH curves were determined over a pH range of 4.0 to 8.0. Sodium acetate/acetic acid buffer was used from pH 4.0

to 5.6, ammonium acetate/ammonium hydroxide buffer for pH 5.8 to 6.5 and Tris/HCl buffer for pH 7.0 to 8.0. The thermostability of each purified enzyme was determined at 60°C in the presence of 1 mg/ml of bovine serum albumin. Cryostability studies were performed by freezing aliquots of the purified enzymes at -5°C for 72 h. The effect of substrate concentration on the activity of the purified human and feline ASB isozymes was measured over a range of 0.625 to 20.0 mM pNCS at pH 5.7 with 0.5 M sodium acetate/acetic acid buffer. The effect of pH on the stability of the normal and MPS VI residual activity was compared by storing each enzyme at 4°C for 72 h over a pH range of 3.5 to 8.5 in 0.14 M sodium acetate/0.14 M sodium barbital buffer, adjusted with 0.1 M HCl.

M. Amino Acid Analysis.

Amino acid analyses were performed on purified human and feline ASB by Dr. Al Smith (University of California). The lyophilized samples were dissolved in 50% formic acid and acid hydrolyses were performed for 24, 48 and 72 h at 110°C in 6 N HCl. Amino acid concentrations were then determined in a Durrum B-500 amino acid analyzer.

N. Cation Exchange Chromatography of MPS VI Residual ASB.

Aliquots of the normal and MPS VI enzyme were dialyzed overnight against 25 mM sodium acetate/acetic acid buffer, pH 5.0, and individually chromatographed over CM-cellulose (10 X 0.8 cm column) which was previously equilibrated in the same buffer. The column was eluted with 30 ml of buffer and then a linear NaCl gradient (0-0.5 M) was applied. Fractions (1.5 ml) were collected at a flow rate of 0.3 ml/min, and assayed for ASB.

O. Production and Partial Purification of Rabbit Anti-Human and Feline ASB Antibodies.

New Zealand rabbits were injected intradermally with 50 µg of homogeneous human or feline ASB in a 1:1 suspension of Freund's complete adjuvant. Booster injections (50 µg) were given at two week intervals.

IgG from rabbit antisera was partially purified as previously described (132). The antibody was precipitated by adding neutralized saturated ammonium sulfate and stirring for 2 h at 4°C. The solution was centrifuged at 2,500 X g for 20 min and the pellet was washed twice with 1.5 M ammonium sulfate. The pellet was resuspended in distilled water and dialyzed extensively against saline. The antibody was aliquoted and stored at -20°C.

P. Carbamylation of Antibody.

The partially purified antibody was carbamylated by the method of Bjerrum et al. (133). Antibody (7.0 mg) in 1 ml of 0.1 M NaCl, 7 ml of borate-buffer, pH 8.0, and 2 ml of 1.0 M KOCN were incubated for 2 h at 45°C. The carbamylation reaction was stopped by desalting on a Sephadex G-25 column (0.5 X 25 cm). Antibody in the first protein peak was concetrated to its original volume and subjected to agarose gel electrophoresis (133) to determine its isoelectric point.

Q. Rocket Immunoelectrophoresis.

Agarose solutions (1%) were prepared in TMED/acetate buffer (0.01 M N,N,N',N'-tetramethyl-1,2-diaminoethane and 0.029 M acetic acid), pH 5.0, and bridges (8.0 X 2.0 cm) containing 4.0 ml of agarose solution were poured into 0.5 X 8.0 cm plastic plates at the anode and cathode

(134). The center of the plate (2.5 X 8 cm) was filled with agarose containing 100 µl (approximately 0.25 mg protein) of partially purified and carbamylated rabbit anti-ASB antibody. Wells (3 mm) were cut at the cathodal end of the antibody containing gel and samples (10 to 25 µl) were applied. Electrophoresis was performed in a tank containing the TMED/ acetate buffer, pH 5.0, at 25 mAmps for 5 h at room temperature.

Following electrophoresis, the gel was washed for 12-15 h in saline. The antibody containing portion was overlaid with 0.3 ml of sheep anti-rabbit peroxidase conjugated IgG, which had been diluted 1:3, and incubated at room temperature for 8-12 h in a moist chamber. Following another 12-15 h wash with saline, the gel was stained with 50 ml of 0.1 M Tris/HCl buffer, pH 7.6, containing 25 mg diaminobenzidine tetrahydrochloride and 0.15 ml of 3% H_2O_2. After 20 min, rockets were visualized using an indirect light source.

R. Dermatan Sulfate Quantitation.

Dermatan sulfate levels were determined by cellulose acetate gel electrophoresis according to the procedure of Schuchman and Desnick (135). Briefly, the leukocyte pellets were subjected to pronase digestion (final concentration 0.5 mg/ml) at 60°C for 2 h. Protein was subsequently assayed by the fluorescamine method (121). Aliquots of each sample containing 200 µg of protein were applied to cellulose acetate gels and electrophoresis was performed for 50 min at 4°C (2.5 mA/cm gel width; 15 volts/cm gel length) using an electrolyte solution containing 10 mM EDTA tetrasodium salt and 50 mM LiCl at pH 8.4. After electrophoresis, the gels were stained for 1 min in 0.1% Alcian Blue 8GN at 23°C. Destaining was accomplished by two serial submersions in 100 ml

of 2% glacial acetic acid for 5 min each. The dermatan sulfate bands were quantitated by a modification of the procedure described by Kimura et al. (136). Each band was cut out and immersed in 2.0 ml of chloroform/methanol 9:1 (vol/vol) which dissolved the cellulose acetate. The absorbance of the solution was determined spectrophotometrically at 625 nm. For control, blank gel slices of the same size were dissolved and their absorbance determined. The standard curve for dermatan sulfate was linear over a range of 1 to 5 µg.

S. Effect of Sulfhydryl Reagents on the Physiokinetic Properties of Purified ASB.

Highly purified normal feline and feline MPS VI ASB activities were determined in the presence and absence of various concentrations of sulfhydryl-reactive agents in 25 mM Tris/HCl buffer, pH 7.5. Kinetic studies were performed at pH 5.7, the pH optimum for both enzymes, in the presence and absence of 0.25 mM DTT. Thermostability (at 60°C) and cryostability (after 72 H at -50°C) of the normal and residual enzymes were determined with and without 0.25 mM DTT. The effect of pH (3.5 to 8.5) on the stability of the normal and MPS VI residual activities was compared by incubating the purified enzymes for 1 h at 37°C in the presence and absence of 0.25 mM DTT in 0.14 M sodium acetate/0.14 M sodium barbital buffer, adjusted to the desired pH with HCl.

Analytical polyacrylamide gel electrophoresis was performed in 7% disc gels (0.5 X 7.0 cm) in 100 mM β-alanine/acetate buffer at pH 4.0 as described by Reisfeld et al. (123). Partially purified normal feline and feline MPS VI ASB were electrophoresed in the presence and absence of 1.0 mM DTT at a constant current of 4 mA/gel for 3 h or until the

tracking dye reached the bottom of the tube. The gels were stained for ASB activity using pNCS as described above. Apparent molecular weight values for each enzyme were determined in the presence and absence of 1.0 mM DTT on 5,6,7 and 8% native polyacrylamide gels according to the method of Hendrick and Smith (127). Apparent molecular weights were also estimated by gel filtration on Sephadex G-200 using a 1.5 X 100 cm column in the presence and absence of 1.0 mM DTT in the enzyme sample and elution buffer.

T. In Vitro Studies.

The effect of DTT, cysteamine and cystamine on ASB activity and dermatan sulfate levels was determined in leukocytes isolated from 30 ml of heparinized whole blood obtained from normal and MPS VI cats. Varying concentrations of each sulfhydryl reagent, freshly prepared in 25 mM Tris/HCl buffer, pH 7.4, were added to 5 ml aliquots of whole blood in 13 X 100 mm screw cap tubes. The tubes were sealed after gassing with N_2 and the suspensions were gently mixed on a rocking platform for 1 h at 37°C. Then the leukocytes were isolated by dextran sedimentation, washed twice with 0.15 M NaCl, suspended in 0.5 ml of distilled water and lysed by 3 cycles of freeze/thaw (-70°C/37°C). Aliquots (50 µl) were removed from each leukocyte homogenate for determination of the dermatan sulfate concentrations and the remainder of the homogenate was centrifuged at 10,000 X g for 10 min. The supernatant was removed, ASA and ASB were separated by batch DEAE-cellulose chromatography as described above and the ASB activities and protein concentrations were determined.

U. In Vivo Studies.

DTT (50 mg/kg) or cysteamine (15 mg/kg) was dissolved in 10 ml of sterile 0.15 M NaCl and passed through an Acrodisc filter (0.2 micron pore size) immediately prior to use. DTT or cysteamine was intravenously administered over 10 min into an 18 month old MPS VI female (2.27 kg) cat and her normal female sibling (3.27 kg). Heparinized blood samples (5 ml) were obtained immediately before and after and at 0, 30, 60, and 120 min intervals after infusion. ASB activity and dermatan sulfate levels were determined in isolated leukocytes as described above. The volume of blood drawn for these studies precluded the assessment of multiple experimental infusions.

V. RESULTS

A. Heterozygote Detection.

Initial studies were undertaken to develop a reliable assay for heterozygote identification. ASA and ASB from leukocyte lysates were totally separated by batch DEAE-cellulose chromatography. The ASB activity did not bind to the resin and was recovered in the buffer wash, whereas the ASA activity was eluted with 0.3 M NaCl.

Figure 7 compares the specific activity of ASB and the activity ratios of ASB to ASA in leukocytes from homozygotes, obligate heterozygotes, relatives of the feline MPS VI families and unrelated normal cats. Note that the values for obligate heterozygotes overlap with those obtained for normal and affected cats when only the ASB activity is determined (Figure 7A). In contrast, when these ASB activities were expressed as the ratio of ASB to ASA, the obligate heterozygotes were clearly discriminated from the normal cats. Although there was no overlap between obligate heterozygotes and affected cats, the low heterozygote ratios were close to the high homozygote values. Several of the related cats were identified as heterozygous for the MPS VI gene.

Analogously, the ASB to ASA ratios provided accurate detection of two obligate heterozygotes for human MPS VI, whereas their values were in the normal range when expressed as ASB specific activity (Figure 8A and B).

B. Purification of Human and Feline Hepatic ASB.

Table 5 summarizes the results of a typical purification procedure. The enzyme was purified approximately 9100-fold with a 13% yield for the feline isozyme and 8400-fold with a 17% yield for the human enzyme. The

Figure 7. Heterozygote detection for feline MPS VI. ASB activities in peripheral leukocytes from feline homozygotes, obligate heterozygotes, relatives of the feline MPS VI families and unrelated normal cats. A, ASB specific activities; B, ratio of ASB to ASA activities.

Figure 7

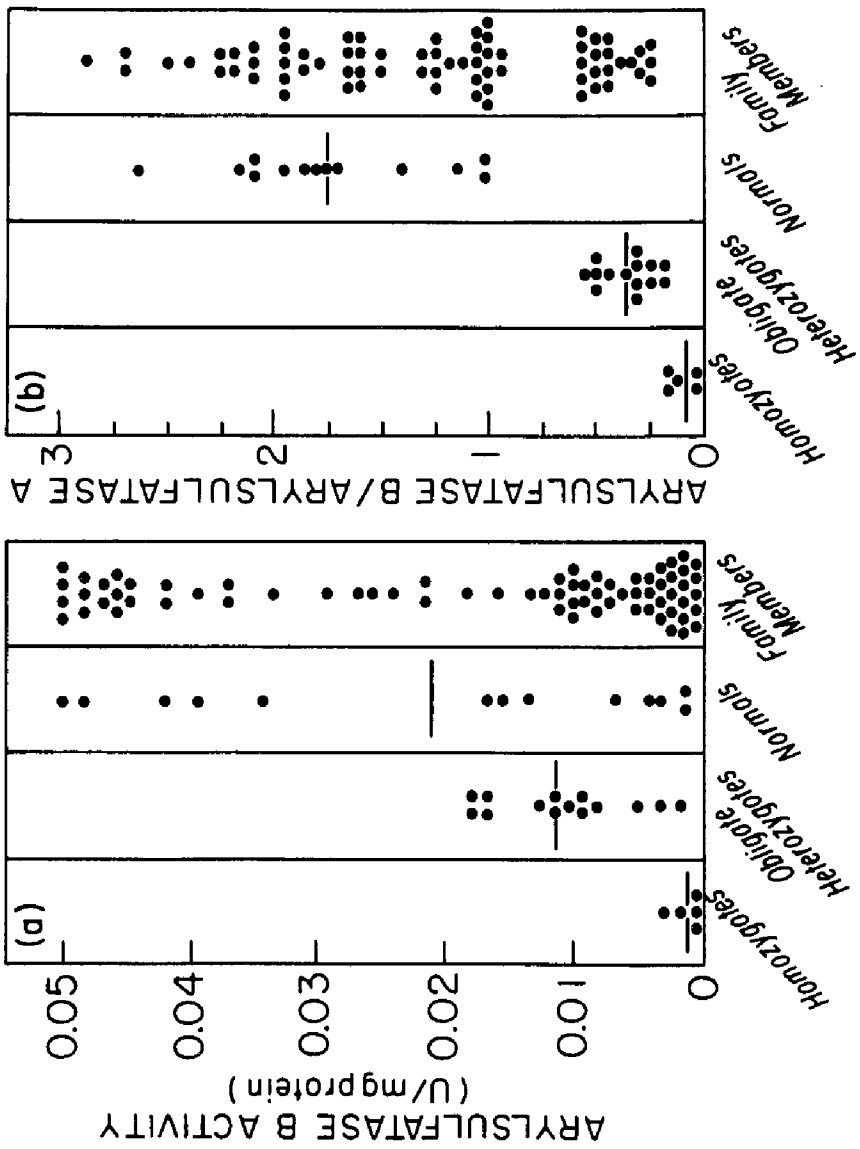

ARYLSULFATASE B ACTIVITY
(U/mg protein)
0.05
0.04
0.03
0.02
0.01
0
(a)
Homozygotes
Obligate Heterozygotes
Normals
Family Members
ARYLSULFATASE B/ARYLSULFATASE A
3
2
1
0
(b)
Homozygotes
Obligate Heterozygotes
Normals
Family Members

Figure 8. Heterozygote detection for human MPS VI. ASB activities in human peripheral leukocytes from a homozygote, her parents (obligate heterozygotes) and relatives of the MPS VI homozygote, as well as unrelated normal individuals. A, ASB specific activity; B, ratio of ASB to ASA activities.

