STUDIES ON BOVINE MANNOSIDOSIS

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ABSTRACT

Bovine mannosidosis is an inborn lysosomal disease of Angus cattle, associated with a deficiency of acidic α-mannosidase and a consequent disorder in the lysosomal catabolism of glycoproteins. Affected calves characteristically show signs of neurological derangement and usually die within their first year of life. The enzymic defect causes abnormal storage of water-soluble oligosaccharide units within membrane-bound vacuoles in various cell-types. Vacuolation is particularly severe in neurones of the central nervous system, reticuloendothelial cells of lymph nodes and liver, and exocrine epithelial cells of the pancreas, salivary glands and lacrimal glands. Certain cell-types of mesenchymal origin, including fibroblasts, mesangial cells, smooth muscle fibres, pericytes and capillary endothelial cells are also affected. There is evidence that the process of crinophagy may be important in contributing glycoprotein substrates to the lysosomal system, at least in the exocrine pancreas, while in some tissues vacuoles appear to develop as dilatations of Golgi apparatus or smooth endoplasmic reticulum. The more usual picture however was that of storage within vacuoles consistent with the structure of enlarged secondary lysosomes.

Mannosidosis is inherited as a simple autosomal recessive disease and whereas acidic α-mannosidase activity is almost completely absent from the tissues and body fluids of affected homozygotes, heterozygous individuals possess a partial deficiency of the enzyme. This gene dosage relationship forms the basis of methods for differentiating heterozygotes from normal animals. A test based on plasma α-mannosidase activity has been evaluated on over 5,000 Angus cattle, and the prevalence of heterozygotes was found to be approximately 10%. Although plasma α-mannosidase activity has been shown to vary between cattle of different age and sex, and significant seasonal, between-herd and even between-mob

differences were demonstrated, the test is suitable for routine use in the control of mannosidosis on a herd basis. A more sophisticated test for mannosidosis heterozygotes, based on α-mannosidase activity in lymphocyte extracts has been developed and evaluated, and is a useful adjunct to the plasma test. Preliminary investigation of a further test, based on enzyme activity in granulocyte extracts, has produced encouraging results.

Included in this thesis is an evaluation of an "experiment of nature" in enzyme replacement therapy, in which a chimeric mannosidosis calf had been endowed with a transplant of lymphocytes from a normal co-twin, due to fused placental blood circulations. This lymphocyte transplant reduced the severity of lesions in the liver, lymph nodes, pancreas and lacrimal glands of the chimeric calf but the vacuolation of neurones in its brain was of the same order of severity as that seen in positive control calves with mannosidosis, and the clinical course of the disease had not been significantly altered. It is concluded that enzyme replacement therapy by infusion of leucocyte suspensions is likely to be most effective in inborn lysosomal diseases with minimal neurological involvement.

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During the pilot testing scheme, assistance in the collection of samples from the field was provided by my wife, Joanne. Automated assays for plasma α-mannosidase activity were performed by Mrs. C. A. Tse and latterly by Mr. C. G. Rammell of the Wallaceville Animal Health Reference Laboratory. Dr. Jolly was ultimately responsible for the designation of genotype of animals tested.

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INTRODUCTION

Mannosidosis of Angus cattle is an inherited lethal disease characterized clinically by progressive neurological deterioration of affected calves, with failure to thrive and death usually within the first year of life. Earlier work in this laboratory has shown that the disease is caused by an almost complete deficiency of the lysosomal enzyme acidic α-mannosidase, and that it fulfills the criteria required for classification as an inborn lysosomal disease. Although only a decade has elapsed since the concept of inborn lysosomal disease was first formulated, by Hers, at least thirty specific human disorders have been classified as such and several analogous or similar diseases have also been described in domestic animals. These have been reviewed in Chapter I which also includes discussion on methods of diagnosis and heterozygote detection.

Preliminary investigations by Jolly et al. (1973) revealed that the mannosidosis genotype was common in Angus cattle, especially in New Zealand, and suggested that a national control programme would be both feasible and desirable. As a consequence, a pilot testing scheme was initiated during January of 1973. An analysis of the results of this scheme is presented in Chapter IV of this thesis.

Due to the high gene frequency for mannosidosis in New Zealand, and the ready availability of affected calves, this disease is the most valuable animal model currently available for research into similar disorders of man. Large numbers of heterozygous animals are available for evaluating various methods of heterozygote detection, and experiments in enzyme replacement therapy which would be unacceptable in human patients may be attempted on diseased calves. A rare opportunity for investigating one aspect of enzyme replacement therapy was provided

during the course of this study by an "experiment of nature", in which a chimeric calf with mannosidosis had received a natural transplant of lymphocytes from a normal co-twin.

The principal objectives of this investigation were (i) to extend the pathological description of bovine mannosidosis; (ii) to analyse the results of data collected during the pilot heterozygote testing scheme; and (iii) to develop an accurate supplementary test for mannosidosis heterozygotes to support the routine plasma test.

CHAPTER I

REVIEW - INBORN LYSOSOMAL DISEASES

I. THE CONCEPT OF INBORN LYSOSOMAL DISEASES

Lysosomes may be defined as cytoplasmic particles consisting of a single lipoprotein membrane enclosing a variety of hydrolases, the majority of which are most active at an acid pH (Novikoff, 1973). They are represented in most cell-types throughout the animal kingdom but show considerable diversity of form and function. Lysosomes form part of an intracellular digestive system capable of catabolizing both exogenous and endogenous macromolecular material, which may enter the vacuolar system by way of endocytosis, autophagy (Hers, 1965; de Duve and Wattiaux, 1966) or crinophagy (de Duve, 1969; Hers, 1973). As such they are involved in the degradation of proteins, mucopolysaccharides, glycoproteins, glycolipids and nucleic acids (Touster, 1973; Vaes, 1973).

Due to the diversity of lysosomal form and function the terminology concerning these organelles has been complicated and at times confusing. A simplified differentiation into primary lysosomes, whose enzymes have yet to partake in digestive activity, and secondary lysosomes in which digestion is proceeding or has occurred, is now preferred (de Duve and Wattiaux, 1966; Novikoff, 1973). Secondary lysosomes are formed when primary lysosomes fuse with endocytic or autophagic vacuoles, or with secretary granules in the process of crinophagy (de Duve, 1969; Farquhar, 1969; Hers, 1973). Secondary lysosomes containing residual undigested particles are referred to as residual bodies (Novikoff, 1973).

Lysosomes are well equipped for their catabolic role by possessing more than 40 hydrolytic enzymes, capable of degrading a wide variety of

macromolecular organic material (Barrett, 1969; Tappel, 1969; Novikoff, 1973). The genetically determined deficiency of just ne of these enzymes, or of a structural lysosomal protein, may generate a blockage at a certain stage of lysosomal digestion, the consequences of which were first predicted by Hers (1965). Hers' concept of inborn lysosomal disease may be summarized as follows:

(i) abnormal storage of undigested material in membrane-bound vacuoles derived from the lysosomal system;

(ii) the stored material need not be homogeneous as a hydrolase may possess a broad substrate-specificity;

(iii) manifestations of the disease may show considerable variation between different cell-types although all cells are presumably affected;

(iv) these diseases are progressive in nature;

(v) "correlations between the basic lysosomal alteration and the clinical manifestations of the disease may be difficult to establish";

(vi) theoretically these diseases should be responsive to enzyme replacement therapy.

After a decade in which our understanding of the biology of inborn lysosomal storage diseases has advanced considerably, these predictions of Hers remain essentially valid, although success with enzyme replacement therapy has yet to be accomplished.

Most mammalian cells, with the possible exception of hepatocytes and renal tubular cells, are unable to eliminate the contents of secondary lysosomes or residual bodies by exocytosis (Hers, 1965; de Duve and Wattiaux, 1966; Hers, 1973; Novikoff, 1973). However, some molecules with molecular weights of around 200 Daltons or less, such as

monosaccharides or small peptides, are able to freely traverse lysosomal membranes (Lloyd, 1973). In patients with mucopolysaccharide storage diseases the incomplete degradation of dermatan sulphate and heparan sulphate results in fragments, some of which are sufficiently small to escape from cells and are excreted in urine (Neufeld et al., 1975). Larger fragments, which are unable to pass through the lysosomal membrane, are retained, resulting in progressive intralysosomal accumulation of substrates which would normally be catabolized by the deficient enzyme.

Not all inborn lysosomal diseases are associated with a complete deficiency of a lysosomal hydrolase. In fact the residual enzymic activity may be as high as 40% of normal. In some cases the enzymic deficiency may not be detected by the use of artificial substrates due to the presence of isoenzymes with similar substrate specificities, but which cannot compensate for the deficient enzyme in the catabolism of natural substrates (Hers, 1973).

Although the enzymic deficiency would be expected to extend to all cell-types the manifestations of the disease in different tissues may be influenced by such factors as: (i) the availability of substrate to the lysosomes; (ii) the possibilities of cellular excretion; and (iii) the rate of turnover of the particular type of cell (Hers and Van Hoof, 1969).

A defect in the catabolism of glycosphingolipids such as gangliosides, which are found predominantly within the brain (Ledeen and Yu, 1973), will lead to the accumulation of these compounds in cells of the central nervous system. When the defect involves the catabolism of soluble circulating materials, or glycolipids of red-cell stroma, e.g. globoside, the intra-lysosomal storage is most severe in cells of the reticuloendothelial system due to their active endocytic properties, and to the ready availability of the substrates.

Cells with a short life-span such as intestinal epithelial cells are unlikely to have sufficient time to accumulate significant quantities of storage material, while longer living cells such as neurones are likely to become severely affected if sufficient substrate is available.

The effect of the congestive enlargement of lysosomes on the physiology and viability of affected cells is open to speculation. Disruption of cellular organization, compressive damage to other cellular components especially where there is little scope for expansion, as in the brain, and the rupture of enlarged lysosomes into the cytoplasm resulting in autolysis are possible sequelae (de Duve and Wattiaux, 1966; Hers and Van Hoof, 1969). Death eventually occurs when the cumulative damage to organ systems is no longer compatible with the performance of essential body functions.

II. THE GENETICS OF INBORN LYSOSOMAL DISEASES

Inborn errors of metabolism are usually associated with simple anomalies of structural or enzymic proteins, which may be caused by either structural gene or controller gene mutations (Aebi, 1967). Controller gene mutations cause an alteration in the rate of synthesis of a protein (e.g. an enzyme) without affecting its structure, whereas structural gene mutations produce a qualitative rather than quantitative effect by causing the synthesis of an abnormal or defective protein (Aebi, 1967). An alteration in the observed level of enzyme activity may also arise through a mutation causing the synthesis of an enzyme with normal catalytic activity but with reduced stability, or indirectly by altering the structure or rate of synthesis of some activator or inhibitor of the enzyme (Harris, 1971). The majority of inborn metabolic errors are

thought to result from mutations to structural genes (Aebi, 1967; Dreyfus, 1969).

With the exception of Fabry's disease and Hunter's syndrome, which show X-linked recessive inheritance, the inborn lysosomal diseases are characterized by an autosomal recessive method of inheritance (Kaback and Howell, 1973). Clinical expression of the disease therefore only occurs in individuals homozygous for the mutant gene. Heterozygous carriers possess both the mutant gene and its normal allele, and while they usually appear phenotypically normal their tissues generally possess intermediate activities of the specific enzyme, between those of the affected and normal homozygotes (Aebi, 1967). A simple gene dosage relationship often applies, with a single normal allele in heterozygotes leading to the formation of approximately 50% of the enzyme formed by normal individuals possessing two normal alleles (Harris, 1971). The gene dosage phenomenon is particularly important as the basis of heterozygote detection methods for many inborn lysosomal diseases.

In spite of a partial deficiency of the specific lysosomal enzyme in heterozygotes, clinical signs of disease are seldom exhibited, suggesting that enzymes are present in excess of functional requirements in normal individuals (Harris, 1971; Rosenberg, 1974).

According to the Hardy-Weinberg Law of population genetics which assumes random mating, and the absence of selection, migration or mutation, the composition of the gene pool will remain unchanged from one generation to the next. In most inborn lysosomal diseases an affected individual will not survive to breeding age, thereby creating a selection pressure against the defective gene. As the frequency of new mutations is likely to be minimal, the gene-frequency for these diseases should theoretically remain low. In the Angus cattle population of New Zealand the incidence of heterozygotes for the mannosidosis genotype is approxi-

mately 10% (Jolly et al., 1973, 1974 and unpublished data), far higher than would be expected for an inherited lethal disease. A similar situation exists with the Tay-Sach's disease genotype in Ashkenazi Jews, where in North America the heterozygote frequency has been shown to be 4.2% (Kaback et al., 1974). The high gene-frequency for both diseases strongly implies that the heterozygous state has had a selective advantage over normal homozygotes and has more than compensated for the loss of defective genes through the inability of homozygous recessive individuals to reproduce.

The classical example of a disease showing heterozygote advantage is sickle-cell anaemia, where affected individuals are homozygous for gene coding for an abnormal haemoglobin molecule (Hb S), which differs from normal human haemoglobin (Hb A) by a single amino acid substitution (Harris, 1971; Lehmann and Huntsman, 1972). The trait is carried by approximately 20% of Negroes over much of West Africa (Allison, 1964) and by 8% of American Negroes (Rucknagel and Arber, 1974), even though affected homozygotes have a low life-expectancy (Allison, 1964). Heterozygotes for the sickle-cell genotype have been shown to enjoy a 24% greater resistance to falciparum malaria than normal, providing them with a survival and reproductive advantage over other members of their population and thereby increasing the gene-frequency for this anomaly (Allison, 1964; Rucknagel and Arber, 1974). Recent evidence has suggested a possible association between heterozygosity for Tay-Sach's disease and an increased resistance to tuberculosis (Myrianthopoulos and Aronson, 1972). Tuberculosis was prevalent throughout north-eastern Europe during the 19th and early 20th centuries, and it is conceivable that an increased resistance to this disease could have provided Tay-Sach's heterozygotes with a sufficient survival and reproductive advantage to significantly increase the frequency of the Tay-Sach's genotype. No

evidence for the existence of a selective advantage for heterozygotes of the mannosidosis genotype in Angus cattle has been established. It is considered more likely that certain heterozygous sires may have had a disproportionate influence on the genetic structure of the Angus breed in this country.

III. SPECIFIC INBORN LYSOSOMAL DISEASES

Many inborn lysosomal diseases have a number of clinical features in common, and the intracellular accumulation of similar compounds may develop from different primary anomalies (Hers and Van Hoof, 1969). It is desirable therefore, that the classification of these diseases should be based on the specific enzymic defect in addition to the nature of the stored material. Two other methods of classification are currently in use - the eponymic and the descriptive. The eponymic system, where a disease carries the name of its "founder" is of limited value, but the descriptive method is useful in describing the characteristics of a disease prior to the establishment of the biochemical anomalies.

Approximately thirty diseases have been described as inborn lysosomal disorders, and the associated enzymic defect has been established in most cases (Hers, 1973; Neufeld et al., 1975).
Although relatively less is known about such diseases in domestic animals, some may be of economic importance and their potential value as models for research into similar human disorders, especially in the field of enzyme replacement therapy, has led to considerable interest. Jolly and Blakemore (1973) reviewed and compared inherited lysosomal storage diseases of domestic animals with those of humans. A summary of the inborn lysosomal disorders of man and domestic animals is presented in Table 1.I. The method of classification is based on that of Neufeld et al. (1975). Heterogeneity of the stored materials has

TABLE 1.I

SUMMARY OF INBORN LYSOSOMAL DISEASES OF MAN AND DOMESTIC ANIMALS

Disorder	Enzymic Defect	Stored Material	Species Affected	Key References
MUCOPOLYSACCHARIDOSES (MPS)				
Hurler Syndrome (MPS I)	α-L-iduronidase	dermatan sulphate heparan sulphate	man	Bach et al. (1972)
Hunter Syndrome (MPS II)	iduronate sulphatase	dermatan sulphate heparan sulphate	man	Bach et al. (1973)
Sanfilippo Syndrome (MPS III)				
Subtype A	heparan N-sulphatase	heparan sulphate	man	Van Hoof (1973a)
Subtype B	N-acetyl-α-glucosaminidase	heparan sulphate	man	Neufeld and Cantz (1973)
Morquio Syndrome (MPS IV)	uncertain	keratan sulphate	man	Van Hoof (1973a) Neufeld et al. (1975)
Scheie Syndrome (MPS V)	α-L-iduronidase	dermatan sulphate heparan sulphate	man	Bach et al. (1972)
Maroteaux-Lamy Syndrome (MPS VI)	arylsulphatase B	dermatan sulphate	man	Stumpf et al. (1973) Van Hoof (1973a) Neufeld et al. (1975)
β-Glucuronidase deficiency mucopolysaccharidosis	β-glucuronidase	chondroitin sulphate	man	Sly et al. (1973) Hall et al. (1973)

Contd.

TABLE 1.I (Contd.)

Disorder	Enzymic Defect	Stored Material	Species Affected	Key References
SPHINGOLIPIDOSES				
G_{M1}-gangliosidosis (Types I and II)	β-galactosidase	G_{M1}-ganglioside, glycoprotein fragments	man	O'Brien et al. (1971) Van Hoof (1973c)
			cat	Baker and Lindsey (1971) Blakemore (1972) Farrell et al. (1973)
			ox	Donnelly et al. (1973a and b) Cheetham et al. (1974)
G_{M2}-gangliosidosis				
Type I (Tay-Sach's disease)	hexosaminidase A	G_{M2}-ganglioside	man	O'Brien et al. (1971)
Type II (Sandhoff's disease)	hexosaminidase A and B	G_{M2}-ganglioside, globoside	man	O'Brien et al. (1971) Sandhoff and Harzer (1973)
Type III (Juvenile G_{M2}-gangliosidosis)	hexosaminidase A	G_{M2}-ganglioside	man dog	O'Brien et al. (1971) Karbe and Scheifer (1967) Karbe (1973), McGrath et al. (1968)
Fabry's disease	β-galactosidase	trihexosylceramide	man	Brady (1974), Kint (1970)
Lactosylceramidosis	lactosylceramide β-galactosidase	lactosylceramide	man	Dawson and Stein (1971)
Gaucher's disease (Infantile, juvenile and adult forms)	β-glucosidase	glucosylceramide	man	Brady (1966, 1974) Brady and King (1973a)
			pig	Sandison and Anderson (1970)
			sheep	Laws and Saal (1968)
			dog	Hartley and Blakemore (1973a)
Metachromatic leucodystrophy	arylsulphatase A	sulphatide	man	Austin et al. (1964), Austin (1973a) Newelt et al. (1972)
Globoid cell leucodystrophy (Krabbe's disease)	galactosylceramide β-galactosidase	galactosylceramide	man	Austin et al. (1968) Suzuki et al. (1971, 1972)
			dog	Fletcher et al. (1966) Fankhauser et al. (1963) Zaki and Kay (1973)
			cat	Johnson (1970)
Niemann-Pick disease	sphingomyelinase	sphingomyelin	man	Brady (1966, 1974) Brady and King (1973b)
			cat	Chrisp et al. (1970) Percy and Jortner (1971)
Farber's disease	ceramidase	ceramide	man	Neufeld et al. (1975)

Contd.

TABLE 1.I (Contd.)

Disorder	Enzymic Defect	Stored Material	Species Affected	Key References
GLYCOPROTEINOSES				
Mannosidosis	α-mannosidase	glycoprotein fragments	man ox	Öckerman (1967, 1969, 1973) Hocking et al. (1972) Jolly (1971) Jolly et al. (1973)
Fucosidosis	α-L-fucosidase	glycoprotein fragments glycolipid	man	Dawson and Spranger (1971) Van Hoof (1973b)
Aspartylglycosaminuria	amidase	aspartyl-2-deoxy-2-acetamido glucosylamine	man	Jenner and Pollitt (1967) Palo et al. (1971, 1972)
OTHER DISORDERS WITH SINGLE ENZYME DEFECT				
Pompe's disease (Type II glycogen storage disease)	α-glucosidase	glycogen	man sheep dog cat ox	Hers (1965) Manktelow and Hartley (1975) Mostafa (1970) Sandstrom et al. (1969) Richards (unpublished data)
Wolman's disease	acid-lipase	cholesterol esters, triglyceride	man	Patrick and Lake (1969, 1973) Sloan and Fredrickson (1972)
Lysosomal acid-phosphatase deficiency	acid-phosphatase	phosphate esters	man	Nadler and Egan (1970)
MULTIPLE ENZYME DEFECTS				
Multiple sulphatase deficiency (Mucosulphatidosis)	arylsulphatase A, B, C; steroid sulphatases; iduronate sulphatase; heparan N-sulphatase	sulphatide, steroid sulphate, mucopolysaccharide	man	Austin (1973b) Van Hoof (1973a)
I-cell disease and pseudo-Hurler polyolystrophy (Mucolipidosis II and III)	most lysosomal enzymes deficient in cultured fibroblasts but present extracellularly	mucopolysaccharide and glycolipids	man	Leroy et al. (1971) Weismann et al. (1971) Hickman and Neufeld (1972) Thomas et al. (1973)

Contd.

TABLE 1.I (Contd.)

Disorders	Enzymic Defect	Stored Material	Species Affected	Key References
SUSPECTED LYSOSOMAL DISORDERS OF UNKNOWN AETIOLOGY				
Mucolipidosis I	unknown	unknown	man	Van Hoof (1973a)
Chediak Higashi Syndrome	unknown	lipofuscin-like pigment	man	White (1966) Padgett (1968)
			ox	Padgett (1968)
			mink	Sung and Okada (1971)
Wobbler disease of mink	unknown	lipofuscin-like pigment	mink	Padgett (1968) Hirano et al. (1971)
Cystinosis	unknown	cystine	man	Seegmiller (1973)
Storage of uncharacterized lipid:				
Neuronal lipidodystrophy	unknown	lipid	ox	Read and Bridges (1969)
Cerebrospinal lipidodystrophy	unknown	lipid	pig	Read and Bridges (1968)
Neuronal lipidosis	unknown	lipid	dog	Ribelin and Kinter (1956)
Visceral histiocytosis with lipid storage	unknown	lipid	pig	Sandison and Anderson (1970)
			ox	Payne et al. (1963)
Neurovisceral cytoplasmic storage disease	unknown	unknown	goat	Hartley and Blakemore (1973b)

often led to confusion regarding the classification of certain diseases. Fucosidosis for example is characterized by the storage of glycolipids and heterosaccharides from glycoproteins (Van Hoof, 1973b), and has been variously classified as a mucolipidosis (Raivio and Seegmiller, 1972), a sphingolipidosis (Brady, 1974) and as a disorder of glycoprotein metabolism (Neufeld et al., 1975). Similarly, in the G_{M1}-gangliosidoses certain glycoprotein fragments are stored in visceral tissues in addition to the storage of G_{M1}-ganglioside in the brain and viscera (O'Brien et al., 1971; Van Hoof, 1973c; Wolfe et al., 1974). An inborn lysosomal disease is usually classified according to the nature of the most abundant storage compound.

1. Mucopolysaccharidoses and mucolipidoses

The mucopolysaccharidoses are inborn lysosomal disorders characterized by an excessive accumulation of mucopolysaccharides and glycolipids in various tissues, and the excretion of mucopolysaccharides in urine (Van Hoof and Hers, 1972; Van Hoof, 1973a; Neufeld, 1974). Clinical features include moderate dwarfism with shortening and thickening of long bones, bone deformities of the head, cardiovascular disease and varying degrees of mental retardation (Van Hoof, 1973a; Dekaban and Constantopoulos, 1973). Although relatively common among the human lysosomal disorders, the heterogeneity of the stored materials and a poor understanding of the biochemistry of dermatan sulphate and heparan sulphate did not facilitate the early identification of the missing enzymes in these diseases. With the exception of Morquio syndrome the enzymic defects of most mucopolysaccharidoses have only recently been established (Van Hoof, 1973a; Neufeld et al., 1975). Dermatan sulphate and heparan sulphate are the predominant mucopolysaccharides found in the urine of patients with the Hurler, Hunter, Sanfilippo, Scheie and Maroteaux-Lamy syndromes, while keratan sulphate

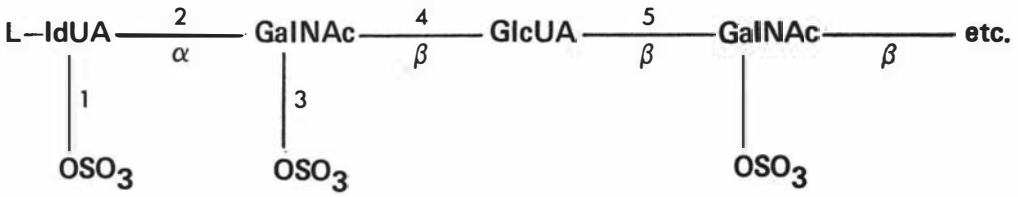
is found in the urine of patients with Morquio syndrome (Van Hoof, 1973a). A syndrome in which the urinary excretion of chondroitin sulphate is a feature has also been described (Philippart and Sugarman, 1969; Thompson et al., 1971; Benson et al., 1972; Sly et al., 1973).

Brachycephalic or "snorter" dwarfism in cattle was suggested as being analogous to the human Hurler syndrome by Lorincz (1960, 1961), Koger et al. (1960) and McIlwaine and Eveleth (1962), who reported increased urinary excretion of acid mucopolysaccharides in these cattle. This suggestion was refuted by Mayes et al. (1964) who were unable to detect any abnormal excretion of mucopolysaccharides in dwarf cattle. In a recent report Hurst et al. (1975) described a 30-fold increase in the level of chondroitin-4-sulphate in the urine of "snorter" dwarf cattle, and suggested a similarity between this condition and the human mucopolysaccharidoses characterized by excessive urinary excretion of chondroitin sulphate. Hurst et al. do not appear to have considered the influence of age on mucopolysaccharidoses as did Mayes et al., and the validity of their report must therefore be regarded with caution.

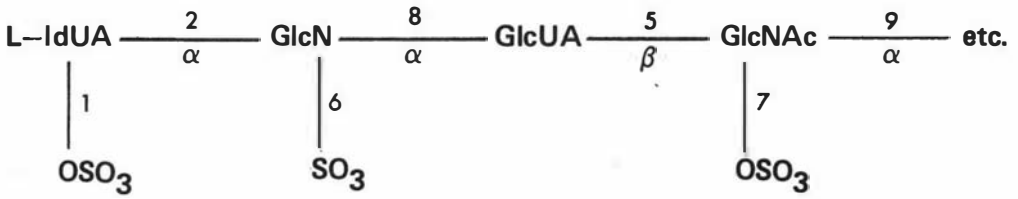
The catabolism of dermatan sulphate and heparan sulphate, the principal storage compounds in the mucopolysaccharidoses, is shown schematically in Figure 1.1 with the enzyme reactions and associated disorders listed (Neufeld et al., 1975). The catabolism of chondroitin sulphate is shown in Figure 1.2 (Muir, 1973).

The mucolipidoses bear a close clinical resemblance to the mucopolysaccharidoses, although the excretion of mucopolysaccharides in urine is not greatly elevated (Van Hoof, 1973a). Mucolipidosis Type I is poorly understood and the nature of the enzymic anomaly is not known. Cultured skin fibroblasts from patients with mucolipidosis Type II (I-cell disease) and Type III (pseudo-Hurler polydystrophy) are deficient

DERMATAN SULPHATE



HEPARAN SULPHATE



	Enzyme involved	Associated disorder
1	iduronate sulphatase	Hunter syndrome
2	α-L-iduronidase	Hurler and Scheie syndromes
3	N-acetylgalactosamine sulphatase	Maroteaux-Lamy syndrome
4	N-acetyl-β-galactosaminidase	
5	β-glucuronidase	
6	heparan N-sulphatase	Sanfilippo syndrome (Type A)
7	N-actylglucosamine sulphatase	
8	N-acetyl-glucosaminidase	Sanfilippo syndrome (Type B)

Key:

L–IdUa - iduronic acid

GalNAc - N-acetylgalactosamine

GlcNAc - N-acetylglucosamine

GlcUA - glucuronic acid

GlcN - glucosamine

Figure 1.1 **Catabolism of heparan sulphate and dermatan sulphate by lysosomal enzymes. The enzyme involved with each reaction, and the disorder associated with its deficiency are listed.**

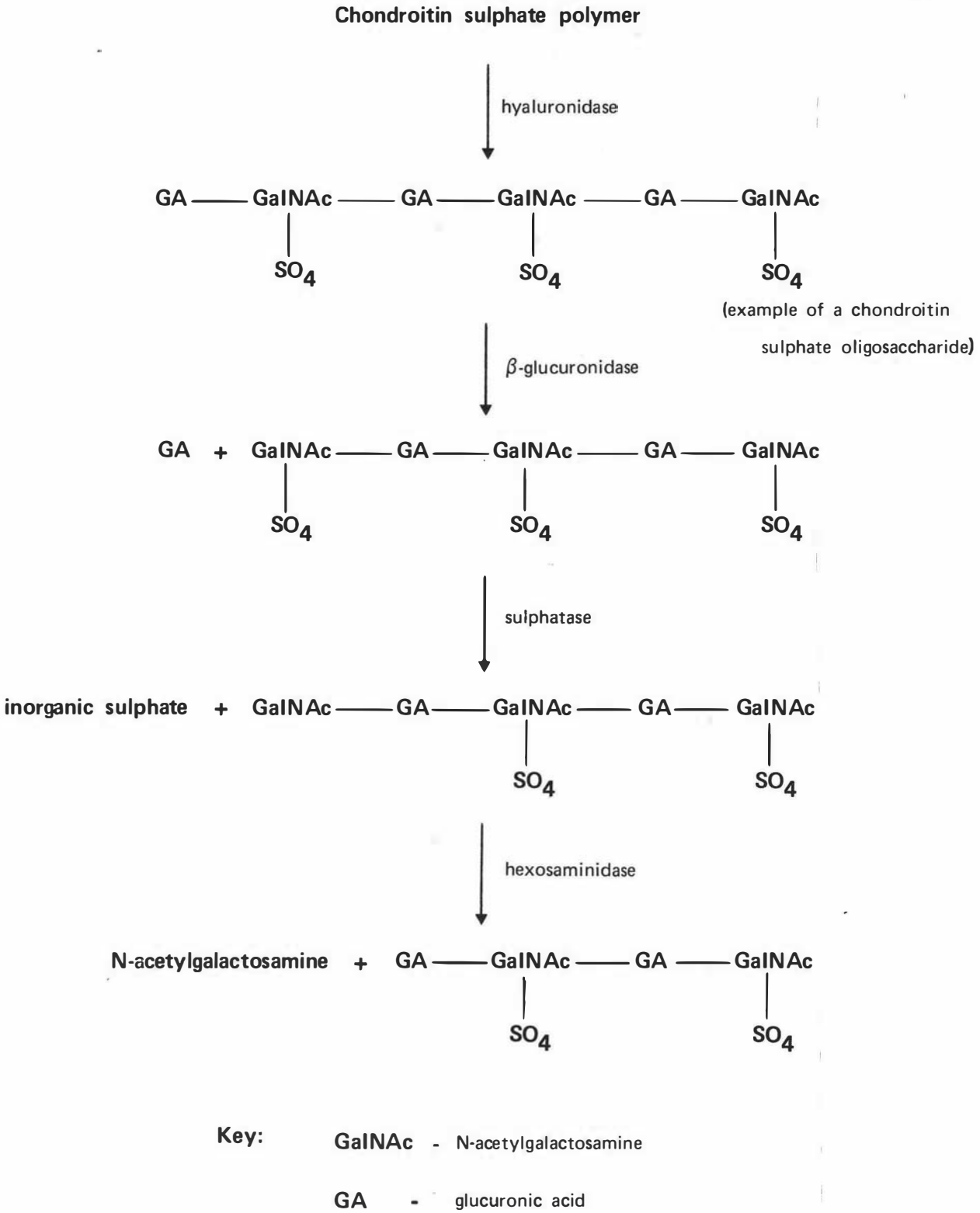


Figure 1.2 **Catabolism of chondroitin sulphate by lysosomal enzymes. Deficiency of β-glucuronidase has been linked with a mucopolysaccharide storage disease in which there was excessive urinary excretion of chondroitin sulphate (Sly *et al.*, 1973)**

in several lysosomal hydrolases, although these enzymes are present in the surrounding medium (Weismann et al., 1971; Leroy et al., 1971; Hickman and Neufeld, 1972; Thomas et al., 1973). Weismann et al. postulated that the multiple lysosomal enzyme deficiency in I-cell disease was due to a defective lysosomal membrane allowing increased leakage of enzymes from fibroblasts. This hypothesis was refuted by Hickman and Neufeld who found that cultured skin fibroblasts from patients with I-cell disease were able to retain ingested enzymes just as efficiently as cells of other genotypes. But cells from patients with various mucopolysaccharidoses were able to take up enzymes derived from I-cells only one-fifth to one-tenth as efficiently as enzymes derived from normal cells. This discovery led Hickman and Neufeld to suggest that the packaging of lysosomal enzymes required their secretion followed by specific recognition and uptake, and that the basic defect in I-cell disease was an alteration in the recognition site on hydrolases. An alternative, and more plausible hypothesis was presented by Ellis et al. (1975). These authors produced evidence to suggest that for several acid hydrolases there is a common biosynthetic reaction leading to the production of isoenzymes destined for incorporation into primary lysosomes rather than secretion by the cell. They suggested that the enzyme catalysing this reaction is deficient in patients with I-cell disease, whereas the synthesis of precursor and secreted isoenzymes is unaffected.

Another multiple-enzyme deficiency disease with certain characteristics in common with the mucopolysaccharidoses is mucosulphatidosis, or multiple sulphatase deficiency, in which the three isoenzymes of arylsulphatase (A, B and C) are deficient (Van Hoof, 1973a; Austin, 1973b). Diseases associated with multiple lysosomal enzyme deficiencies have yet to be diagnosed in domestic animals.

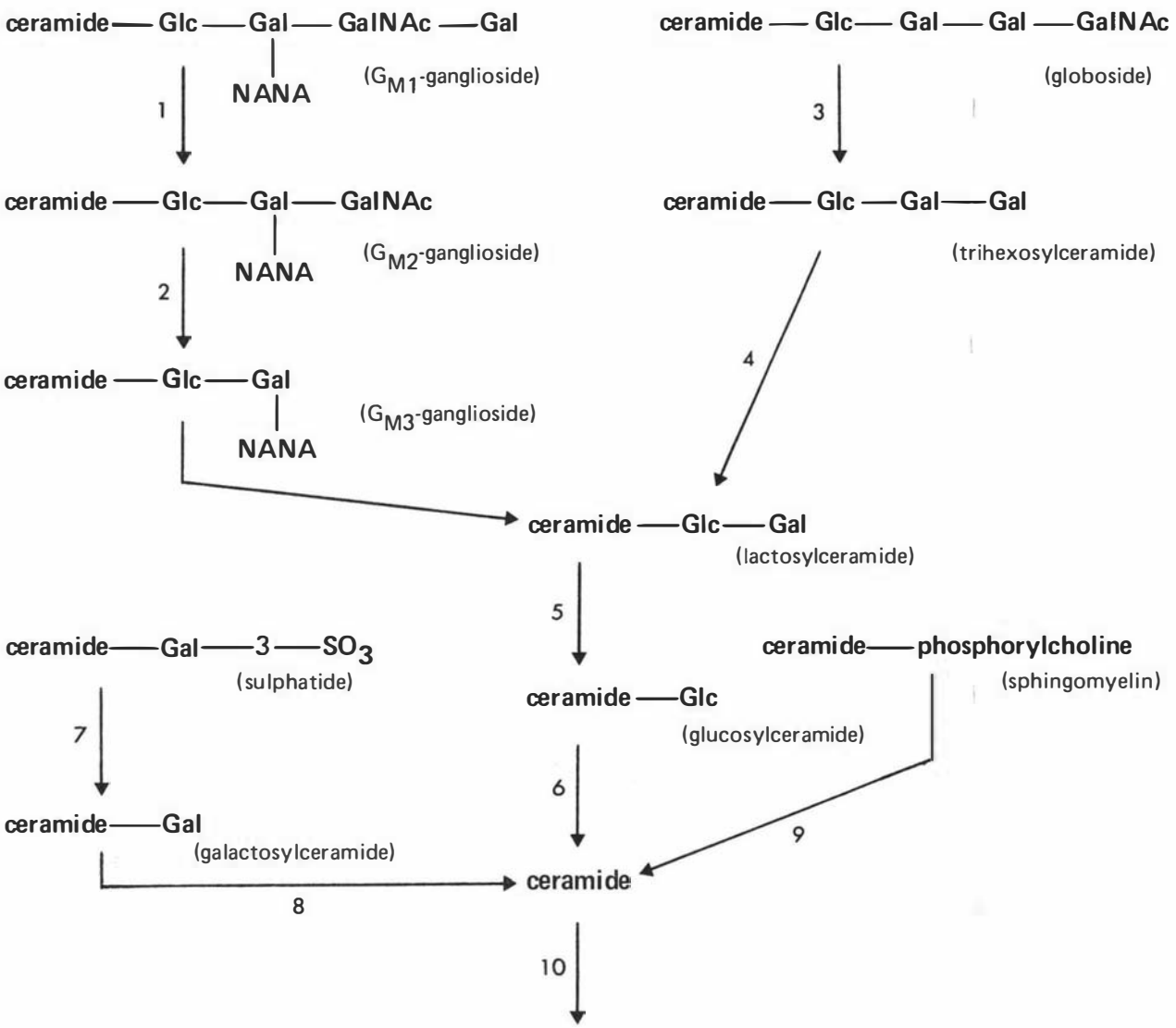
2. The Sphingolipidoses

The sphingolipidoses are the most comprehensively documented group of lysosomal storage diseases in human medicine, with the enzymic defect having been established in each case (Raivio and Seegmiller, 1972; Hers, 1973; Brady, 1974; Neufeld et al., 1975). Our knowledge of these diseases in domestic animals however is less complete, and where the missing enzyme is not known, classification is based on histological features and the nature of the stored substance.

Sphingolipids are structural compounds, located primarily within membranous elements of most mammalian cell-types and catabolized principally by lysosomal enzymes (Brady, 1974; Ledeen and Yu, 1973). Certain glycosphingolipids (e.g. cerebrosides, sulphatides and gangliosides) are present in unusually high concentrations in the brain, while globoside is an important glycolipid of leucocytes and erythrocytes (Raivio and Seegmiller, 1972; Ledeen and Yu, 1973). The metabolism of these compounds is illustrated schematically in Figure 1.3, which also lists the lysosomal enzyme involved with each reaction and the disorder associated with its deficiency (Dawson and Stein, 1971; Touster, 1973).

(a) Ganglioside storage diseases of at least five types have been described in children (O'Brien et al., 1971, 1972). Two are associated with the storage of G_{M1}-ganglioside and three with G_{M2}- ganglioside storage.

A deficiency of β-galactosidase in patients with G_{M1}-gangliosidosis Types I and II leads to the accumulation of G_{M1}-gangliosides in the brain and oligosaccharide units, derived from glycoproteins, in the viscera (O'Brien et al., 1971; O'Brien, 1972; Van Hoof, 1973c; Wolfe et al., 1974). G_{M1}-ganglioside is also stored in the viscera of patients with the Type I disease, but this is not a feature of G_{M1}-gangliosidosis Type II (O'Brien et al., 1971).



	Enzyme involved	Associated disorder
1	β-galactosidase	generalized G_{M1}-gangliosidosis
2	hexosaminidase A	Tay-Sach's disease (G_{M2}-gangliosidosis Type I)
3	hexosaminidase A and B	Sandhoff's disease (G_{M2}-gangliosidosis Type II)
4	α-galactosidase	Fabry's disease
5	β-galactosidase	lactosylceramidosis
6	β-glucosidase	Gaucher's disease
7	arylsulphatase A	metachromatic leucodystrophy
8	β-galactosidase	globoid-cell leucodystrophy
9	sphingomyelinase	Niemann-Pick disease
10	ceramidase	Farber's disease

Key: **Glc** – glucose; **Gal** – galactose; **GalNAc** – N-acetylgalactosamine; **NANA** – N-actylneuraminic acid (sialic acid)

Figure 1.3 **Catabolism of sphingolipids by lysosomal enzymes. The enzyme involved with each reaction, and the disorder associated with its deficiency are listed.**

A G_{M1}-gangliosidosis with similar clinical, histopathological and biochemical lesions to G_{M1}-gangliosidosis Type II of children has been described in a Siamese cat (Baker and Lindsey, 1971; Farrell et al., 1973). No visceral involvement was observed in this case. A further case of G_{M1}-gangliosidosis in a cat was reported by Blakemore (1972). The excessive storage of glycolipid in the brain and liver of the cat, in addition to storage of a highly labile compound in the liver, suggested that this case was analogous to G_{M1}-gangliosidosis Type I (generalized gangliosidosis) of children. Cheetham et al. (1974) reported a similar case to that described by Blakemore and demonstrated a deficiency of β-galactosidase activity at pH 5.0 in liver from the affected cat.

G_{M1}-gangliosidosis has also been reported in Friesian calves, where the absence of visceral ganglioside storage suggested a similarity with the human Type II form of the disease (Donnelly et al., 1973a and b). A partial deficiency of β-galactosidase was reported by Donnelly et al. (1973b). A complete deficiency of one isoenzyme of β-galactosidase was later demonstrated by Cheetham et al. (1974) in tissues from affected calves.

In G_{M2}-gangliosidosis Type I (Tay-Sach's disease) a complete deficiency of hexosaminidase A is associated with the accumulation of G_{M2}-ganglioside in the brain (O'Brien et al., 1971). The accumulation of G_{M2}-ganglioside is less severe in the brain of patients with G_{M2}-gangliosidosis Type III, where a partial deficiency of hexosaminidase A has been demonstrated (O'Brien et al., 1971). In G_{M2}-gangliosidosis Type II (Sandhoff's disease) both hexosaminidase A and B are deficient and there is storage of globoside in the viscera in addition to G_{M2}-gangliosides in the brain (O'Brien et al., 1971; Sanhoff and Harzer, 1973). A canine

G_{M2}-gangliosidosis has been described in German short-haired Pointers (Karbe and Scheifer, 1967; Karbe, 1973). The actual enzymic defect has yet to be confirmed for the canine disease, which appears on histopathological observations (Karbe, 1973) to be intermediate between the human Types I and III, suggesting an anomaly of hexosaminidase A. Ganglioside storage, along with the storage of cerebrosides, sulphatides and lipofuscin-like material, also occurs in mink suffering from a condition known as Wobbler disease (Padgett and Kanfer, cited by Hirano et al., 1971). Morphological evidence suggests that Wobbler disease is an inborn lysosomal disorder (Hirano et al., 1971), however the deficiency of a lysosomal enzyme has yet to be recorded.

(b) Gaucher's disease is one of the most frequently encountered of the sphingolipodoses in human medicine (Brady and King, 1973a; Brady, 1974) and may occur in three distinct forms, i.e. infantile, juvenile and adult. The infantile form is characterized by mental retardation in addition to splenomegally, hepatomegally and erosion of the cortices of long bones and the femoral head. Patients with the two other forms tend to be free from neurological manifestations. β-Glucosidase is almost completely absent from tissues of patients with the infantile form of the disease, but the residual activity of this enzyme may be as high as 17% of normal in the juvenile form and 40% of normal in patients with the adult form (Brady, 1966; Brady and King, 1973a; Brady, 1974). The glucosylceramide which accumulates in the reticuloendothelial system appears to originate from glycolipids of aged leucocytes and erythrocytes, while gangliosides are probably the major precursors of glucosylceramide stored in the brain of patients with infantile Gaucher's disease (Brady and King, 1973a). Characteristic "Gaucher cells", histiocytes

containing the stored glucosylceramide, are present throughout the reticuloendothelial system.

A condition closely resembling Gaucher's disease has been diagnosed in an 8-month old Sydney silky dog showing signs of progressive central nervous damage (Hartley and Blakemore, 1973a). Typical "Gaucher cells" were present in the liver, lymph nodes and cerebellum but not in the spleen, and large amounts of glucosylceramide were detected in the brain and liver. Gaucher-like disorders have also been reported in a sheep (Laws and Saal, 1968) and in a pig (Sandison and Anderson, 1970), the diagnosis in both cases being made from tissues submitted from abattoirs.

(c) Globoid cell leucodystrophy (Krabbe's disease) is an inborn lysosomal disorder of the nervous system, with symptoms generally appearing within the first year of life and following a rapidly progressive fatal course (Austin et al., 1968; Suzuki and Suzuki, 1972, 1973, 1974; Wenger et al., 1974). Degenerative changes to axons and myelin sheaths are observed in the peripheral nervous system. In the central nervous system the pathological changes are largely confined to the white matter, where there is a severe deficiency of myelin, astrocytic gliosis, plus many "globoid cells". The characteristic "globoid cells" are lipid-filled macrophages, thought to originate from non-neural mesodermal cells. They stain positively with periodic acid-Schiff (PAS) and are often found in the vicinity of blood vessels (Austin et al., 1968; Suzuki and Suzuki, 1972, 1973).

The primary enzymic anomaly in globoid cell leucodystrophy is a deficiency of galactosylceramide β-galactosidase (Suzuki and Suzuki, 1971, 1972, 1973, 1974; Suzuki et al., 1971). Galacto-

sylceramide is an important component of myelin sheaths, therefore its biosynthesis and degradation might be expected to occur within oligodendroglial cells of the central nervous system. The abnormal accumulations of this compound in globoid cell leucodystrophy however, are found within globoid cells, where they are seldom surrounded by a single limiting membrane (Suzuki and Suzuki, 1973).

Globoid cell leucodystrophy is well recognized in the Cairn and West Highland White terrier breeds of dog (Fankhauser et al., 1963; Fletcher et al., 1966, 1971; Austin et al., 1968; Fletcher, 1970; Kurtz and Fletcher, 1970; Suzuki et al., 1971, 1972, 1974), and has recently been reported in a miniature poodle (Zaki and Kay, 1973), and three Bluetick Hound pups (Boysen et al., 1974). Although the canine and human forms of globoid-cell leucodystrophy show similar clinical and histopathological characteristics and exhibit the same enzymic deficiencies, they appear to differ in the nature of the mutations underlying their enzymic anomalies (Suzuki et al., 1972, 1974). In spite of this basic difference Suzuki et al. (1974) have suggested that canine globoid cell leucodystrophy may serve as a valuable model for certain aspects of research into the human disease.

Globoid cell leucodystrophy has also been reported in the cat, where two female kittens with a history of inbreeding in their immediate ancestry, were shown to be affected (Johnson, 1970).

(d) Niemann-Pick disease is characterized by the excessive accumulation of a phosphosphingolipid, sphingomyelin, in the central nervous system and in large lipid-filled cells throughout the spleen, bone marrow, liver, lungs, lymph nodes and ganglion cells (Brady, 1966, 1974; Fredrickson and Sloan, 1972; Brady and King,

1973b). Four clinical categories are recognized in human medicine due to variations in the degree of central nervous system involvement and the rate of progression of the disease (Brady, 1974). The basic enzymic defect is a deficiency of the lysosomal enzyme sphingomyelinase, the deficiency being less pronounced in patients with the more chronic forms of the disease (Brady, 1966; Brady and King, 1973b). Niemann-Pick disease has also been reported in a Siamese cat (Chrisp et al., 1970) and in a domestic cat (Percy and Jortner, 1971). Biochemical studies revealed increased levels of sphingomyelin in tissues, and histopathological features were similar to those described for Niemann-Pick disease in humans, however the actual enzymic defect was not confirmed in either case.

(e) Metachromatic leucodystrophy is associated with a deficiency of arylsulphatase A, resulting in the accumulation of sulphatides in the brain, kidney, gall bladder epithelium and leucocytes (Austin et al., 1964; Hers and Van Hoof, 1969; Newelt et al., 1972). Metachromatic leucodystrophy may become clinically manifest at any age, but the disease prevails in infancy (Haberland et al., 1973; Austin, 1973a). Histopathological characteristics include extensive demyelination in both central and peripheral nervous systems, in addition to deposition of metachromatically staining substances in the central nervous system and other systemic organs (Newelt et al., 1972; Haberland et al., 1973; Austin, 1973a).

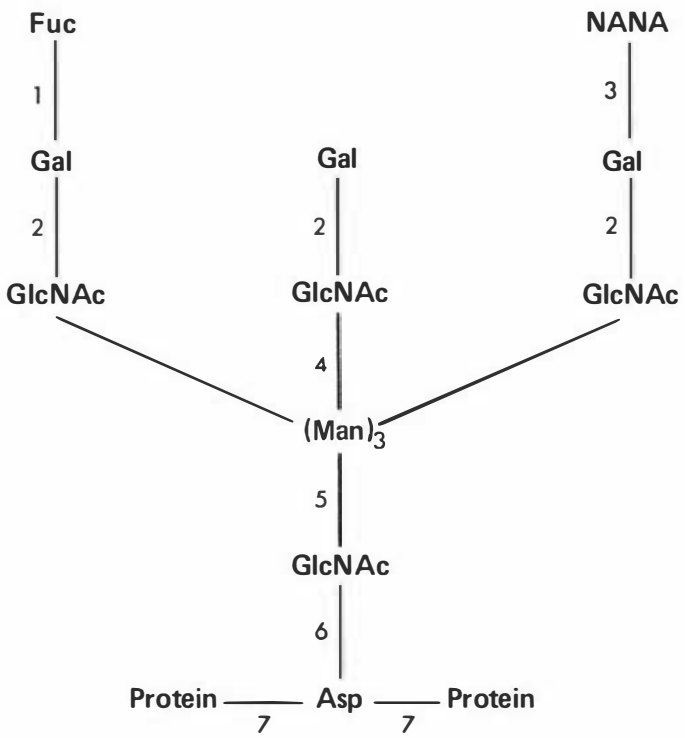
A type of metachromatic leucodystrophy was reported in mink (Christensen and Pulladan, 1965; Andersen and Pulladan, 1968) but unlike the human disorder there was no excessive sulphatide storage and no evidence to suggest that it was an inborn lysosomal disease.

The three other human sphingolipidoses, Fabry's disease, Farber's disease and lactosyl-ceramidosis (Table 1.I) have not been reported in domestic animals and will not be discussed further in this review.

3. Glycoproteinoses

Glycoproteins are a diverse and heterogeneous group of compounds consisting of carbohydrates covalently linked to proteins. They are represented in animal tissues by such essential components as mucus, enzymes, hormones, antibodies, membranes and collagen (Spiro, 1969a and b). The catabolism of both carbohydrate and peptide portions of glycoproteins is a function of lysosomal enzymes (Mahadevan and Tappel, 1967, 1968; Aronson and de Duve, 1968; Mahadevan et al., 1969; Spiro, 1969a) and is illustrated diagramatically in Figure 1.4 (Mahadevan and Tappel, 1967; Thomas and Winzler, 1971). Inborn lysosomal disorders associated with the defective catabolism of glycoproteins have been linked with deficiencies of the lysosomal enzymes α-mannosidase, α-fucosidase and aspartylglycosamine amido hydrolase. Oligosaccharides derived from the incomplete catabolism of glycoproteins are also stored in certain tissues and in cultured skin fibroblasts from patients with G_{M2}-gangliosidosis Type II (Sandhoff's disease) and G_{M1}-gangliosidosis Types I and II (Tsay and Dawson, 1973, 1975; Wolfe et al., 1974) but due to the more abundant storage of gangliosides in these disorders they are classified as sphingolipidoses.

(a) Aspartylglycosaminuria is characterized clinically by skeletal abnormalities, coarse facial features, mental retardation and recurrent infections (Jenner and Pollitt, 1967; Palo and Mattsson, 1970). The disease appears to be most common among the Finnish population (Palo and Mattsson, 1970; Aula et al., 1973). There is storage of aspartylglycosamine and an aspartyloligosaccharide in



	Enzyme involved	Associated disorder
1	α-fucosidase	fucosidosis
2	β-galactosidase	G_{M1}-gangliosidosis Types I and II
3	N-acetylneuraminidase	
4	hexosaminidase	G_{M2}-gangliosidosis Type II
5	α- mannosidase	mannosidosis
6	β-aspartylglucosylamine amido hydrolase	aspartylglycosaminuria
7	lysosomal proteases and peptidases	

Key:

Fuc - fucose

Man - mannose

Gal - galactose

Asp - asparagine

NANA - N-acetylneuraminic acid

GlcNAc - N-acetylglucosamine

Figure 1.4 **Catabolism of a glycopeptide unit by lysosomal enzymes. The enzyme involved with each catabolic step and the disorder associated with its deficiency are listed.**

the brain, liver and kidney (Palo and Savolainen, 1972, 1973), and large amounts of aspartylglycosamine are excreted in the urine (Jenner and Pollitt, 1967; Pollitt et al., 1968; Palo and Mattsson, 1970; Palo and Savolainen, 1973). The latter compound represents the glycopeptide linkage region of many glycoproteins and is not normally detected in a free form (Spiro, 1969b). A reduction in the activity of the lysosomal enzyme aspartylglycosamine amido hydrolase (N-aspartyl-β-glucosaminidase) has been reported in plasma, seminal fluid (Pollitt et al., 1968), brain, liver (Palo et al., 1971, 1972) and cultured fibroblasts (Aula et al., 1973) from patients with aspartylglycosaminuria. Ultrastructural features include numerous electron-lucent vacuoles, limited by a single membrane, in neurones, hepatocytes and renal tubular cells (Arstila et al., 1972; Haltia et al., 1975).

(b) Fucosidosis has many features in common with Hurler's syndrome and the differentiation of these two diseases may be difficult in the early stages (Van Hoof, 1973b). Clinical characteristics of fucosidosis include severe progressive cerebral degeneration, spinal abnormalities, hepatomegally, cardiomegally, thickened skin, abundant sweating and recurrent respiratory infections (Durand et al., 1966; Van Hoof, 1973b). Glycolipids and glycoprotein residues containing an excess of fucose are stored in the brain and liver due to a deficiency of α-fucosidase (Durand et al., 1968; Van Hoof, 1973b). The heterogeneity of the storage product in fucosidosis has led to inconsistencies in the classification of this disease (see page 14). Clear vacuoles surrounded by a single membrane are observed in all types of brain cells and in hepatocytes and Kupffer cells of patients with fucosidosis. Hepatocytes also possess vacuoles containing lamellar material (Van Hoof, 1973b).

(c) Mannosidosis is the only disorder of glycoprotein catabolism that has been reported both in humans (Öckerman, 1967, 1969, 1973) and domestic animals (Hocking et al., 1972; Jolly et al., 1973; Jolly, 1975). Although mannosidosis is considered to be of economic importance to the New Zealand beef industry (Jolly et al., 1974; Jolly, 1975) its high incidence provides an excellent opportunity for its use as a model for research into similar inborn lysosomal diseases of humans, especially with regard to heterozygote testing and enzyme replacement therapy. Human and bovine mannosidosis are discussed in detail in section V of this chapter.

4. Other lysosomal disorders with a single enzymic defect

(a) Pompe's disease (Type II glycogenosis) was the prototype lysosomal storage disease on which the original concepts of Hers (1965) (see page 4) were based. A deficiency of acidic α-glucosidase leads to the accumulation of excessive glycogen within lysosomes in most tissues including liver, smooth and skeletal muscle, heart, lung, spleen, kidney and central nervous system (Hers, 1965; Hers and de Barsy, 1973).

Abnormal accumulations of glycogen in membrane-bound vacuoles within neurones, neuroglia and microglia were reported as an incidental finding in a cat which had no history of clinical disease (Sandstrom et al., 1969). Further cases resembling Pompe's disease have been described in a dog (Mostafa, 1970), in a sheep (Manktelow and Hartley, 1975), and more recently in Shorthorn cattle (Richards, unpublished data). In each case the diagnosis was based on histochemical studies and on the distribution of lesions, without demonstration of the actual enzymic defect.

(b) Wolman's disease is characterized biochemically by the accumulation of neutral lipids, especially cholesteryl esters and triglycerides, in most organs, due to the deficiency of an acid esterase (Patrick and Lake, 1969, 1973; Sloan and Fredrickson, 1972).

(c) Lysosomal acid-phosphatase deficiency has recently been reported in children (Nadler and Egan, 1970; Nadler, 1973). Excessive levels of material staining positively with the PAS method were observed within hepatocytes and renal tubular cells of patients (Nadler, 1973).

5. Suspected lysosomal disorders of unknown aetiology

(a) The Chediak-Higashi syndrome is a disease of children, Hereford cattle, mink and the beige mouse, characterized by defective pigmentation, increased susceptibility to infection, and anomalous enlarged lysosomes in leucocytes and many other cell-types (Padgett, 1968; Sung and Okada, 1971; Van Hoof and Hers, 1973; Essner and Oliver, 1974; Windhorst and Padgett, 1973). Although the disease is rare in humans, cattle and mice it is frequently seen in mink where it is associated with homozygosity for the recessively inherited aleutian coat-colour genotype (Padgett, 1968; Sung and Okada, 1971).

White (1966) demonstrated a positive relationship between acid-phosphatase activity and the giant granules in circulating leucocytes of patients with Chediak-Higashi syndrome, and suggested that this was a lysosomal disease. This suggestion was endorsed by Sung and Okada on the basis of ultrastructural studies, and a partial deficiency of a lysosomal enzyme was postulated. A proteinase deficiency at pH 3.0 and 7.5 in leucocytes of four human

patients has been claimed (Gralnick, 1972 cited by Jolly and Blakemore, 1973) but is yet to be substantiated. Ultrastructural evidence was presented by Essner and Oliver (1974) to suggest that the anomalous lysosomes arose directly from dilated portions of golgi-associated smooth endoplasmic reticulum (GERL).

(b) Wobbler disease of mink is a similar condition to the Chediak-Higashi syndrome, characterized by the accumulation of membranous cytoplasmic bodies and lipofuscin-like material in various nerve cells (Hirano et al., 1971). This disease has been mentioned among the ganglioside storage diseases (page 22) due to the presence of excessive levels of cerebrosides, sulphatides and gangliosides in the brain of affected mink.

(c) Cystinosis is a human disorder of cystine metabolism in which an elevation of intracellular cystine levels results in the deposition of cystine crystals in lysosomes, primarily of reticuloendothelial cells (Schneider et al., 1967, 1969; Raivio and Seegmiller, 1972; Seegmiller, 1973). The primary genetic defect has yet to be established for this disease, which has not been reported in domestic animals.

(d) Several other suspected inborn lysosomal disorders associated with intracytoplasmic accumulation of lipid material have been described in domestic animals.

(i) Neuronal lipidosis has been reported in five dogs of Cocker Spaniel or part Cocker Spaniel breeding (Ribelin and Kinter, 1956; Fankhauser, 1965; Koppang, 1970). The involvement of a single breed suggests that these different reports may reflect a specific disease of Cocker Spaniels.

(ii) A "neuronal lipidodystrophy" with involvement of macrophages in spleen and lymph nodes was reported in a 19-month old bull from an inbred strain of cattle (Read and Bridges, 1969). Neurones contained granular cytoplasmic inclusions which reacted positively with various lipid stains suggesting an error of lipid metabolism, and the history of a similar clinical syndrome having occurred previously in the herd implied a genetic aetiology.

(iii) A "cerebral lipidodystrophy" was reported by Read and Bridges (1968) in two Yorkshire swine. Pathological changes included membranous cytoplasmic inclusions similar to those seen in human G_{M2}-gangliosidosis Type I (Tay-Sach's disease).

(iv) Storage disorders involving visceral histiocytosis have been recorded in a two-year old Shorthorn bull (Payne et al., 1963), a six-month old pig and an adult Ayreshire cow (Sandison and Anderson, 1970), and may represent inborn lysosomal diseases.

(e) A neurovisceral cytoplasmic storage disease was recently described in two neonatal goats (Hartley and Blakemore, 1973b). A similarity was drawn between the histopathological features of this disease and the human mucopolysaccharidoses and mannosidosis of Angus calves.

IV. LYSOSOMAL STORAGE DISEASES WITHOUT PRIMARY LYSOSOMAL DEFECTS

Although the concept of inborn lysosomal diseases, as formulated by Hers (1965), is based on the genetically determined deficiency of a lysosomal enzyme or structural protein, such a deficiency is not an essential prerequisite to the accumulation of a compound within lysosomes.

Since lysosomes are the ultimate site for the digestion of most bodily substances the lysosomal system may become overloaded by excessive levels of indigestible or poorly digestible compounds of endogenous or exogenous origin. Jolly and Hartley (1976) recognized six further types of lysosomal storage diseases in addition to those caused by the deficiency of a lysosomal enzyme:

(i) Such a disease may be associated with the injection or ingestion of compounds which cannot be digested within lysosomes. For example, the experimental introduction of indigestible materials such as Triton WR-1339, sucrose or dextran to an individual or to cells in tissue culture causes marked lysosomal enlargement, simulating a storage disease (Lloyd, 1973). Intoxications by the ingestion of the plants Swainsona spp., Astragalus spp. and Oxytropis spp. may have this pathogenesis (Huxtable, 1970, 1972; Jolly and Hartley, 1976).

(ii) The inherited deficiency of a non-lysosomal enzyme may lead to overproduction of a poorly digestible compound which accumulates in secondary lysosomes, e.g. neuronal ceroid-lipofuscinosis (see below).

(iii) Certain substrates, when ingested, may be formed into indigestible polymeric compounds within cells and become stored within secondary lysosomes. Chronic Phalaris spp. intoxication is thought to represent this type of pathogenesis (see below).

(iv) The lysosomal system may become overloaded with normal cellular constituents following the enhancement of autophagy by drugs such as chloroquine.

(v) Inorganic compounds may become stored as organic complexes

within lysosomes under certain conditions. Examples include iron (as haemosiderin) and copper pigments.

(vi) The storage of normal cellular material may follow inhibition of lysosomal enzymes. This is largely a theoretical concept.

Examples of some of the above types of lysosomal storage disease are well recognized and will be discussed in more detail.

1. Neuronal ceroid-lipofuscinosis

The autofluorescent lipopigments ceroid and lipofuscin accumulate in lysosomes of neurones, astrocytes and reticuloendothelial cells in the neuronal ceroid-lipofuscinoses, a well documented group of diseases of humans (Zeman and Dyken, 1969; Zeman and Saikotos, 1973), English Setter dogs (Koppang, 1970; Bernheimer and Karbe, 1970), Chihuahuas (Rac and Giesecke, 1975; Jolly and Stevenson, pers. comm.; Hartley, pers. comm.) and Siamese cats (Green and Little, 1974).

Ceroid and lipofuscin are originally formed within the cytoplasm, possibly from the breakdown of mitochondria, and become incorporated into autophagic vacuoles (Zeman and Siakotos, 1973). As lysosomes are not equipped with enzymes capable of degrading these polymers their formation leads to accumulation within secondary lysosomes, and in fact the gradual accumulation of lipofuscin, especially in long-lived post-mitotic cells, is a normal process associated with senescence (Zeman and Siakotos, 1973). In neuronal ceroid-lipofuscinosis of man and English Setter dogs the increased formation of ceroid and lipofuscin and the excessive storage of these pigments, especially in the brain, is associated with a genetically induced deficiency of a phenylenediamine-mediated peroxidase (Armstrong et al., 1974; Patel et al., 1974a). This enzyme is not a recognised component of lysosomes, therefore neuronal ceroid-lipofuscinosis cannot be considered a true inborn lysosomal disease according to the original concept of Hers (1965).

2. Storage disorders associated with plant intoxications

Storage diseases have been described in domestic animals following the ingestion of toxic plants, including Swainsona spp., Astragalus spp., Oxytropis spp. and Phalaris spp. Swainsona poisoning has been reported in sheep, cattle and horses in Australia. Clinical characteristics include stiffness of hind limbs, ataxia, emaciation and eventual prostration (Gardiner et al., 1969). Histologically there is widespread vacuolation of neurones, pancreatic exocrine cells, epithelial cells in proximal convoluted tubules of the kidney, and macrophages within lymph nodes (Laws and Anson, 1968; Gardiner et al., 1969; Hartley, 1971). The vacuoles are lined by a single membrane, and as such are considered to represent secondary lysosomes (Huxtable, 1970). A yellow lipopigment is also stored in the brain and macrophages of the liver and lymph nodes in some cases of Swainsona poisoning.

The ingestion of the legumes Astragalus spp. and Oxytropis spp. produces a disease known as "locoism" in sheep, cattle, horses and deer in Western U.S.A. (Oehme et al., 1968; Van Kampen and James, 1969; James et al., 1970). Clinical and histological features are similar to those described for Swainsona poisoning. The nature of the storage compound has not been established in either Swainsona poisoning or "locoism", but is apparently very soluble and is removed during routine preparation of tissues for histological investigations. It is interesting to speculate whether the storage disorder in these plant intoxications is due to the ingestion of compounds which cannot be catabolized by the normal complement of lysosomal enzymes, or whether a compound is ingested which inhibits the normal action of a certain lysosomal enzyme. Jolly and Hartley (1976) favour the former hypothesis.

Chronic intoxication by ingestion of Phalaris spp. has been reported in sheep and cattle in Australia and New Zealand, and is characterized by

the storage of yellow-brown pigment granules in neurones of the brain stem, and in liver, kidney and lymph nodes (Milne, 1955; Hartley and Kater, 1962; Gallagher et al., 1966, 1967; Simpson et al., 1969). The toxic principles in Phalaris spp. are thought to be tryptamine alkaloids, which competitively inhibit the breakdown of serotonin by monoamine oxidase, thereby causing an accumulation of serotonin and catecholamines (Gallagher et al., 1966). The pigment is thought to be a melanin derived from the tryptamine alkaloids, and although it is initially formed within mitochondria, it would be expected to find its way into secondary lysosomes by the process of autophagy (Jolly and Hartley, 1976).

3. Lysosomal storage of inorganic substances

Certain inorganic substances bound to organic components may become stored within lysosomes of parenchymal cells and macrophages. Intracellular deposits of some heavy metals such as iron and copper, which are important enzyme constituents, act as reservoirs which may be readily mobilized when required. Excessive quantities of inorganic compounds may however become stored in lysosomes under certain circumstances. For example, haemosiderin forms when a cell contains more iron than can be bound by apoferritin, a protein which prevents condensation of iron micelles and facilitates mobilization while protecting the cytoplasm from the toxic effects of iron. Abnormally high levels of organically bound copper may be stored in lysosomes after excessive administration of therapeutic copper compounds, or in Wilson's disease which is caused by a defect in copper metabolism (Gedigk, 1969; Slater, 1969). In chronic copper poisoning of sheep, copper may become deposited in macrophages and reticuloendothelial cells of the liver giving rise to the characteristic "copper cells" which appear grey-brown in sections stained with haemotoxylin and eosin (Jolly and Hartley). Several other organic compounds such as silicon dioxide derivatives, lead and silver may be similarly stored within the lysosomes of various tissues (Gedigk, 1969).

V. MANNOSIDOSIS

1. Human mannosidosis

Human mannosidosis was first described as a "Hurler-like" syndrome by Öckerman (1967) and has since been reported in 19 further children (Norden et al., 1973a; Autio et al., 1973; Tsay et al., 1974; Gehler et al., 1974; Taylor et al., 1975; Farriaux et al., 1975). Clinical characteristics include psychomotor retardation, discrete skeletal changes, hepatosplenomegally, corneal or lenticular opacities and vacuolation of lymphocytes (Öckerman, 1967, 1973; Norden et al., 1973a; Autio et al., 1973). Histological studies on the original patient (Kjellman et al., 1969) revealed a generalized ballooning of the cytoplasm of neurones throughout the cerebral cortex, brainstem and spinal cord, apparent swelling of reticuloendothelial cells of the liver, spleen and lymph nodes and there was fine vacuolation of lymphocytes in the spleen and lymph nodes. Ultrastructural studies on liver biopsies from two later patients (Autio et al., 1973) revealed storage vacuoles, surrounded by a single membrane, in hepatocytes, Kupffer cells and bile-duct epithelium.

In human mannosidosis the storage of mannose-rich oligosaccharides (Öckerman, 1967, 1969 , 1973) is secondary to an almost complete deficiency of the lysosomal enzyme acidic α-mannosidase (pH 3.5 - 4.5) (Carroll et al., 1972; Tsay et al., 1974; Masson et al., 1974; Taylor et al., 1975). Increased levels of mannose-containing oligosaccharides are excreted in the urine of affected children (Norden et al., 1973a and b, 1974; Autio et al., 1973). The most abundant compound was a trisaccharide with the structure: α-D-mannopyranoside-(1 -> 4)-2-acetamido-2-deoxy-D-glucose. A tetrasaccharide and a pentasaccharide were also excreted in large amounts. These oligosaccharides were thought to be derived from the inner core of glycoprotein chains (Norden et al., 1973b, 1974).

2. Bovine mannosidosis

A disease in Angus calves characterized by intention tremor, ataxia, an aggressive tendency, failure to thrive and death usually within the first year of life, was first described by Whittem and Walker (1957) in Australia. Histopathological findings included widespread vacuolation of neurones of the central nervous system and reticuloendothelial cells of lymph nodes, hypertrophy of Purkinje cell dendrites, and the presence of numerous eosinophilic bodies throughout the brain. This disease had certain features in common with the lipidodystrophies of man, but as Whittem and Walker were unable to demonstrate the presence of stored lipid, they described the condition as a "neuronopathy" and "pseudo-lipidosis".

Since this early report "pseudolipidosis" has been regularly diagnosed in both Australia and New Zealand. A comprehensive investigation into the pathology of the central nervous system in this disease was described by Jolly (1971), who extended the findings of Whittem and Walker. Electron microscopic studies revealed that the cytoplasmic vacuoles in neurones were lined by a single unit membrane, and appeared empty except for membranous fragments and a variable amount of amorphous electron-dense material. Their structure was compatible with that of secondary lysosomes. The eosinophilic bodies mentioned by Whittem and Walker were shown to be spheroidal swellings of axons caused by local accumulations of dense bodies, mitochondria, and a local proliferation of neurofilaments. PAS-positive lipofuscin-like globules were reported as occurring frequently within astrocytes, microglia and pericytes of blood vessels. As a result of this study Jolly postulated that the disease was associated with an anomaly of glycoprotein metabolism.

The epidemiology of "pseudolipidosis" suggested an inherited aetiology (Whittem and Walker, 1957; Jolly. 1970) and further support

was gained by mating cows which had previously produced affected calves to bulls which had sired such calves. Ten out of forty-one calves resulting from these matings developed the disease (Jolly et al., 1973, and unpublished data), closely approximating the 25% frequency expected if the disease was inherited as a simple autosomal recessive condition.

An oligosaccharide containing mannose and glucosamine in the ratio of three to one was extracted from lymph nodes of calves with pseudolipidosis (Hocking et al., 1972). The compound was water soluble and would be expected to be readily leached out of tissues during preparation for microscopy. It is not surprising therefore that Whittem and Walker and Jolly (1971) were unable to demonstrate any specific substance within the vacuoles in neurones and reticuloendothelial cells of lymph nodes.