Figure 8

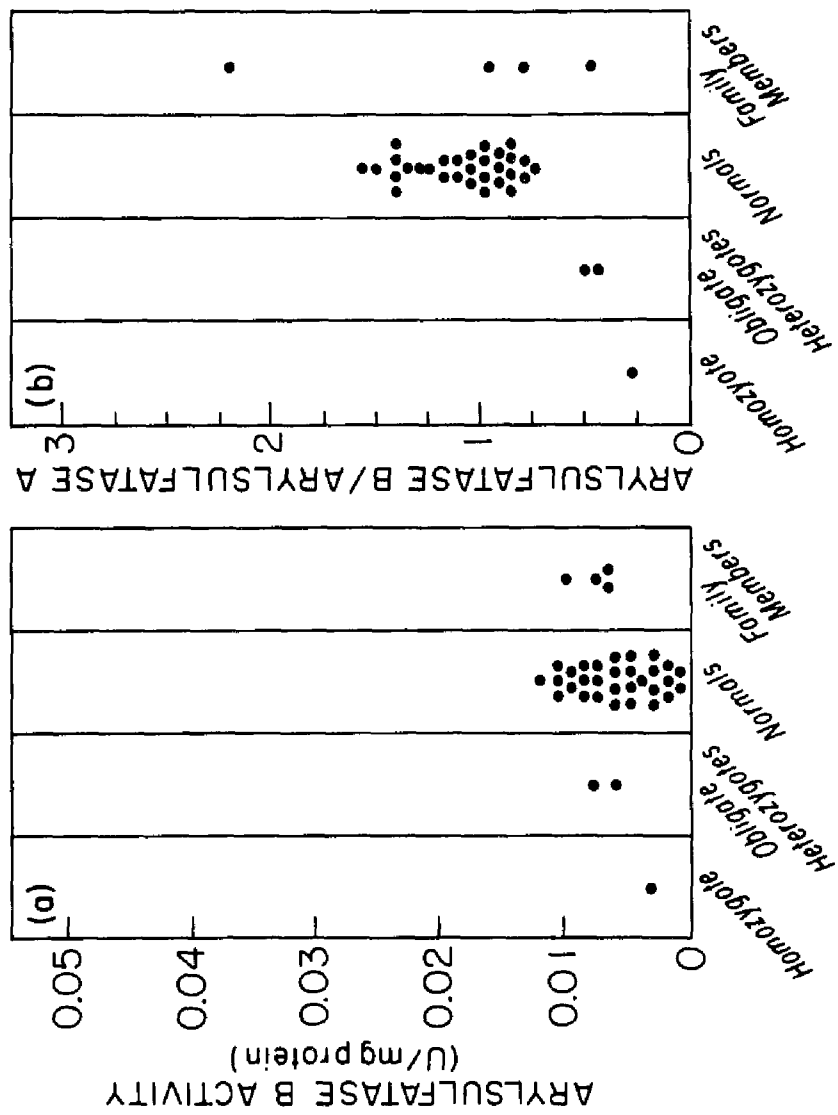

ARYLSULFATASE B ACTIVITY
(U/mg protein)
0.05
0.04
0.03
0.02
0.01
0
(a)
Homozygote
Obligate Heterozygotes
Normals
Family Members
ARYLSULFATASE B/ARYLSULFATASE A
3
2
1
0
(b)
Homozygote
Obligate Heterozygotes
Normals
Family Members

TABLE 5

Purification of ASB from Normal Feline and Human Liver

Step	Feline			Human		
	Specific Activity	Fold	Yield (%)	Specific Activity	Fold	Yield (%)
Crude Homogenate	120	-	100	95	-	100
Preparative DEAE-Cellulose	200	1.7	93	130	1.4	97
Concanavalin A Sepharose	95,000	790	71	35,000	368	68
Blue Sepharose	150,000	1250	50	394,000	4150	60
CM-Cellulose	300,000	2500	30	710,000	7475	31
Sephadex G-200	420,000	3500	25	*	*	*
DEAE-Cellulose	850,000	7080	19	*	*	*
Sephadex G-200	1,100,000	9100	13	800,000	8400	17

*Steps not performed

initial preparative DEAE-cellulose chromatographic step resulted in complete separation of ASA from ASB from both sources. Both human and feline activities bound to Con A-Sepharose (~80% for the human and 85% for the feline) and were eluted with 1 M 1-O-methyl-α-D-glucopyranoside. Although both feline and human ASB bound to Blue Sepharose (Figure 9), the feline enzyme was eluted at 60 mM NaCl (Figure 9A), whereas human ASB activity was eluted at 155 mM NaCl (Figure 9B). Both enzymes also bound to CM-cellulose and were eluted with a salt gradient, 25 mM NaCl for the feline and 32 mM NaCl for the human enzyme.

C. Characterization of Human and Feline ASB.

1. Electrophoretic Studies. The purified ASB activities from the human and feline hepatic tissues each appeared homogeneous by native polyacrylamide gel electrophoresis at pH 4.0. The feline activity migrated less cathodally than the human enzyme (Figure 10), however, both migrated as a single band which stained for protein and activity. Under denaturing conditions in SDS polyacrylamide gels, each preparation migrated as a single protein band. Consistent with the polyacrylamide gels, the human ASB migrated more cathodally than the feline activity on cellulose acetate gel electrophoresis (Figure 11).

2. Isoelectric Point. Flat bed isoelectric focusing showed a slight difference in pI values for the purified ASB activities from human and feline liver. Human ASB had a pI of 8.0, whereas the pI of the feline enzyme was 7.8.

3. Molecular Weight and Subunit Composition Determinations. The

Figure 9. Blue Sepharose chromatographic profiles of feline (A) (■——■) and human (B) (●——●) ASB isozymes. The columns were washed with 25 mM sodium acetate/acetic acid buffer, pH 6.0, and the activities were eluted with a NaCl gradient.

Figure 9

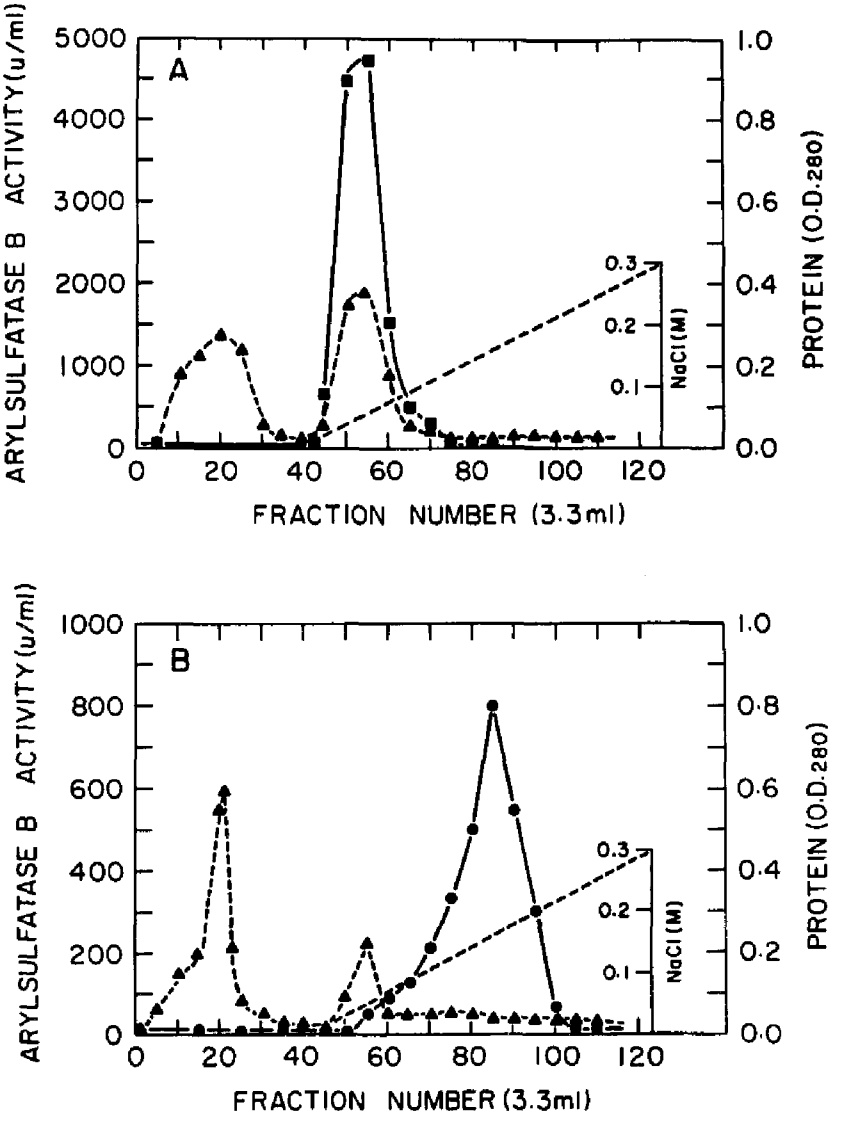

A
ARYLSULFATASE B ACTIVITY(u/ml)
PROTEIN (O.D.280)
NaCl(M)
FRACTION NUMBER (3.3ml)
B
ARYLSULFATASE B ACTIVITY(u/ml)
PROTEIN (O.D.280)
NaCl(M)
FRACTION NUMBER (3.3ml)

Figure 10. Analytical polyacrylamide disc gel electrophoresis of purified feline and human hepatic ASB isozymes. Gel 1, feline ASB; Gel 2, human ASB. The protein band shown (stained with Coomassie Brilliant Blue) coincided with the enzymatic activity band. Arrow, point of application.

Figure 10

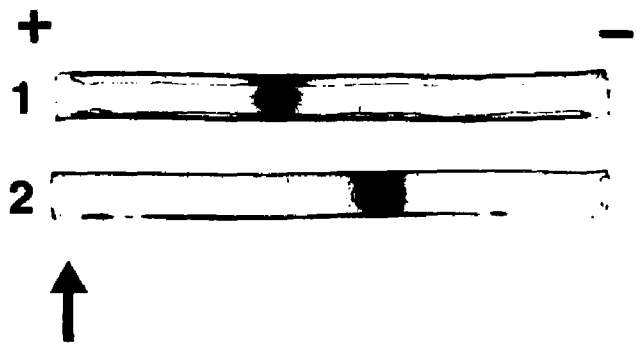
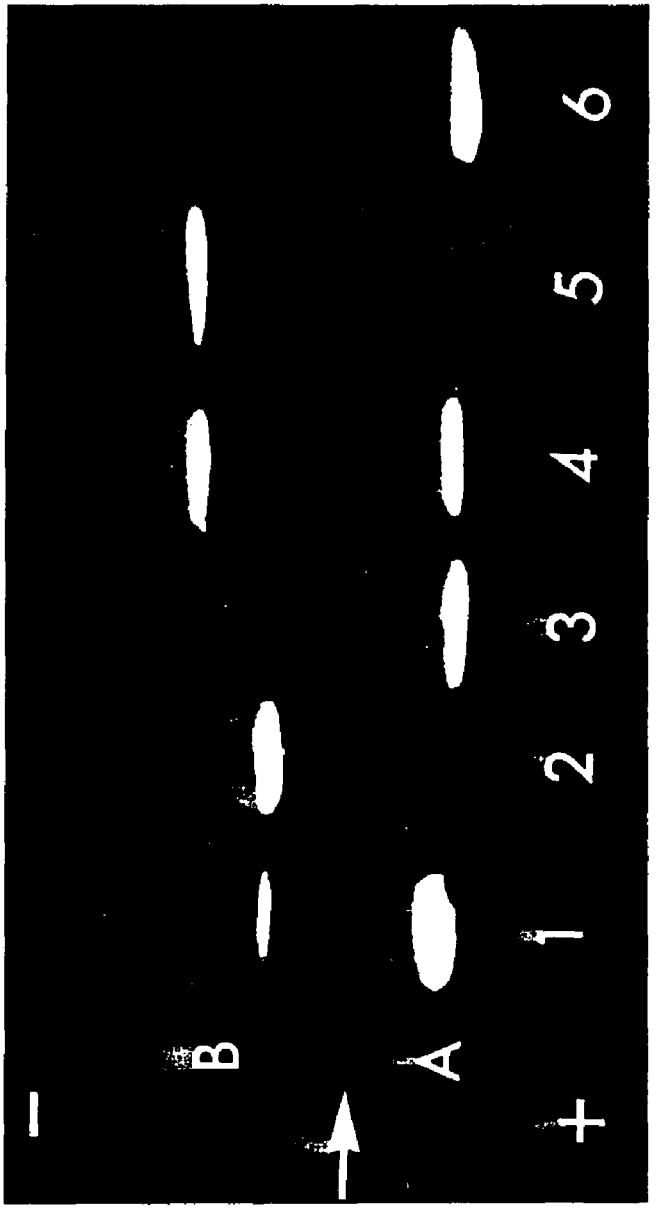


Figure 11. Cellulose acetate gel electrophoresis of normal feline and human leukocyte ASA and ASB activities. ASA and ASB were separated by batch DEAE-cellulose chromatography. Lanes 1-3, feline leukocyte lysates; lanes 4-6, human leukocyte lysates; lanes 1 and 4, total leukocyte lysate; lanes 2 and 5, unbound activity, lanes 3 and 6, bound activity eluted from DEAE-cellulose. Note the difference in electrophoretic mobility for the human and feline ASB isozymes. Arrow, point of application; A, ASA; B, ASB.

Figure 11



apparent molecular weights of the feline and human ASB activities, determined by gel filtration on Sephadex G-200, were 100,000 and 48,000, respectively (Figure 12). The native molecular weights of the two enzymes were also determined by the analytical polyacrylamide gel electrophoresis method of Hendrick and Smith (127) using 5, 6, 7 and 8 percent gels (Figure 13). The plots of gel concentration versus log R_m for each enzyme preparation are shown in Figure 13A. The molecular weights estimated from the slopes of these plots were 166,000 for the feline enzyme and 80,000 for the human isozyme (Figure 13B). Molecular weight values determined by sucrose-density gradient centrifugation were 105,000 and 55,000 for the feline and human enzymes, respectively (Figure 14). When each enzyme was electrophoresed under denaturing conditions in SDS polyacrylamide gels, the human isozyme had a single band with a molecular weight of 38,000, whereas the feline isozyme had a single band with a molecular weight of 41,000 (Figure 15).

Figure 16 shows the polyacrylamide electrophoretic gels of the reassociated thiol-treated subunits of feline ASB. The iodoacetate-treated enzyme (gel 1) migrated less cathodally, as expected, than the iodoacetamide-treated enzyme (gel 2) whose mobility was unaltered. Electrophoresis of the reassociated mixture of the two alkylated preparations (e.g., dissociated, thiol-treated, mixed, and then reassociated) revealed a third activity band which migrated to a position intermediate to those of each alkylated enzyme preparation (gel 3). This intermediate band represents a hybrid molecule containing one iodoacetate- and one iodoacetamide-treated subunit. Also, a control mixture of the reassociated iodoacetate- and iodoacetamide-treated enzymes (mixed after reassociation) had only the respective bands (not shown) seen in gels 1

Figure 12. Determination of the molecular weights of the feline and human ASB isozymes by gel filtration. Each isozyme was chromatographed separately. Enzyme (500 U) and 5 mg of each protein standard were applied to a column of Sephadex G-200. The elution points of the feline and human ASB are indicated (X).