The disease appeared to have many characteristics of an inborn lysosomal disorder, and the nature of the storage compound suggested a possible deficiency of α-mannosidase. Hocking et al. measured the activity of this enzyme, plus eight other lysosomal enzymes, in tissues from affected calves and found an almost complete deficiency of α-mannosidase activity at pH 4.3, while the activities of the other enzymes were elevated. In studies on the pH dependence of α-mannosidase activity in plasma and serum, Hocking et al. discovered that while the optimum activity of this enzyme in a control calf was around pH 4.0 - 4.5, there was an absolute deficiency of α-mannosidase activity below pH 4.75 in diseased calves. Control and diseased calves both possessed activity between pH 4.75 and pH 6.5, suggesting the presence of at least two enzymes with α-mannosidase activity in normal animals, with pH optima at 4.3 and 5.5 respectively, and only the acidic form being deficient in "pseudolipidosis". These findings were confirmed and extended by Phillips et al. (1974b). Similarities between this disease in Angus cattle and

human mannosidosis prompted Hocking et al. to introduce the name "mannosidosis" of Angus cattle to replace the less accurate descriptive term "pseudolipidosis".

Mannose-containing oligosaccharides are excreted in the urine of calves with mannosidosis (Norden et al., 1973c; Lundblad et al., 1975). At least five oligosaccharide fractions were detected by paper chromatography, the most abundant compound having the structure: α-D-mannopyranoside-(1->6)-β-D-mannopyranoside-(1->4)-β-D-GlcNAcp-(1->4)-β-D-GlcNAcp-(1->4)-D-GlcNAc (Lundblad et al., 1975). This pentasaccharide is distinct from the oligosaccharides previously reported in brain (Norden et al., 1973c) and lymph nodes (Hocking et al., 1972) of affected calves, and has not been observed in the urine of patients with human mannosidosis (Norden et al., 1973b, 1974; Lundblad et al., 1975).

3. α-Mannosidase (EC 3.2.1.24, α-D-mannoside mannohydrolase)

α-Mannosidase is widely distributed in nature. Its activity has been detected in virtually every mammalian tissue or fluid, with the male genital tract being a particularly abundant source (Conchie et al., 1956, 1959a and b; Conchie and Mann, 1957; Barrett, 1972; Snaith and Levvy, 1973). It is a very stable enzyme, dependent on zinc for maximum activity, and is inhibited by EDTA and certain cations including cobalt, cadmium, mercury, silver and copper (Conchie and Hay, 1959; Snaith and Levvy, 1968, 1969; Crow, 1971). A pH optimum of 5.0 was reported by Conchie and Hay (1959) for α-mannosidase activity in a variety of mammalian tissues, while Crow (1971) found optimal activity at pH 4.0 in bovine liver homogenates. Its localization within lysosomes was demonstrated by Conchie and Hay (1963) and Bowers and de Duve (1967).

In normal human and bovine tissues there are two components of acidic α-mannosidase with pH optima at approximately 4.0 - 4.3, an

intermediate form with optimum activity at approximately pH 5.5 and a more neutral form with activity at pH 6.5 (Hocking et al., 1972; Dewald and Touster, 1973; Phillips et al., 1974a; Winchester et al., unpublished data). The acidic α-mannosidase isoenzymes are presumably of lysosomal origin and both are absent from the tissues and body fluids of children and Angus calves with mannosidosis. The neutral and intermediate isoenzymes persist in mannosidosis patients and contribute to the residual α-mannosidase activity (Carroll et al., 1972; Phillips et al., 1974a and b; Masson et al., 1974).

The intermediate form of α-mannosidase (pH 5.5) appears to be associated with the Golgi apparatus (Dewald and Touster, 1973), and there is evidence to suggest that the neutral form (pH 6.5) localizes in the soluble cytoplasmic fraction, at least in rat liver (Marsh and Gourlay, 1971).

VI. RECOGNITION OF INDIVIDUALS HOMOZYGOUS AND HETEROZYGOUS FOR INBORN LYSOSOMAL DISEASES

Until the last decade the inborn lysosomal disorders had been poorly understood and their diagnosis had depended largely on clinical signs and histopathology. A greater understanding of their pathogenesis has led to the development of biochemical tests which have facilitated the differentiation of phenotypically similar diseases, and in many cases made possible the detection of heterozygous carriers.

1. Diagnosis of homozygous individuals

(a) Clinical signs: The inborn lysosomal diseases are generally characterized by progressive neurological deterioration, but a variety of other signs, including musculo-skeletal disorders, may also be exhibited. In some cases relatively specific abnormalities,

such as the cherry-red spot in the retina of children with certain gangliosidoses (O'Brien et al., 1971), may assist a clinical diagnosis, but due to the similarity of the clinical manifestations in many closely related lysosomal storage diseases an accurate diagnosis cannot depend solely on such features.

(b) Pathology: A more precise diagnosis may be obtained from histopathological and histochemical studies of tissues from affected individuals, obtained either by biopsy or after necropsy. A variety of intracytoplasmic inclusions have been revealed by light and electron microscopy and the nature of the storage products may, in some cases, be identified either by their histochemical staining properties or their ultrastructural features. In some diseases the ultrastructural characteristics of the inclusions may be relatively specific, such as the tubular inclusions of Gaucher's disease (Brady and King, 1973a) and Krabbe's disease (Yunis and Lee, 1969; Suzuki and Suzuki, 1973), and the characteristic cystine crystals in cystinosis (Seegmiller, 1973). In others, such as aspartylglycosaminuria (Haltia et al., 1975), mannosidosis (Kjellman et al., 1969; Jolly, 1971; Autio et al., 1973) and the mucopolysaccharidoses (Van Hoof, 1973a), the storage product is very soluble and is usually removed during routine fixation and embedding procedures, leaving relatively clear vacuoles surrounded by a single membrane. Many storage diseases therefore may not be differentiated on the basis of clinical and pathological findings, and in such cases biochemical tests to determine the characteristics of the stored substance and the enzymic defect are required.

(c) Chemistry of storage product: Characterization of the stored material has been accomplished for most recognized inborn lysosomal diseases (Neufeld et al., 1975). This knowledge not only allows

partial classification of the disease, but also provides an insight into the nature of the enzymic defect. The accumulation of storage products commonly occurs in cells of the central nervous system and reticuloendothelial system. The relative unavailability of these tissues for chemical analyses restricts the value of storage product estimations in ante-mortem diagnosis. Heterogeneity of the storage material is a feature of many inborn lysosomal diseases (Hers, 1973), and the fact that different compounds may be stored in different organs must be considered when making a diagnosis. If the storage product is water-soluble or of relatively small molecular weight then it may be excreted in increased amounts in the urine (Robinson, 1974). This occurs, for example, in the mucopolysaccharidoses (Van Hoof, 1973a; Neufeld, 1974), aspartylglycosaminuria (Jenner and Pollitt, 1967) and in mannosidosis of man (Norden et al., 1973a and b, 1974; Autio et al., 1973) and Angus cattle (Norden et al., 1973c; Lundblad et al., 1975). Identification of these compounds in urine by rapid and inexpensive techniques such as thin-layer chromatography (Humbel and Collart, 1975) may allow widespread screening for such diseases in children.

The same clinical syndrome and the intracellular accumulation of the same materials may arise from different basic enzyme deficiencies therefore a knowledge of the specific enzymic anomaly is necessary for a precise diagnosis of an inborn lysosomal disease (Hers and Van Hoof, 1969).

(d) Enzymic deficiency: Partial or complete deficiencies of one or more lysosomal enzymes have now been established for almost all the recognized inborn lysosomal diseases (Hers, 1973; Neufeld et al., 1975). The demonstration of such a deficiency in the tissues of a patient is the most reliable method of diagnosis and allows

relatively precise classification of the disease. Lysosomal enzymes are present in most tissues, plasma and certain other body fluids. Because of accessibility, plasma (serum) and peripheral leucocytes have proven most convenient for routine investigations. Cultured skin fibroblasts also reflect the lysosomal enzyme complement of an individual. Most lysosomal storage diseases may be reliably diagnosed by assays for the activity of specific enzymes in serum, leucocytes or cultured skin fibroblasts.

Prenatal diagnosis of inborn lysosomal diseases by enzyme assays on amniotic fluid or amniotic cells obtained by paracentesis has been an important development (Kaback and Howell, 1973). Heterozygous couples who have a 25% chance of producing an affected child may choose to have only unaffected children by allowing the monitoring of each pregnancy and the selective abortion of diseased foetuses.

2. Detection of heterozygous individuals

Although heterozygotes for most inborn lysosomal disorders do not exhibit clinical manifestations of the disease, they possess a partial deficiency of the enzyme. A simple gene-dosage relationship often applies with the average level of enzyme activity in heterozygotes being approximately 50% of that found in normal homozygotes (Aebi, 1967; Harris, 1971). This gene-dosage phenomenon provides a convenient basis for methods of heterozygote detection. Variations in enzyme activities due to physiological, pathological and genetic factors may however cause overlapping in enzyme activities between normal and heterozygous populations, therefore the identification of heterozygotes can not always be made with certainty (Aebi, 1967; Harris, 1971; Kaback and Zeiger, 1972; Jolly et al., 1973; Kaback et al., 1974).

(a) Heterozygote detection using serum or plasma: Assays of enzyme activity in serum or plasma have been successfully employed in detecting heterozygotes for several inborn lysosomal diseases (Table 1.II).

TABLE 1.II

Inborn lysosomal storage diseases for which serum or plasma have been successfully employed in heterozygote detection

Disease	Key references
G_{M2}-gangliosidosis Type I (Tay-Sach's disease)	O'Brien et al. (1970, 1972), Kaback and Zeiger (1972)
G_{M2}-gangliosidosis Type II (Sandhoff's disease)	Suzuki et al. (1973)
Juvenile G_{M1}-gangliosidosis	O'Brien et al. (1972)
Sanfilippo syndrome (Subtype B)	Von Figura et al. (1973)
Fabry's disease	Kint (1970)
Globoid cell leucodystrophy	Suzuki et al. (1972)
Mannosidosis of Angus cattle	Hocking et al. (1972), Jolly et al. (1973)

O'Brien et al. (1972) were able to differentiate obligate Tay-Sach's heterozygotes from healthy controls by expressing serum hexosaminidase A activity as a percentage of total serum hexosaminidase activity. A 5% overlap between the two populations was reduced to 1% on retesting of equivocal cases. This test was unreliable when employed on pregnant women, women on oral contraceptives and on individuals suffering from severe illnesses such as diabetes mellitus. In such cases the relative percentage of

hexosaminidase A in serum is reduced and there is a greater overlap in values between heterozygotes and normal controls (Saifer et al., 1972; Navon et al., 1973; Kaback et al., 1974).

A test for bovine mannosidosis heterozygotes has been developed on the basis of acidic α-mannosidase activity (pH 4.3) in plasma (Hocking et al., 1972; Jolly et al., 1973). Plasma was preferred to serum as variable increases in α-mannosidase activity were detected in serum relative to plasma samples from the same animals.

(b) Heterozygote detection using peripheral leucocytes: The ready availability of leucocytes has encouraged their use in heterozygote testing for many inborn lysosomal diseases. Table 1.III lists the diseases for which enzyme assays on extracts of circulating leucocytes have been employed with success in heterozygote detection.

The status of pregnant women with regard to the Tay-Sach's genotype may be more reliably determined by assays of hexosaminidase activity in leucocytes than in serum (Saifer et al., 1972; Navon et al., 1973).

Leucocytes offered no advantage over serum in the detection of heterozygotes for human globoid cell leucodystrophy. Suzuki et al. (1972) found considerable overlap in leucocyte extract galactocerebroside β-galactosidase activities between heterozygotes and normal controls, despite significantly different mean values. Similarly, Hocking et al. (1972) found a significant difference between mean leucocyte α-mannosidase activities in heterozygotes for bovine mannosidosis and control animals, but again there was some overlap between the two populations.

Masson et al. (1974) were unable to separate normal individuals from human mannosidosis heterozygotes on the basis of their serum

TABLE 1.III

Inborn lysosomal storage diseases for which heterozygote detection based on enzyme assays of leucocyte extracts has been successful

Disease	Key references
Fabry's disease	Kint (1970)
Gaucher's disease	Beutler and Kuhl (1970), Brady *et al*. (1971)
Pompe's disease	Koster *et al*. (1974)
G_{M2}-gangliosidosis Type I	Kaback and Zeiger (1972), Navon *et al*. (1973)
G_{M2}-gangliosidosis Type II	Harzer (1973)
G_{M1}-gangliosidosis Type I	O'Brien *et al*. (1971)
G_{M1}-gangliosidosis Type II	Patel *et al*. (1974b)
Niemann-Pick's disease	Brady *et al*. (1971), Brady and King (1973b)
Metachromatic leucodystrophy	Bass *et al*. (1970), Taniguichi and Nanba (1970), Hackett *et al*. (1971)
Fucosidosis	Matsuda *et al*. (1973)
Lysosomal acid-phosphatase deficiency	Nadler and Egan (1970)
Wolman's disease	Patrick and Lake (1973)

or leucocyte α-mannosidase activities (pH 4.0) alone, but claimed that heterozygotes and normal individuals could be differentiated when α-mannosidase activity was expressed relative to total hexosaminidase activity. The validity of employing hexosaminidase as a reference enzyme in this manner is questionable (see Chapter V).

In some situations it appears that the ability to detect carriers using peripheral leucocytes may be enhanced by stimulating the lymphocytes with phytohaemagglutinin, thereby selecting a more homogeneous population of cells and possibly amplifying the genetic differences by accelerating cellular metabolism (Kaback and Howell, 1973). Success with this method has been reported in detecting carriers for Pompe's disease (Hirschhorn et al., 1969) and lysosomal acid-phosphatase deficiency (Nadler and Egan, 1970).

(c) Heterozygote detection using cultured fibroblasts: The culture of human fibroblasts from skin biopsies has become a routine and valuable aid in the diagnosis and experimental investigation of many inherited disorders of man. Enzyme assays on cultured fibroblasts have enabled the successful detection of heterozygotes for many human inborn lysosomal disorders (Table 1.IV).

Although a number of variables may affect the metabolism and enzymic composition of fibroblasts in culture (Beutler et al., 1970; Milunsky et al., 1972; Galjaard et al., 1974) it has been claimed that the uniformity of the cell population provides a more accurate method of differentiating heterozygotes from normal individuals than is obtained with either serum or leucocytes (Kaback and Howell, 1970; Beutler et al., 1971). In G_{M2}-gangliosidosis Type II (Sandhoff's disease) obligate heterozygotes can

be differentiated from normal controls on the basis of total hexosaminidase activity in cultured skin fibroblasts, but not in serum.

The use of cultured skin fibroblasts in testing for heterozygotes has not been reported in domestic animals.

TABLE 1.IV

Human inborn lysosomal storage diseases for which heterozygote detection using cultured skin fibroblasts has been successful.

Disease	Key references
G_{M2}-gangliosidosis Type I	Okada et al. (1971)
G_{M2}-gangliosidosis Type II	Okada et al. (1971)
Gaucher's disease	Beutler et al. (1971) O'Brien et al. (1972)
Globoid cell leucodystrophy	Suzuki and Suzuki (1971)
Metachromatic leucodystrophy	Kaback and Howell (1970) Kaback et al. (1972) Newelt et al. (1972)
Lactosyl ceramidosis	Dawson et al. (1971)
Aspartylglycosaminuria	Aula et al. (1973)
Fabry's disease	O'Brien et al. (1972)

(d) Heterozygote detection by other methods: The success of enzyme assays on serum (plasma), leucocytes and cultured skin fibroblasts in heterozygote detection has not encouraged the use of less accessible tissues or body fluids for this purpose. Tay-Sach's heterozygotes may however be differentiated from normal controls on the basis of hexosaminidase A to hexosaminidase B

ratios in tear fluid (Carmody et al., 1973; Singer et al., 1973). In contrast, enzyme assays on saliva did not successfully differentiate the heterozygotes (Singer et al.).

VII. THE PRACTICAL APPLICATION OF HETEROZYGOTE TESTING

The practical application of methods for detecting heterozygous carriers of human inborn lysosomal diseases lies in the identification of "heterozygous couples", who have a 25% chance of producing a diseased child. The development of methods for the prenatal diagnosis of most of these disorders offers such couples the opportunity of having unaffected children, by allowing the monitoring of each pregnancy and the selective abortion of diseased foetuses.

The initiation of widespread screening programmes to test for heterozygotes is not practical unless the gene frequency is suspected to be relatively high and within a defined population. As most inborn lysosomal disorders are rare, testing for heterozygous carriers is usually confined to the family and relatives of affected individuals. Tay-Sach's disease however is carried by 4.3% of Ashkenazi Jews, at least in North America (Kaback et al., 1974), thus allowing the practical application of a heterozygote testing programme on a population basis. Kaback and Zeiger (1972) initiated a mass screening programme aimed at the prevention of Tay-Sach's disease in the North American Jewish population, and stated three specific requirements which made this scheme feasible:

(i) the disorder was known to occur with a high frequency in a finite population.

(ii) a simple, accurate and inexpensive heterozygote detection test was available.

(iii) prenatal detection of the disease was possible at a stage early enough for termination of pregnancy if necessary.

These requirements were modified and extended by Jolly et al. (1974) to make them applicable for the control of similar inherited diseases in animals, and may be summarized as follows:

(i) the disease must occur in a finite population with a gene-frequency of sufficient magnitude to make it economically important.

(ii) a simple, inexpensive and accurate test for heterozygotes is required, and a reliable back-up test for equivocal animals should be available.

(iii) control of the disease by culling of certain heterozygotes should not be to the detriment of the breed's genetic structure, or cause excessive loss of valuable breeding stock.

(iv) the scheme should be acceptable to breeders, and should not interfere with other important disease-control programmes.

(v) there should be provision for adequate genetic counselling and either breed society rules or legislation to ensure that control measures are instigated.

The gene frequency for mannosidosis in New Zealand's Angus cattle population appears to be considerably higher than that for Tay-Sach's disease in Ashkenazi Jews (Hocking et al., 1972; Jolly et al., 1973), and the disease is considered to be of economic importance to the New Zealand beef industry (Jolly and Blakemore, 1973; Jolly et al., 1974; Jolly, 1975). The development of a simple and relatively accurate method of heterozygote detection (Jolly et al., 1973) introduced the possibility of initiating a widespread screening programme aimed at the

rapid and economical control of this disease. The initial step towards such a scheme was a pilot testing programme in which several thousand cattle throughout the country were tested in an effort to gain further information on the incidence of heterozygotes, and to assess the accuracy and reliability of the test. An analysis of the results of this pilot scheme is presented in Chapter IV.

CHAPTER II

MATERIALS AND METHODS

Standard techniques employed throughout this study are presented in this chapter. Information regarding the animals used in each experiment and specific details of other methods are included within each chapter.

COLLECTION AND PREPARATION OF SAMPLES

1. Collection of plasma

Blood was collected from the jugular vein or the middle coccygeal blood vessels into heparinized Vacutainers*. Plasma was separated by centrifugation at 2,000 g x 5 min, usually within 4 hr of collection, and stored at -12°C until required for assay. Transport of samples involved the use of either dry ice or refrigerated van at -20°C.

2. Separation of lymphocytes

Lymphocyte separation was based on the method of Boyum (1968). Two x 10 ml EDTA Vacutainers of blood were collected from each animal and thoroughly mixed with the anticoagulant. After centrifugation of Vacutainers at 2,000 g x 10 min, the buffy coats were removed and diluted with 2.0 ml of 0.85% NaCl. This cell suspension was gently layered upon 3.0 ml of Lymphoprep** in a centrifuge tube (Figure 2.1a) and centrifuged at 400 g x 30 min. After centrifugation the lymphocytes were deposited at the interface, whereas erythrocytes and granulocytes were deposited at the bottom of the tube (Figure 2.1b). The lymphocytes were removed and washed twice in 0.85% NaCl at 160 g x 5 min to remove platelets, and once in 0.15M-NH_4Cl to lyse any contaminating erythro-

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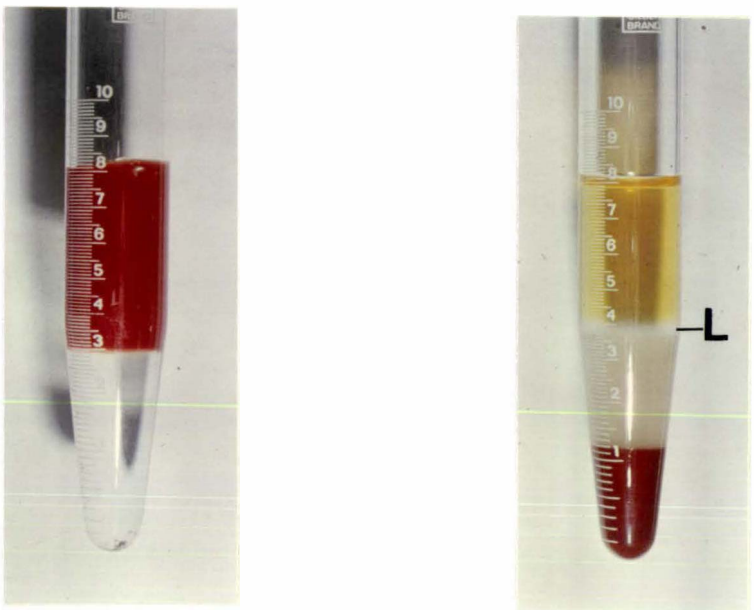
* Becton Dickinson & Co. Ltd., Dublin, Ireland.

** Nyegaard & Co., Oslo, Norway.



a b

Figure 2.1: Method for the separation of lymphocytes from whole blood.
(a) Buffy coat suspension layered upon 3.0ml of Lymphoprep.
(b) After centrifugation at 400g x 30min lymphocytes (L) are deposited at the interface.

cytes. The lymphocytes were then stored as a pellet at -12°C until required for assay.

3. Separation of thrombocytes

Thrombocytes were obtained as a by-product of the above procedure for separating lymphocytes. Instead of discarding the supernatant from the first wash of lymphocytes in 0.85% NaCl, this suspension, which contained a relatively pure population of thrombocytes, was retained. After centrifugation at 160 g x 5 min to remove any remaining lymphocytes and erythrocytes, the supernatant was again retained and centrifuged at 400 g x 5 min to sediment the thrombocytes. The supernatant was discarded and the thrombocytes were stored as a pellet at -12°C until required for assay.

4. Separation of granulocytes

Two x 10 ml EDTA Vacutainers of blood from each animal were centrifuged at 2,000 g x 10 min. After complete removal of the buffy coat and plasma the remaining cells from each tube, consisting of erythrocytes and granulocytes, were pooled. Erythrocytes were flash-lysed with 10 ml of distilled water for 30 s. Isotonicity was restored by the addition of 10 ml of 1.7% NaCl. The granulocytes were washed once in 0.15M-NH_4Cl to lyse any remaining erythrocytes, and once in 0.85% NaCl, at 160 g x 5 min and were stored as a pellet at -12°C until required for assay.

PREPARATION OF TISSUES FOR HISTOLOGICAL STUDIES

1. Light microscopy

Tissues for light microscopy were fixed in either 10% formal saline or in Bouin's solution, and routine procedures for paraffin embedding and sectioning were employed. Sections were stained with haemotoxylin and eosin, and by periodic acid-Schiff (PAS) and Masson's trichrome methods.

Sections of 0.5 to 1.0 μm in thickness were also cut for light microscopy from resin-embedded tissues prepared as for electron microscopy. These sections were stained for 20 s with 1% toluidine blue in 0.1M-phosphate buffer (pH 7.2) on a hot-plate at 80°C, and counterstained with 4% basic fuchsin for 10 s at the same temperature.

2. Electron microscopy

Tissues for electron microscopy were obtained as soon as possible after euthanasia of the animal and were sliced into small cubes (approximately 1 mm^3) in modified Karnovsky's solution containing 3% glutaraldehyde and 2% formaldehyde in 0.1M-phosphate buffer (pH 7.2). After 3 hr in this fixative at 4°C, the tissues were washed in 0.1M-phosphate buffer (pH 7.2), and post-fixed for a further 3 hr in 1% osmium tetroxide. Following fixation the tissues were again washed in the buffer, dehydrated by passage through a series of graded alcohol solutions, and embedded in epoxy resin*. Sections of approximately 7,000 nm in thickness were cut on an LKB ultramicrotome and mounted on unsupported copper grids. The sections were stained in 50% ethanol with saturated uranyl acetate for 10 min, and in lead citrate for a further 10 min.

CULTURE OF SKIN FIBROBLASTS

1. Biopsy technique

An area of skin in the dorso-lateral neck region was shaved and disinfected with 1% Hibitaine**. After subcutaneous injection of 2% lignocaine along the dorsal and anterior borders of the shaved area, several plugs of skin approximately 1.5 mm in diameter were removed with the aid of a shortened non-bevelled 12-gauge needle. The epidermis was dissected from each plug of skin and discarded. The dermis was sectioned

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* Fluka durcupan, Switzerland.

** ICI Ltd., Macclesfield, England.

into fragments, less than 1.0 mm in diameter, and placed in approximately 5.0 ml of T199 culture medium* containing 20% foetal bovine serum plus antibiotics (penicillin, streptomycin and kanamycin).

2. Establishment of primary cultures

The plasma-clot embedding technique of Hyman (1968) was employed in establishing primary cultures. After 72 hr at 37°C in culture medium, the fragments of dermis were attached to the surface of 50 ml tissue culture flasks** in clots of fresh plasma. The addition of 0.2 ml sterile 2% calcium chloride to 1.0 ml of oxalated plasma induces clotting after approximately 5 min. Once the clot had formed about each explant 5.0 ml of the same culture medium used above was added to each flask. The primary cultures were incubated at 37°C and the culture medium was changed every 3 days.

The initial outgrowth of fibroblasts from explants usually occurred within 2 or 3 days and by 7 days most explants were usually surrounded by massive numbers of fibroblasts. At this stage the primary cultures were considered to be ready for subculture.

3. Subculture of primary cultures

Following the removal of culture medium and two washes with phosphate-buffered saline, 2.0 ml of 0.05% trypsin*** containing 0.02% EDTA was added to each flask. These were then incubated at 37°C for a further 10 min or until the majority of fibroblasts had become detached from the surface of the flask. The cell suspension was removed and placed in a sterile centrifuge tube. Following centrifugation at approximately 200 g x 5 min most of the supernatant was removed and discarded. The fibroblasts were resuspended in approximately 0.5 ml of remaining supernatant

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* Wellcome Reagents Ltd., Beckenham, England.

** Falcon, Oxnard, California, U.S.A.

*** Trypsin 1:250, Difco Laboratories, Detroit, Michigan, U.S.A.

and divided between two new culture flasks. After the addition of 5.0 ml of culture medium each subculture was incubated for 5 days, by which time a uniform monolayer of fibroblasts had developed.

4. Harvesting of cultured fibroblasts

Once the subcultures had formed monolayers, the fibroblasts were again removed with the aid of trypsin and placed in centrifuge tubes. Following centrifugation at 200 g x 5 min the supernatant was completely removed and discarded. The fibroblasts were washed twice in phosphate-buffered saline and stored as a pellet at -12°C until required for assay.

ENZYME ASSAYS AND PROTEIN ESTIMATIONS

1. Automated assay for α-mannosidase activity in plasma

During the pilot testing scheme assays for α-mannosidase activity in plasma were automated. A Technicon autoanalyser was employed, using 0.47mM-4-methylumbelliferyl-α-D-mannopyranoside as substrate in 37mM-sodium acetate buffer (pH 4.3). The incubation mixture consisted of 0.05 ml of plasma and 1.6 ml of substrate solution. Following a 12 min incubation at 42°C the reaction was stopped by the addition of 2.5 ml of glycine buffer (pH 10.0) and fluorescence was measured in a flow-through cell on a Turner model 111 fluorometer using primary filter 7-60 and secondary filter 22.

2. Assays for five lysosomal enzymes in tissue extracts

Frozen pellets were thawed on the day of assay and diluted with 0.85% NaCl containing 0.1% Triton X-100*. The volumes of diluent added were 0.5 ml to thrombocyte pellets, 1.5 ml to lymphocyte and fibroblast pellets and 3.0 ml to granulocytes. Samples were then sonicated for 30 s at 3.0 amperes using a Dawe Soniprobe and centrifuged at 2,000 g x 5 min, the supernatant being retained for assay.

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* BDH Chemicals Ltd., Poole, England.

Methods employed in assays for the activities of five lysosomal enzymes in tissue extracts are summarized in Table 2.I. These methods were based on those of various authors and were developed after a study of the principal kinetic characteristics of each enzyme in bovine lymphocyte extracts (see Chapter V).

Fluorogenic substrates were used for assays of α-mannosidase, β-galactosidase and hexosaminidase activity. Fluorescence was measured on a Turner model 111 fluorometer using the same filters as mentioned previously and the release of 4-methylumbelliferone was determined by reference to a standard curve. Assays for α-fucosidase and arylsulphatase A activity involved colour reactions, and absorbance was measured at 400 nm and 515 nm respectively on a Spectronic 20 spectrophotometer.

For pH dependence studies substrates were prepared in distilled water and buffers were added independently to each incubation mixture. For pH dependence investigations of α-mannosidase activity a range of 0.2M-Na_2HPO_4/citric acid buffers replaced the sodium acetate/acetic acid buffer employed in routine assays.

3. Protein estimations

The folin phenol method of Lowry et al. (1951) was employed in estimating the protein concentration of tissue extracts.

TABLE 2.I

Assay methods for five lysosomal enzymes in bovine tissue extracts

Enzyme	Substrate and concentration	Buffer	Incubation mixture	Incubation time	Reaction terminated with:
α-mannosidase	6mM-4-methyl-umbelliferyl-α-D-mannopyranoside	2M-CH_3COONa/CH_3COOH (pH 4.3)	0.05 ml buffered substrate 0.01 ml tissue extract	20 min	2.0 ml 0.5M-glycine buffer (pH 10.0)
β-galactosidase	1mM-4-methyl-umbelliferyl-β-galactopyranoside monohydrate	0.2M-Na_2HPO_4/citric acid (pH 4.5)	0.5 ml buffered substrate 0.05 ml tissue extract	20 min	1.5 ml 0.5M-glycine buffer (pH 10.0)
hexosaminidase	6mM-4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside	0.2M-Na_2HPO_4/citric acid (pH 4.5)	0.05 ml buffered substrate 0.01 ml tissue extract	20 min	2.0 ml 0.5M-glycine buffer (pH 10.0)
α-fucosidase	2mM-p-nitrophenyl-α-L-fucoside	0.2M-Na_2HPO_4/citric acid (pH 5.25)	0.5 ml buffered substrate 0.1 ml tissue extract	2 hr	1.5 ml 0.5M-glycine buffer (pH 10.0)
arylsulphatase A	6mM-nitrocatechol sulphate	1M-CH_3COONa/CH_3COOH containing 0.5mM-$Na_4P_2O_7$ plus 10% NaCl (pH 4.75)	0.5 ml buffered substrate 0.1 ml tissue extract	2 hr	0.5 ml 3M-NaOH

CHAPTER III

THE PATHOLOGY OF MANNOSIDOSIS IN ANGUS CATTLE

INTRODUCTION

The pathological features of mannosidosis in Angus cattle were initially described by Whittem and Walker (1957) after examination of four affected calves ranging from 6 to 15 months of age. Slightly enlarged superficial lymph nodes were noted in one calf and mild internal hydrocephalus in another, but no gross abnormalities were common to all four calves. Microscopic features included widespread swelling and vacuolation of neurones in the brain, hypertrophy of Purkinje cell dendrites and axons, and numerous slightly eosinophilic bodies scattered throughout the brain. The medullary sinusoids of lymph nodes were packed with vacuolated macrophages, the pancreatic exocrine epithelium was vacuolated and convoluted tubules in the kidney cortex contained occasional vacuolated epithelial cells.

The above findings were confirmed by Jolly (1970) who recorded the occurrence of this disease in New Zealand. This report was followed by a more detailed study of the pathology of the central nervous system in mannosidosis based on observations made on 30 affected Angus calves up to 18 months of age (Jolly, 1971). Mild internal hydrocephalus was a frequent macroscopic observation, while the primary microscopic lesion was vacuolation of neurones. This neuronal vacuolation was observed at all levels of the brain but those in certain areas were more severely affected than others. Ultrastructural evidence was presented suggesting that the vacuoles arose from saccular dilatations of the Golgi apparatus. The eosinophilic bodies described by Whittem and Walker were found to be swellings of axons due to local accumulations of electron-dense bodies, mitochondria and local proliferation of neurofilaments. The storage

compound in bovine mannosidosis was identified by Hocking et al. (1972) as an oligosaccharide containing mannose and glucosamine. As this compound is water-soluble it would be expected to be removed during the dehydration of tissues prior to embedding in paraffin or epoxy resin or during aqueous staining procedures, and it is not surprising that neither Whittem and Walker nor Jolly (1971) were able to demonstrate any specific compounds within vacuoles in routinely prepared tissues, in spite of employing a variety of histochemical staining techniques.

Considerable interest has been shown recently in enzyme replacement therapy for inborn lysosomal diseases of man. It is the intention of this laboratory to exploit bovine mannosidosis as an animal model for research in this field. A more detailed study of the pathology of this disease is a necessary prerequisite to such investigations. Because the pathology of the central nervous system in bovine mannosidosis was reported by Jolly (1971), it was not reinvestigated in detail, and emphasis was placed on the study of other tissues.

MATERIALS AND METHODS

Tissues from six mannosidosis calves ranging from 3 days to 12 months of age were embedded in epoxy resin and sectioned for light microscopy and ultrastructural studies following the procedures outlined in Chapter II. Similar tissues from three normal Angus control calves were prepared in the same manner. Paraffin-embedded tissues from a further 30 calves with mannosidosis were sectioned for examination by light microscopy.

RESULTS

1. Gross pathology

Calves with mannosidosis were generally undersized and in relatively poor condition. The majority showed a mild internal hydrocephalus, but

in one calf, which appeared blind and died at six days of age, the internal hydrocephalus was severe. Lymph nodes throughout the body were almost invariably enlarged to approximately twice normal size. No other consistent macroscopic abnormalities could be attributed to the disease.

2. Histopathology

The outstanding histological lesion was an intracytoplasmic vacuolation of many tissues, varying in severity between different cell-types and tending to be more pronounced in older calves. Ultrastructurally vacuoles were seen to be surrounded by a single tripartate membrane of approximately 900 nm in thickness (Figures 3.1, 3.14). Although most vacuoles were electron-lucent they often contained a moderate amount of amorphous electron-dense material and occasional membranous fragments and fibrillar elements. Large vacuoles in apposition to each other often appeared to be connected (Figure 3.22).

(a) Reticuloendothelial system

In medullary sinusoids of lymph nodes there were large numbers of highly vacuolated free macrophages and fixed reticuloendothelial cells (Figures 3.2, 3.3). Vacuoles in these cells were approximately 1-2 μm in diameter but some were up to 12 μm in diameter in free macrophages. Ultrastructurally they were bounded by a single membrane (Figure 3.4). In some macrophages, vacuoles appeared to represent "sausage-like" dilatations of smooth endoplasmic reticulum (Figure 3.5). Empty saccules, resembling the intracellular vacuoles, were often observed extracellularly in medullary sinusoids (Figure 3.4) but it is not known whether these represented the release of vacuoles from cells ante-mortem or during the preparation of tissues for microscopy. Vacuolation was not commonly observed in either mature or immature lymphocytes.

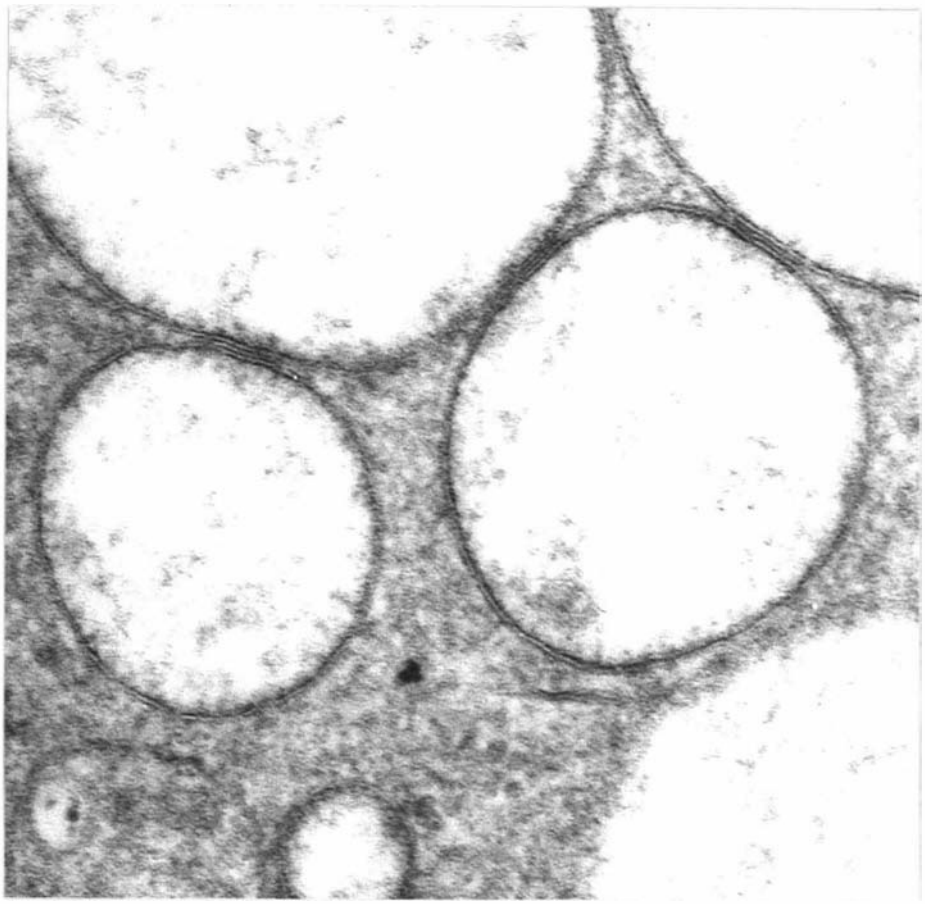
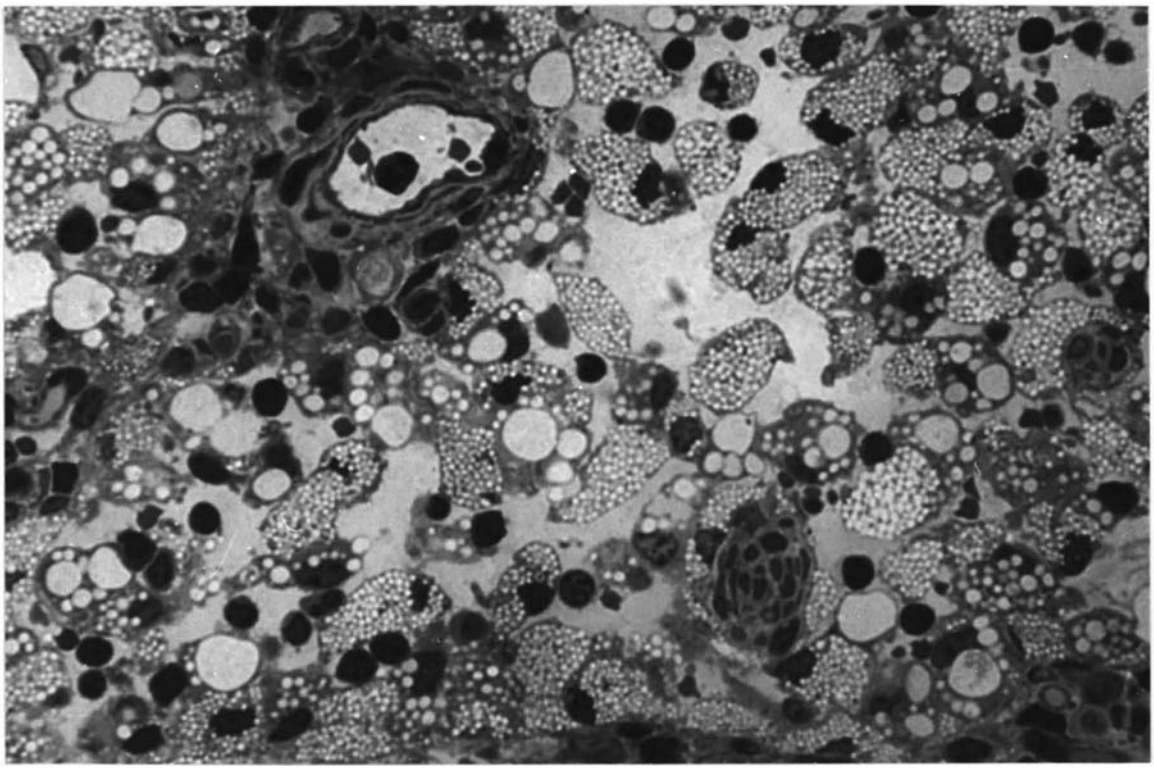


Figure 3.1: Vacuoles, bounded by a tripartate membrane approximately 900 nm in thickness, within a neurone of a calf with mannosidosis. (E.M. x 140,000)

Figure 3.2: Medullary area of a lymph node from a calf with mannosidosis, showing highly vacuolated free macrophages within sinusoids. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 750)

Figure 3.3: Medullary area of a lymph node from a calf with mannosidosis showing highly vacuolated fixed reticuloendothelial cells and free macrophages. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)



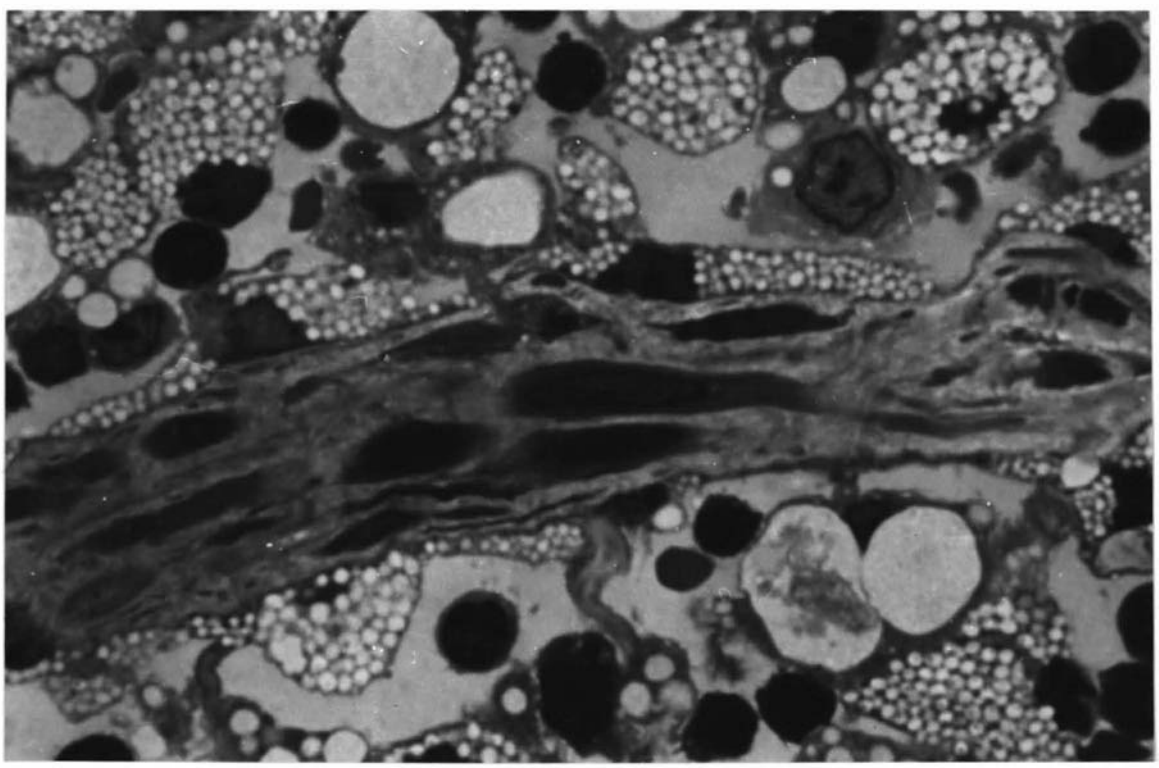
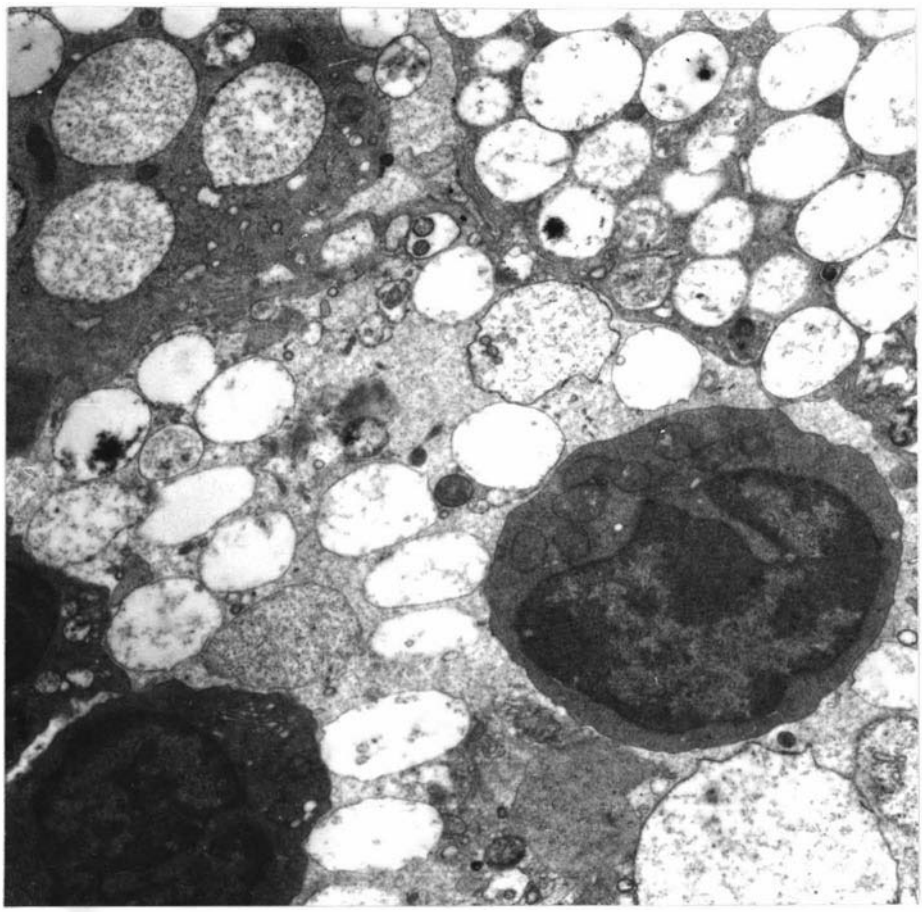
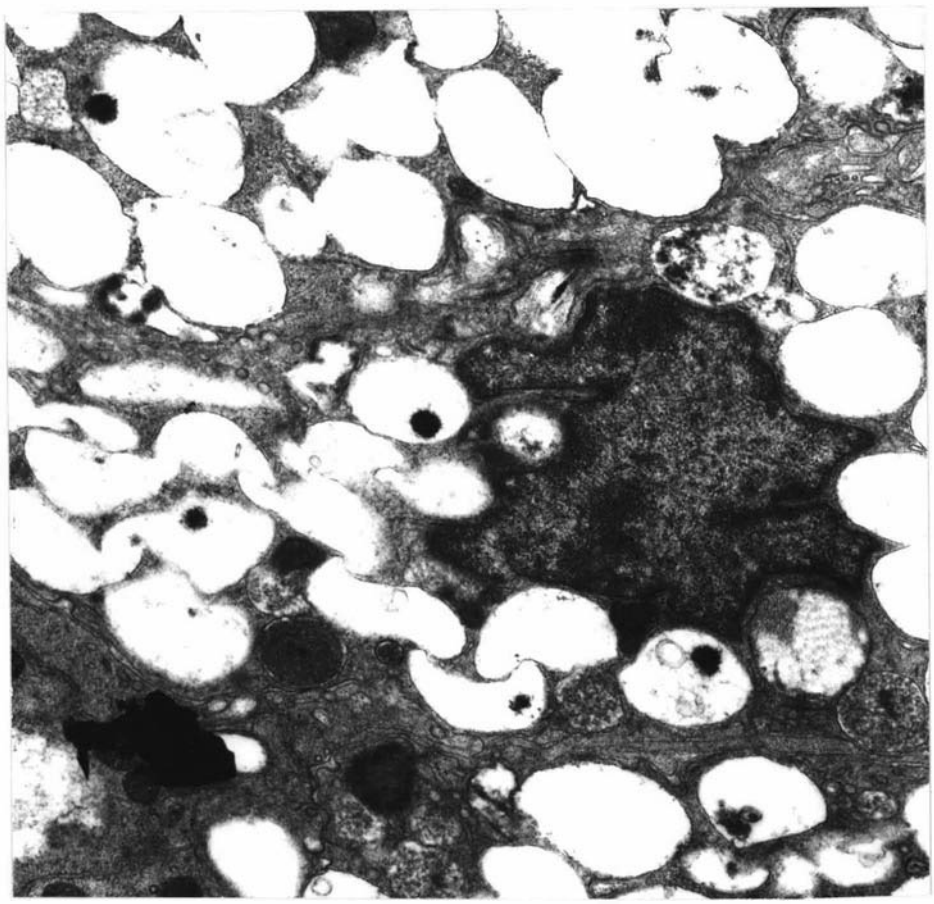


Figure 3.4: Lymph node from a calf with mannosidosis showing membrane-bound vacuoles within macrophages, and saccules within the medullary sinusoid with a similar appearance to intracellular vacuoles. (E.M. x 10,500)

Figure 3.5: Lymph node macrophage from a mannosidosis calf containing vacuoles and "sausage-like" dilatations of smooth endoplasmic reticulum. (E.M. x 12,000)





Kupffer cells in the liver were packed with vacuoles 1-2 μm in diameter, and their cytoplasm occupied considerably more space in hepatic sinusoids than normal (Figures 3.6, 3.7, 7.4b). This extensive vacuolation of Kupffer cells was relatively inapparent in sections prepared for light microscopy from paraffin embedded liver tissue.

Reticuloendothelial cells in the spleen were only moderately affected. Plasma cells observed in sinusoids sometimes contained a small number of vacuoles approximately 1 μm in diameter (Figure 3.8).

(b) Central nervous system

The pathological changes noted in the central nervous system confirmed those reported by Jolly (1971) and therefore will be described only briefly. Neurones in most areas of the brain and spinal cord contained large numbers of small clear vacuoles (Figure 3.9). As was observed in the previous study, vacuoles often appeared to be continuous with Golgi apparatus (Figure 3.10). Spheroids were present throughout the grey and white matter of the brain and spinal cord and were particularly common in the cerebellar white matter.

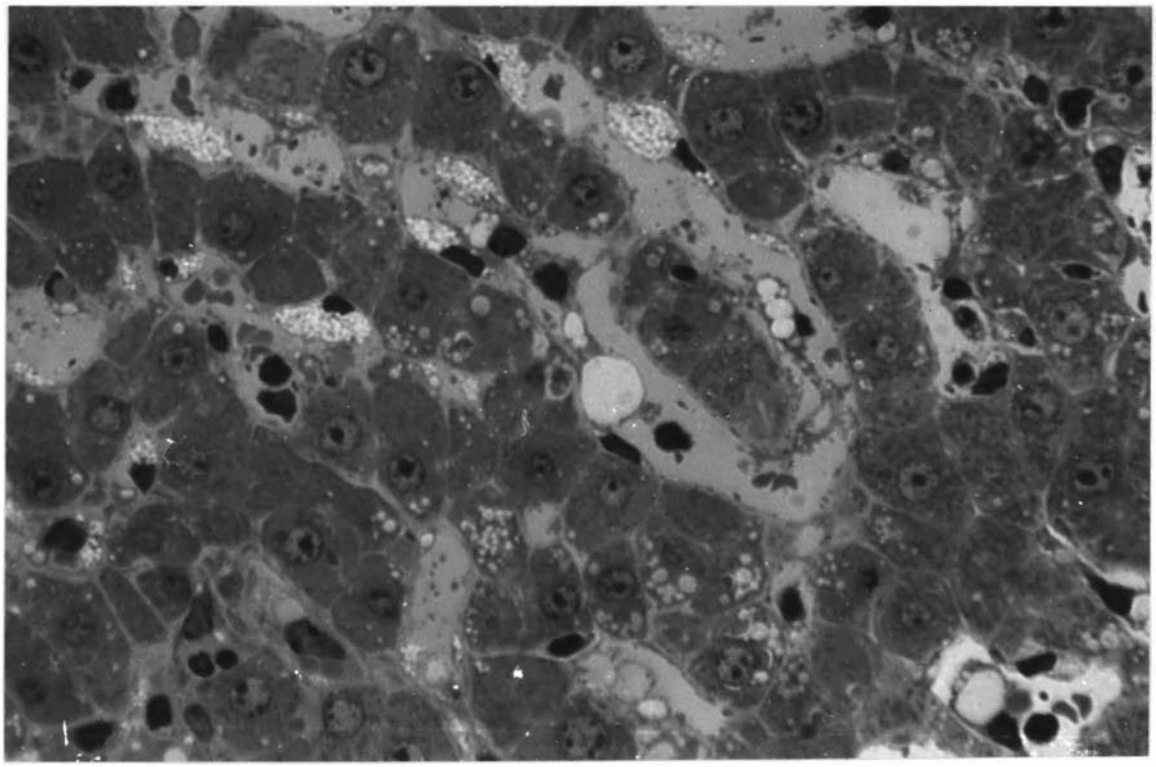
Ganglion cells of myenteric plexuses in the gastrointestinal tract also showed pronounced vacuolation. Other autonomic ganglia were not examined.

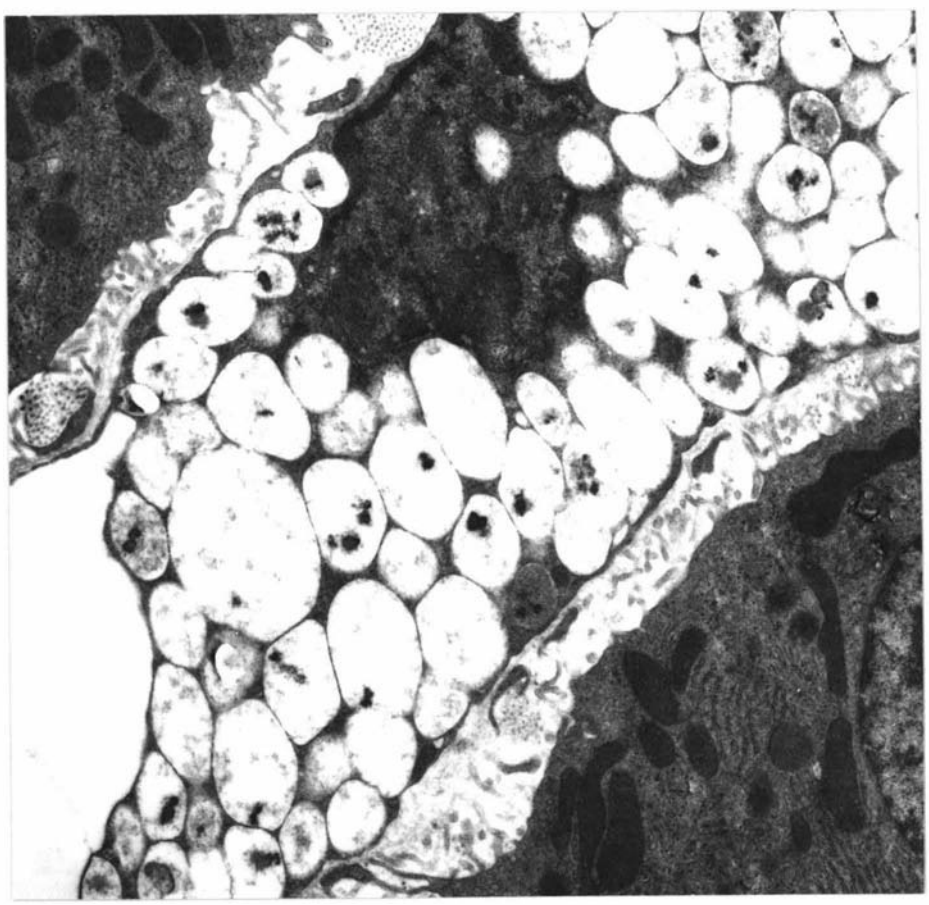
(c) Epithelial tissues

There was vacuolation of exocrine epithelial cells in the pancreas, lacrimal glands, submaxillary salivary glands and abomasum. In the pancreas large clear vacuoles, mainly in the basal areas of cells, contrasted with the darker staining zymogen granules in apical zones (Figures 3.11, 3.12). The severity of

Figure 3.6: Liver from a mannosidosis calf showing highly vacuolated Kupffer cells lining sinusoids. Vacuoles are also present within some hepatocytes. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 625)

Figure 3.7: Highly vacuolated Kupffer cell from the liver of a calf with mannosidosis. (E.M. x 10,500)





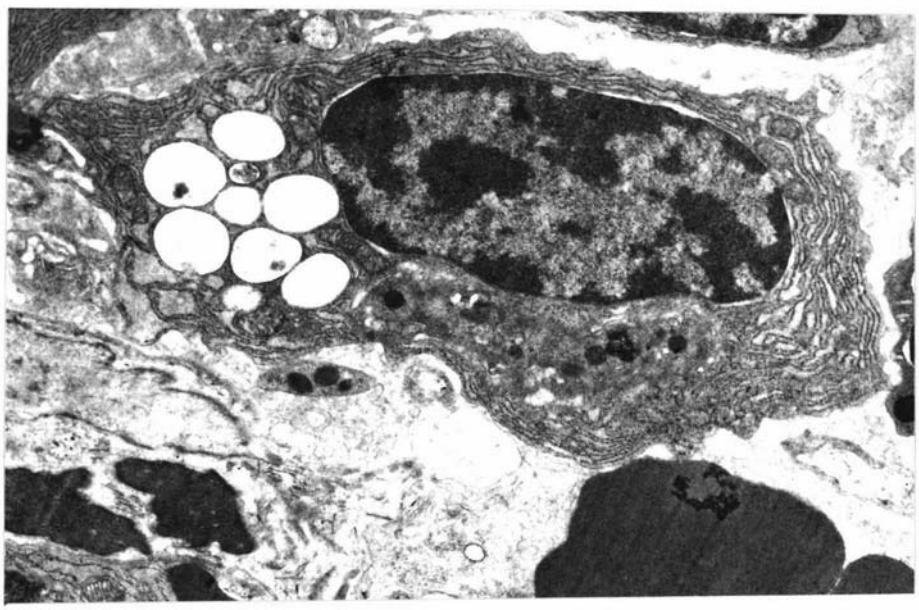
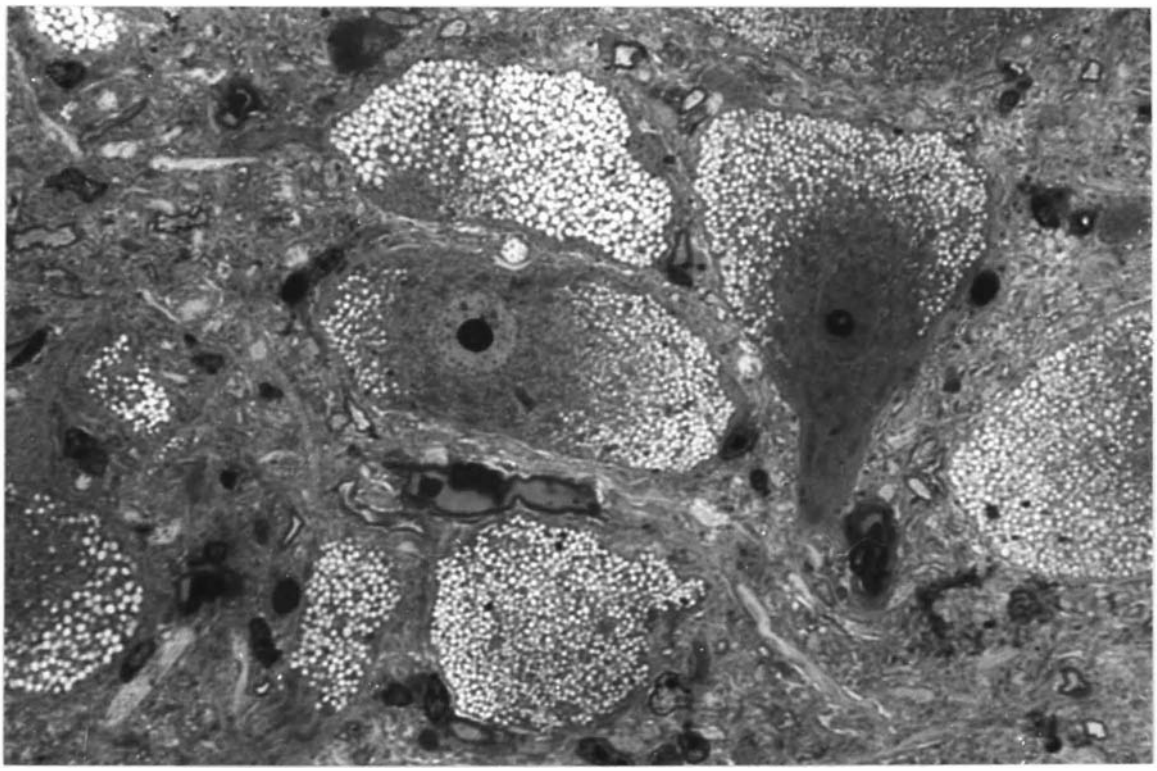


Figure 3.8: Vacuoles within a plasma cell in the spleen of a calf with mannosidosis. (E.M. x 7,500)

Figure 3.9: Finely vacuolated neurones in the medulla of the brain of a calf with mannosidosis. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 15,60)

Figure 3.10: Vacuole appearing to show continuity with a Golgi apparatus (G) in a neurone of a calf with mannosidosis. (E.M. x 30,600)



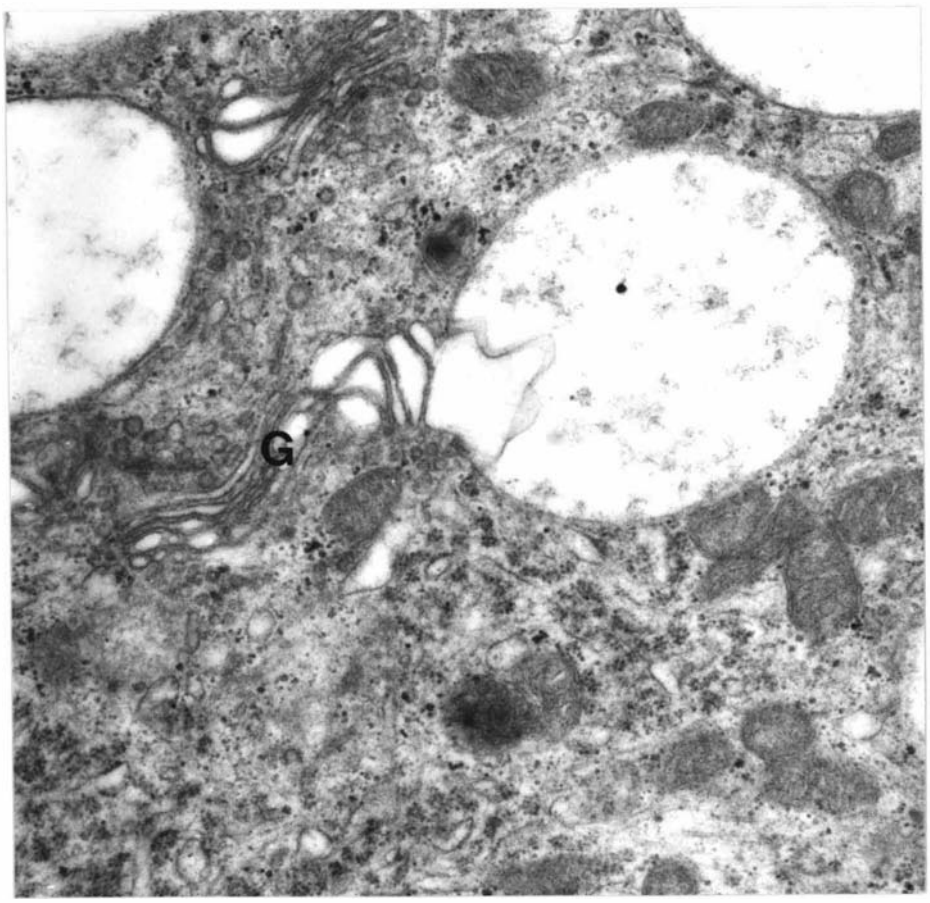
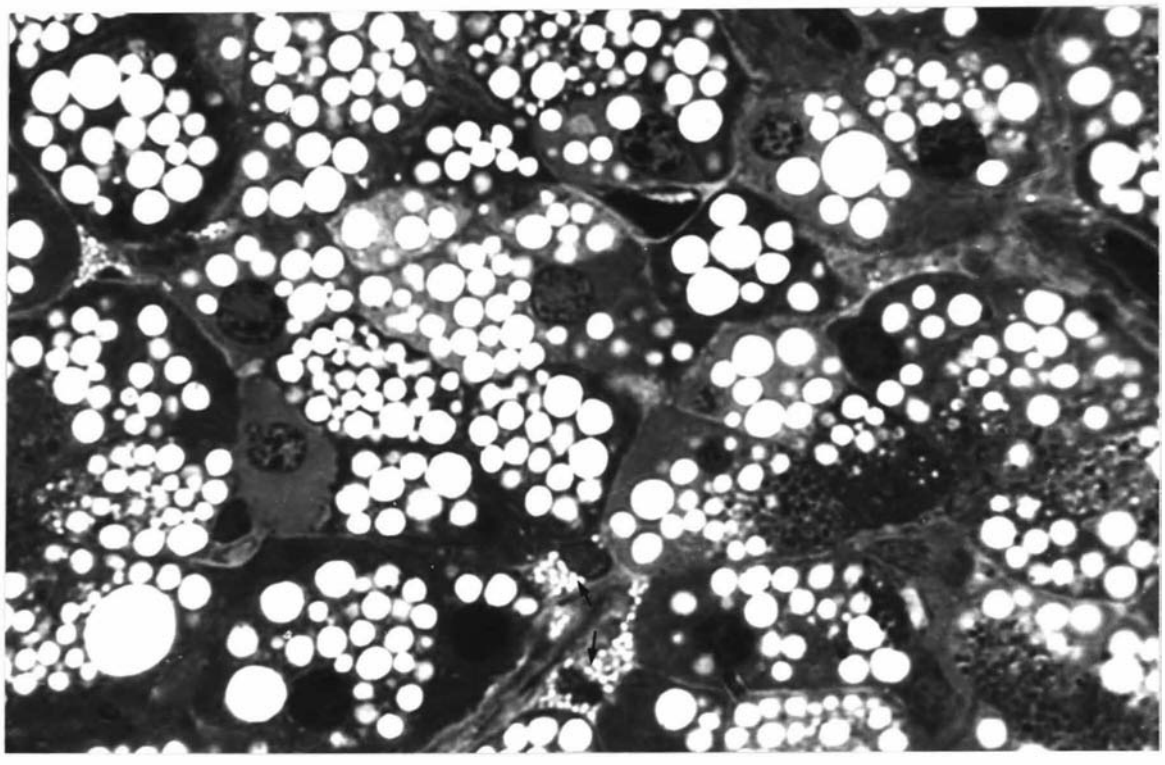
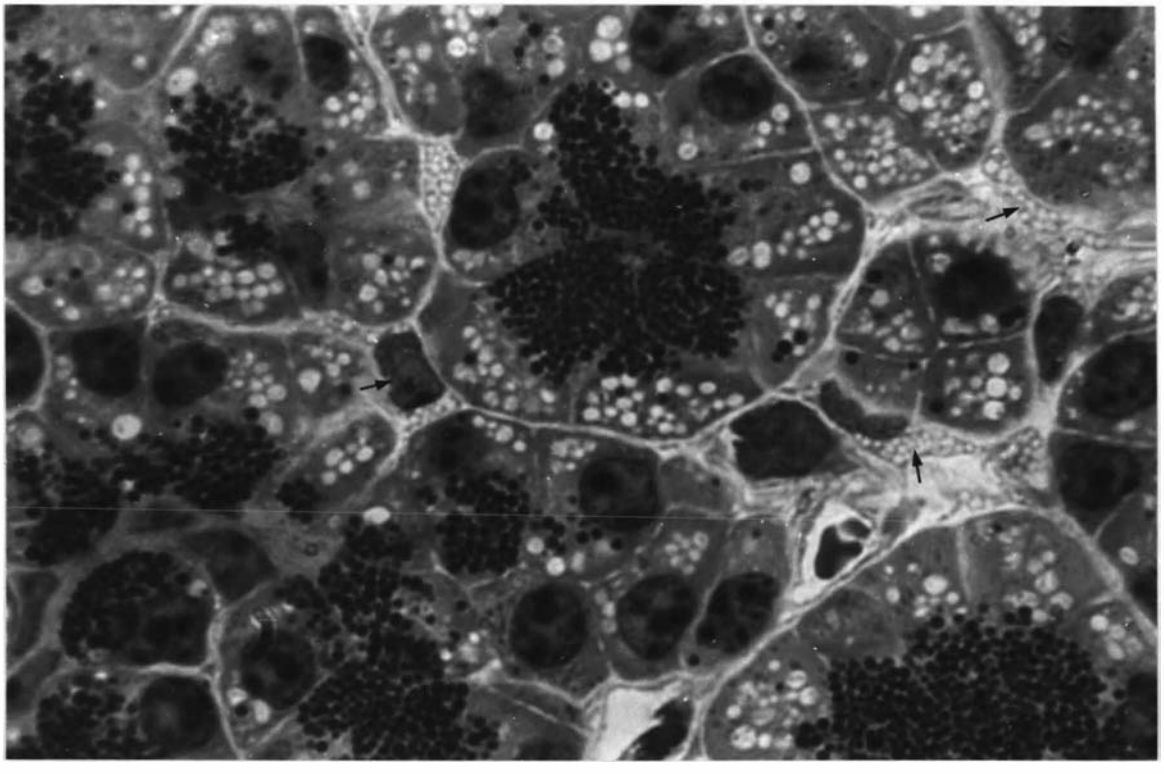

G

Figure 3.11: Pancreas from a six-month old calf with mannosidosis showing numerous large vacuoles in exocrine epithelial cells and finely vacuolated fibroblasts (arrows) in the interstitium. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)

Figure 3.12: Pancreas from a three-day old calf with mannosidosis showing considerably less vacuolation of exocrine epithelial cells than Figure 3.11. Fibroblasts (arrows) in the interstitial tissues are still highly vacuolated. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)





vacuolation increased with the age of affected calves (c.f. Figures 3.11, 3.12). Vacuoles in pancreatic exocrine cells varied in size from 1-6 μm in diameter. Ultrastructurally these vacuoles initially appeared to occur in rough endoplasmic reticulum (Figure 3.13), but at higher magnifications the presence of a single three-layered limiting membrane, unassociated with rough endoplasmic reticulum, was noted (Figure 3.14). Variable amounts of fibrillar material and recognizable zymogen granules were occasionally observed within electron-lucent storage vacuoles in the pancreas (Figure 3.15).