Figure 12

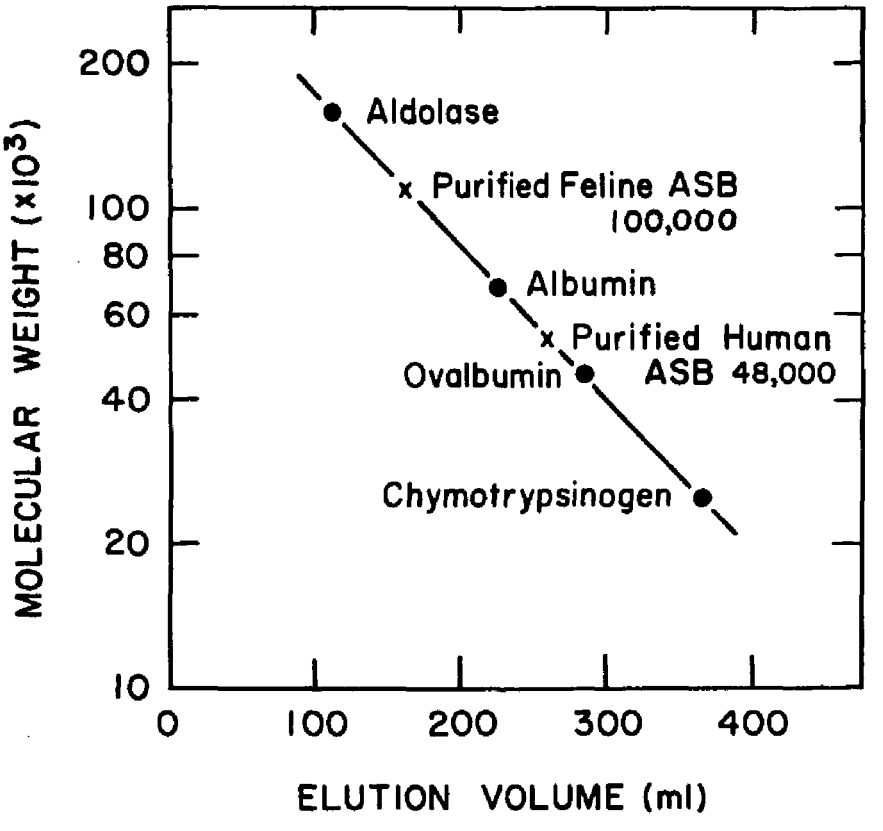

MOLECULAR WEIGHT (x10³)
200
100
80
60
40
20
10
0
100
200
300
400
ELUTION VOLUME (ml)
Aldolase
Purified Feline ASB
100,000
Albumin
Purified Human
ASB 48,000
Ovalbumin
Chymotrypsinogen

Figure 13. Native molecular weights of purified feline and human hepatic ASB isozymes determined by polyacrylamide gel electrophoresis according to the method of Hendrick and Smith (127). A) Gel concentration versus log R_m for feline (■—■) and human (●—●) ASB. B) Estimation of the molecular weights from the slopes of the feline and human ASB actvities are indicated (X).

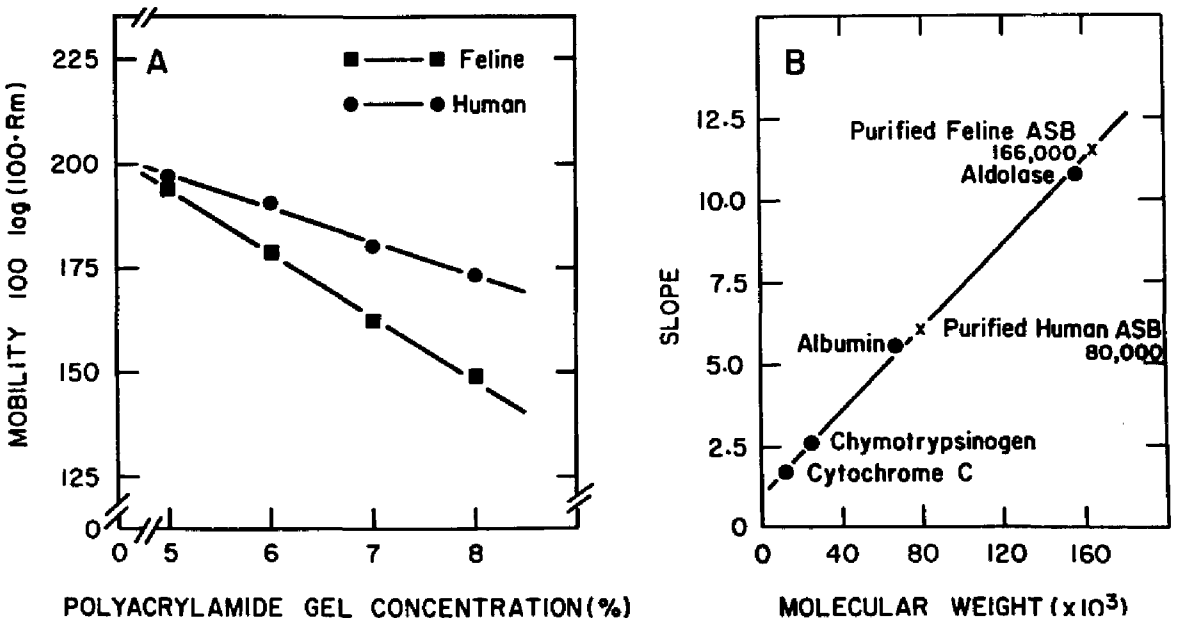

A
Feline
Human
MOBILITY 100 log(100·Rm)
225
200
175
150
125
0
0 5 6 7 8
POLYACRYLAMIDE GEL CONCENTRATION(%)
B
Purified Feline ASB
166,000
Aldolase
Albumin
Purified Human ASB
80,000
Chymotrypsinogen
Cytochrome C
SLOPE
12.5
10.0
7.5
5.0
2.5
0
0 40 80 120 160
MOLECULAR WEIGHT (x10³)

Figure 13

Figure 14. Sucrose-density gradient centrifugation of human and feline hepatic ASB. Each enzyme (50 μg) and marker proteins were layered onto the top of the gradient and centrifuged as described in Methods.

Figure 14

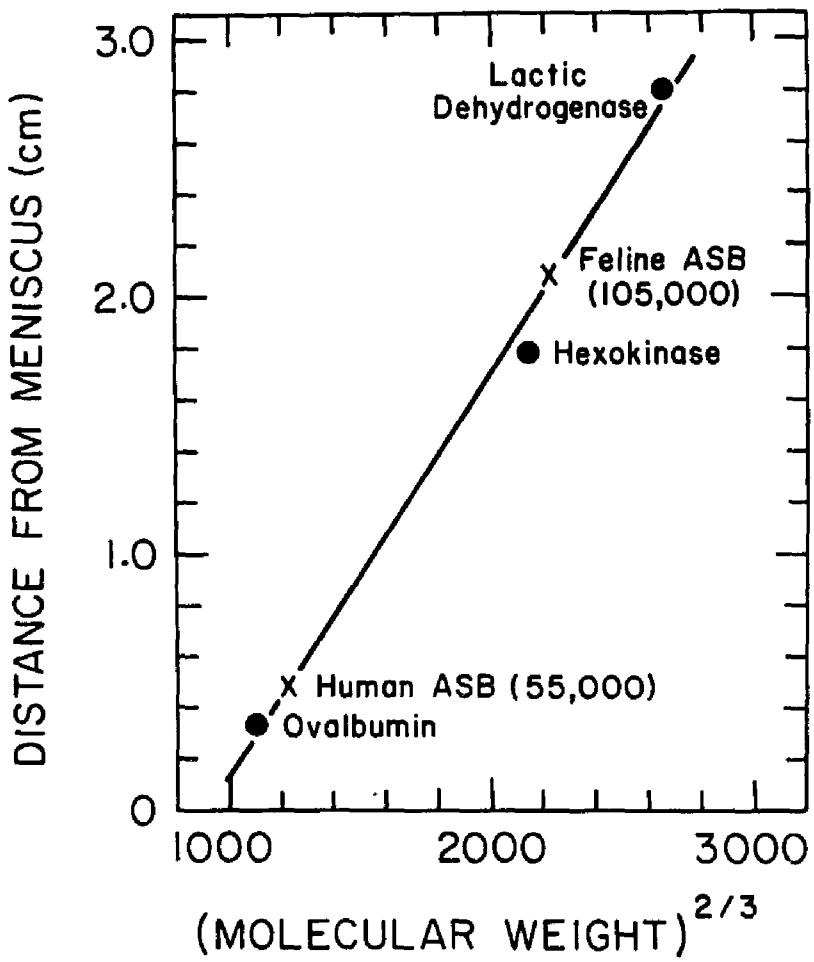

3.0
2.0
1.0
0
DISTANCE FROM MENISCUS (cm)
Lactic Dehydrogenase
Feline ASB (105,000)
Hexokinase
Human ASB (55,000)
Ovalbumin
1000
2000
3000
(MOLECULAR WEIGHT)2/3

Figure 15. SDS polyacrylamide gel electrophoresis of feline and human hepatic ASB. Each standard (40 μg) and 40 μg of homogeneous human and feline ASB were treated and electrophoresed as described in Methods.

Figure 15

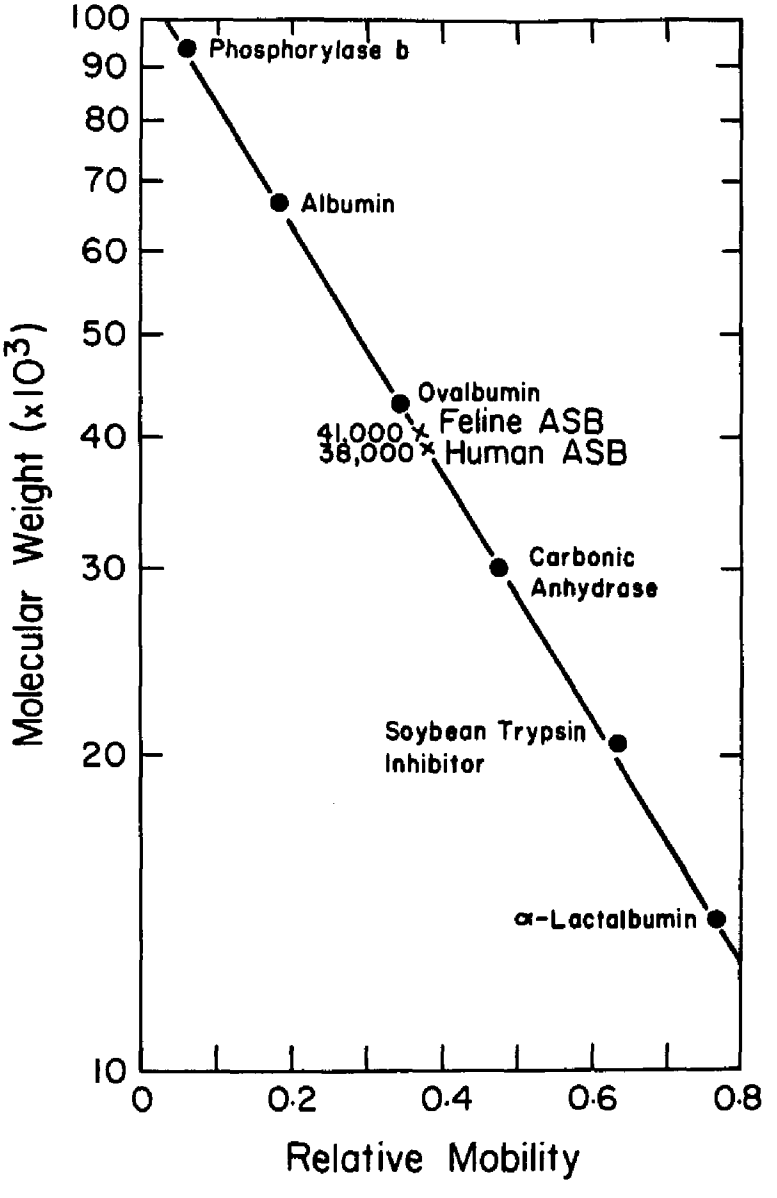
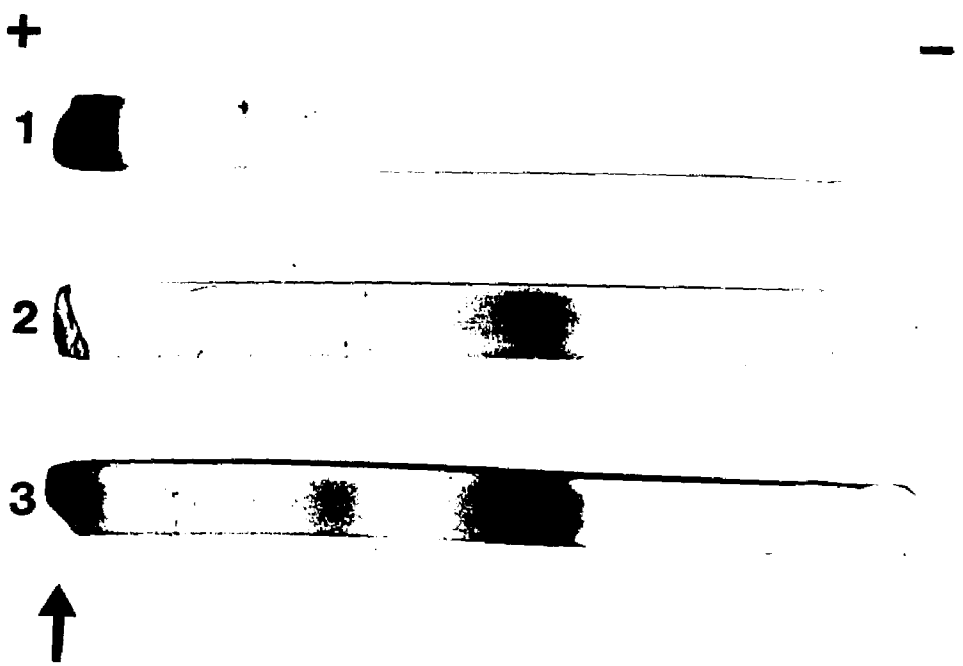

Molecular Weight (x10³)
100
90
80
70
60
50
40
30
20
10
0
0·2
0·4
0·6
0·8
Relative Mobility
Phosphorylase b
Albumin
Ovalbumin
41,000 × Feline ASB
38,000 × Human ASB
Carbonic Anhydrase
Soybean Trypsin Inhibitor
α-Lactalbumin

Figure 16. Analytical polyacrylamide gel electrophoresis of purified feline hepatic ASB treated with iodoacetate (gel 1) and iodoacetamide (gel 2) to generate two electrophoretically different forms of the enzyme. The hybrid sample is a 50:50 mixture of the two preparations (gel 3). The arrow indicates the point of application. The minor band in gel 1 represents partially alkylated enzyme.