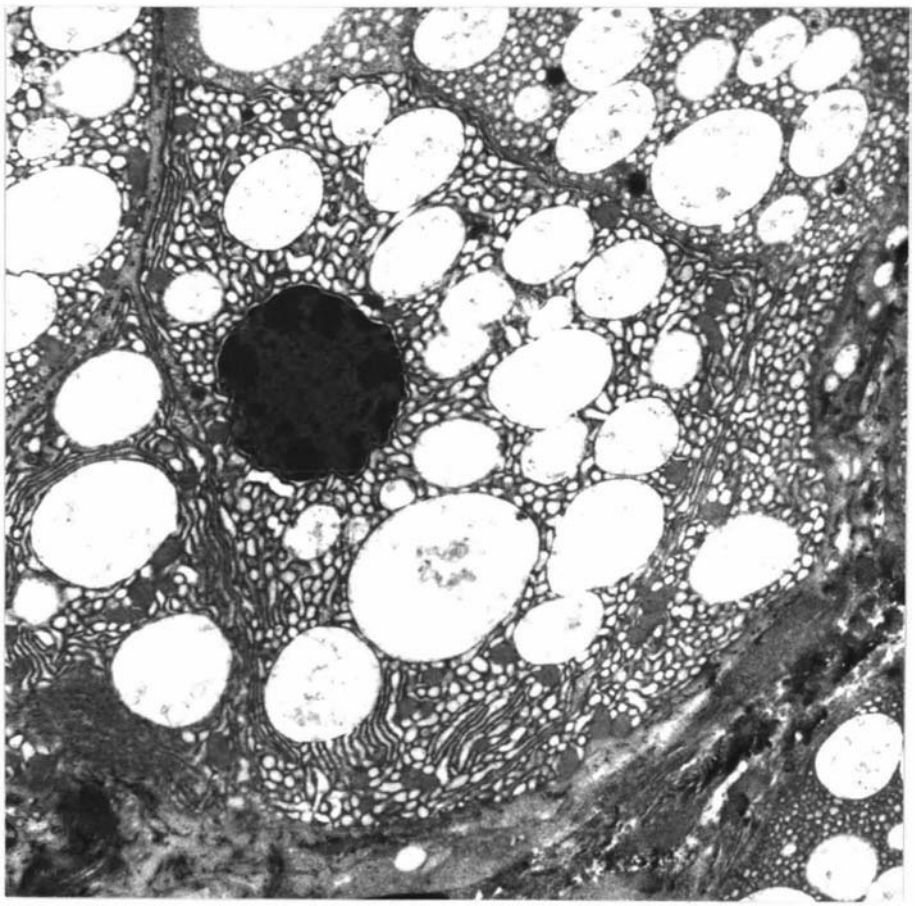
Compared to the controls, the exocrine epithelium of lacrimal glands in mannosidosis calves was also highly vacuolated, with lesions appearing similar to those observed in the pancreas (Figures 3.16, 3.17, 3.18). Similarly, in the abomasum, chief cells were consistently vacuolated and smaller numbers of vacuoles were often seen in parietal cells (Figures 3.19, 3.20, 3.21, 3.22). The mucus secreting cells of submaxillary salivary glands of mannosidosis calves often contained large vacuoles up to 15 μm in diameter in addition to the many small mucus containing vacuoles normally present in these cells (Figures 3.23, 3.24, 3.25, 3.26). The serous cells of these salivary glands contained vacuoles of various sizes up to 6 μm in diameter, but the serous epithelium of the parotid salivary glands was relatively unaffected (Figure 3.31). Evidence for an association between secretory granules and storage vacuoles was found only in the pancreas.

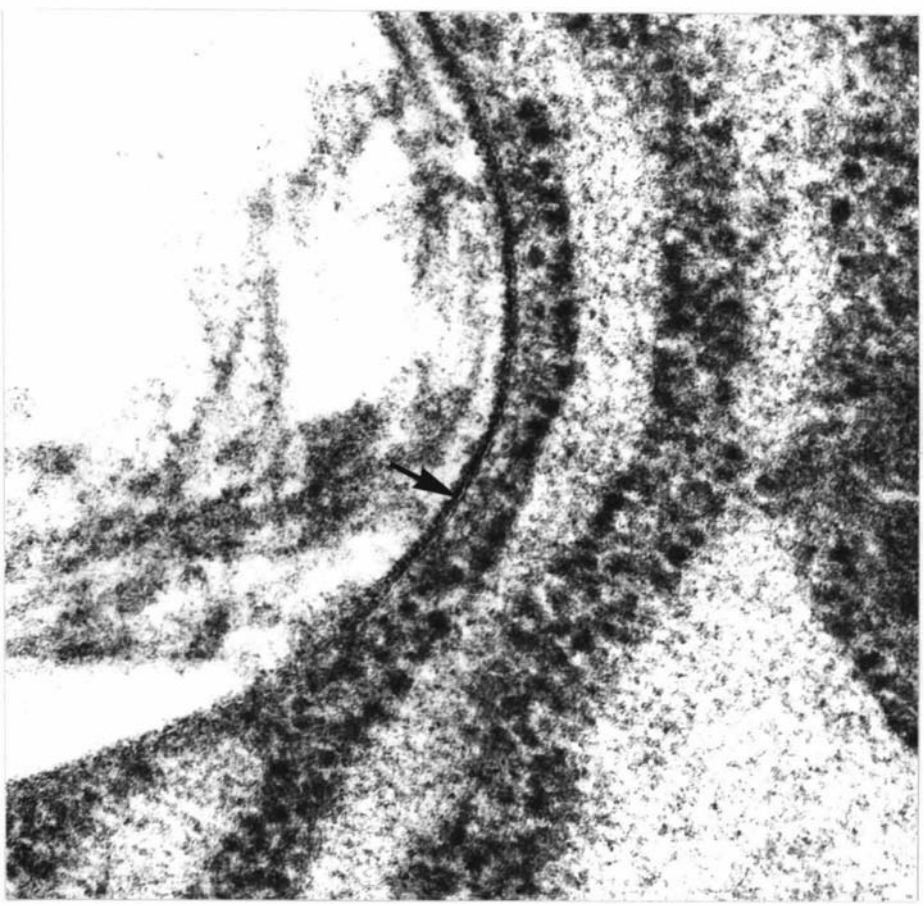
The endocrine epithelial cells of the pancreas (islets of Langerhans), thyroid and adrenal glands appeared to be unaffected on investigation by light microscopy.

Renal convoluted tubules and collecting ducts showed varying degrees of vacuolation. A severely vacuolated distal convoluted

Figure 3.13: Pancreatic exocrine epithelial cell from a mannosidosis calf showing clear vacuoles, some of which appear to occur within areas of rough endoplasmic reticulum. (E.M. x 6,400)

Figure 3.14: Single three-layered membrane (arrow) approximately 900 nm in thickness defining a vacuole in a pancreatic exocrine cell. (E.M. x 115,000)





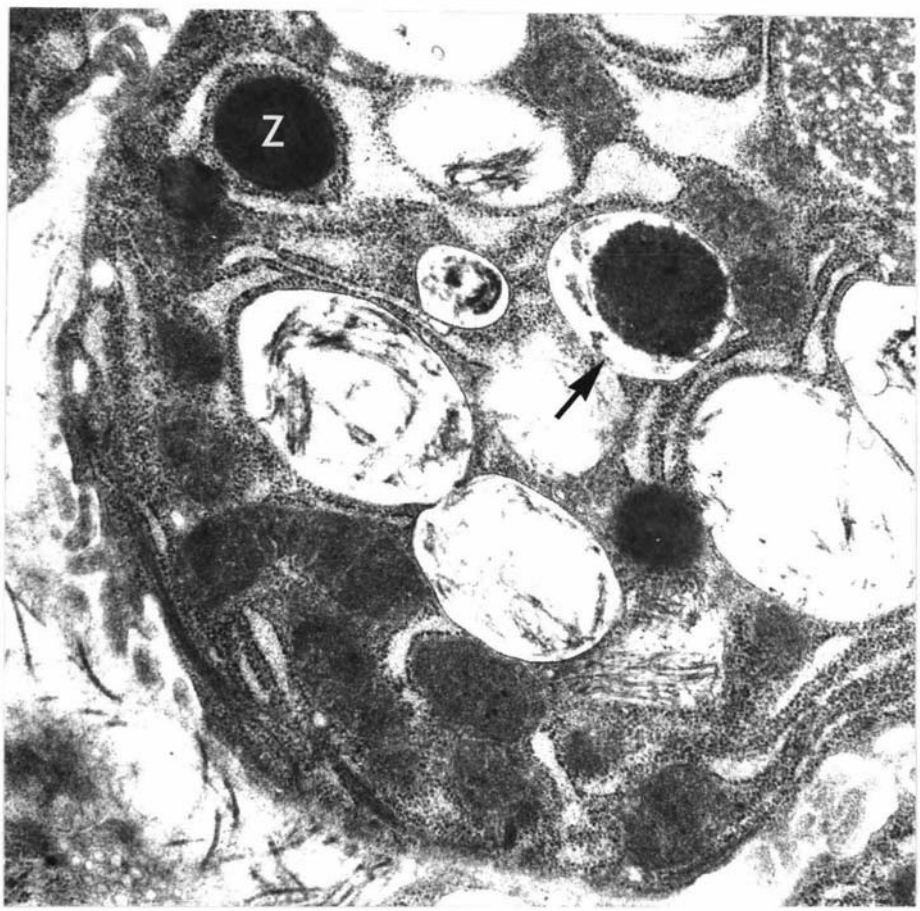
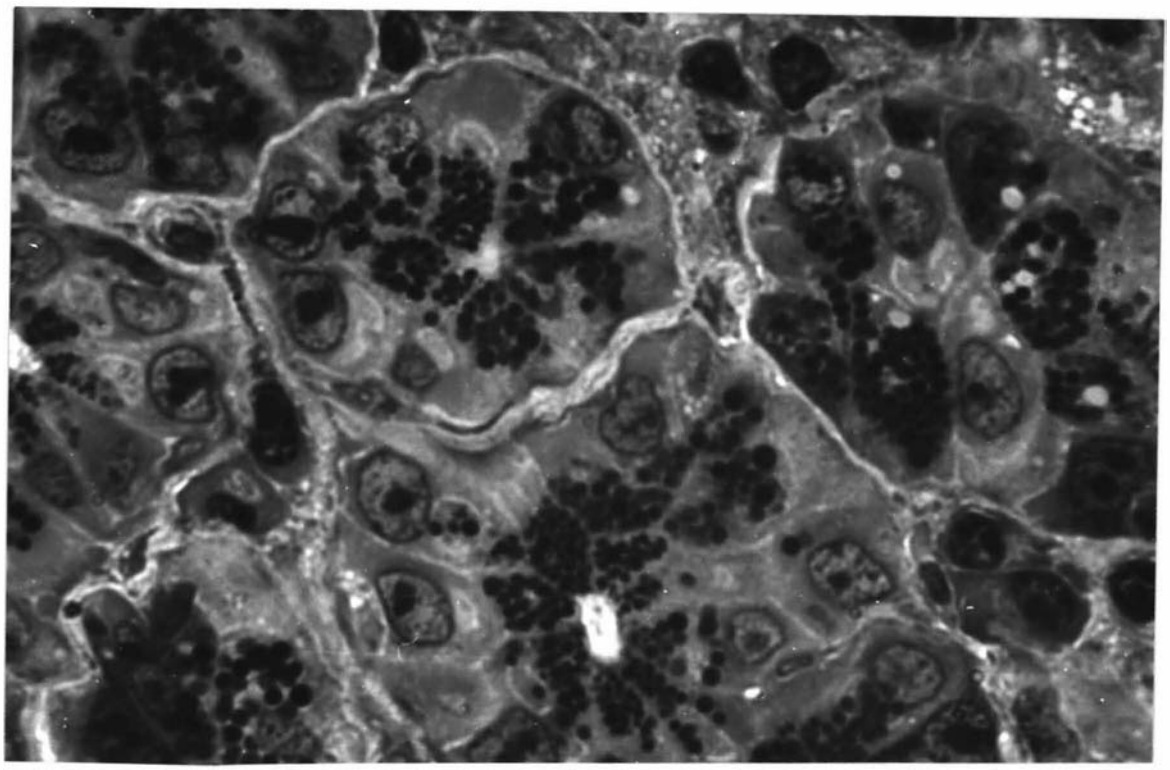
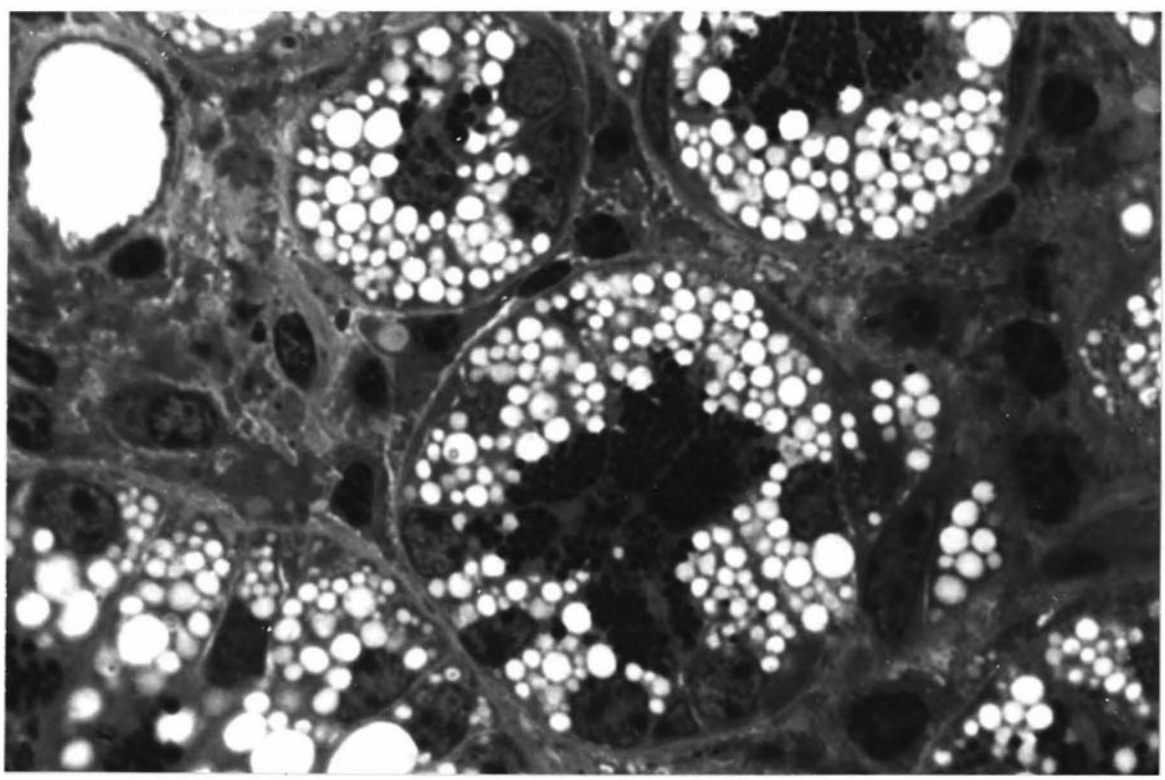


Figure 3.15: Portion of a pancreatic exocrine cell showing vacuoles containing fibrillar elements and one containing a zymogen granule (arrow). A zymogen granule (Z) unassociated with storage vacuoles is also present. (E.M. x 22,500)

Figure 3.16: Lacrimal gland from a normal control calf. Numerous dark-staining secretory granules are present within apical zones of exocrine epithelial cells. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)

Figure 3.17: Lacrimal gland from a calf with mannosidosis. Numerous large clear vacuoles in basal areas of exocrine cells contrast with the darker staining secretory granules. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)





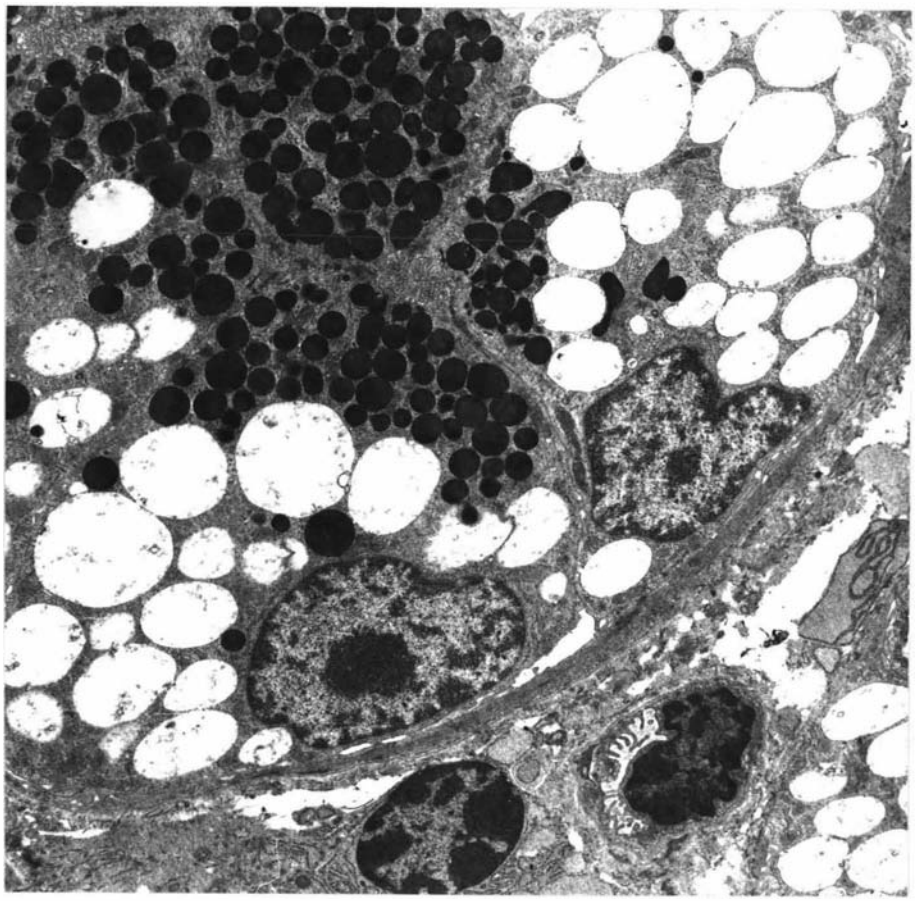
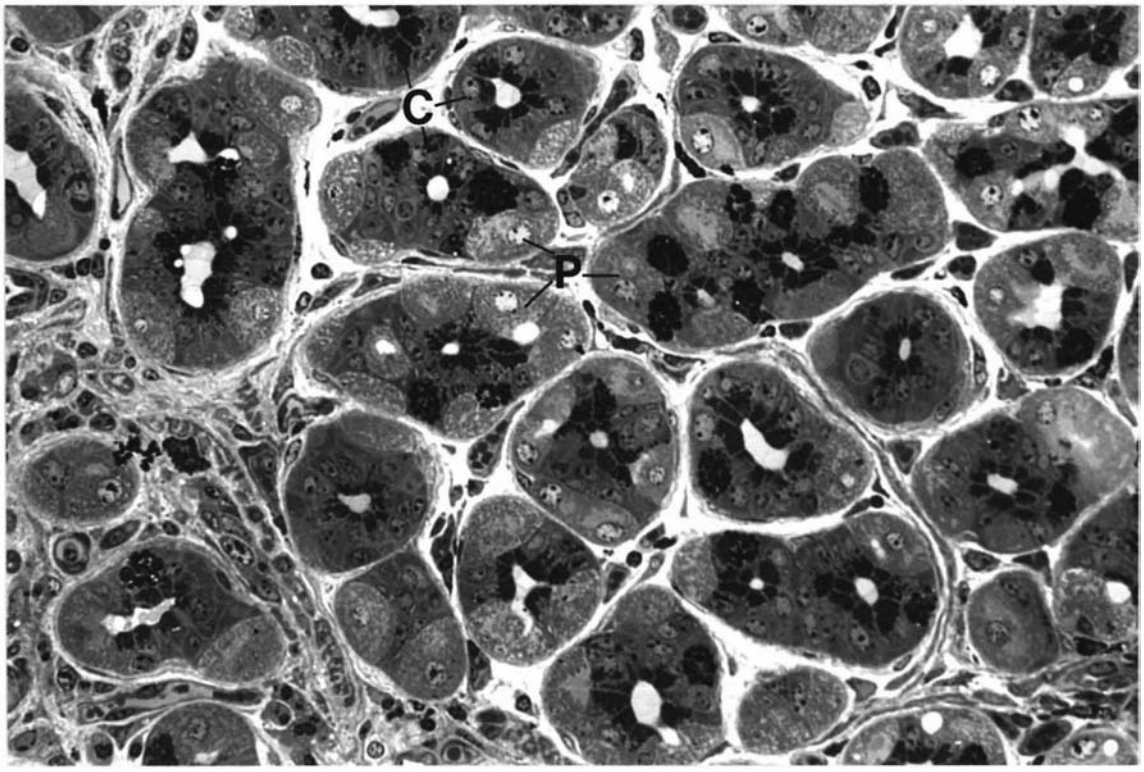


Figure 3.18: Lacrimal gland from a calf with mannosidosis showing membrane-bound vacuoles in exocrine epithelial cells. Darker staining secretory granules are present in apical zones of these cells. (E.M. x 6,400)

Figure 3.19: Abomasal mucosa of a normal control calf showing parietal cells (P) and chief cells (C), which contain dark-staining secretory granules. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 625)

Figure 3.20: Abomasal mucosa of a calf with mannosidosis showing widespread vacuolation of chief cells (C) and less frequent vacuolation of parietal cells (P). (x 625)


C
P

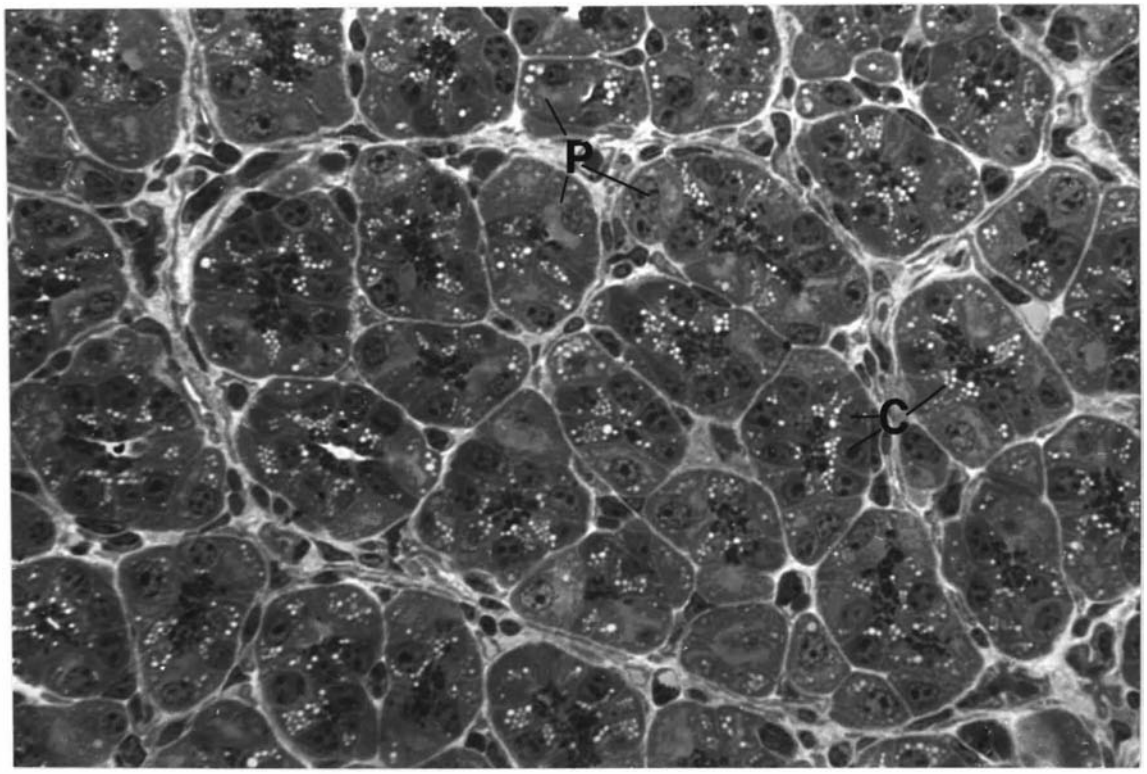
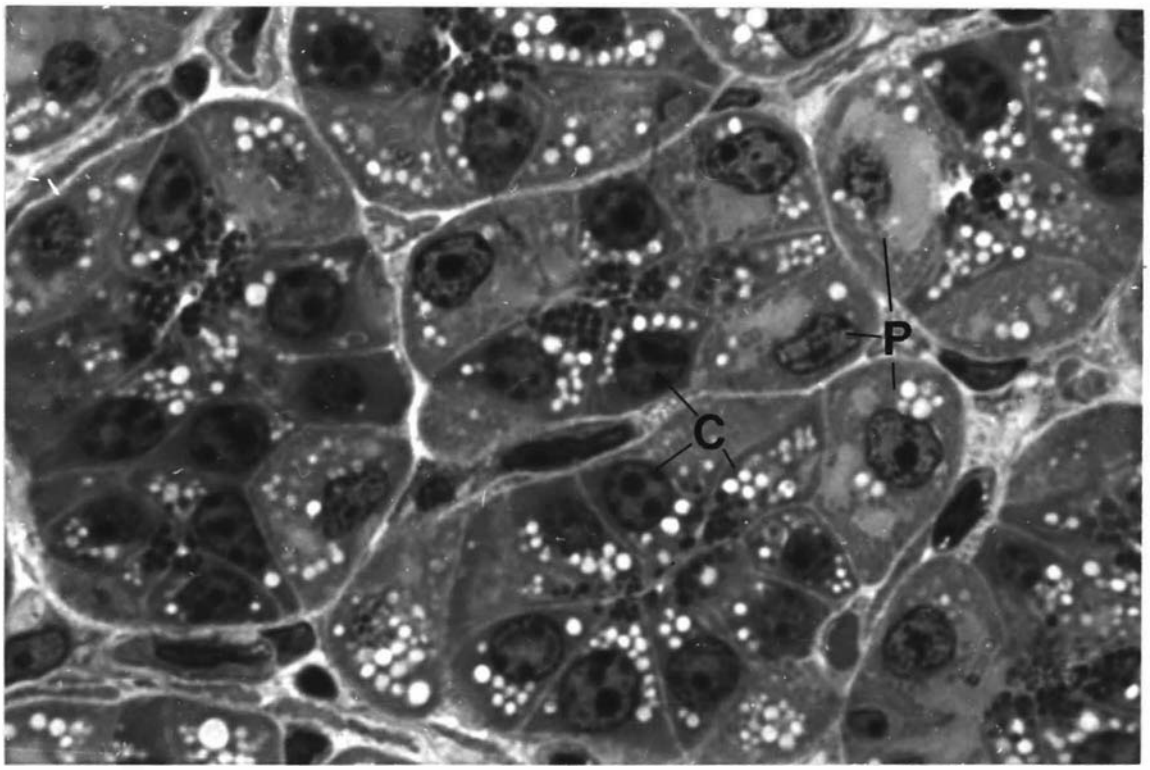

P
C

Figure 3.21: Abomasal mucosa of mannosidosis calf showing vacuolation of both chief (C) and parietal (P) cells. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)

Figure 3.22: Chief cells with dark-staining secretory granules and clear vacuoles containing a variable amount of electron-dense material. An apparent connection (arrow) is observed between two vacuoles. (E.M. x 13,500)


P
C

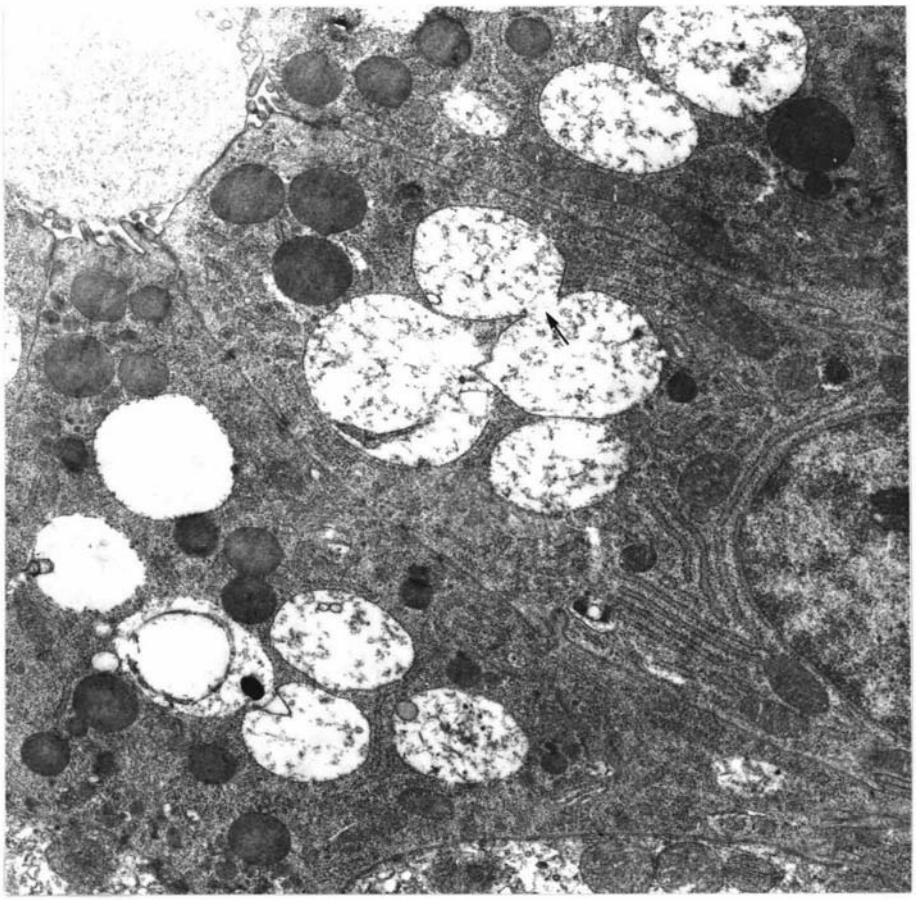
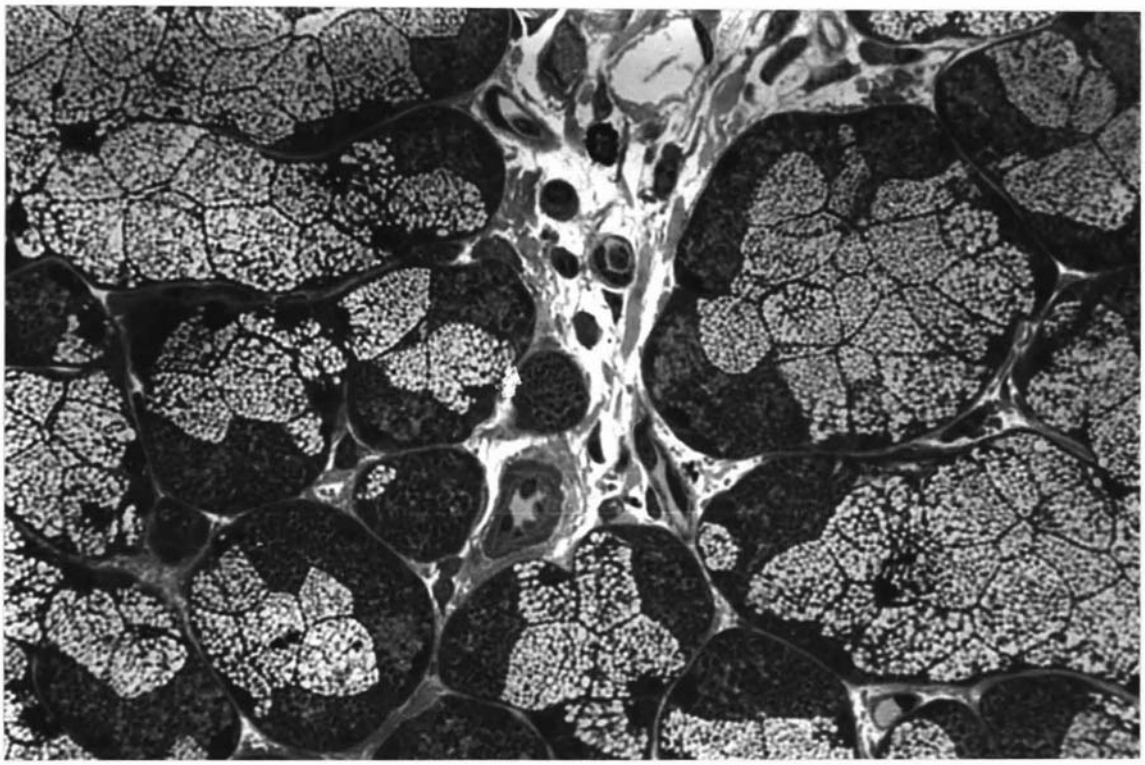


Figure 3.23: Submaxillary salivary gland of a normal control calf showing dark-staining serous demilunes and finely vacuolated mucus secreting cells. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 625)

Figure 3.24: Submaxillary salivary gland from a calf with mannosidosis. Abnormally large vacuoles are present within many mucous cells (M). Vacuoles are also present within the cells of serous demilunes (S) and the epithelium of a glandular duct (D). (Epoxy resin embedded, toluidine blue-basic fuchsin, x 625)