Figure 16



and 2. When the human enzyme was treated with these alkylating agents, the expected change in its charge with iodoacetate was not observed,

Figure 17 shows the SDS electrophoretic gels of the dimethyl suberimidate-treated (gel 2) and untreated (gel 1) feline ASB. The untreated feline enzyme migrated as a single band to the expected position corresponding to a molecular weight of approximately 41,000. The treated enzyme displayed two major bands corresponding to molecular weights of 41,000 and 80,000, respectively. The presence of two bands in the treated sample indicated that the feline enzyme was a dimer. Identical studies on the human enzyme revealed a single band in both the treated and untreated samples, indicating that the human enzyme was a monomer.

4. Kinetic and Stability Studies. Figure 18 shows the effect of pH on the purified ASB enzymes. Both human and feline enzymes had pH optima of 5.7. Kinetic studies performed at the optimal pH demonstrated that the feline activity was maximal at 5 mM pNCS and the human activity at 10 mM pNCS (Figure 19). Approximate Km values, calculated from Lineweaver-Burk plots, were 1.2 mM for the feline activity, and 3.6 mM for the human enzyme. V_{max} values for the feline and human isozymes were 4,000 and 5,100 U/mg, respectively. Figure 20 shows the effect of thermal inactivation at 60°C on the purified feline and human enzymes. The feline enzyme had a half-life of 68 min, whereas the human enzyme had a half-life of 30 min. Following 72 h at -55°C, 90% of the initial activity was recovered for the feline and 84% for the human enzyme. Table 6 shows the effect of various compounds on the activities of the feline and human isozymes. None of the divalent cations tested had a large inhibitory effect on either the human or feline ASB. However, 50

Figure 17. SDS gel electrophoresis of dimethyl suberimidate-treated feline ASB. Gel 1, untreated enzyme; Gel 2, treated enzyme. Arrow indicates point of application.

Figure 17

Figure 18. Effect of pH on feline (■—■) and human (●—●) ASB activity.

Figure 18

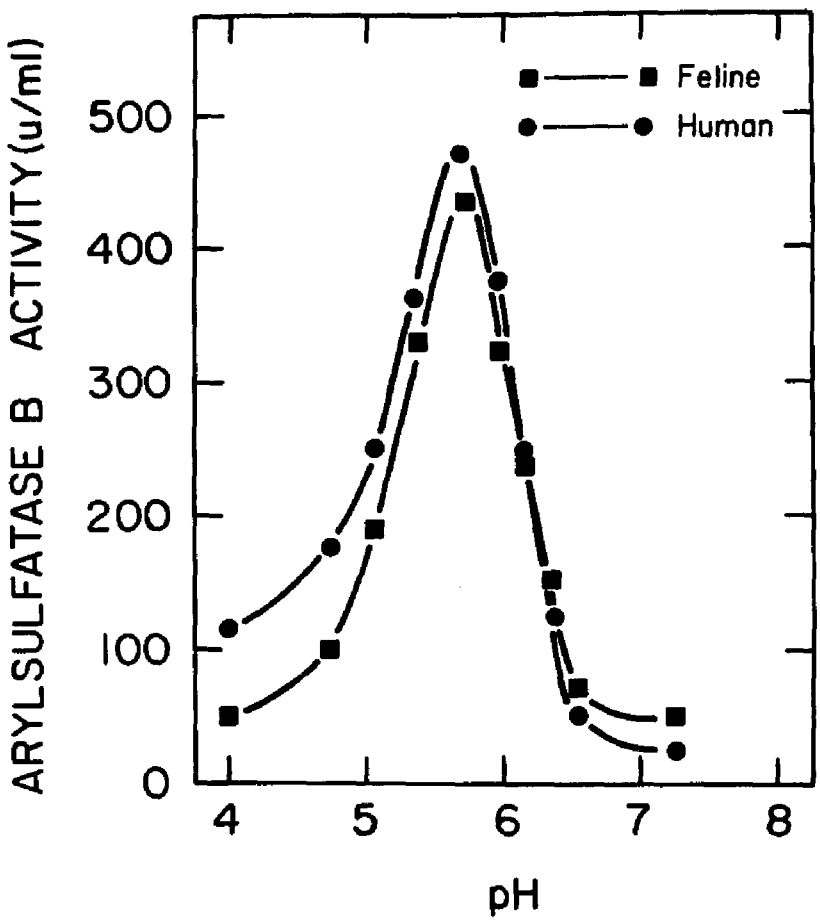

ARYLSULFATASE B ACTIVITY(u/ml)
500
400
300
200
100
0
4
5
6
7
8
pH
Feline
Human

Figure 19. A) Effect of substrate concentration on purified human and feline hepatic ASB activities. (■—■), feline ASB; (●—●), human ASB, B and C) Lineweaver-Burk plots for normal feline and human hepatic ASB isozymes.

A
B
C
Feline
Human
ARYLSULFATASE B ACTIVITY(u/ml)
pNCS (mM)
1/V (u/ml⁻¹)
1/S (mM⁻¹)
0
100
200
300
400
500
0
5
10
15
20
0.002
0.004
0.006
0.008
0.003
-0.8
0
0.8
1.6

Figure 19

Figure 20. Thermal inactivation of feline (■—■) and human (●—●) ASB at 60°C.

Figure 20

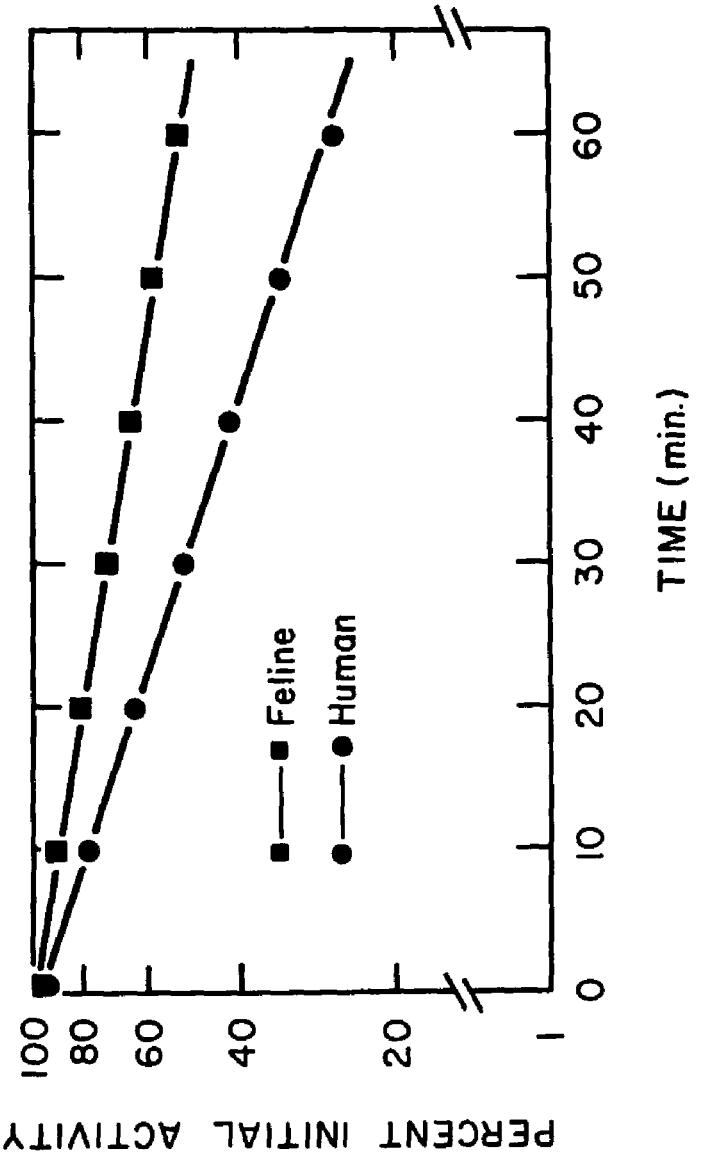

PERCENT INITIAL ACTIVITY
100
80
60
40
20
1
0
10
20
30
40
50
60
TIME (min.)
Feline
Human

TABLE 6

Effect of Bivalent Cations and Other Compounds on the Activity of Feline and Human ASB

Compound	Final Concentration	Feline Activity	Human Activity
	mM	% of Initial Activity	
$BaCl_2$	25	95	90
$CaCl_2$	10	88	90
$MgCl_2$	10	97	130
$MnCl_2$	10	112	200
Na_2SO_4	10	78	95
EDTA	10	90	170
	50	80	145
K_2PO_4	5	12	12

mM EDTA had a stimulatory effect on the human activity, whereas the feline enzyme was inhibited. In addition, both the feline and human activities were markedly inhibited by phosphate ions.

5. Amino Acid Composition of Human and Feline ASB. Table 7 summarizes the results of the amino acid analyses of homogeneous human and feline ASB. The enzymes had little methionine and similar amounts of cysteine.

6. Natural Substrate Assay. Both the human and feline purified ASB preparations displayed activity toward the natural substrate. Equal amounts of each enzyme activity, determined by the artificial substrate, pNCS, hydrolyzed similar quantities of dermatan sulfate.

D. Purification of Residual MPS VI ASB Activity.

Table 8 summarizes the typical results obtained for the purification of ASB activity from normal and MPS VI livers. As noted above, batch DEAE-cellulose chromatography with 0.1 M Tris/HCl buffer, pH 7.5, readily separated the ASB and ASA activity. Both the normal and residual MPS VI ASB activities were bound to Con A-Sepharose ($\sim$80% of applied activity) and elution resulted in 50% recovery with 140- and 60-fold purification, respectively. Subsequent column chromatography on DEAE-cellulose equilibrated with 25 mM Tris/HCl, pH 7.5, resulted in elution of the normal feline enzyme at 50 mM NaCl, whereas the MPS VI residual ASB activity did not bind to the resin and was recovered in the buffer wash. Following the gel filtration step, the final specific activities were 106,000 U/mg protein for the normal feline ASB and 2,750

TABLE 7. Amino Acid Composition of Feline and Human ASB

Amino Acid	Feline ASB	Human ASB
	Residues per Subunit Molecule	
Aspartic Acid/Asparagine	31.5	33.9
Glutamic Acid/Glutamine	34.9	47.3
Threonine	21.8	21.0
Serine	32.5	46.1
Proline	19.3	15.5
Glycine	30.6	45.7
Alanine	20.5	33.3
Valine	21.7	20.9
Methionine	3.9	3.5
Isoleucine	12.8	12.8
Leucine	26.9	25.3
Tyrosine	15.7	13.6
Phenylalanine	13.3	12.5
Histidine	13.9	22.3
Lysine	19.0	16.0
Arginine	13.2	12.9
Cysteine	4.8	6.0
Tryptophan	34.8	15.1

TABLE 8

Purification of ASB from Normal and MPS VI Feline Livers

Step	Normal Feline*			Feline MPS VI*		
	Specific Activity **U/mg	Purification fold	Yield %	Specific Activity U/mg	Purification fold	Yield %
Crude Homogenate	39.2	1	100	1.5	1	100
Preparative DEAE-Cellulose	46.0	1.2	97	3.1	2	97
Con A-Sepharose	6,380	172	46	185	123	52
DEAE-Cellulose	21,800	589	35	820	547	31
Sephadex G-100	106,000	2,840	20	2,750	1,880	16

*Based on 80 and 30 g of normal and MPS VI liver, respectively.
**U = nmoles/h.

U/mg protein for the feline MPS VI enzyme, which represented purifications of 2,840- and 1,800-fold, respectively. The final yields were 20% and 16% for the normal and MPS VI hepatic enzymes. On native polyacrylamide gel electrophoresis, these preparations each contained two major protein bands, only one of which stained for ASB. These partially purified preparations were used for characterization of the kinetic and physical properties as described below.

E. Characterization of the Residual MPS VI ASB.

1. Electrophoretic Studies. Charge differences between the normal and MPS VI ASB enzymes were revealed by both cation exchange chromatography and polyacrylamide gel electrophoresis. On CM-cellulose chromatography, both the partially purified normal and MPS VI ASB activities bound to the resin; however, the normal feline activity eluted at 25 mM NaCl, whereas the MPS VI residual activity eluted at an NaCl concentration of 14 mM (Figure 21). The normal enzyme bound to DEAE-cellulose, but the residual enzyme did not bind, contrary to expectation. The anomalous behavior of MPS VI ASB activity on anion exchange chromatography may be due to the fact that the residual enzyme's charge and/or binding properties may have been altered at pH 7.5. On polyacrylamide gel electrophoresis, the normal feline activity migrated more cathodally than the feline MPS VI enzyme (Figure 22), consistent with the more negative charge of the MPS VI activity on cation exchange chromatography. In addition, the normal and residual enzymes were shown to be charge isomers by the method of Hendrick and Smith (127) as described below.

Figure 21. CM-cellulose chromatography of normal feline (■—■) and feline MPS VI (●—●) hepatic ASB. The column was washed with 25 mM sodium acetate/acetic acid buffer, pH 5.0, and the activities were eluted with a NaCl gradient.