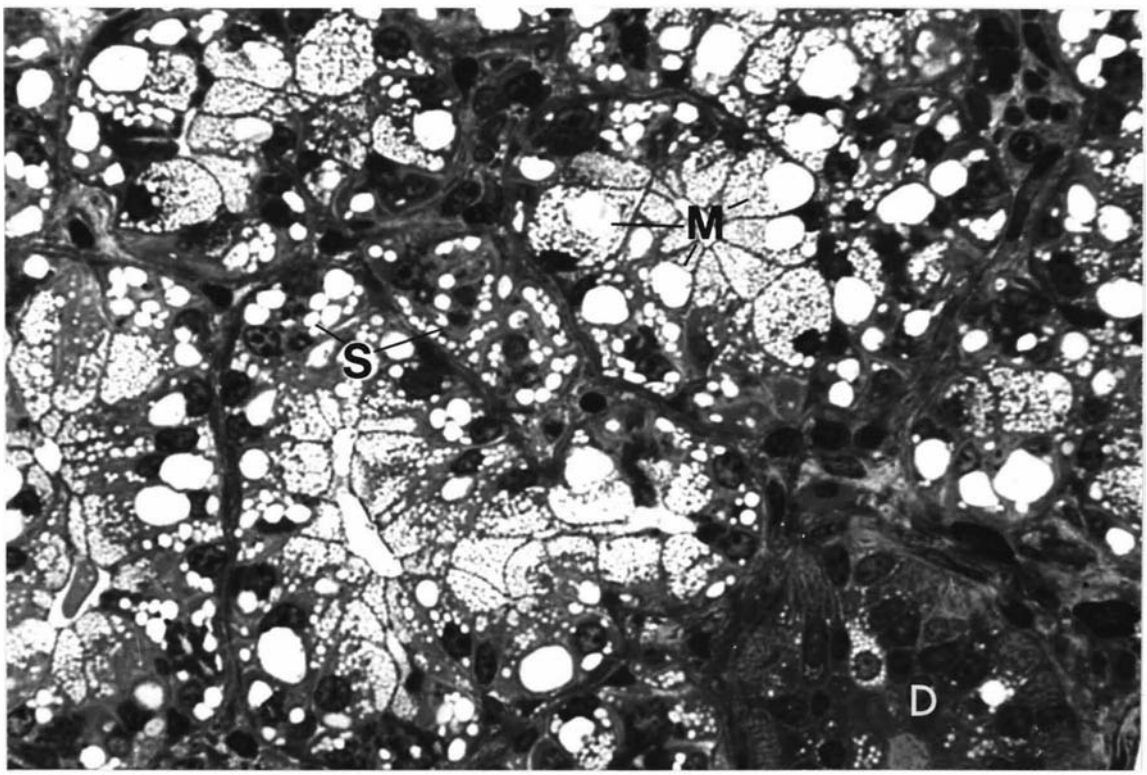
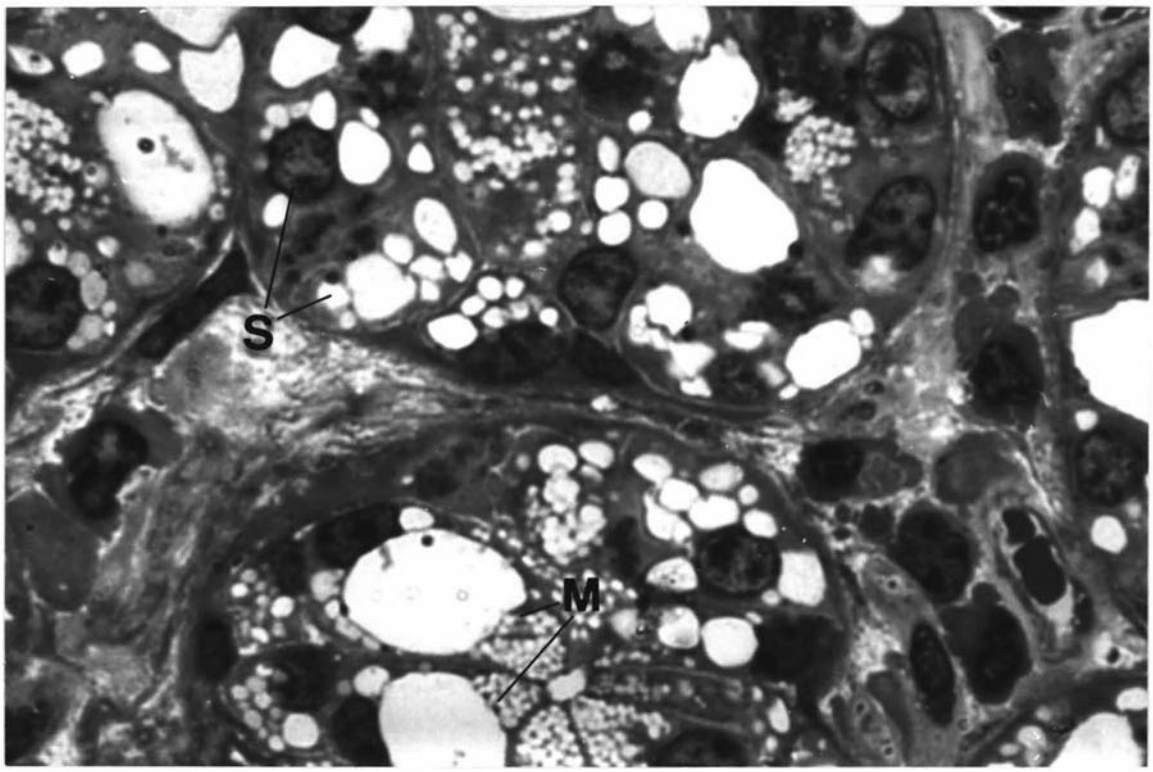
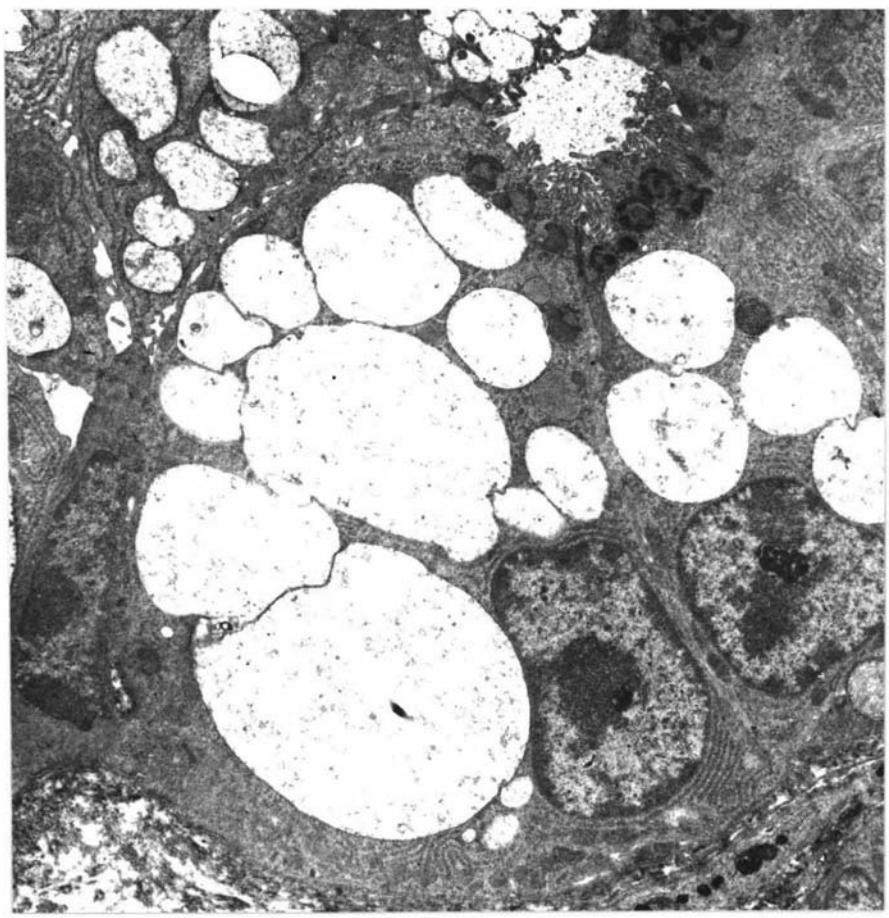

M
S
D

Figure 3.25: Submaxillary salivary gland of a mannosidosis calf showing abnormal vacuolation of both mucous (M) and serous (S) cells. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)

Figure 3.26: Serous cells from the submaxillary salivary gland of a calf with mannosidosis. Large vacuoles up to 6 μm in diameter occupy a considerable area of their cytoplasm. (E.M. x 6,400)


S
M



tubule is shown in Figure 3.27. Ultrastructurally podocytes in renal glomeruli sometimes contained a few small vacuoles. Other epithelial tissues showing vacuolation in calves with mannosidosis were type II epithelial cells in the lungs, bronchiolar epithelium (Figure 3.28) and the epithelium of ducts from certain glandular tissues such as the submaxillary salivary gland (Figure 3.24). Hepatocytes were not consistently affected but sometimes contained a small number of vacuoles (Figure 3.29), some of which included fibrillar elements similar to those seen in the pancreas.

(d) Mesenchymal tissues

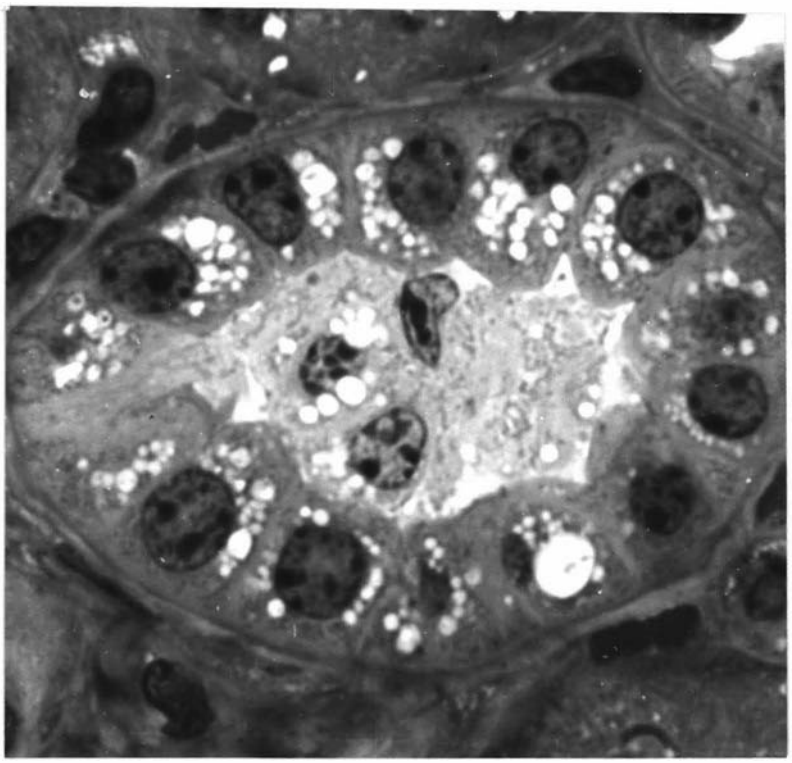
Many fibroblasts in tissues examined from calves with mannosidosis were packed with small electron-lucent vacuoles of approximately 0.5 to 1.0 μm in diameter. These were most obvious in the interstitial tissues of the pancreas and kidney, and between smooth muscle fibres in the external muscular layers of the gastrointestinal tract (Figures 3.11, 3.12, 3.32). Pericytes of small blood vessels were often vacuolated (Figure 3.30), as were capillary endothelial cells (Figure 3.31). Smooth muscle fibres in the gastrointestinal tract and in the walls of large blood vessels frequently contained vacuoles in their perinuclear zones (Figures 3.32, 3.33). Vacuolation was also noted in mesangial cells of renal glomeruli from affected calves (Figure 3.34).

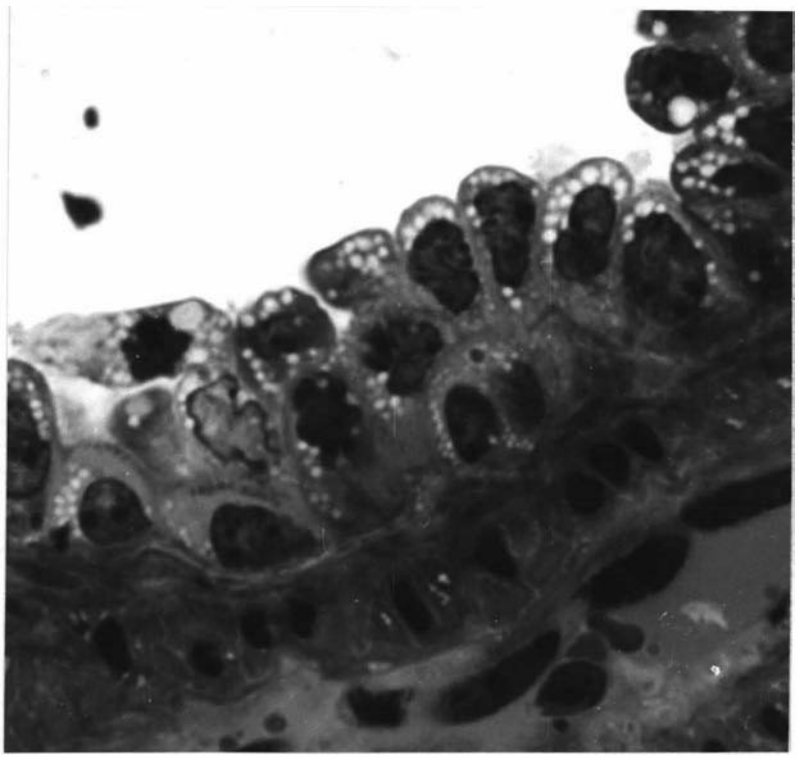
DISCUSSION

Ultrastructural studies have confirmed the presence of a single three-layered membrane defining vacuoles in tissues of calves with mannosidosis (Figure 3.1). This membrane was approximately 900 nm in thickness and as such is consistent with the identification of these vacuoles as secondary lysosomes (Ericsson et al., 1965; Maunsbach, 1969).

Figure 3.27: Vacuolation of the epithelium of a renal distal convoluted tubule in a calf with mannosidosis. Desquamated epithelial cells, also containing vacuoles, are present within the lumen of this tubule. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)

Figure 3.28: Vacuolated bronchial epithelial cells in a calf with mannosidosis. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)





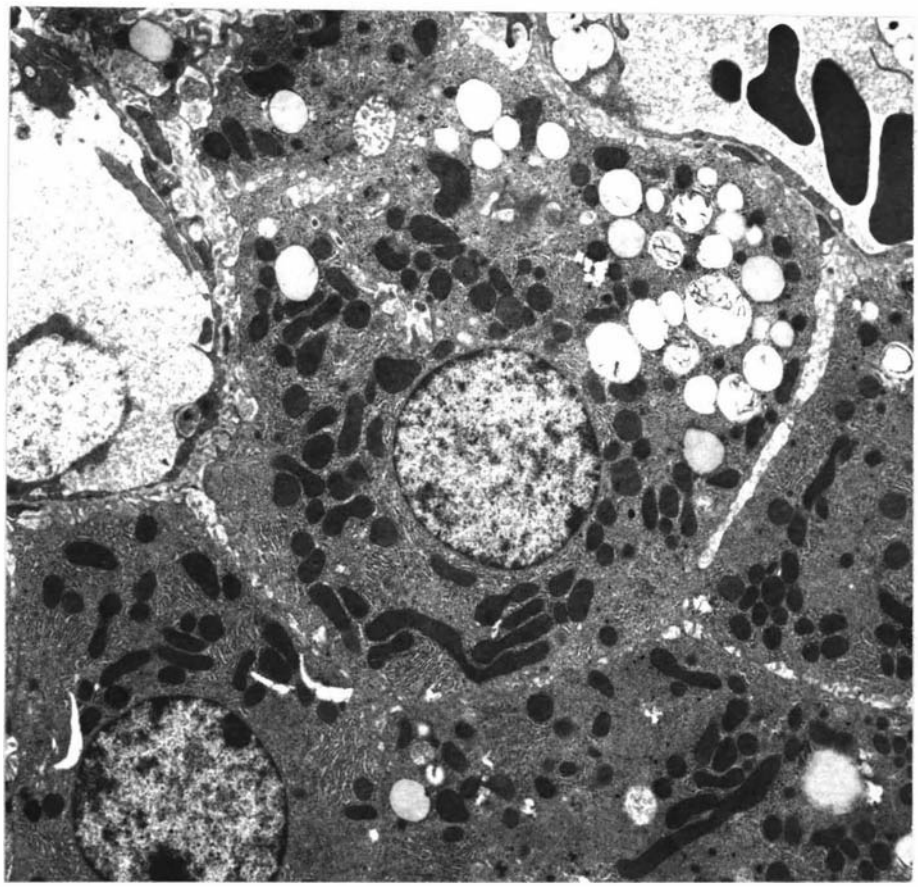
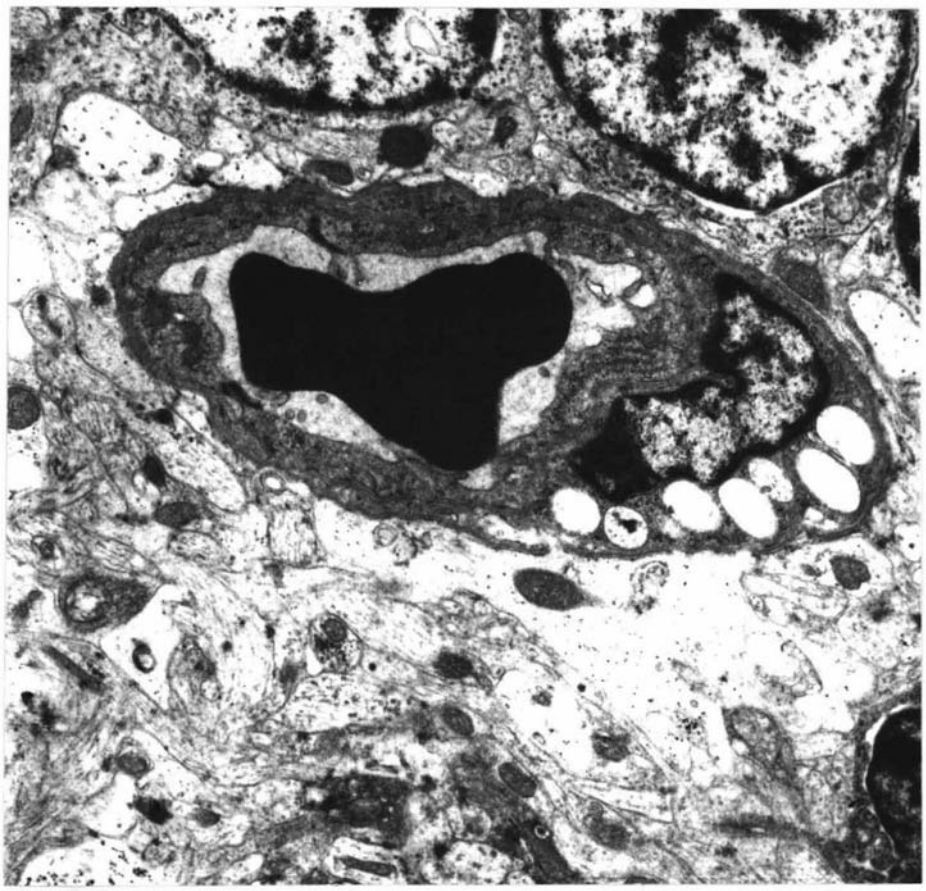


Figure 3.29: Clear vacuoles in a hepatocyte from a calf with mannosidosis. Some vacuoles contain fibrillar elements (c.f. Figure 3.15). (E.M. x 4,700)

Figure 3.30: Vacuoles in a pericyte of a blood capillary in the brain of a calf with mannosidosis. (E.M. x 10,500)

Figure 3.31: Vacuoles in two capillary endothelial cells in the parotid salivary gland of a mannosidosis calf. Dark staining secretory granules are present within exocrine epithelial cells but very few clear vacuoles are observed within these cells. (E.M. x 6,400)



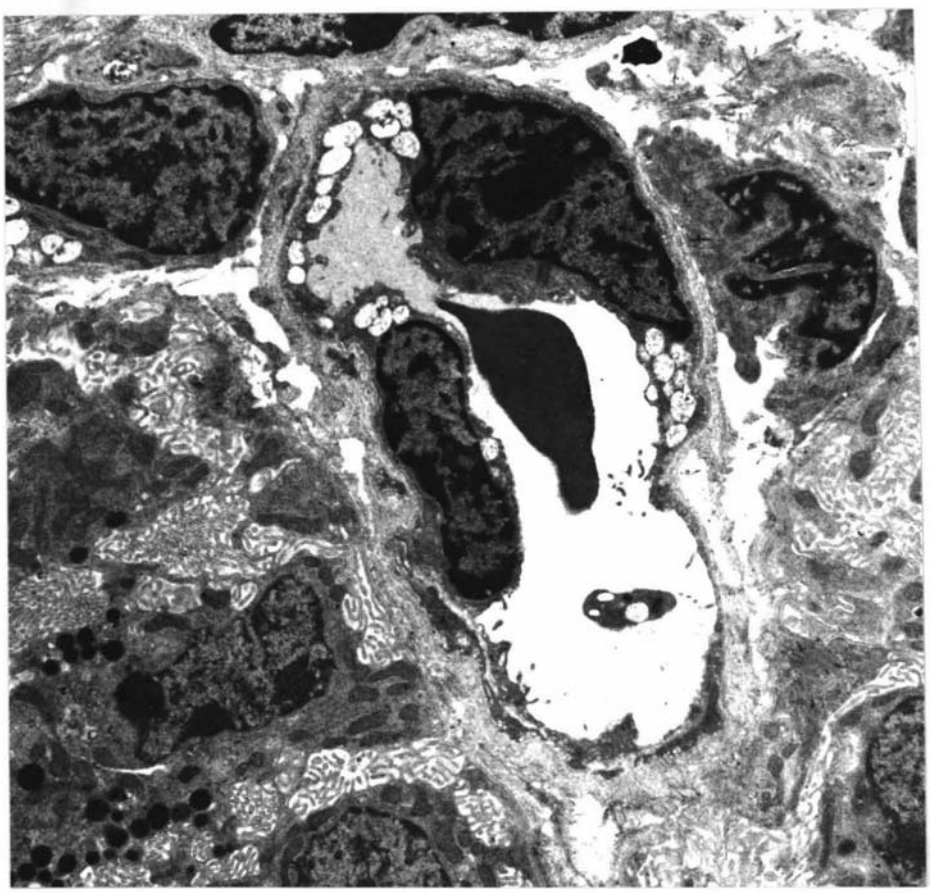
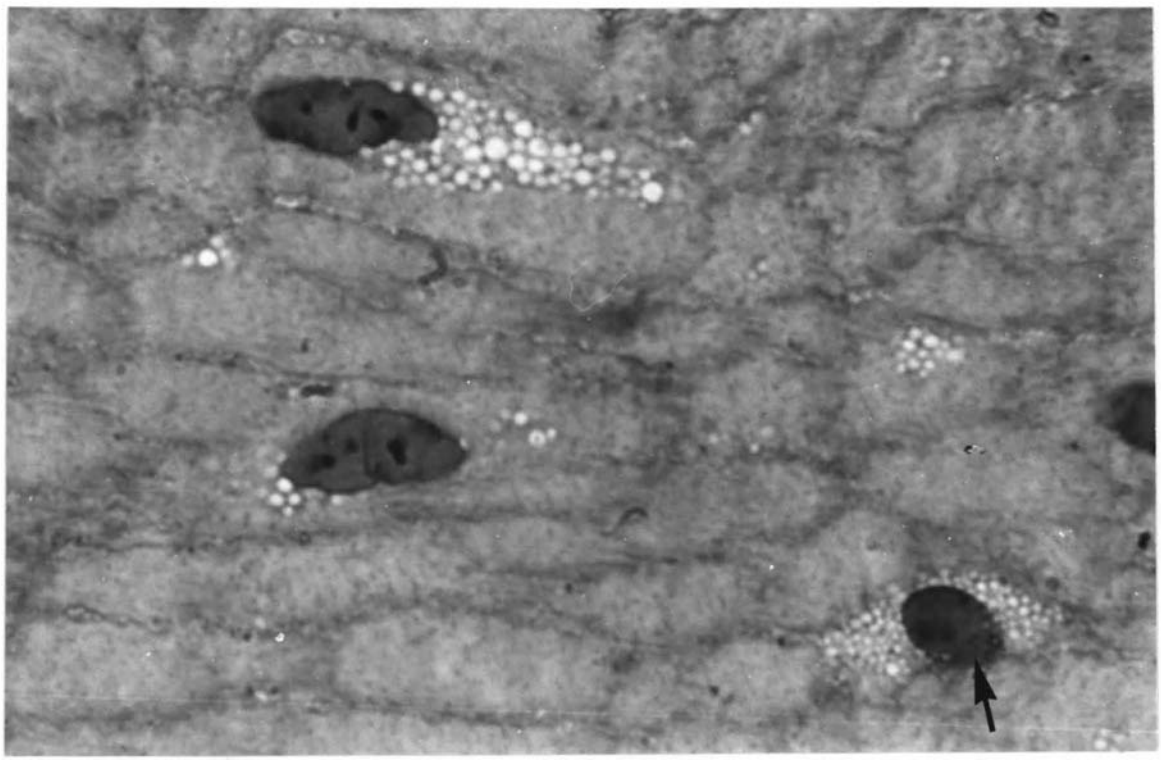
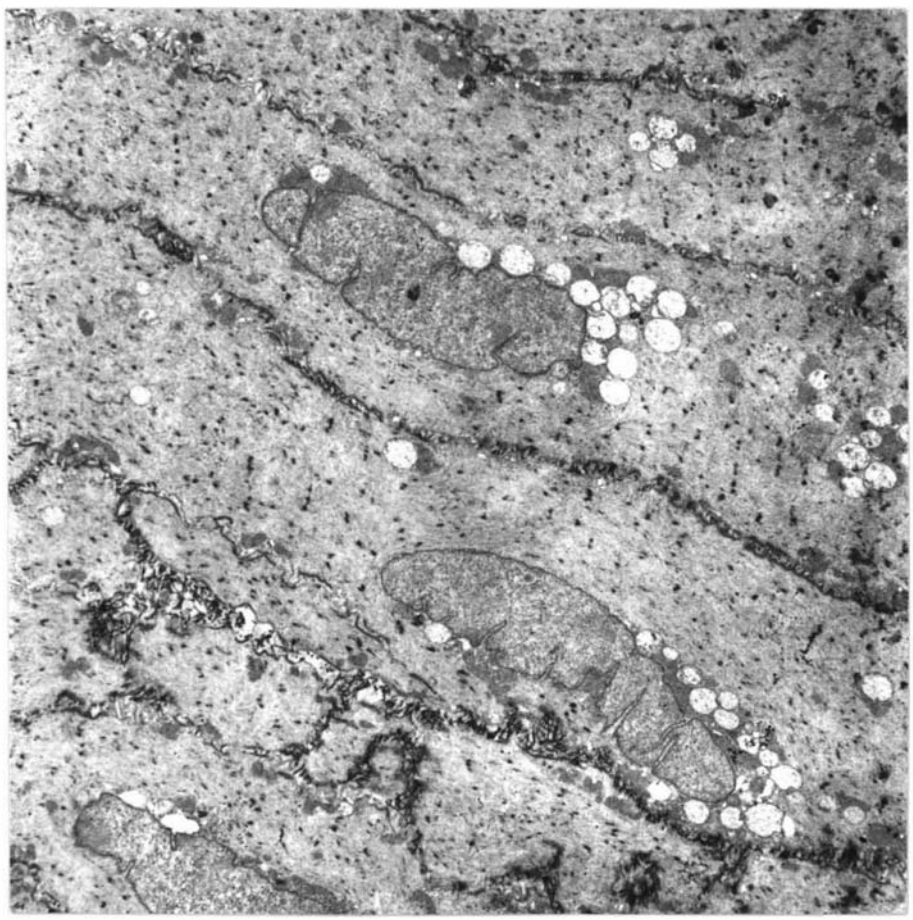


Figure 3.32: Vacuoles within smooth muscle fibres in the external muscular layer of the small intestine of a calf with mannosidosis. A fibroblast (arrow) containing relatively smaller vacuoles is present between smooth muscle fibres. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,560)

Figure 3.33: Vacuoles in perinuclear zones of smooth muscle fibres in the external muscular layer of the small intestine of a calf with mannosidosis. Mitochondria are also particularly common in these areas. (E.M. x 3,400)





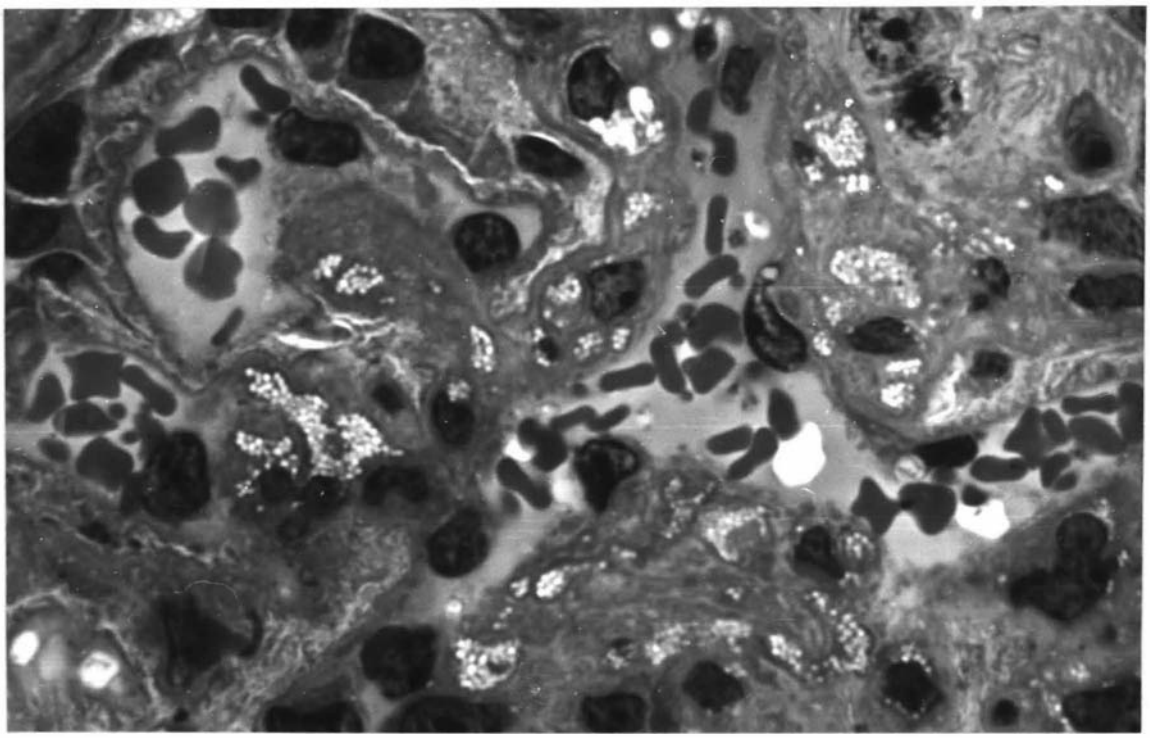


Figure 3.34: Vacuolation of mesangial cells in a renal glomerulus of a calf with mannosidosis. (Epoxy resin embedded, toluidine blue-basic fuchsin x 1,560)

According to the basic concepts of inborn lysosomal diseases, as discussed in Chapter I (pages 1-4), the most severe lesions would be expected in (i) relatively long-lived cell-types; (ii) in cells with a high endocytic activity, such as those of the reticuloendothelial system, and (iii) in cells with ready access to substrates normally catabolized by the deficient enzyme. Mannosidosis is associated with an error in glycoprotein catabolism, and as glycoproteins are widely distributed throughout the mammalian body it is not surprising that the accumulation of glycoprotein fragments within lysosomes is widespread. As in several other inborn lysosomal diseases, reticuloendothelial cells of the liver and lymph nodes show severe vacuolation, presumably due to their high phagocytic activity.

Involvement of exocrine epithelial tissues in storage diseases has not been widely reported although the vacuolation of such tissues in bovine mannosidosis would suggest the possibility of similar lesions in human mannosidosis and closely related disorders such as fucosidosis, aspartylglycosaminuria and possibly in certain of the mucopolysaccharidoses. Exocrine epithelial cells are not long-lived when compared to neurones or muscle fibres therefore their vacuolation is unlikely to be related to this factor, but they might be expected to possess a relatively high endocytic (or pinocytic) activity to supply the materials and energy required for synthesis of their secretory products. However, the recognition of zymogen granules within storage vacuoles in the pancreas of calves with mannosidosis (Figure 3.15) suggests that vacuolation of this tissue may be associated with a ready source of substrate provided by the process of crinophagy. Crinophagy is a process whereby secretory products superfluous to functional requirements are directed into the lysosomal system and undergo intracellular digestion (Farquhar, 1969; de Duve, 1969). It has been suggested that this process is an important factor in the overloading of lysosomes in glandular cells, fibroblasts

and capillary endothelial cells (Van Hoof, 1973a and b), but direct evidence to support this hypothesis has not previously been reported. Glycoproteins are major components of secretory granules (Neutra and Leblond, 1969), therefore crinophagy would be expected to contribute indigestible oligosaccharide substrates to the lysosomal system in disorders of glycoprotein catabolism. An association between secretory granules and storage vacuoles was observed only in the pancreas but the occurrence of crinophagy in other exocrine epithelial tissues cannot be discounted. It is not known why the exocrine epithelium of parotid salivary glands is relatively unaffected in mannosidosis when similar serous cells in submaxillary salivary glands are highly vacuolated.

Although endocrine epithelial cells might be expected to possess a similar endocytic activity to exocrine cells, and crinophagy has been demonstrated in various endocrine cell-types (Farquhar, 1969), vacuolation was not observed in islets of Langerhans, thyroid or adrenal glands of calves with mannosidosis. This is probably due to the fact that the hormonal secretions from these glands are not glycoproteins and therefore the process of crinophagy would not be expected to contribute indigestible material to the lysosomal system in cells of these tissues.

The vacuolation of hepatocytes and epithelial cells of renal tubules in mannosidosis calves was relatively mild and would not have been expected to impair the function of either organ.

Finely vacuolated fibroblasts were observed in almost every tissue examined. Fibroblasts are responsible for the production of acid mucopolysaccharides and collagen precursors, some of which probably find their way into the lysosomal system of fibroblasts by the process of crinophagy (Van Hoof, 1973a) where they accumulate in disorders of glycoprotein and mucopolysaccharide metabolism. Pericytes of small blood vessels and mesangial cells in renal glomeruli are also thought to be

capable of producing collagen precursors and are believed to possess phagocytic properties (Ham, 1965). The vacuolation of these cell-types in affected calves is not therefore surprising. Smooth muscle fibres in the walls of large blood vessels and in the external muscular layers of the gastrointestinal tract were often vacuolated (Figures 3.32, 3.33), but vacuoles were not observed in skeletal muscle and cardiac muscle. Muscle fibres are long-lived cells and therefore the accumulation of storage product in their lysosomes is not surprising but the absence of vacuoles from skeletal and cardiac muscle fibres is difficult to explain.

The central nervous system was not examined in detail in this study, but the widespread and severe vacuolation of neurones, previously reported by Whittem and Walker (1957) and Jolly (1971), was confirmed (Figures 3.9, 7.8b). Hers (1973) suggested that neurones probably have a low endocytic activity and that the storage of undigested material in their secondary lysosomes arises largely through the process of autophagy, but Hers has possibly underestimated the endocytic capabilities of these cells. Neurones are responsible for supplying nutrients to a large volume of cytoplasm, in the form of axons, and might therefore be expected to possess a relatively high endocytic activity. Both methods probably contribute substrates to the lysosomal system but the severity of neuronal vacuolation is most likely a reflection of the longevity of these cells. In contrast, short-lived cell-types, such as intestinal epithelial cells, would be expected to have insufficient time to accumulate significant quantities of storage compounds in their lysosomal system prior to exfoliation. As expected, vacuoles were only occasionally observed in the intestinal epithelium of affected calves. Storage-type inclusions have however been observed in intestinal epithelial cells of patients with G_{M1}-gangliosidosis and mucopolysaccharidosis Types, I, II and III. These inclusions are thought to develop as a result of a defect in the crinophagic regulation of the secretion of the cell coat material

of these cells due to the deficiency of a lysosomal enzyme (Daems et al., 1973).