Figure 21

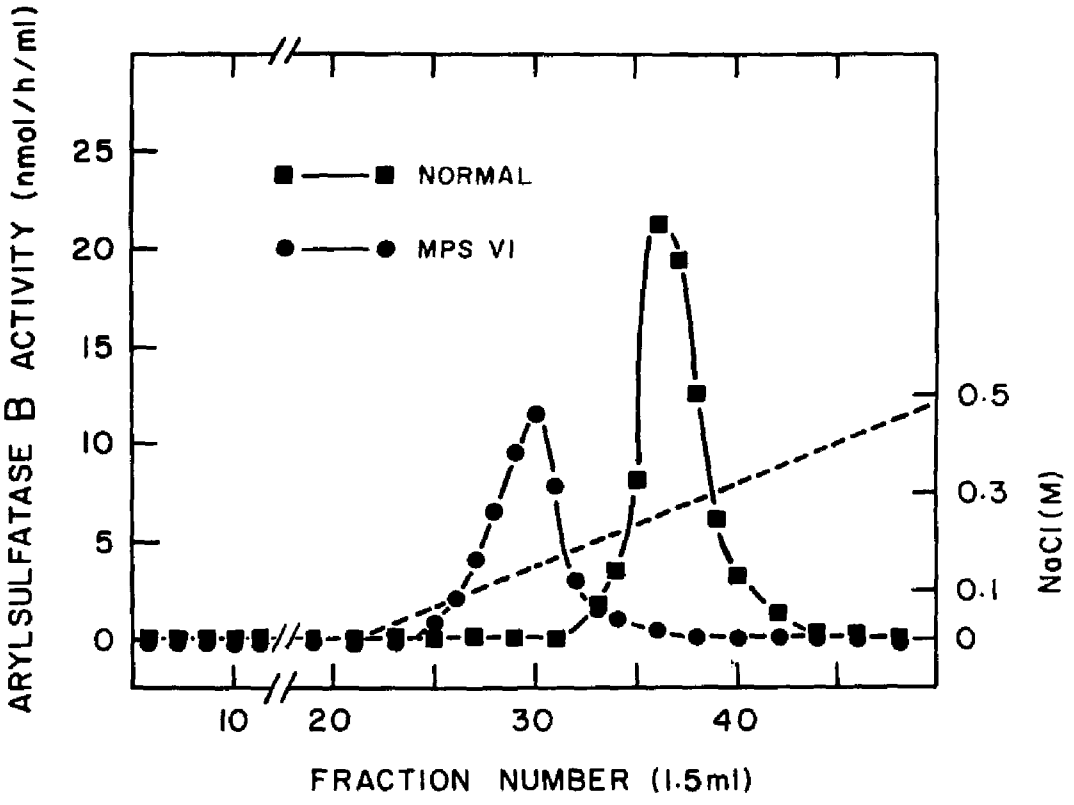
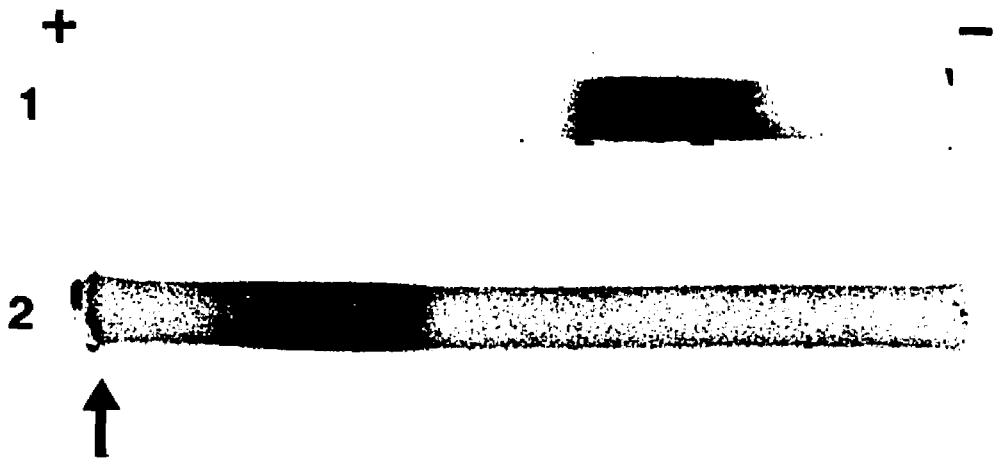

ARYLSULFATASE B ACTIVITY (nmol/h/ml)
25
20
15
10
5
0
NORMAL
MPS VI
0.5
0.3
0.1
0
NaCl (M)
10
20
30
40
FRACTION NUMBER (1.5ml)

Figure 22. Analytical polyacrylamide gel electrophoresis of partially purified (post Con A-Sepharose) normal feline and feline MPS VI hepatic ASB. Gel 1, normal feline ASB; gel 2, MPS VI ASB. The arrow indicates point of application.

Figure 22



2. Kinetic and Stability Studies. pH activity profiles were determined using pNCS as substrate. The pH optimum of both the freshly purified normal and MPS VI ASB activities was 5.7. However, when the profiles were repeated following several days of storage at 4°C, the MPS VI activity had a triphasic curve with optima at pH 5.2, 5.7 and 6.2; after further storage at 4°C, the pH profile was biphasic, with optima at pH 5.2 and 6.2. In contrast, the pH optimum of the normal hepatic enzyme was not affected by storage at 4°C.

Kinetic studies at pH 5.7 demonstrated that the purified MPS VI activity could not be saturated at final substrate concentrations up to 20 mM (the maximum solubility of the substrate), while the normal feline activity was maximal at 2.5 to 5.0 mM pNCS and was inhibited at higher substrate concentrations (Figure 23). K_m values calculated for pNCS from Lineweaver-Burk plots were 0.5 mM for the normal feline enzyme (Figure 23B) and 50 mM for the mutant enzyme (Figure 23C). Based on these studies, the final substrate concentrations used in the routine assay for the normal and MPS VI activities were 5 and 20 mM pNCS, respectively. Figure 24 shows the effect of thermal inactivation at 60°C on the partially purified normal and MPS VI ASB activities. The normal enzyme had a half-life of about 50 min, while the mutant enzyme was markedly more cryolabile than the normal feline enzyme. Following 72 h of freezing, 85% of the initial normal activity was retained, while only 42% of the MPS VI activity was recovered. The normal activity was stable when stored at pH values ranging from 3.5 to 8.5. In contrast, the mutant activity was rapidly lost when stored below pH 4.5 or above 7.5 (Figure 25). Therefore, the partially purified preparations were routinely stored at pH 6.5 at 4°C.

Figure 23. A) Effect of substrate concentration on partially purified (post Sephadex G-200) normal and MPS VI feline hepatic ASB activities; (■—■) normal, (●—●) MPS VI. B and C) Lineweaver-Burk plots for hepatic ASB from normal feline (Km = 0.5 mM) and feline MPS VI (Km = 50 mM), respectively.

Figure 23

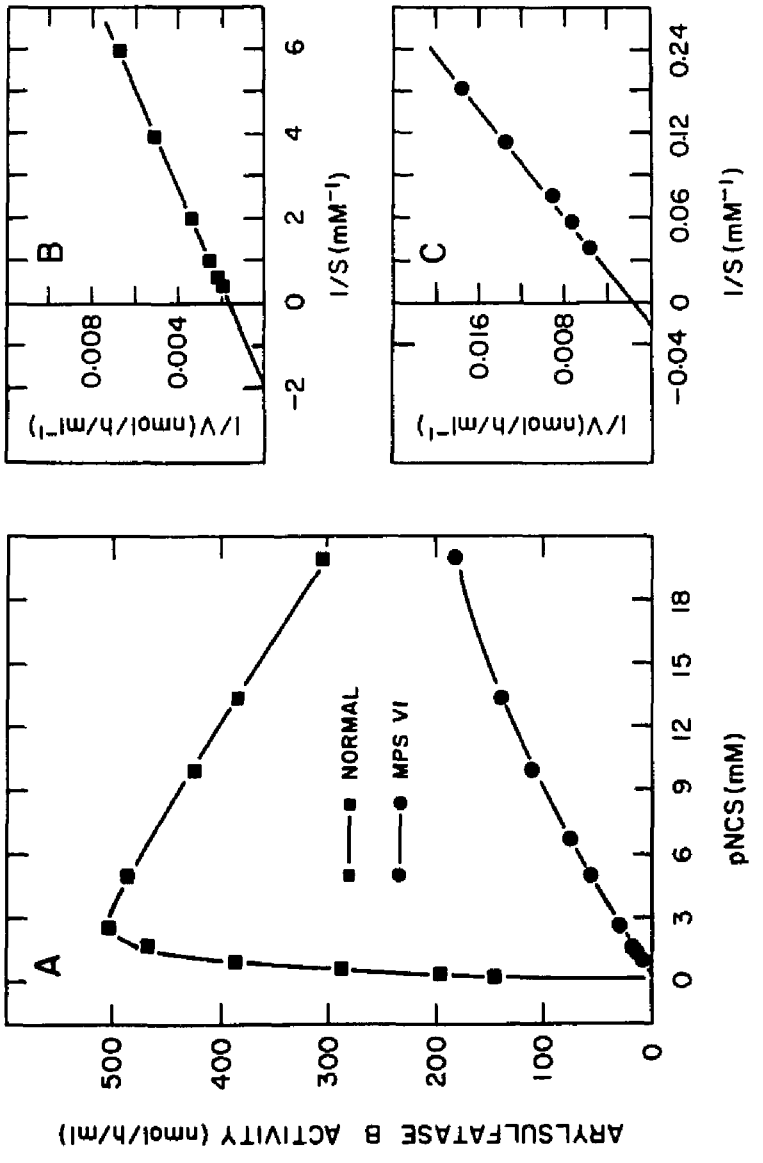

A
B
C
NORMAL
MPS VI
ARYLSULFATASE B ACTIVITY (nmol/h/ml)
pNCS (mM)
1/V (nmol/h/ml⁻¹)
1/S (mM⁻¹)
0
100
200
300
400
500
3
6
9
12
15
18
0.004
0.008
0.016
0.06
0.12
0.24
-2
-0.04

Figure 24. Thermal inactivation of partially purified (post Sephadex G-200) normal feline (■—■) and feline MPS VI (●—●) hepatic ASB at 60°C, pH 7.5.

Figure 24

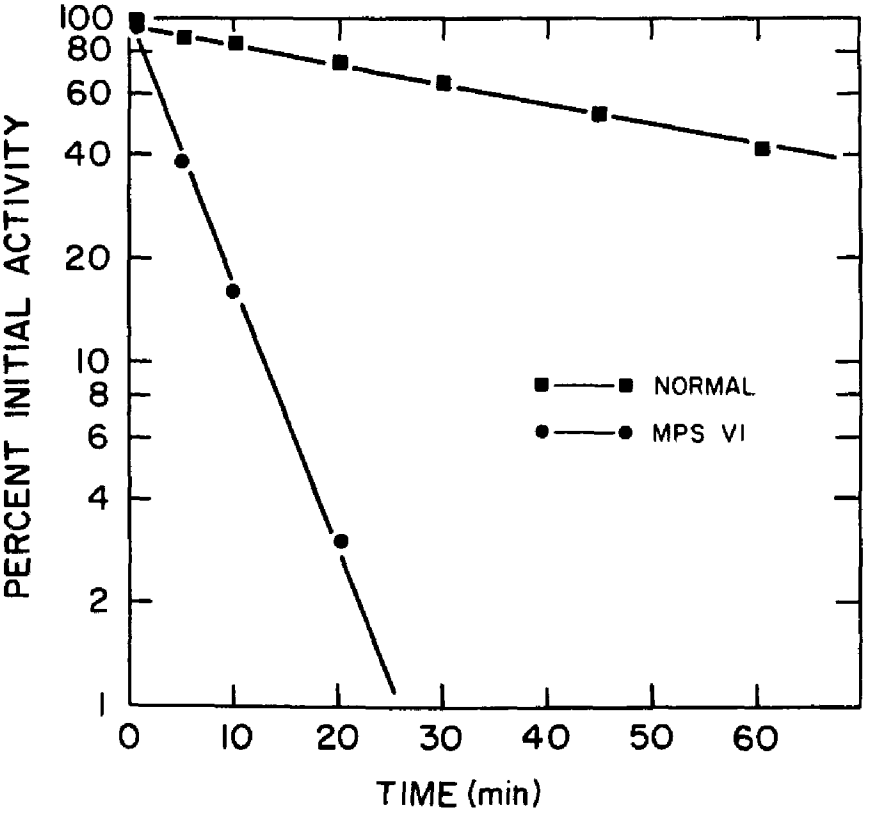
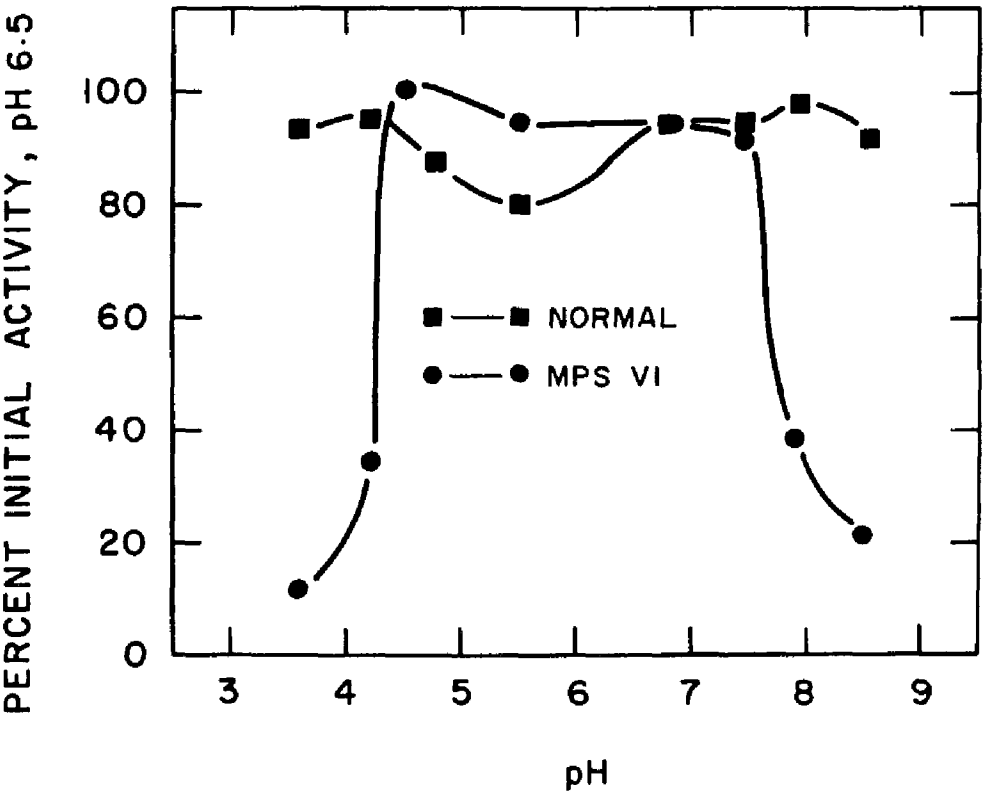

PERCENT INITIAL ACTIVITY
100
80
60
40
20
10
8
6
4
2
1
0
10
20
30
40
50
60
TIME (min)
NORMAL
MPS VI

Figure 25. Effect of pH on stability of normal feline (■—■) and feline MPS VI (●—●) hepatic ASB activities.

Figure 25


PERCENT INITIAL ACTIVITY, pH 6.5
100
80
60
40
20
0
3
4
5
6
7
8
9
pH
NORMAL
MPS VI

3. Molecular Weight Determinations. The apparent molecular weights of the normal and mutant ASB activities determined by gel filtration on Sephadex G-200 were 110,000 and 53,000, respectively (Figure 26). The native molecular weights of the two enzymes also were determined by the analytic polyacrylamide gel electrophoretic method of Hendrick and Smith (127) using 5, 6, 7 and 8% gels (Figure 27). The plots of gel concentration versus log Rm for the two enzyme preparations were not parallel and did not intersect at the ordinate, indicating that the normal and residual activities differed in molecular weight as well as charge (Figure 27A). Molecular weights estimated from the slopes of these plots were 80,000 for the residual ASB activity and 166,000 for the normal feline enzyme (Figure 27B).

F. Rocket Immunoelectrophoresis. A rocket immunoelectrophoretic system was used to quantitate immunoreactive ASB protein. As shown in Figure 28, various dilutions of leukocyte cell lysate gave proportionate decreases in rocket peak height. Similarly, decreasing activity in cell lysates resulted in proportionately decreasing rocket heights. Preliminary results demonstrated the presence of non-catalytic immunologically cross-reacting material (CRM-positive) in both human and feline MPS VI. The amount of immunoreactive protein in feline MPS VI was 11.1-fold that of normal feline lysates, assuming identical antibody activities.