The lesions of mannosidosis may be observed in affected foetuses as early as the sixth month of gestation (Jolly, personal communication). Although vacuolation of most cell-types was already widespread at birth, lesions tended to be more severe in older calves (Figures 3.11, 3.12) due to the progressive accumulation of storage compounds.

The average size of vacuoles varied between different tissues. In epithelial cells they were often greater than 10 μm in diameter, while in mesenchymal cells and reticuloendothelial cells they were smaller (0.5 to 2.0 μm in diameter) and of a more uniform size.

A detailed study of the histopathology of human mannosidosis has yet to be described, but sufficient evidence has been reported to suggest that the lesions are similar, though not identical, to those of the bovine disease. Widespread ballooning of neurones in the brain and spinal cord was described by Kjellman et al. (1969) in a child with mannosidosis, and clear vacuoles, surrounded by a single membrane, were reported in hepatocytes and Kupffer cells (Autio et al., 1973). Vacuolation of lymphocytes is a feature of human mannosidosis (Kjellman et al.) that was not observed in affected calves. Another human disorder of glycoprotein metabolism, aspartylglycosaminuria, has several histological features in common with mannosidosis of Angus cattle, including vacuolation of neurones, hepatocytes, Kupffer cells, endothelial cells and pericytes (Arstila et al., 1972; Haltia et al., 1975). Vacuoles were surrounded by a single membrane and no specific compounds could be identified within them by histochemical staining (Haltia et al.). Similar clear vacuoles also occur in various tissues of patients with fucosidosis (Van Hoof, 1973b) and with certain mucopolysaccharide storage disorders (Van Hoof, 1973a). In Type II glycogenosis (Pompe's disease) there is vacuol-

ation of neurones and several other tissues including liver, kidney, spleen, pancreas, smooth muscle, skeletal muscle and cardiac muscle. The presence of glycogen within these vacuoles may be demonstrated by standard histochemical techniques or by the characteristic ultrastructural appearance of glycogen (Sandstrom et al., 1969; Hers and de Barsy, 1973).

Vacuolation of cells within renal glomeruli has been reported in Fabry's, Neimann-Pick and Gaucher's diseases and in an unclassified mucopolysaccharidosis. In Neimann-Pick and Gaucher's diseases the "foam-cells" were present within glomerular capillaries, but in Fabry's disease and in the mucopolysaccharidosis visceral epithelial cells (podocytes) were vacuolated (Rosenmann and Aviram, 1973). There was no mention of vacuolation of mesangial cells in these diseases.

Jolly (1971) described vacuoles apparently developing from saccular dilatations in Golgi apparatus in neurones of calves with mannosidosis. This was confirmed during the present study (Figure 3.10) and although a similar relationship between vacuoles and Golgi apparatus was not observed in any other tissue, vacuoles in lymph node macrophages sometimes appeared as continuous elongated swellings which may represent dilated portions of smooth endoplasmic reticulum (Figure 3.5). Large vacuoles in apposition to each other often appeared to be connected (Figure 3.22) suggesting that the vacuoles may, in some cases, be grossly dilated sections of smooth endoplasmic reticulum. These observations suggest that, at least in some tissues, storage material may accumulate within GERL, a specialized portion of Golgi-associated smooth endoplasmic reticulum from which lysosomes appear to form, and from which certain autophagic vacuoles are thought to be derived (Novikoff et al., 1966, 1971; Ericsson, 1969; Novikoff, 1973). Material entering the cell by endocytosis would not be expected to become associated with the Golgi apparatus or endoplasmic reticulum, but would theoretically be digested or stored within secondary

lysosomes. Haltia et al. (1975) were unable to establish any relationship between vacuoles and either smooth or rough endoplasmic reticulum or Golgi apparatus in tissues of patients with aspartylglycosaminuria.

In this study the advantage of using sections of resin embedded tissues for light microscopy was amply demonstrated. The vacuolation of smooth muscle fibres, fibroblasts, mesangial cells, pericytes and capillary endothelial cells was inapparent in sections from paraffin embedded tissues but was clearly visible in resin embedded sections. This investigation of the pathology of bovine mannosidosis is by no means complete. Tissues which have not yet been examined but which may prove to be of interest include skin, bone, bone marrow, cartilage and reproductive organs.

SUMMARY

Mannosidosis in Angus cattle is characterized pathologically by widespread vacuolation of neurones in the central nervous system, reticuloendothelial cells in the liver, lymph nodes and spleen, exocrine epithelial cells of the pancreas, abomasum, salivary glands and lacrimal glands, and certain cells of mesenchymal origin including fibroblasts, mesangial cells, smooth muscle cells, pericytes, and endothelial cells of blood capillaries. Vacuoles are surrounded by a single tripartate membrane of approximately 900 nm thickness and although most vacuoles are electron-lucent they often contain variable amounts of amorphous electron-dense material and membranous or fibrillar elements. Evidence was obtained to suggest that the process of crinophagy contributes to the formation of vacuoles in the pancreas. It is thought likely that crinophagy is also responsible for the severity of lesions noted in other exocrine tissues and possibly in fibroblasts, pericytes and capillary endothelial cells. An apparent continuity between some vacuoles and

Golgi apparatus, and the presence of "sausage-like" dilatations of smooth endoplasmic reticulum suggest that at least some lesions may be due to the accumulation of storage materials within GERL and not merely within secondary lysosomes as traditionally appreciated.

CHAPTER IV

EVALUATION OF A PILOT TEST AND CONTROL PROGRAMME FOR THE MANNOSIDOSIS GENOTYPE

INTRODUCTION

The high frequency of the mannosidosis genotype in New Zealand's Angus cattle population, plus the development of a simple and inexpensive test for mannosidosis heterozygotes, based on plasma α-mannosidase activity (Hocking et al., 1972; Jolly et al., 1973), suggested that a test and control programme would be both desirable and feasible. Consequently a pilot scheme planned to involve 5,000 animals was initiated in January 1973 with the following principal aims: (i) to provide a more accurate estimate of the gene frequency for this disease in New Zealand, (ii) to determine the accuracy and reliability of the plasma test for heterozygosity, (iii) to provide a nucleus of two-year old bulls free from the mannosidosis genotype for breeders who required them, and (iv) to stimulate the interest of breeders in the control of mannosidosis. Testing continued on a lesser scale during 1974 and 1975 while arrangements were being made for a national test and control scheme to be run by the Ministry of Agriculture and Fisheries in conjunction with the New Zealand Angus Association.

In this chapter the results obtained from the 1973 pilot scheme plus a limited number of later results are analysed and discussed.

MATERIALS AND METHODS

1. Animals used and collection of samples

All cattle bled as part of the pilot scheme were of the Angus breed, and were voluntarily presented by stud breeders from throughout New

Zealand. Details of the scheme were distributed to all members of the Angus Association and as many applicants as possible were included. Preference was given to herds with a history of mannosidosis, therefore the selection of animals could not be regarded as random. A total of 4,799 cattle from 38 different herds were tested in the pilot scheme, with samples being collected by the author mainly during January, February and March of 1973. Plasma was separated by an assistant within four hours of collection.

A pedigree extending back at least two generations was obtained for each animal tested in order to facilitate diagnosis and to allow a check on the validity of results. Samples from a herd of 57 mixed-aged Jersey cows, bled monthly over a period of one year, have been included to show seasonal variations in plasma α-mannosidase activities. Mannosidosis has never been recorded in this breed.

2. α-Mannosidase assays

Assays for α-mannosidase activity were performed by an assistant on a Technicon autoanalyser using the fluorogenic substrate 4-methyl-umbelliferyl-α-D-mannopyranoside. Details of the assay method are presented in Chapter II.

3. Method of dealing with data

Due to the influence of factors such as age, sex and environment on plasma α-mannosidase activities (Jolly et al., 1973) the results from each herd were analysed separately and in three groups, i.e. (i) mature cows (2 years and older), (ii) 14-22-month females, and (iii) 14-22-month males. For simplicity, animals aged 14-22 months are hereafter referred to as being 18 months of age.

Histograms, with class intervals of five fluorimetric units, were constructed for each group within a herd. For comparative studies between different herds fluorimetric units were converted to "standard" units by reference to a standard plasma sample which was run concurrently with all assays.

Further analysis of data included estimates of skewness of distribution curves (Snedecor and Cochran, 1967) and conversion of these curves to estimates of straight lines by either probit or rankit transformation (Finney, 1952; Fisher and Yates, 1963). Rankit transformation was used when the number of animals in the population was less than thirty.

RESULTS

1. Characteristics of the distribution curve for plasma α-mannosidase activity in normal animals

The characteristics of the distribution of plasma α-mannosidase activities of normal animals were investigated by examining in detail the results from 12 herds of Angus cows plus one herd of Jersey cows. Only Angus herds containing 50 or more cows and with a clear separation between normal and heterozygous populations were selected for this investigation.

A feature of many histograms was an apparent positive skewness in the distribution of plasma α-mannosidase activity in normal populations (Figure 4.1a). In six of the thirteen herds the distributions were significantly skewed, and in all but one case the skewness could be corrected by logarithmic transformation of values (Table 4.I, Figure 4.1b).

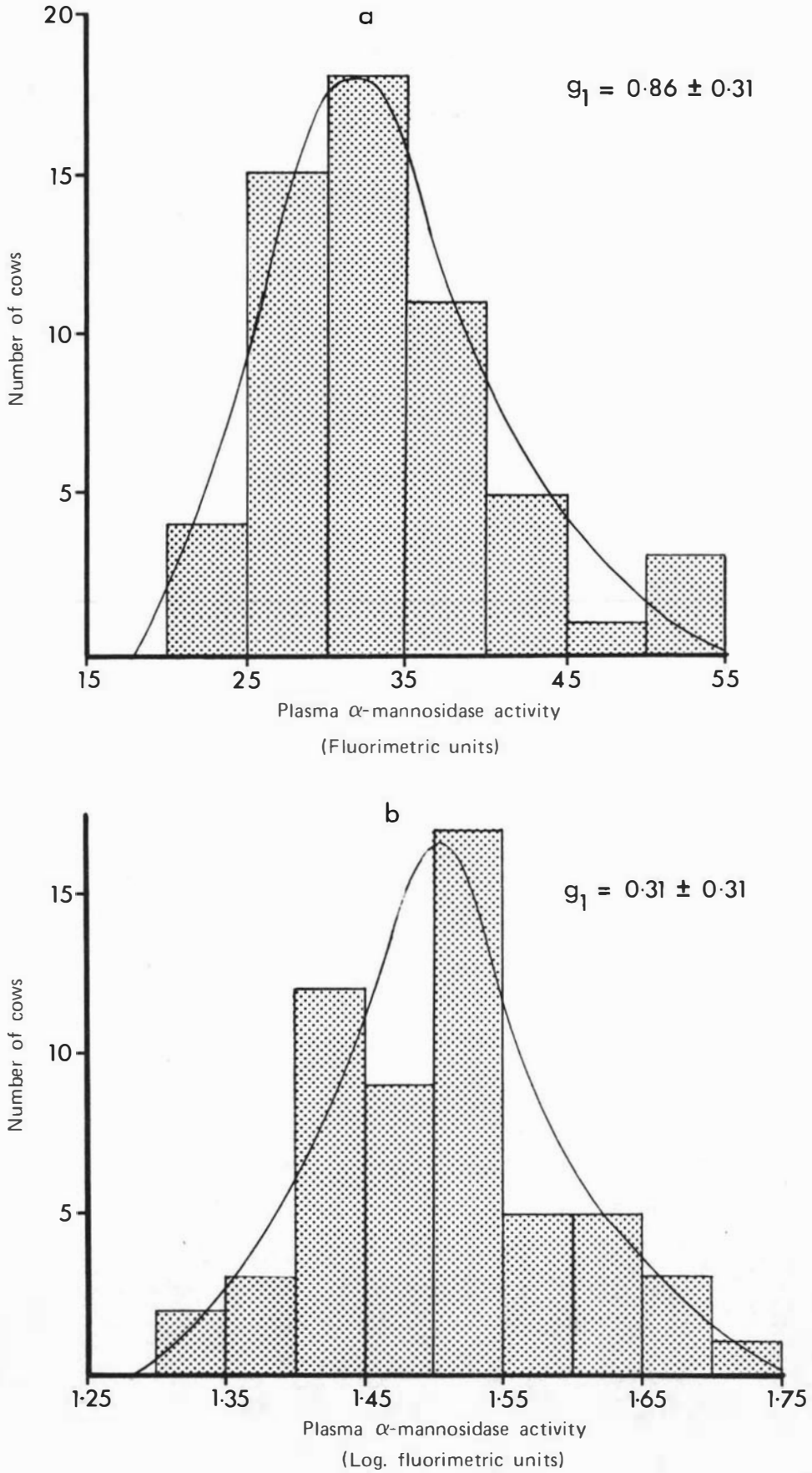


Figure 4.1 **Distribution of plasma α-mannosidase activities showing positive skewness and correction by logarithmic transformation of values, in a herd of 57 Jersey cows.**

TABLE 4.I

Tests for skewness in distributions of plasma α-mannosidase activity in herds of normal adult cows

Herd and no. animals			Estimates of skewness ($g_1 \pm$ standard error)	
			Fluorimetric units	Log. fluorimetric units
1	Angus	(101)	0.27 ± 0.24	- 0.06 ± 0.24
2	"	(89)	0.46 ± 0.26 *	0.13 ± 0.26
3	"	(108)	0.29 ± 0.23	0.07 ± 0.23
4	"	(142)	0.53 ± 0.20 *	0.03 ± 0.20
5	"	(83)	0.47 ± 0.26 *	0.29 ± 0.26
6	"	(135)	2.37 ± 0.21 *	0.59 ± 0.21 *
7	"	(65)	0.94 ± 0.30 *	0.46 ± 0.30
8	"	(50)	0.43 ± 0.34	- 0.37 ± 0.34
9	"	(92)	- 0.11 ± 0.25	- 0.14 ± 0.25
10	"	(83)	0.23 ± 0.26	- 0.25 ± 0.26
11	"	(134)	- 0.04 ± 0.21	- 0.29 ± 0.21
12	"	(99)	0.19 ± 0.24	- 0.37 ± 0.24
13	Jersey	(57)	0.86 ± 0.31 *	0.31 ± 0.31

* Significantly skewed distribution ($p < 0.05$)

A between-herd comparison of mean plasma α-mannosidase activities and distribution of log. values about the mean is shown in Figure 4.2 after probit transformation of distribution curves for the 12 Angus herds. A value of "5" on the probit scale represents the mean of the distribution, while each probit unit represents one standard deviations from the mean. Ninety-nine per cent. of animals would be expected to have plasma α-mannosidase activities within ± 2.58 standard deviation of the mean. Significant differences ($p < 0.05$) between mean values, as indicated by an absence of overlap between 5% fiducial limits, were shown for several herds. The distribution of log. values about each mean were similar, as demonstrated by the similarity between the slopes of most of the probit lines. Only one line had a significantly different slope than the average ($p < 0.05$). The value below which only 1% of the normal population would be expected to fall varied from 56% to 70% of the mean, with an average of 63%.

2. Plasma α-mannosidase activity in heterozygotes

The separation of certain Angus herds into two distinct populations on the basis of plasma α-mannosidase activities is demonstrated in Figures 4.3a and 4.4a. The animals contributing to the population with low enzyme activities are considered to be heterozygous for the mannosidosis genotype. In many groups there was no clear separation between heterozygous and normal populations thereby creating difficulties in deciding the status of animals in the low normal and high heterozygous areas. After logarithmic transformation of values to remove positive skewness (Figures 4.3b and 4.4b) the distribution curves were sometimes converted to probit lines (Figures 4.3c and 4.4c). The theoretical cut-off point between the heterozygous and normal populations is the point of inflection of the probit line (indicated by an arrow in Figures 4.3c and 4.4c), where the line representing one population

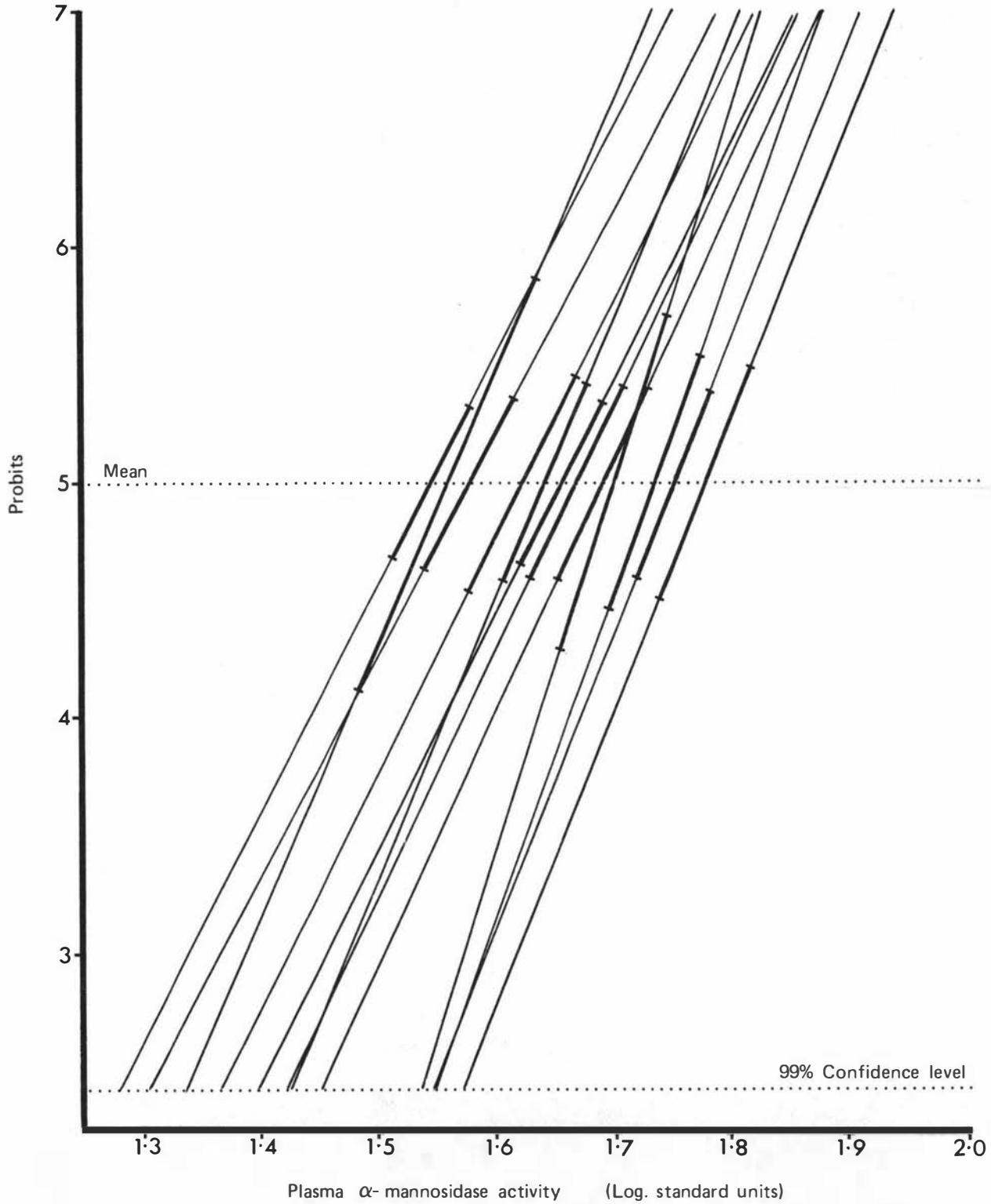


Figure 4.2 **Distribution of plasma α-mannosidase activities, expressed on a probit scale, for normal adult cows from twelve Angus herds. Superimposed on each probit line is the 5% fiducial limit about the mean.**

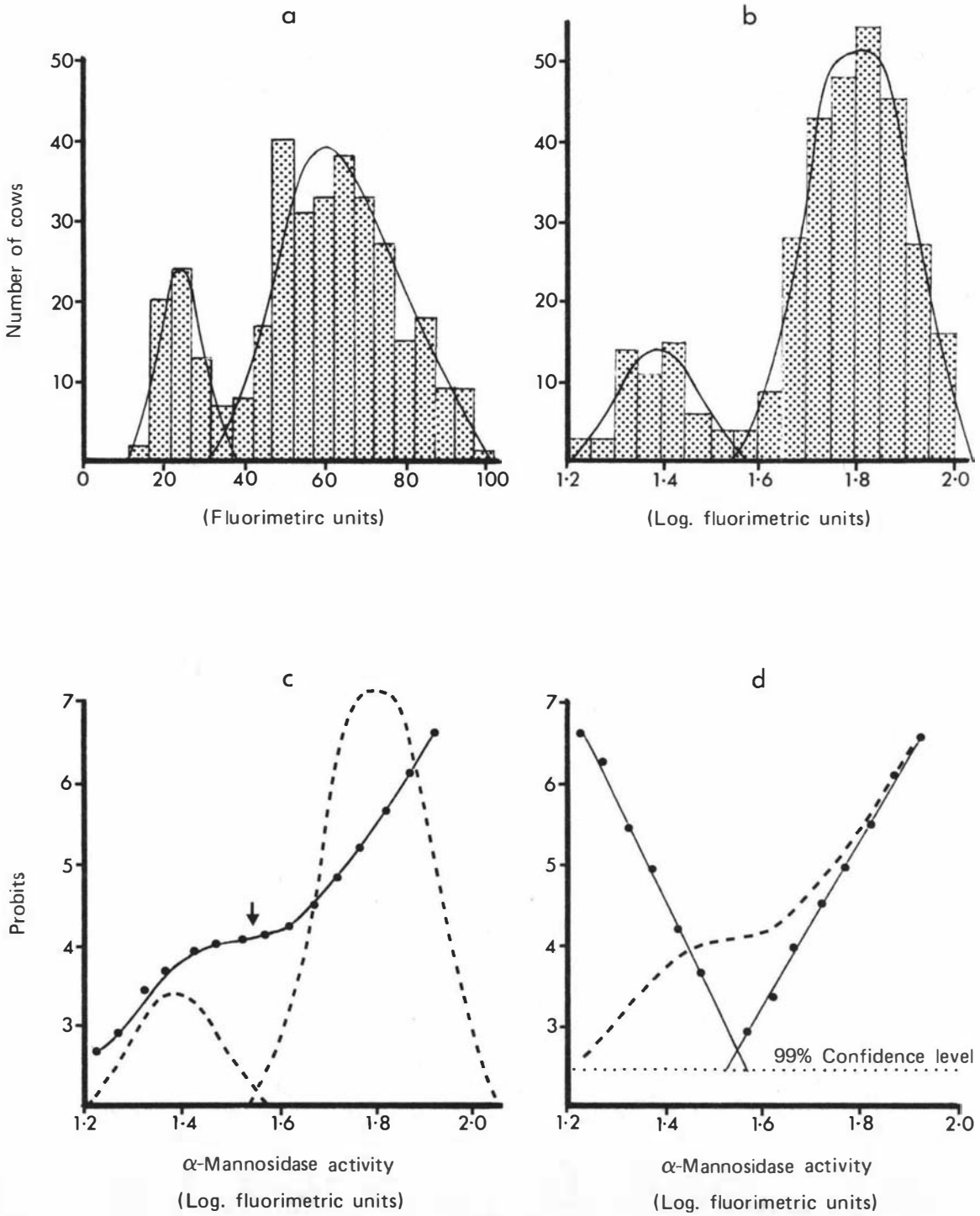


Figure 4.3 **Graphical treatment of plasma α-mannosidase activities in a herd of Angus cows to determine the probable status of individuals with regard to the mannosidosis genotype.**

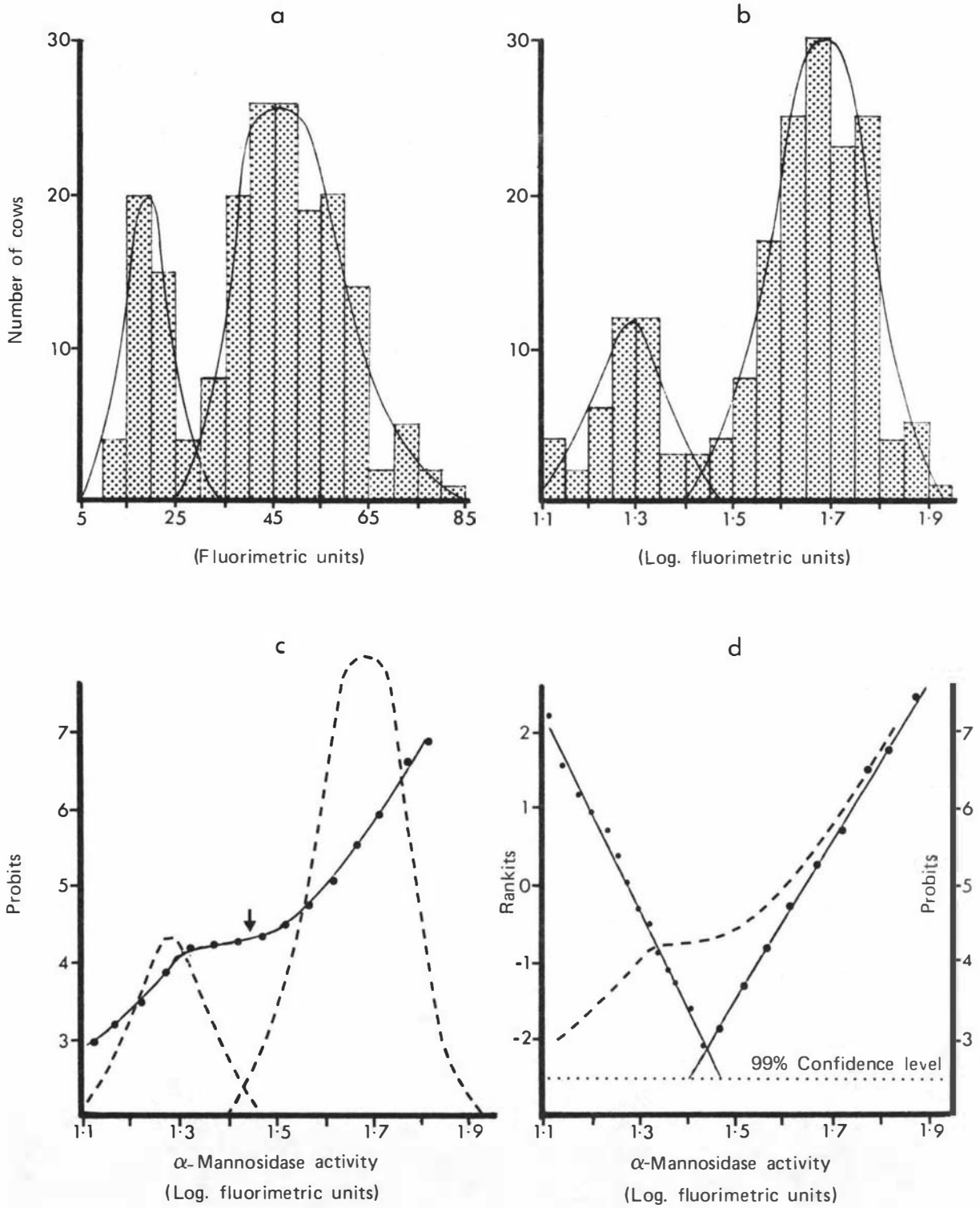


Figure 4.4 **Graphical treatment of plasma α-mannosidase activities in a herd of Angus cows to determine the probable status of individuals with regard to the mannosidosis genotype.**

changes to that representing the other. The two populations may then be redrawn separately using either probit or rankit transformation. Each is fitted with the line of best fit which may be extended to the 99% confidence level (Figures 4.3d and 4.4d), providing an estimate of the upper and lower values expected to be shown by 1% of the heterozygous and normal populations respectively.

Five of the 12 Angus herds discussed previously, contained a sufficient number of heterozygotes to allow a comparison between plasma α-mannosidase activities of normal and heterozygous animals. By placing fiducial limits on the differences between the log. means of both populations in each herd it was possible to show that the means of the heterozygous populations were significantly ($p < 0.05$) less than half the means of the normal populations (Table 4.II). The fiducial limits did not include 0.30 (log. of 2.0) in any of the five herds. The unweighted average of plasma α-mannosidase activity in heterozygotes was 37.5% of normal with a range of 31.8% to 41.2%.

TABLE 4.II

The relationship between plasma α-mannosidase activity in normal and heterozygous cows

Herd	Mean plasma α-mannosidase activity (fluorimetric units)		Fiducial limits of difference (log. scale)	Heterozygous value as % of normals
	Normal population	Heterozygous population		
	*			
3	48.5 (114)	20.0 (10)	0.3384 - 0.4332	41.2%
4	43.3 (142)	17.3 (42)	0.3713 - 0.4271	40.0%
6	38.0 (135)	12.1 (33)	0.3683 - 0.6315	31.8%
11	59.4 (134)	23.3 (18)	0.3607 - 0.4513	39.2%
12	41.6 (98)	14.6 (19)	0.4094 - 0.5004	35.1%

* Number of cows per group

3. Overall results of the pilot test and control programme

A complete list of results obtained from the pilot scheme is presented in Table 4.III. Of 4,799 animals tested, 13.1% were designated heterozygous. Only two herds, both of which were relatively small, contained no heterozygous animals, while the prevalence of heterozygotes was greater than 20% in eight of the 38 herds tested. The geographical distribution of the mannosidosis genotype formed no obvious pattern, and was widespread throughout both North and South Islands of New Zealand. Of the animals tested, 41 (0.85%) were listed as equivocal, 21 of which occurred in a large herd (herd 38) with an abnormally high prevalence of heterozygotes (28.9%). Considerable difficulty was encountered in deciding the status of many of the 18-month females in this herd, as indicated by the 10 equivocal cases recorded (Table 4.III). These heifers were retested 12 months later and on this occasion the situation was more clear, with all 10 equivocal animals appearing normal.

The prevalence of heterozygotes among animals from herds with a history of mannosidosis was 22% compared with 9.8% in herds with no recorded history of the disease (Table 4.IV).

TABLE 4.IV

Incidence of mannosidosis heterozygotes in herds with and without a history of mannosidosis

	Herds with history of mannosidosis	Herds without history of mannosidosis
No. herds	8	30
No. animals	1,277	3,522
No. heterozygotes	281	346
% heterozygotes	22.0%	9.8%

TABLE 4.III

Overall results of the pilot testing programme for mannosidosis heterozygotes

Herd	18-month females				18-month males				Mature females				Mature males				Total % of H
	N	H	E	% H	N	H	E	% H	N	H	E	% H	N	H	E	% H	
1	34	-	-	0	32	1	-	3.0	102	1	-	1.0	3	-	-	0	1.2
2	43	-	-	0	37	-	-	0	90	2	-	2.2	4	-	-	0	1.1
3	46	4	-	8.0	35	2	-	5.4	114	10	1	8.0	3	-	-	0	7.4
4	47	11	-	19.0	43	7	1	13.7	142	42	1	22.7	2	-	-	0	20.3
5	30	-	-	0	22	-	-	0	82	2	-	2.4	2	-	-	0	1.5
6	53	4	-	7.0	49	5	-	9.0	135	33	1	19.5	9	-	-	0	14.5
7	19	1	-	5.0	24	-	-	0	65	2	-	3.1	2	-	-	0	2.7
8	17	-	-	0	14	-	-	0	50	-	-	0	1	-	-	0	0
9	40	-	-	0	25	1	-	3.8	92	2	-	2.1	1	1	-	50	2.5
10	22	1	-	4.4	32	-	-	0	83	5	-	5.7	3	-	-	0	4.1
11	48	-	-	0	57	3	1	4.9	134	18	-	12.0	6	-	-	0	7.9
12	42	3	-	6.7	32	6	1	15.4	98	19	1	16.0	4	-	-	0	13.6
13	-	-	-	-	31	3	-	8.8	-	-	-	-	3	-	-	0	8.1
14	9	-	-	0	6	-	-	0	24	-	-	0	-	-	-	-	0
15	6	1	-	14.3	11	1	-	8.3	24	4	-	14.3	1	-	-	0	12.5
16	23	7	-	23.3	8	3	-	27.3	116	35	4	23.0	6	1	1	12.5	22.6
17	9	5	-	35.7	15	2	1	11.1	39	-	-	0	-	-	-	0	9.9
18	6	-	-	0	-	-	-	-	17	1	-	5.6	1	-	-	0	4.0
19	9	-	-	0	10	-	-	0	25	1	-	3.9	1	-	-	0	2.2
20	14	1	-	6.7	4	1	-	20.0	35	9	-	20.5	1	1	1	33.3	17.9

Contd.

TABLE 4.III (Contd.)

Herd	18-month females				18-month males				Mature females				Mature males				Total % of H
	N	H	E	% H	N	H	E	% H	N	H	E	% H	N	H	E	% H	
21	18	-	-	0	15	2	-	11.8	50	6	-	10.7	1	-	-	0	8.7
22	24	-	-	0	15	1	-	6.3	59	2	-	3.3	1	-	-	0	2.9
23	15	-	-	0	13	1	-	7.1	55	7	-	13.5	3	-	-	0	8.5
24	17	6	-	26.1	9	4	-	30.8	28	10	1	25.6	-	-	-	-	26.7
25	13	3	-	18.8	24	5	1	16.7	-	-	-	-	-	-	-	-	17.4
26	14	1	-	6.7	21	-	-	0	56	1	1	1.7	2	-	-	0	2.1
27	11	3	-	21.4	-	-	-	-	34	2	1	5.4	-	1	-	100	11.5
28	15	13	-	46.4	14	4	-	22.2	58	1	-	1.7	-	1	-	100	17.9
29	-	-	-	-	14	1	-	6.7	-	-	-	-	3	-	-	0	5.6
30	-	-	-	-	31	8	-	20.5	-	-	-	-	1	1	-	50	22.0
31*	25	5	-	17.0	56	18	-	24.3	-	-	-	-	10	2	1	15.4	21.4
32*	11	1	-	8.3	3	-	-	0	61	5	-	7.6	-	-	-	-	7.4
33*	24	3	-	11.1	-	-	-	-	12	-	-	0	1	-	-	0	7.5
34*	10	1	-	9.1	10	2	-	16.7	54	17	-	23.9	1	-	-	0	21.1
35*	38	5	-	11.6	37	3	1	7.3	114	18	-	13.6	3	-	-	0	11.9
36*	50	14	-	21.9	32	17	-	34.7	-	-	-	-	-	-	-	-	29.2
37*	-	-	-	-	1	-	-	0	51	5	-	8.9	3	-	-	0	8.3
38*	36	22	10	32.4	61	26	7	27.7	262	116	3	30.5	7	1	1	11.1	28.9
Totals	838	115	10	11.9	843	127	13	12.9	2361	376	14	13.7	89	9	4	8.8	13.1

* Herds with history of mannosidosis.

Total no. animals = 4,799
Total no. heterozygous = 627 (13.1%)
Total no. equivocal = 41 (0.85%)

N = Normal
H = Heterozygous
E = Equivocal

4. Reliability of the plasma test

Several cattle tested prior to the commencement of the pilot scheme were retested to provide an indication of the reliability of the plasma test for mannosidosis heterozygotes. These results are presented in Table 4.V.

No alterations were recorded in the status of 84 adult and 29 18-month cattle from three properties. On four properties where 365 animals were tested initially as calves (4 to 8 months of age) and again at 18 months of age, the diagnosis changed from normal to heterozygous, or vice versa, in seven cases. Nine animals with equivocal test results were resolved by the second test but the diagnosis became equivocal in a further five cases.