G. Effect of Sulfhydryl Reagents on Highly Purified Normal and MPS VI ASB.

Table 9 summarizes the effect of varying concentrations of selected sulfhydryl-reactive compounds on the highly purified normal feline and

Figure 26. Determination of the molecular weight of normal feline and feline MPS VI hepatic ASB by gel filtration. The normal and MPS VI enzymes (post Sephadex G-200) were analyzed in separate chromatographic runs. Enzyme (2,000 U) and 2 mg of each protein standard were applied to a column of Sephadex G-200. The protein standards used were aldolase (M_r = 158,000), bovine serum albumun (68,000), ovalbumin (45,000) and chymotrypsinogen (24,000). The elution points of the normal feline and feline MPS VI ASB are indicated (X).

Figure 26

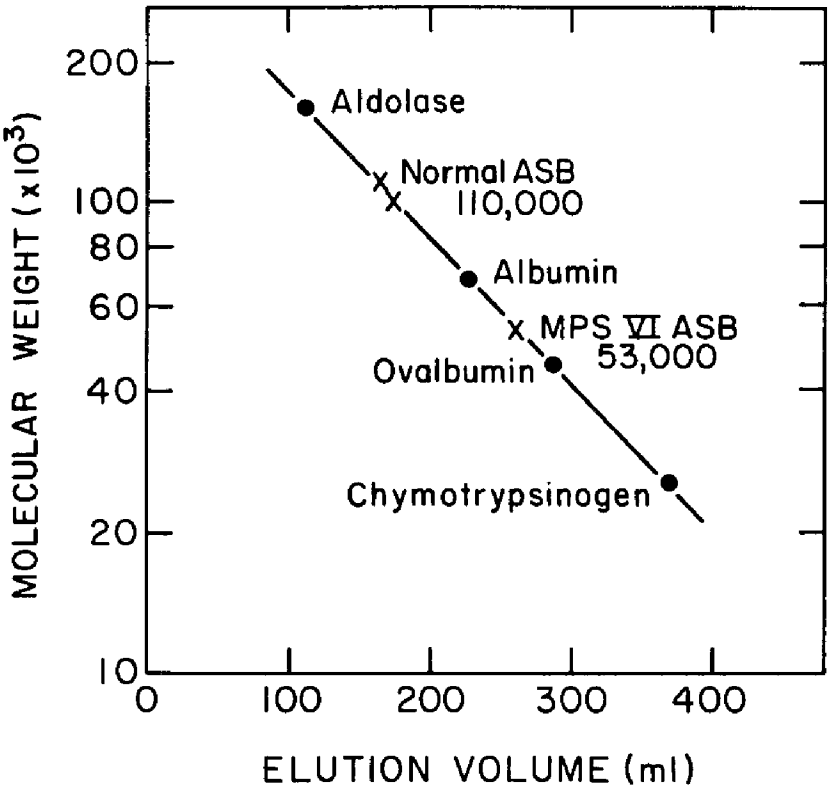

MOLECULAR WEIGHT (x10³)
200
100
80
60
40
20
10
0
100
200
300
400
ELUTION VOLUME (ml)
Aldolase
Normal ASB
110,000
Albumin
MPS VI ASB
53,000
Ovalbumin
Chymotrypsinogen

Figure 27. Native molecular weights of partially purified (post Sephadex G-200) normal feline and feline MPS VI hepatic ASB determined by polyacrylamide gel electrophoresis according to the method of Hendrick and Smith (127). A) Gel concentrations versus log Rm for normal feline (■—■) and feline MPS VI (●—●) ASB. Note that the lines do not intersect at the ordinate, indicating that the enzymes are charge isomers. B) Estimation of the molecular weights from the slopes of the normal feline (slope = 6) and feline MPS VI (slope = 11.5) ASB. The protein standards used were aldolase (M_r = 158,000), bovine serum albumin (68,000), chymotrypsinogen (24,000) and cytochrome C (12,400). Normal feline and feline MPS VI ASB are indicated (X).

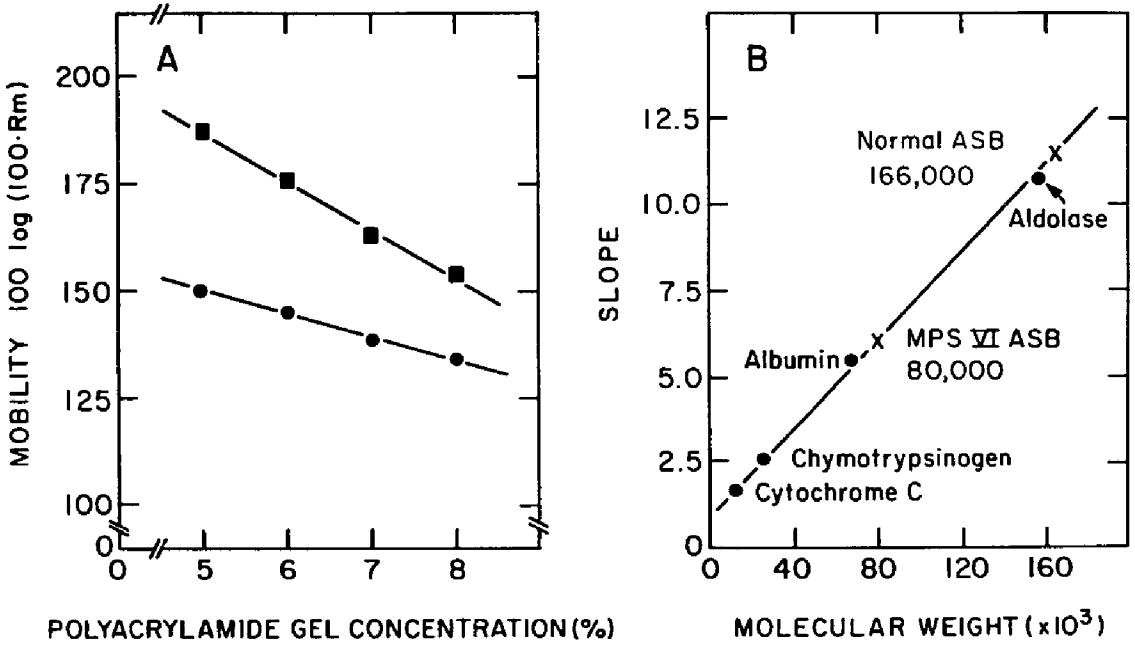
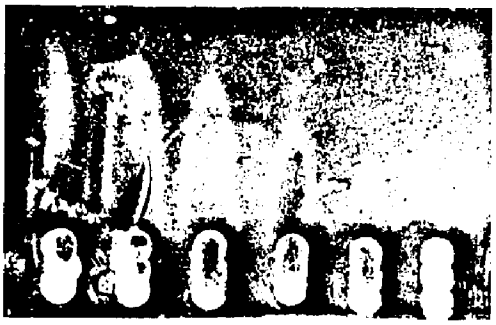

A
MOBILITY 100 log (100·Rm)
200
175
150
125
100
0
0
5
6
7
8
POLYACRYLAMIDE GEL CONCENTRATION (%)
B
SLOPE
12.5
10.0
7.5
5.0
2.5
0
Normal ASB
166,000
Aldolase
MPS VI ASB
80,000
Albumin
Chymotrypsinogen
Cytochrome C
0
40
80
120
160
MOLECULAR WEIGHT (x10³)

Figure 27

Figure 28. Rocket immunoelectrophoresis of feline ASB from leukocyte lysates. Application of 20 µl of cell lysate in dilutions of 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 (600 to 18.7 ng protein) resulted in progressive decreases in rocket height.

Figure 28



feline MPS VI residual ASB activities. The thiol-reducing compounds, DTT and cysteamine, had a marked stimulatory effect on the residual activity. For example, DTT, at concentrations ranging from 0.025 to 0.25 mM, increased the MPS VI residual hepatic activity about 4-fold; at higher DTT concentrations, less stimulation was obtained. In marked contrast, DTT had no effect or slightly inhibited the purified normal enzyme. In addition, similar concentrations of alkylating or other reducing agents consistently resulted in stimulation of the residual activity, whereas the normal enzyme was not affected or was slightly inhibited. The greatest enhancement was obtained with 0.25 mM DTT and 0.25 mM cysteamine (4- and 12-fold, respectively). The inhibitory effect of EDTA on the residual activity (Table 9) indicated that the depletion of divalent cations was not the mechanism responsible for residual enzyme stimulation.

The effect of DTT on the physical and kinetic properties of the highly purified normal feline and feline MPS VI hepatic ASB enzymes is summarized in Table 10. DTT had essentially no effect on all the properties of the normal feline enzyme studied. However, DTT altered the electrophoretic mobility, apparent V_{max} and apparent native molecular weight values of the highly purified MPS VI residual activity. Although the apparent K_m value of the residual activity was unchanged in the presence of DTT, this thiol-reducing agent increased the apparent V_{max} about 3-fold (Figure 29). Analytical polyacrylamide gel electrophoresis of the residual feline activity in the presence of 1.0 mM DTT (Figure 30) or 1.0 mM cysteamine (data not shown) resulted in the migration of the residual ASB to a position similar to that of the normal enzyme, which was not changed by the presence of DTT. DTT had no effect on the

TABLE 9

Effect of Various Thiol Reagents and EDTA on the Highly Purified Normal Feline and Feline MPS VI ASB Activities

Thiol Reagent	Concentration	% Initial Activity*	
		Normal Feline	Feline MPS VI
	(mM)		
None		100	100
DTT	0.025	102	411
	0.25	90	420
	2.5	77	154
Cysteamine	0.025	96	270
	0.25	95	1200
	2.5	81	800
Mercaptoethanol	0.25	86	197
p-Chloromercuri-benzoate	0.25	92	152
Iodoacetate	0.25	78	152
Iodoacetamide	0.25	82	147
EDTA	5	92	74
	250	87	62
	500	88	57

*Initial activities of the normal feline and feline MPS VI residual enzymes were 313.1 and 20.3 nmol/h/ml, respectively.

TABLE 10

Effect of DTT on the Properties of the Highly Purified Normal and MPS VI Residual ASB Activities

Property	Feline MPS VI	Feline MPS VI +DTT*	Normal Feline ±DTT*
Electrophoretic Mobility: (Rm on PAGE)	0.32	0.65	0.74
Cryostability: (% Initial Activity after 72 h, -50°C)	42	43	85
Thermostability: ($t_{1/2}$ at 60°C, min)	5	5	50
pH Stability Range:	4.5-7.5	4.5-7.5	3.5-8.5
K_m: (mM for pNCS)	50	50	0.5
V_{max}: (nmol/h/ml)	667	2000	715
(nmol/h/mg)	1962	5880	3967
Molecular Weight:			
Sephadex G-2000	53,000	100,000	110,000
Native PAGE	80,000	166,000	166,000

*The physical and kinetic properties were determined in the absence and presence of 0.25 mM DTT with the exception of the molecular weight studies which were performed with and without 1.0 mM DTT.

Figure 29. Effect of substrate concentration on normal and feline MPS VI hepatic ASB activities ± DTT. (A) Velocity vs pNCS concentration. (B) Lineweaver-Burk plots. V_{max} values expressed as nmol/h/ml.

Figure 29

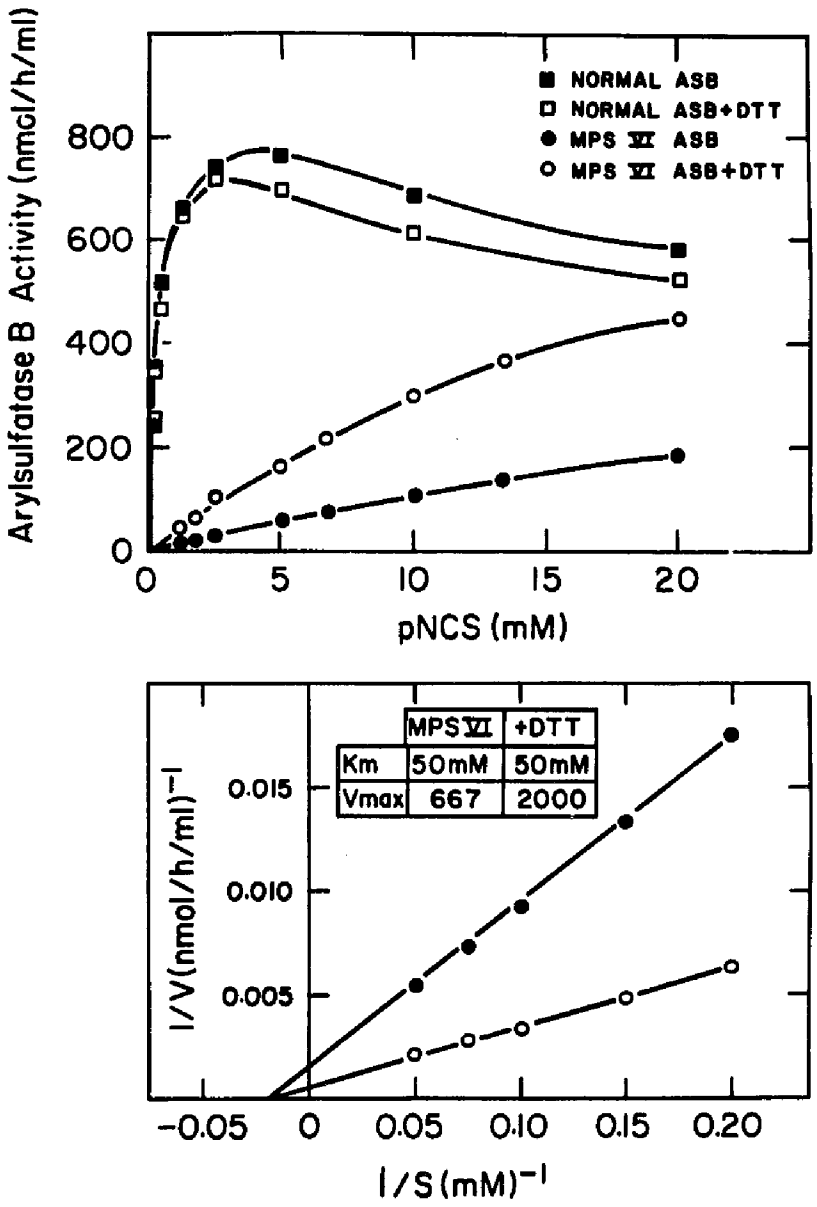

Arylsulfatase B Activity (nmol/h/ml)
800
600
400
200
0
0
5
10
15
20
pNCS (mM)
NORMAL ASB
NORMAL ASB+DTT
MPS VI ASB
MPS VI ASB+DTT
MPS VI
+DTT
Km
50mM
50mM
Vmax
667
2000
1/V(nmol/h/ml)⁻¹
0.015
0.010
0.005
-0.05
0
0.05
0.10
0.15
0.20
1/S(mM)⁻¹

Figure 30. Analytical polyacrylamide gel electrophoresis of purified normal and feline MPS VI hepatic ASB ± DTT. Gels 1 and 2, normal feline ASB in the absence and presence of 1.0 mM DTT, respectively. Gels 3 and 4, feline MPS VI ASB in the absence and presence of DTT, respectively. Samples were applied at the anode.

Figure 30

altered thermo-, cryo- or pH-stabilities of the residual MPS VI enzyme.

Most intriguingly, the apparent native molecular weight values of the residual enzyme were essentially the same as those of the normal enzyme when estimated in the presence of 1.0 mM DTT by gel filtration or by analytical polyacrylamide gel electrophoresis using increasing acrylamide concentrations according to the procedure of Hendrick and Smith (127). Analysis of the latter studies indicated that the two native enzymes had the same apparent molecular weight values (Figure 31B) and similar, but not identical, charges in the presence of DTT (Figure 31A). In the absence of DTT, the molecular weight data were consistent with the residual MPS VI enzyme having approximately half the apparent molecular weight of the normal enzyme; additionally, in the absence of the thiol-reducing agent, the untreated enzymes were charge isomers. The molecular weight of the normal enzyme was estimated at 110,000 by Sephadex G-200 chromatography in the absence of DTT, while that of the mutant was 53,000. When 1.0 mM DTT was added to the highly purified enzyme sample and to the elution buffer, the molecular weight of the residual ASB was increased to 100,000, whereas that of the normal feline enzyme remained unchanged (Figure 32). In the presence of 1.0 mM cysteamine, the residual enzyme also had an estimated molecular weight of 100,000 by gel filtration (data not shown).

H. <u>In Vitro Effect of Thiol-Reducing Agents on MPS VI Residual ASB</u>.

Tables 11 and 12 summarize the changes in leukocyte ASB activity and dermatan sulfate concentration following incubation of whole blood samples from an 18 month old affected female cat and her normal female sibling with various concentrations of DTT and cysteamine (0.1 mM to 5

Figure 31. Estimation of the native molecular weights of normal and feline MPS VI hepatic ASB ± DTT by polyacrylamide gel electrophoresis (127). (A) The effect of different gel concentrations on the mobility of normal feline and feline MPS VI ASB ± DTT. Note that the slopes of the normal enzyme ± DTT and the MPS VI enzyme + DTT are parallel indicating that they have the same molecular weights, but are charge isomers. The slope and mobility of the MPS VI enzyme - DTT is consistent with a different molecular weight and charge. (B) The indicated molecular weight estimates were determined from the slope-molecular weight relationship for the feline enzymes ± DTT compared to protein standards (127). The feline enzymes ± DTT are indicated (X).

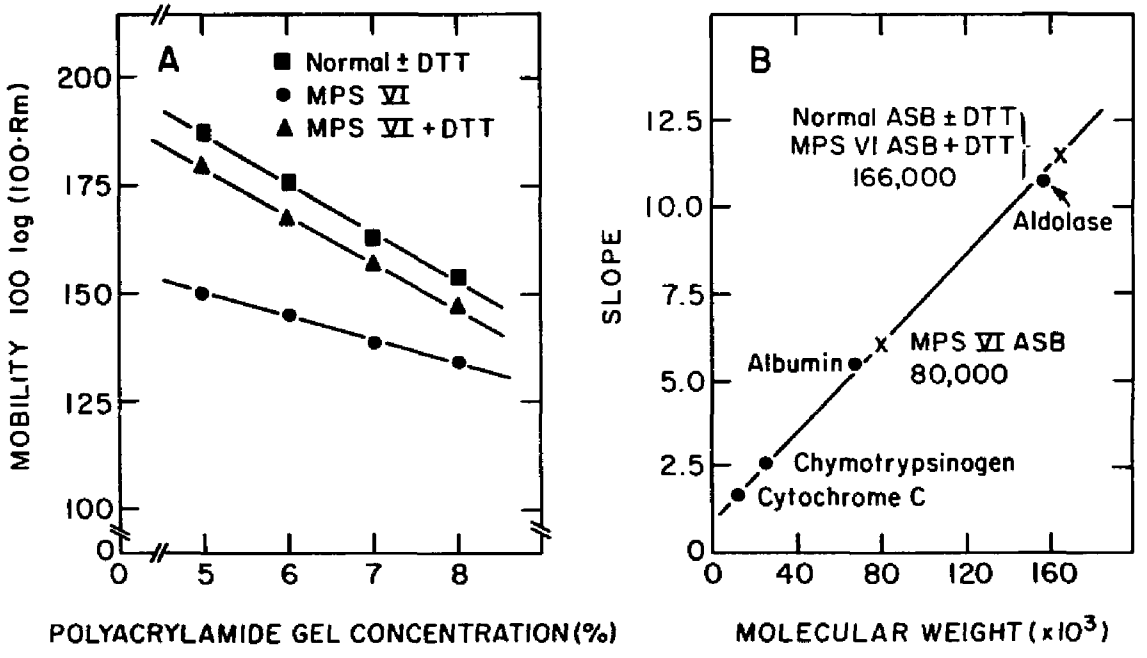
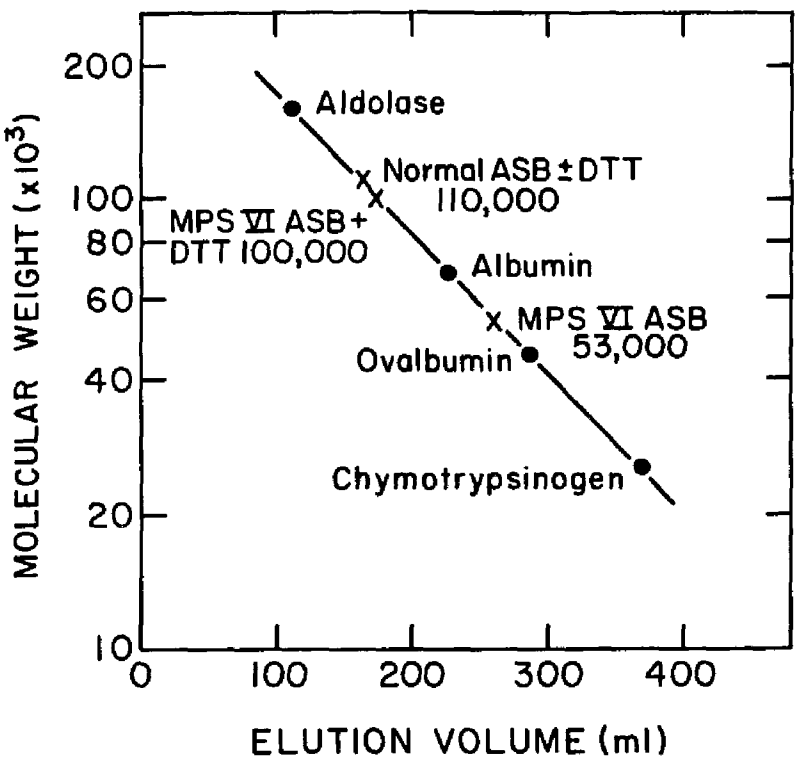

A
Normal ± DTT
MPS VI
MPS VI + DTT
MOBILITY 100 log (100·Rm)
200
175
150
125
100
0
0
5
6
7
8
POLYACRYLAMIDE GEL CONCENTRATION (%)
B
Normal ASB ± DTT
MPS VI ASB + DTT
166,000
Aldolase
MPS VI ASB
80,000
Albumin
Chymotrypsinogen
Cytochrome C
SLOPE
12.5
10.0
7.5
5.0
2.5
0
0
40
80
120
160
MOLECULAR WEIGHT (x10³)

Figure 31

Figure 32. Estimation of the native molecular weights of normal and MPS VI feline hepatic ASB ± DTT by gel filtration. Each enzyme (500 U) and protein standards (5 mg each) were applied to a Sephadex G-200 column and chromatographed in the presence and absence of 1.0 mM DTT. The elution points of the normal and MPS VI ASB are indicated (X).

Figure 32


MOLECULAR WEIGHT (x10³)
200
100
80
60
40
20
10
0
100
200
300
400
ELUTION VOLUME (ml)
Aldolase
Normal ASB ± DTT
110,000
MPS VI ASB +
DTT 100,000
Albumin
MPS VI ASB
53,000
Ovalbumin
Chymotrypsinogen

mM) for 1 h at 37°C. The MPS VI leukocyte residual activity was increased up to 11- and 20-fold with DTT and cysteamine, respectively. Maximal stimulation of leukocyte residual activity occurred when either thiol reagent concentration in blood was 2 mM. The dermatan sulfate concentration in MPS VI leukocytes was markedly decreased ($\sim$ 5% of initial) or non-detectable at all concentrations of both sulfhydryl-reducing agents. A comparable increase in residual leukocyte activity and decrease in dermatan sulfate concentration was observed when whole blood was incubated with 5.0 mM cystamine. In contrast, none of these reagents had a significant effect on the ASB activity in normal leukocytes.

I. In Vivo Effect of Thiol-Reducing Agents on MPS VI Residual ASB.

Intravenous infusions of DTT (50 mg/kg) or cysteamine (15 mg/kg) were well tolerated by the animals. Heart and respiratory rates, as well as glucose and hemoglobin levels, remained stable throughout the infusions. No vomiting or seizure activity was observed.

Table 13 compares the ASB activities and dermatan sulfate concentrations in leukocytes from the normal and MPS VI cats before and after intravenous administration of DTT. Immediately following infusion of DTT, the MPS VI residual ASB activity in leukocytes was increased almost 5-fold. A transient decrease occurred in the dermatan sulfate level at zero time, returning to pre-infusion values at 30 min.

When cysteamine was intravenously infused (Table 14), the residual activity was increased more than 4-fold immediately after infusion, increasing to almost 7-fold at 30 min, and then returning to pre-infusion levels by 60 min. Dermatan sulfate concentrations in peripheral leuko-

TABLE 11

Effect of DTT on Normal and MPS VI Feline Leukocyte ASB Activities and Dermatan Sulfate Concentrations *In Vitro*

Concentration	% Initial ASB Activity*		% Initial Dermatan Sulfate Level+	
mM	Normal	MPS VI	Normal	MPS VI
0	100	100	ND	100
0.1	100	170	ND	ND
1.0	110	510	ND	ND
2.0	110	1120	ND	ND
5.0	110	810	ND	ND

*Initial ASB specific activity were 341.9 and 49.2 nmol/h/mg protein in the normal and MPS VI leukocytes, respectively.

+Initial dermatan sulfate concentration was 14.5 μg/mg protein in MPS VI leukocytes. No dermatan sulfate was detected (ND) in normal feline leukocytes within the sensitivity of the assay (0.01 μg/mg protein).

TABLE 12

Effect of Cysteamine and Cystamine on Normal and MPS VI Feline Leukocyte ASB Activities and Dermatan Sulfate Concentrations *In Vitro*

Thiol Reagent	Concentration mM	% Initial ASB Activity* Normal	% Initial ASB Activity* MPS VI	% Initial Dermatan Sulfate Level+ Normal	% Initial Dermatan Sulfate Level+ MPS VI
None	-	100	100	ND	100
Cysteamine	0.1	100	600	ND	ND
	1.0	130	1580	ND	ND
	2.0	120	2070	ND	5
	5.0	110	1680	ND	5
Cystamine	5.0	110	1820	ND	ND

*Initial ASB specific activities were 268 and 16 nmol/h/mg protein in the normal and MPS VI leukocytes, respectively.

+Initial dermatan sulfate concentration was 105 µg/mg protein in MPS VI leukocytes. No dermatan sulfate was detectable (ND) in normal feline leukocytes.

TABLE 13

Effect of Intravenous Infusion of DTT on Normal and MPS VI Feline Leukocyte ASB Activities and Dermatan Sulfate Concentrations

Time (min)	% Preinfusion ASB Activity*		% Preinfusion Dermatan Sulfate Level+	
	Normal	MPS VI	Normal	MPS VI
pre	100	100	ND	100
0	96	490	ND	73
30	94	140	ND	92
60	102	93	ND	100
120	98	140	ND	100

*Preinfusion ASB specific activities were 758 and 14 nmol/h/mg protein in the normal and MPS VI leukocytes, respectively.

+Preinfusion dermatan sulfate concentration was 64.0 μg/mg protein in MPS VI leukocytes. No dermatan sulfate was detected (ND) in the normal feline leukocytes.

TABLE 14

Effect of Intravenous Infusion of Cysteamine on Normal and MPS VI Feline Leukocyte ASB Activities and Dermatan Sulfate Concentrations

Time (min)	% Preinfusion ASB Activity*		% Preinfusion Dermatan Sulfate Level+	
	Normal	MPS VI	Normal	MPS VI
Pre	100	100	ND	100
0	111	430	ND	34
30	98	680	ND	39
60	86	60	ND	46
120	70	80	ND	46

*Preinfusion ASB specific activities were 705 and 12 nmol/h/mg protein in the normal and MPS VI leukocytes, respectively.

+Preinfusion dermatan sulfate concentration was 147 µg/mg protein in the MPS VI leukocytes. No dermatan sulfate was detected (ND) in normal feline leukocytes.

cytes were decreased to about 35% of pre-infusion levels after infusion and remained at about 45% of initial levels for at least 120 min. In contrast, cysteamine had an inhibitory, if any, effect on normal ASB activity; dermatan sulfate in normal feline leukocytes remained undetectable.

VI. DISCUSSION

It is likely that the development of effective therapy to ameliorate the metabolic pathology in inborn errors of metabolism will be facilitated by animal model systems. In addition to removing the restraints which exist in human experimentation, animal models of diseases which have residual enzyme activity, like MPS VI, allow the purification of the residual activity and comparison of its properties with those of the normal enzyme. Such studies may provide insight into the molecular nature of the enzymatic defect and, based on these results, various therapeutic strategies can be developed for evaluation in the animal model system.

In order to conduct these studies using the feline analogue of MPS VI, it was necessary to develop a reliable method of heterozygote detection. The ability to accurately identify carriers would allow appropriate selection of animal mates for the efficient establishment of a breeding colony. In addition, the reliable detection of carriers for human MPS VI would permit improved risk assessment for the genetic counseling of unaffected members of MPS VI families.

The demonstration of deficient ASB activity in isolated leukocytes (21,24,137,138), plasma (139), cultured skin fibroblasts (21,138,140) or cultured lymphoblasts (21) has proven diagnostic for homozygotes affected with human or feline MPS VI. However, the detection of obligate heterozygotes for human MPS VI has been difficult (21,24,138,139). Determination of ASB in the presence of ASA activity using the differential inhibitor assay of Baum (141) has limited specificity due to the interaction of both arylsulfatases with the artificial substrates (138, 142). Therefore, several investigators have used ion exchange chromato-

graphy to separate ASA and ASB activities prior to assay (137,138). Even then, the varying amounts of ASB activity in the cell lysates have apparently complicated accurate identification of obligate heterozygotes (138 and Figures 7A and 8A).

Recognizing these difficulties, studies were undertaken to develop a rapid and reliable method for heterozygote detection by first separating the ASB and ASA activities from peripheral leukocytes and then expressing the ASB and ASA activities as a ratio. This method has proven reliable for the discrimination of all obligate heterozygotes studied for feline MPS VI using isolated peripheral leukocytes (Figure 7B). In addition, obligate heterozygotes for the human disease were readily detected. This represented the largest series of obligate heterozygotes studied to date for either the human or feline disorder. Although the range of ASB/ASA ratios in obligate heterozygotes and homozygotes were close, homozygote diagnosis can be substantiated by clinical examination (e.g., corneal opacities, dysostosis multiplex) in both the human and feline disease. Thus, efficient breeding of cats in the MPS VI colony was possible and provided a continual source of experimental animals.

Since one objective of these studies was to compare the residual ASB activities in human and feline MPS VI, it was necessary to characterize the properties of the respective normal enzymes. Therefore, feline and human hepatic ASB were purified to apparent homogeneity with final specific activities of 1,100,000 and 800,000 nmoles/h/mg protein, respectively. Previously, several investigators, using conventional chromatographic techniques, have purified human hepatic ASB (103-106). However, the specific activities of these preparations have varied greatly (7,200 to 5,600,000 nmoles/h/mg protein). Thus, when purifying

human and feline ASB by the procedure described here, particular attention has been taken with regard to the reliability and reproducibility of each step. Several subsequent purifications of either human or feline ASB have consistently resulted in similar specific activity and fold purification values (Table 15). Therefore, characterization studies using these homogeneous enzymes were performed. Although both isozymes had the same pH optimum (5.7), the purified feline enzyme had a lower K_m toward pNCS (1.2 versus 3.6 mM), a slightly lower isoelectric point (pI 7.8 versus 8.0) and was more thermostable at 60°C ($T_{1/2}$ 68 min versus 30 min) than the human enzyme. In addition, electrophoretic studies in cellulose acetate and polyacrylamide gels demonstrated that the feline enzyme was less electropositive than the human enzyme. Amino acid composition analyses revealed few significant differences between the two enzymes.

Several experimental findings suggested that the feline enzyme was a homodimer, whereas the human enzyme was a monomer. These included: 1) the molecular weight of the purified feline enzyme was about twice that determined for the human enzyme by gel filtration (100,000 versus 48,000), analytical polyacrylamide gel electrophoresis (166,000 versus 80,000), and sucrose-density gradient centrifugation (105,000 versus 55,000). The differences in the apparent molecular weights obtained for each enzyme may have been due to the known anomalous behavior of glycoproteins in molecular weight studies (143); 2) both isozymes were found to have similar subunit molecular weights on SDS polyacrylamide gel electrophoresis; 3) alkylation experiments with the feline enzyme resulted in a single hybrid form consistent with a homodimeric structure; and 4) cross linking studies using the feline enzyme resulted in two

TABLE 15

Specific Activity and Fold Purification Values for Several Preparations of Feline and Human Hepatic ASB

Feline		Human	
Specific Activity	Fold Purification	Specific Activity	Fold Purification
(nmoles/h/mg prot.)		(nmoles/h/mg prot.)	
800,000	9100	1,100,000	8400
710,000	8900	900,000	7600
740,000	9060	960,000*	7800*
840,000	8100		

*From human lung.

bands on polyacrylamide gels representing the cross linked dimer and the unlinked monomer. The subunit structure of ASB had not been previously reported in any other species. Thus, this finding may represent the first evidence suggesting that this enzyme evolved by reducing its molecular weight while retaining its catalytic function.

Most attempts to study the molecular pathology of inborn errors of metabolism have focused on the characterization of the primary enzymatic defect. The presence of residual enzymatic activity permits the purification of the defective enzyme and comparison of its physical and kinetic properties to those of the normal enzyme. Among the human lysosomal storage diseases, several have defective enzymes with residual activity including Gaucher Type 1 disease (144), mannosidosis (52), and MPS VI (13,19-21). However, purification and characterization of the residual activity usually has been precluded by the unavailability of adequate fresh, human material. Thus, animal analogues of human enzyme deficiency disorders provide the opportunity to characterize the defective enzyme and gain insight into the molecular pathology of the enzymatic lesion.

Therefore, the residual ASB activity in feline MPS VI was purified, and comparison of its properties was made to those of the normal feline enzyme. The MPS VI enzyme differed from its normal counterpart in electrophoretic mobility, kinetic properties, stability and molecular weight. Compared to the partially purified normal hepatic enzyme, the MPS VI ASB activity had at least a 100-fold greater K_m value and was markedly more thermo-, cryo-, and pH-labile. In addition, the molecular weight of the native MPS VI residual activity was approximately half that of the native normal feline enzyme as determined by both gel fil-

tration and polyacrylamide gel electrophoresis. These results and the demonstration that the normal feline enzyme was a homodimer suggest that the mutation in the structural gene for feline ASB altered the gene product such that it was unable to maintain its normal dimeric subunit conformation. Although the defective enzyme retained partial activity, the inability for subunit association (e.g., dimerization) presumably rendered the MPS VI protein more defective catalytically and markedly unstable.

It is notable that human MPS VI also has been shown to have about 4-15% of normal ASB activity in liver (13), cultured fibroblasts (19, 20), and leukocytes (21). In contrast to the defective ASB activity in the feline disease, the human hepatic residual activity was similar to the normal human enzyme in pH optimum, apparent K_m, electrophoretic mobility, and thermostability at 60°C (19,20). Immunologic studies of the human MPS VI enzyme demonstrated that the ratio of immunoreactive protein to residual activity was 6.7 (20). Thus, it can be concluded that the structural gene mutations which cause the defective ASB activities in human and feline MPS VI differ in their molecular nature.

Since the residual enzyme was more thermo-, cryo- and pH-labile, particularly when highly purified, efforts were made to stabilize the enzyme. When the thiol-reducing reagent, DTT, was added to the highly purified residual enzyme, its activity was increased several fold (Table 9). This observation stimulated the evaluation of other sulfhydryl-reactive agents. Cysteamine, a strong reducing agent which also is lysosomotropic (145), markedly increased the residual activity, whereas the alkylating and mercurial reagents were stimulatory, but to a lesser degree. Since these reagents had no effect or slightly inhibited

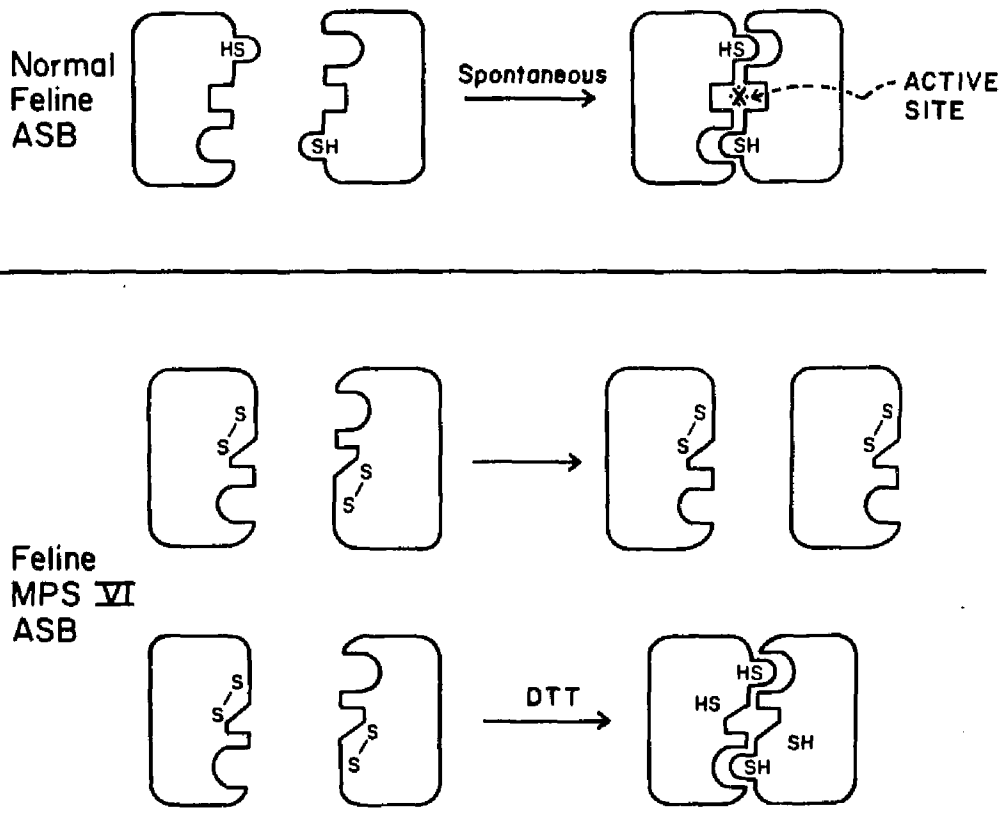
the normal ASB activity, an investigation was undertaken to determine the effect of DTT, the prototype reducing agent, on the physical and kinetic properties of the purified normal feline and feline MPS VI residual activities.

Intriguingly, in the presence of DTT, the residual activity had an increased V_{max} value and an electrophoretic mobility and native molecular weight similar to those of the normal enzyme (Figures 29 and 30). In the presence of cysteamine, the native molecular weight of the residual activity also was similar to that of the normal enzyme. Previous studies of the purified native and denatured enzymes indicated that the MPS VI residual activity was a monomer, whereas the normal feline enzyme was a homodimer. Thus, these findings suggested that DTT and cysteamine facilitated the dimerization of the residual enzyme. However, DTT did not alter the apparent K_m or stability of the dimerized residual activity.

The following model is proposed to explain the effect of the thiol-reducing agents on the MPS VI residual enzyme (Figure 33). In the normal feline enzyme, spontaneous dimerization of the isologous subunits occurs to form the active homodimer. In feline MPS VI, a point mutation in the structure gene may result in the substitution of a cysteinyl residue at or near the substrate binding site - or a conformational change in the protein occurs due to a different amino acid substitution which alters substrate binding and, in addition, exposes a normally unavailable cysteine. This substituted or exposed cysteine forms an intramolecular disulfide bridge with another cysteinyl residue. The formation of this disulfide bridge causes a further conformational change which prevents subunit dimerization. In the presence of the

Figure 33. Model of the molecular defect in feline MPS VI. Upper panel, the normal isologous subunits spontaneously dimerize to form the active homodimer; center panel, due to a structural gene mutation, a substituted cysteine or previously unavailable cysteinyl residue at or near the substrate binding site forms a disulfide bridge with another cysteine causing a conformational change which prevents subunit dimerization; lower panel, in the presence of DTT or cysteamine, the disulfide bond is reduced allowing subunit association to occur, with a concomitant enhancement of residual ASB activity.

Figure 33


Normal
Feline
ASB
HS
SH
Spontaneous
HS
SH
ACTIVE
SITE
Feline
MPS VI
ASB
S
S
S
S
DTT
HS
HS
SH
SH

thiol-reducing reagents, DTT and cysteamine, the disulfide bond is reduced, permitting subunit association as well as normalizing several other properties of the MPS VI enzyme (Table 10).

Since DTT and cysteamine have been safely administered as experimental therapeutic agents in patients with cystinosis (145-151), the therapeutic use of these compounds was evaluated in the feline model. Initially, *in vitro* studies were undertaken to determine if thiol-induced dimerization could enhance the MPS VI residual ASB activity in leukocytes and catabolize the accumulated substrate. Following incubation of fresh heparinized whole blood with DTT or cysteamine, the leukocyte residual ASB activity was increased up to 11- and 20-fold, respectivey, and most importantly, the accumulated dermatan sulfate was degraded (Tables 11 and 12). Based on these encouraging *in vitro* results, *in vivo* trials were conducted. Intravenously administered DTT resulted in an immediate, but transient, increase in leukocyte residual ASB activity and had little, if any, effect on the leukocyte dermatan sulfate levels (Table 13). In contrast, cysteamine infusion not only enhanced the residual leukocyte activity for at least 1 h, but also resulted in the clearance of leukocyte dermatan sulfate; the accumulated substrate was reduced to 35% of the pre-infusion level immediately after administration and remained at about 45% of the pre-infusion level for the 120 min period studied. The differential effectiveness of these thiol-reducing reagents may have been due to the rapid inactivation (i.e., oxidation, plasma clearance, etc.) of DTT, whereas cysteamine, an aminothiol, may have been protected by its preferential uptake by lysosomes (145). The effectiveness of cysteamine and the fact that cystamine (the disulfide of cysteamine) enhanced the residual leukocyte

activity *in vitro* (Table 12) suggests that the disulfide may be of therapeutic value since it is reduced to cysteamine presumably by glutathione or other reducing agents (145).

In summary, these studies provide the prototype for the treatment of inborn errors by enhancement of the residual activity via subunit reassociation. This approach may be useful in the design of therapeutic endeavors in human diseases in which the enzymatic defect results from mutations which alter subunit association and enzyme function. Furthermore, these findings emphasize the value of characterizing the nature of the enzymatic defect, particularly in disorders with residual enzymatic activity, for the design of novel strategies to manipulate and therapeutically enhance the function and/or stability of the defective enzyme.

VII. CONCLUDING REMARKS

1. Accomplishments:

The development of a reliable assay for heterozygote detection for MPS VI has permitted: 1) the efficient establishment of a breeding colony for feline MPS VI which provided a continual source of affected animals for these studies; 2) the accurate measurement of ASB in cell lysates during *in vitro* and *in vivo* experiments designed to evaluate therapeutic strategies, and 3) risk assessment for families of human MPS VI patients. Purification and characterization of normal human, normal feline and feline MPS VI ASB has provided an understanding of the molecular defect in feline MPS VI. These studies suggested that the mutation in the structural gene for feline ASB altered the gene product such that it was unable to maintain its normal dimeric conformation. Subsequent studies indicated that thiol-reducing agents, particularly DTT and cysteamine, were capable of restoring the enzyme's dimeric conformation and enhancing its activity. More importantly, *in vitro* and *in vivo* experiments have shown that these compounds enhance the residual ASB activity up to 20-fold, resulting in a decrease in the levels of dermatan sulfate, presumably due to increased catabolism. Finally, a rocket immunoelectrophoretic system was developed which readily detected CRM in both human and feline MPS VI.

2. Future Studies:

The heterozygote detection assay has proven useful in the monitoring of ASB levels in cell lysates during therapeutic trials. Thus, this assay may provide a means to evaluate the ASB levels in human patients as therapy becomes available. The encouraging *in vivo* results detailed

here provide the rationale for long-term treatment of an affected cat with cysteamine, or its disulfide, cystamine. It is anticipated that this treatment would result in the amelioration of the clinical course of the disease. Furthermore, these studies demonstrate the need to carefully characterize the molecular defect in those enzyme deficiency diseases with residual activity in order to develop novel therapeutic strategies. Lastly, the rocket immunoelectrophoretic system provides a method to study mutants from various feline and human families in order to assess genetic heterogeneity in this disease.

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