TABLE 4.V

Comparison of diagnosis for cattle tested for the mannosidosis genotype in successive years

Age at first test	No. animals	Animals with same diagnosis	Animals with different diagnosis					
			N → H	N → E	H → N	H → E	E → N	E → H
2 years and older	84	84						
18 months	29	29						
6 months	365	344	5	2	2	3	5	4

N = Normal plasma test

E = Equivocal plasma test

H = Heterozygous plasma test

5. Inheritance of the mannosidosis genotype

The relationships between parents and their progeny with regard to mannosidosis genotype, as determined by plasma α-mannosidase activities, are presented in Table 4.VI. Animals were included in this analysis only if the presumed genotype of both parents was available.

TABLE 4.VI

Genotype of parents and progeny as determined by plasma α-mannosidase activity

	Normal progeny	Heterozygous progeny
Normal sire x normal dam (1,714 matings)	1,691 (98.7%) (Expected = 100%)	23 (1.3%) (Expected = 0%)
Normal sire x heterozygous dam (316 matings)	156 (49.4%) (Expected = 50%)	160 (50.6%) (Expected = 50%)

In 316 matings of normal sires to heterozygous dams, 156 (49.4%) of the progeny were normal and 160 (50.6%) were heterozygous. Normal parents would not be expected to produce any heterozygous progeny, but from 1,714 matings between presumed normal animals, 23 (1.3%) heterozygotes were recorded. It was possible to reinvestigate seven of these anomalies, and in each case the plasma test produced the same result. Blood-typing was also performed on these animals and their parents to check the stated parentage, the results being listed in Table 4.VII. A parentage cannot be proven by blood-typing but it can be excluded if all blood characters present in the offspring are not present in one or both of the parents (Johansson and Rendel, 1968). The recorded parentages of four animals with anomalous results were found to be incorrect and a fifth animal could have been the progeny of either the stated sire or a closely related heterozygous sire on the same property.

TABLE 4.VII

Investigation of seven anomalous results by blood-typing to check alleged parentage

Animal identification	Result of blood-typing
645	Alleged parentage not possible
67	" " " "
642	" " " "
698	" " " "
349	Parentage possible both for the alleged sire and a closely related heterozygous sire on same property
296	Alleged parentage possible
292	" " "

6. Comparison between plasma tests and progeny tests on herd sires

During the pilot test and control programme 102 mature bulls were tested. A significant number of progeny was also tested from 52 of these sires and from 21 sires tested immediately subsequent to the pilot scheme, allowing a comparison to be made between individual plasma tests and progeny tests. These results are presented in Table 4.VIII.

One inconsistency was found between the two tests. A bull which appeared clearly heterozygous on the basis of its plasma α-mannosidase activity had a progeny test consisting of one heterozygote and thirteen normal offspring. On checking with the New Zealand Angus Association, it was discovered that at least six of the progeny in question were sired by another bull. Permission for further investigation was denied by the breeder. Of ten bulls whose genotype could not be determined on the basis of plasma α-mannosidase activity alone, only one remained

equivocal after progeny testing. The progeny tested from this bull included 12 normal and six heterozygous animals. Five of the latter had heterozygous dams, suggesting that the bull was probably normal but with one of its progeny being wrongly recorded. It was necessary however to record such a progeny test as equivocal. This bull was subsequently designated normal on the basis of a supplementary test on lymphocytes (see Chapter V).

TABLE 4.VIII

Comparison between plasma tests of sires and tests on their progeny, in assessment of genotype

Genotype of sires				
Determined by plasma tests		Determined by genotype of progeny*		
		Normal	Equivocal	Heterozygous
Normal	51	51	-	-
Equivocal	10	7	1	2
Heterozygous	12	1	-	11

* To be designated normal on progeny test, a sire must have produced at least 7 normal calves and no suspected heterozygotes unless from heterozygous cows. A sire could be designated heterozygous on a smaller number of calves if these included heterozygotes from normal cows.

7. Genetic influence on plasma α-mannosidase activity in normal animals

In eleven Angus herds the relationship between plasma α-mannosidase activity of normal dams and their progeny was examined. Correlation coefficients were calculated for this relationship in each herd and averaged by means of the weighted z transformation (Snedecor and Cochran, 1967). The average correlation coefficient (r) was 0.27, and the heritability of plasma α-mannosidase activity is therefore 0.54 (2 x r).

Figure 4.5 shows the mean plasma α-mannosidase activities (with 5% fiducial limits) of 18-month males and females sired by seven different bulls in a large herd tested during 1975. The 18-month male progeny of bull A had a significantly lower ($p < 0.05$) mean value than the male progeny of bulls F and G. The male progeny of bull B also had a significantly lower mean value than those of bull G. The 18-month female progeny of bulls A, C and E all had significantly lower mean values than the female progeny of bull G.

The 18-month bulls in this herd were run in five separate mobs, none of which had significantly different mean plasma α-mannosidase activities, and all 18-month females were run together, therefore between-mob variation in plasma α-mannosidase activity is unlikely to have influenced the above results.

8. Between-sex differences in plasma α-mannosidase activity

When the mean plasma α-mannosidase activity of 18-month female cattle was compared with that of 18-month males in the same herd , the females had lower mean values in 15 of 17 herds examined (Table 4.IX). In six cases this difference was significant ($p < 0.01$). In one herd the males had a higher mean value than the females but the difference was nonsignificant.

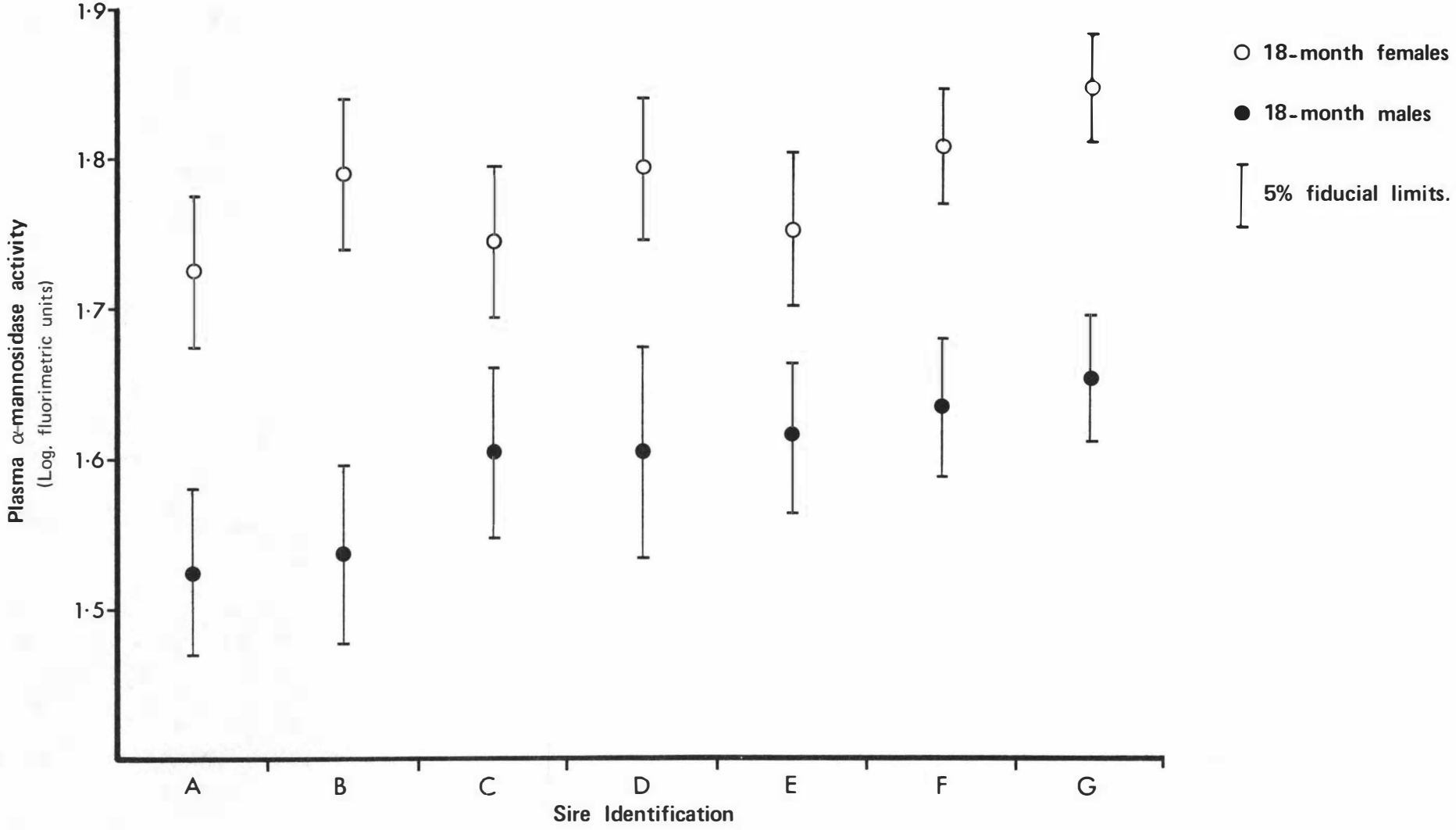


Figure 4.5 Variation in mean plasma α-mannosidase activity of 18-month male and female progeny from seven sires in an Angus herd.

TABLE 4.IX

Between-sex differences in mean plasma α-mannosidase activity of 18-month cattle from 17 Angus herds

Herd	Mean plasma α-mannosidase activity (fluorimetric units)		Significance of difference
	18-month females	18-month males	
1	51 (34)*	39 (32)	$p < 0.01$
2	34 (43)	30 (37)	N.S.
3	30 (46)	29 (35)	$p < 0.01$
5	52 (30)	49 (22)	N.S.
6	40 (53)	33 (49)	$p < 0.01$
7	42 (19)	41 (24)	N.S.
9	57 (40)	44 (25)	$p < 0.01$
10	49 (22)	43 (32)	N.S.
11	44 (48)	46 (57)	N.S.
16	54 (23)	34 (8)	$p < 0.01$
17	52 (9)	45 (15)	N.S.
21	41 (18)	34 (15)	N.S.
24	46 (17)	39 (9)	N.S.
25	57 (13)	35 (24)	$p < 0.01$
26	39 (14)	39 (21)	N.S.
28	43 (15)	42 (14)	N.S.
34	48 (10)	41 (10)	N.S.

* Number of animals

9. The effect of pregnancy on plasma α-mannosidase activity

The effect of pregnancy on plasma α-mannosidase activity was investigated in two of the herds discussed above, where there were sufficient numbers of pregnant and non-pregnant 18-month females for a comparison to be made. As can be seen from Table 4.X, in neither herd was there a significant difference in mean plasma α-mannosidase activity between pregnant and non-pregnant cattle.

TABLE 4.X

The effect of pregnancy on plasma α-mannosidase activity in 18-month female cattle

Herd	Mean plasma α-mannosidase activity (fluorimetric units)		Significance
	Non-pregnant	Pregnant	
2	55.1 (16)*	52.7 (27)	N.S.
11	72.8 (28)	69.9 (20)	N.S.

* Number of animals in group

10. Seasonal variation in plasma α-mannosidase activity

Plasma α-mannosidase activities were assayed in a herd of 57 Jersey cows sampled at approximately 4-week intervals for 12 months. The variation in the herd mean log. values, with 5% fiducial limits about each mean, is shown in Figure 4.6. Superimposed on this figure is the herd milk production for the period of sampling, which approximated the rise and fall in plasma α-mannosidase activity.

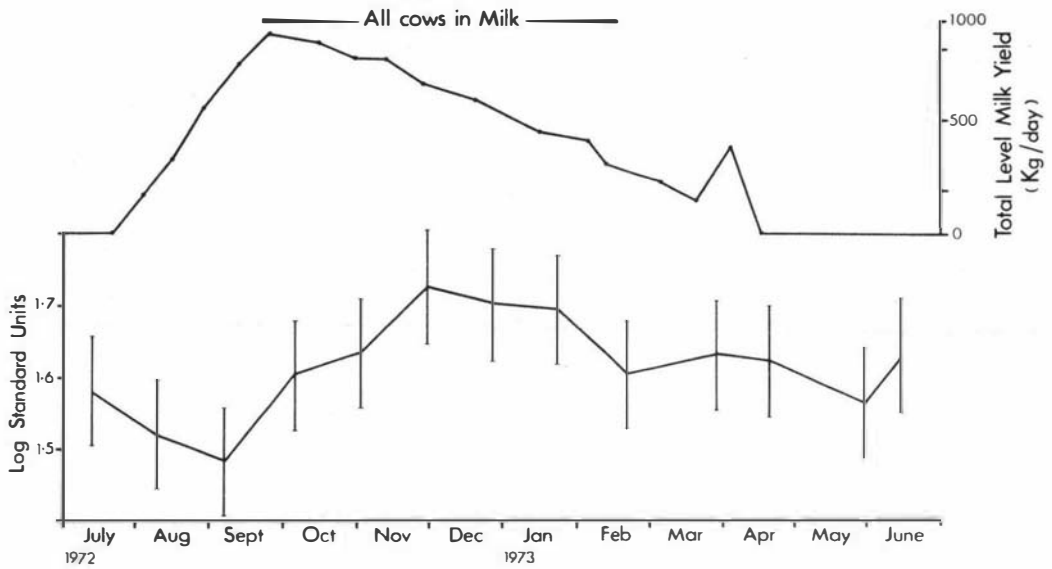


Figure 4.6 Seasonal variation in mean plasma α-mannosidase activity in a herd of 57 Jersey cows. The 5% fiducial limits are included for each mean. The upper curve shows seasonal milk production for the herd.

When expressed as probit lines (Figure 4.7) the slopes did not differ significantly, indicating that while the mean plasma α-mannosidase activity varied throughout the year, the distribution of individual log. values about the mean remained relatively constant.

11. Between-mob differences in plasma α-mannosidase activity within a herd

In many herds the adult cows are run in separate mobs on the basis of their age, stage of lactation or pregnancy, on the sex of their calf, or on the basis of the bull to which they are mated. Cows in late pregnancy or early lactation are likely to be on a relatively high plane of nutrition, especially when compared with dry cows, and cows with male calves may be fed preferentially to those with female calves. Between-mob differences in mean plasma α-mannosidase activity therefore might be expected and may complicate the overall separation of normal and heterozygote populations on histograms of plasma α-mannosidase activity.

In a large herd tested subsequent to the completion of the pilot scheme, the adult cows were in eight mobs. Probit or rankit lines representing the distribution of plasma α-mannosidase activities of the presumed normal animals in each mob are shown in Figure 4.8. Highly significant between-mob differences in mean plasma α-mannosidase activity were observed, but the distribution of values about each mean (as judged by the slopes of the lines) appeared to be relatively constant.

The highest mean plasma α-mannosidase activity was recorded in a mob of late-calving cows which would consist of cows in early lactation. One of the two mobs with significantly lower mean values than the others consisted entirely of dry cows.

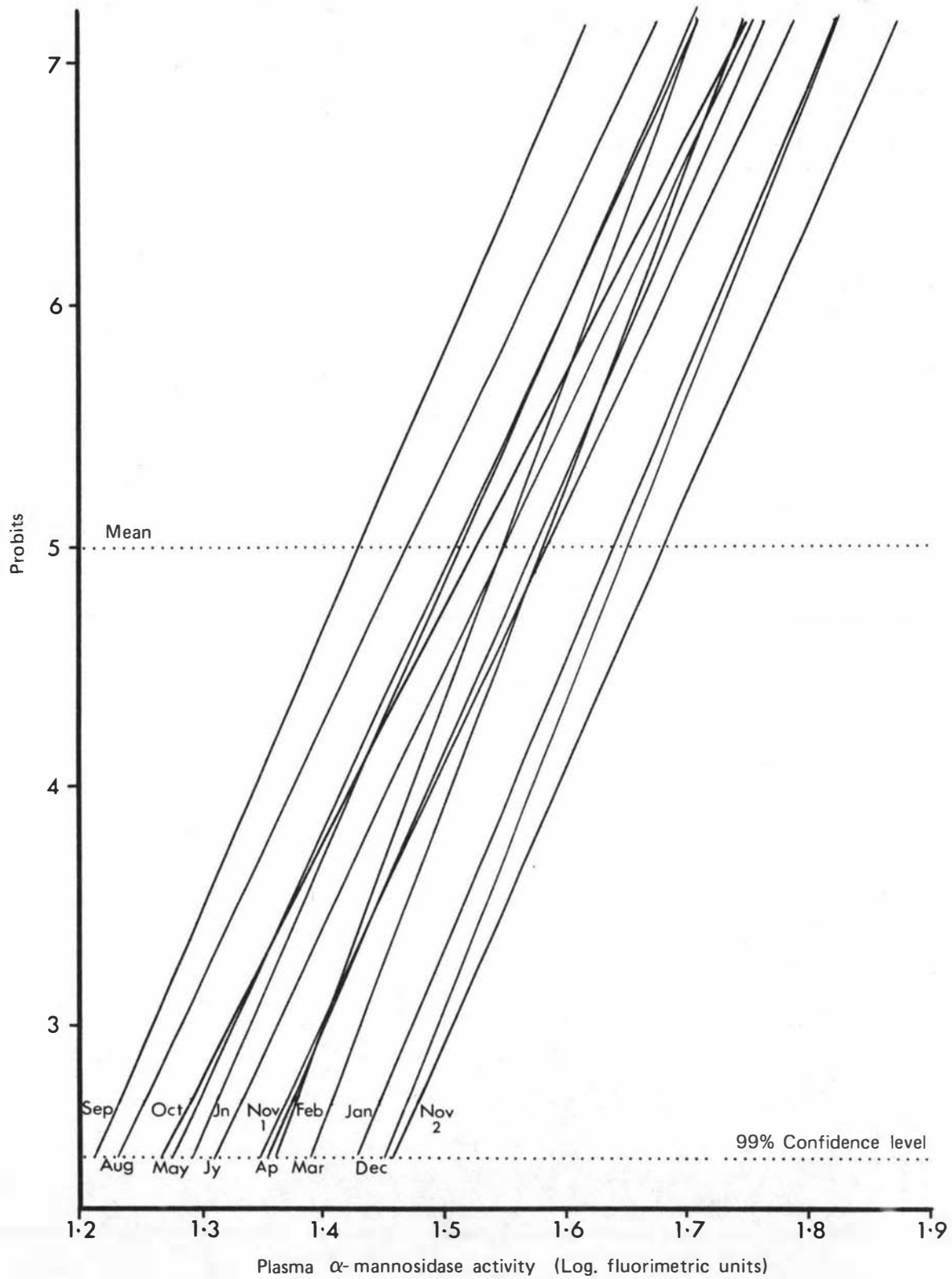


Figure 4.7 Distribution of plasma α-mannosidase activities, at each month of the year, for a herd of 57 Jersey cows, with results being expressed on a probit scale.

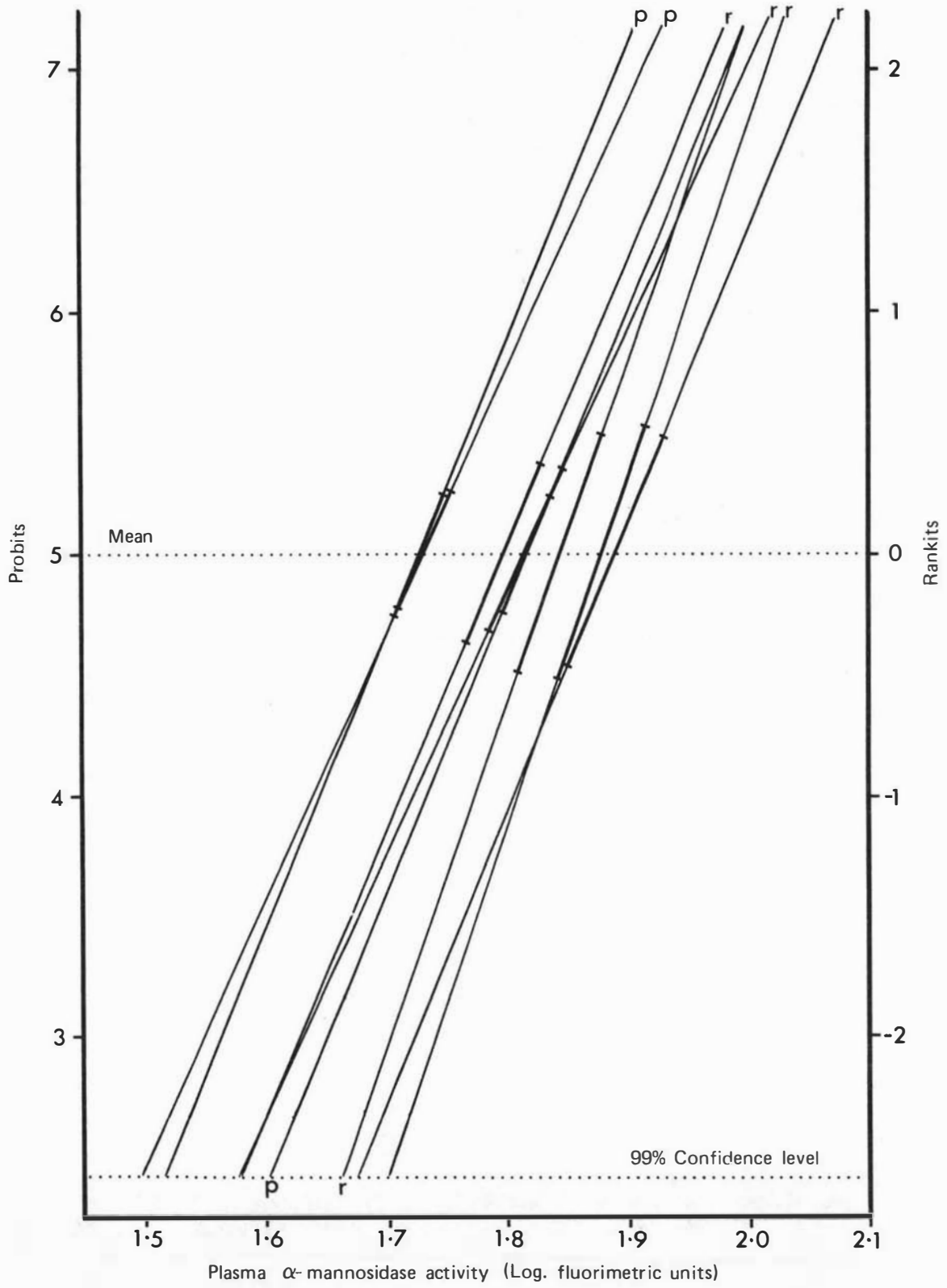


Figure 4.8 **Distribution of plasma α-mannosidase activities, expressed as probit (p) or rankit (r) lines, for eight separate mobs of adult Angus cows on the one property. The 5% fiducial limits about each mean are superimposed on each line.**

DISCUSSION

The plasma test for mannosidosis heterozygotes is based on the gene dosage phenomenon, and heterozygotes should therefore be expected to possess approximately 50% of the normal level of α-mannosidase activity in their tissues and body fluids (Chapter I, page 7). The mean activity of α-mannosidase in the plasma of heterozygotes was in fact found to be significantly less than 50% of the mean for normal animals, and was estimated as being, on average, 37.5% of normal (Table 4.II). A similar observation was reported by Hocking et al. (1972). In spite of this large difference between the population means, the distribution of individual values about each mean were such that there was often some overlap between the two populations, and the differentiation of heterozygotes from normal animals was not always straightforward.

The results obtained from a herd of Jersey cows, and from 12 Angus herds in which there was clear separation between presumed heterozygous and normal populations, were used to study the characteristics of the distribution curve for plasma α-mannosidase activity in normal animals. Many of these distributions showed an apparent positive skewness (Figures 4.1, 4.3a and 4.4a) which was significant in six herds and which could usually be corrected by logarithmic transformation of values (Table 4.I, Figures 4.1, 4.3b and 4.4b). The skewed distribution of values about the mean reflects heterogeneity of the population, possibly through the presence of cows at various stages of lactation or oestrus, or a small number of animals suffering from sub-clinical disease. Alternatively it may be due to the existence of two or more "normal" alleles for α-mannosidase, each with different average levels of activity within the normal range. In a population of individuals several different alleles, either normal or abnormal, may occur for a given gene locus (Harris, 1971). If for example there were two "normal"

alleles, E_A and E_B, coding for α-mannosidase in Angus cattle, and if these alleles produced different average levels of α-mannosidase activity, then the following hypothetical situation might be imagined:

Proposed genotype	Average α-mannosidase activity (arbitrary units)
$E_A E_A$	50
$E_B E_B$	80
$E_A E_B$	65

In a situation where the allele E_A was considerably more common than E_B the distribution curve for α-mannosidase activity in a normal population would be expected to show a slight positive skewness. The activity of α-mannosidase in animals heterozygous for the abnormal mannosidosis allele would also vary according to which of the "normal" alleles they possessed.

A relatively strong heritability ($h = 0.54$) was estimated for plasma α-mannosidase activity in normal animals, from the relationship between enzyme activities in dams and their progeny. Direct evidence for this genetic influence on the level of plasma α-mannosidase activity in normal animals was obtained by demonstrating significant differences in mean enzyme levels between the progeny of different bulls on the same property (Figure 4.5). The inheritance of different "normal" levels of α-mannosidase activity would be expected if there were more than one allele coding for activity within the normal range as discussed above. Alternatively the genetic differences may be due to the inheritance of different modifying genes responsible for regulating enzyme synthesis or activity.

The mean plasma α-mannosidase activity of normal animals in the 12 Angus herds varied considerably, with the differences often being highly significant (Figure 4.2). The distribution of log. values about each mean however was relatively constant, as illustrated by the similarity of slopes of the probit lines in Figure 4.2. By extending probit lines to the 99% confidence level it was possible to gain an estimate for each herd of the lower limit of the normal population, below which only 1% of normal animals would be expected. This value varied from 56% to 70% of the mean, with an average of 63%. While this figure is useful in providing an indication as to the lower limit of normality it does not demonstrate the degree of overlap between normal and heterozygous populations. When sufficient heterozygous animals were present in a group to allow rankit or probit transformation of their values, the lines representing the normal and heterozygous populations almost invariably overlapped before reaching the 99% confidence level. Thus a small percentage of heterozygotes would be expected to have plasma α-mannosidase activities greater than 63% of the mean for the normal population. The extent of overlap varied between herds and only a small percentage of animals, if any, were included in this area. The designated genotype of animals within this indefinite area was influenced by information on the genotype of their parents, if available. If either parent was regarded as heterozygous then the animal in question was taken to be either heterozygous or equivocal, but if both parents had normal plasma tests then the animal was designated normal. A small percentage of animals in the overlapping area were listed as "equivocal" when information on the genotype of their parents was unavailable, or if it was felt that a diagnosis could not be made with an acceptable degree of probability. An attempt to slightly over-call heterozygotes was made, so as to minimize the number of carriers escaping detection.

Of the animals tested in the pilot scheme only 0.85% were finally listed as equivocal, and many of these occurred in one herd (herd 38 in Table 4.III). In this herd the percentage of heterozygotes was extremely high (28.9%) and both normal and heterozygous populations appeared to have a greater than usual distribution about their mean. This may have been due partly to between-mob differences in mean plasma α-mannosidase activity, which had not been established at this stage but which would be expected to contribute to the heterogeneity of the populations. The number of animals in the area of overlap was therefore greater than might be expected and the cut-off point more difficult to determine. Furthermore, the extensive and difficult conditions under which this herd was managed meant that less emphasis could be placed on the pedigrees of equivocal animals, as errors were likely to be more frequent than in a more easily managed herd. Results for the 18-month female group were not considered "official" due to the uncertainty of the status of almost 14% of these animals. This group was retested 12 months later, at which time the situation was more clear, and all 10 equivocals were designated normal.

Probit and/or rankit transformation of distribution curves was not considered necessary for routine diagnosis of genotype in herd groups. In many cases the separation between normal animals and heterozygotes was quite distinct on histograms of plasma α-mannosidase activity. The mean value of the presumed normal population was calculated for each group, and any animals with plasma α-mannosidase activities less than 60% of this mean were initially considered heterozygous. In groups where the separation on histograms was less distinct a second or even third mean often had to be calculated for animals considered normal, each time disregarding those animals below 60% of the new mean and listing them as heterozygous. All animals designated heterozygous were then

checked against the apparent genotype of their parents, if tested, to ensure that both parents had not been previously diagnosed as normal.

Prior to the commencement of this pilot test and control programme, Jolly et al. (1973) reported an average heterozygote frequency of 16% in 2,500 animals, many of which were from herds with a history of mannosidosis. Of 4,799 animals tested in the pilot scheme, 13.1% were designated heterozygous, but this figure is also biased by the inclusion of several herds selected because of their previous history of the disease. The incidence of heterozygotes in herds with no reported occurrence of mannosidosis was 9.8% (Table 4.IV) and this is probably a more accurate estimate of the overall frequency of Angus cattle carrying the mannosidosis genotype in New Zealand. From the data collected it is apparent that the genotype is spread widely throughout New Zealand, with very few herds being completely free, suggesting that it is not a recently introduced disease. Geneological studies support this hypothesis (Jolly, personal communication) and indicate that the major spread of the genotype occurred approximately 30 years previous to this study.

Possible reasons for the mannosidosis genotype having attained such a high prevalence include a selection or survival advantage of the heterozygous state or, more likely, certain heterozygous sires may have had a disproportionate influence on the genetic structure of New Zealand's Angus population. A single bull may sire 50 or more progeny per year, therefore a heterozygous bull with particularly desirable phenotypic characteristics could rapidly spread its defective genotype throughout the country's breeding herds. With the advent of artificial breeding it becomes possible for a single bull to sire many thousands of progeny per year, and it is therefore of extreme importance that every Angus bull employed in artificial breeding should be tested for the mannosidosis genotype.

The unreliability of the plasma test when employed on young calves was expressed by Jolly et al. (1973) and has been confirmed by results presented in Table 4.V. Plasma α-mannosidase activity increases considerably with age until maturity and the activity for a normal calf corresponds more or less with that of a heterozygous adult (Jolly et al., 1973). A group of calves may include animals from 4 months to 8 months of age when tested, and this relatively great variation in age is thought to contribute largely to the inaccuracy of the test in this age-group. The test does appear to be reliable in adult and 18-month cattle, where no change was necessary in the designated genotype of 113 cattle tested in consecutive years (Table 4.V).

The majority of inborn lysosomal disorders in both man and animals are characterized by an autosomal recessive nature of inheritance. Breeding experiments in which known heterozygous bulls were mated to heterozygous cows have provided strong evidence that this method of inheritance also applies to the mannosidosis genotype in Angus cattle (Jolly et al., 1973). Further support for this method of inheritance, and the validity of the plasma test in heterozygote detection, has been obtained from the pilot testing programme. In matings of heterozygous dams to normal bulls 50% of the progeny would be expected to be heterozygous and 50% normal. In 316 such matings, 156 (49.4%) of the progeny were considered normal and 160 (50.6%) were designated heterozygous (Table 4.VI), closely approximating the expected percentages in spite of an attempt to slightly over-call heterozygotes. Of 1,714 matings between normal animals, 23 (1.3%) heterozygous progeny were recorded (Table 4.VI). Seven of these anomalies were reinvestigated and the recorded parentage was found to be impossible in four cases. In a fifth case the parentage was possible both for the alleged sire and a closely related heterozygous bull on the same property (Table 4.VII). Although

the stated parentage of the remaining two animals was possible, it need not be correct. Alternatively these animals may possess very low "normal" levels of plasma α-mannosidase activity which might be expected to be shown by a small percentage of the normal population. The occurrence of a small percentage of anomalous results is not unexpected and in fact some breed societies accept that 4 to 5% of recorded pedigrees may be incorrect. Errors may occur in the management of sires, in the tagging and recording of newborn calves, or even during the actual recording of pedigrees. Another possible source of error is the adoption of newly-born calves by foster mothers, a phenomenon which is well recognised in sheep and which probably occurs to a lesser extent in cattle. It is not surprising that the frequency of anomalous results was greater on properties where management appeared, on subjective assessment, to be poor or difficult. In fact, more than half of the anomalies (12) occurred on one property, run under very extensive conditions, where the owner freely admitted to the likelihood of errors in the recorded parentages.

Problems were encountered when herd sires were presented for testing, due to the reduced accuracy of the plasma test on small groups or individual animals. To check the accuracy of the test in such cases a comparison was made between plasma tests and progeny tests on 73 mature bulls (Table 4.VIII). Progeny tests involved the screening of at least seven offspring from each bull by the plasma test, thereby providing a more reliable indication of a bull's status than was possible by an individual plasma test. Only one inconsistency between the two methods was discovered. A bull whose plasma α-mannosidase activity was well below that expected for a normal animal had an apparently normal progeny test, but, as mentioned previously, at least six of the 14 progeny forwarded for testing were not in fact sired by the bull in question. This does not fully explain the anomaly as the bull still

appears to have produced an unbalanced ratio of normal to heterozygous offspring, but it does cast some doubt as to the validity of this particular progeny test. The opportunity of further investigation by the testing of other possible progeny on the property was denied. Although 13.7% of the progeny-tested sires had equivocal plasma tests this figure is biased by an increased effort to obtain progeny tests from equivocal bulls. During the pilot testing scheme less than 4% of herd sires had equivocal plasma tests.

Seasonal variation in mean plasma α-mannosidase activity of a herd of 57 Jersey cows is shown in Figure 4.6. The highest mean values were recorded during late November, December and January, these values being significantly higher ($p < 0.05$) than the mean values recorded during August and September. Although the seasonal variation in plasma α-mannosidase activity tended to approximate the rise and fall in the total herd milk production, it was not possible to assume any casual relationships as the overall nutritional status of the animals, under New Zealand's pastoral farming conditions would be expected to follow a similar pattern.

In 15 of 17 herds where mean plasma α-mannosidase activities of 18-month female cattle were compared with those of 18-month males, the females had higher mean values, and in six herds the difference was significant ($p < 0.01$) (Table 4.IX). In humans, total serum hexosaminidase activities are elevated in pregnant women and in women on oral contraceptives (Kaback and Zeiger, 1972). This led Jolly et al. (1973) to suggest that the effects of pregnancy may be responsible for the increased level of α-mannosidase activity observed in young female cattle. In the current study however, in two herds where a direct comparison could be made between pregnant and non-pregnant heifers, the mean plasma α-mannosidase activities were not significantly different

(Table 4.X). There is doubt as to whether the apparent difference is due to sex per se, or whether it is a reflection of different planes of nutrition or management techniques. Significant differences in mean plasma α-mannosidase activity have been shown to occur between mobs of cattle on the same property (Figure 4.8) and as 18-month female and male cattle are run in separate mobs, factors associated with this between-mob variation may be responsible for the apparent between-sex differences. Furthermore, most breeders admitted that their 18-month male cattle were enjoying a higher plane of nutrition than their 18-month females.

Due to the demonstration of significant between-mob differences in plasma α-mannosidase activity of similarly aged cattle on the same property, it is desirable that information regarding the separation of cattle into mobs should be obtained for each herd tested. When feasible, separate histograms should be constructed for each mob to minimize the degree of heterogeneity and, theoretically, to reduce the distribution of values about the mean. Differences in the average stage of lactation may be a factor contributing to variation in mean plasma α-mannosidase activity between cows of similar age.

It is thought probable that seasonal variations, between-herd differences and between-mob differences in mean plasma α-mannosidase activity may be related to variation in the nutritional status of animals in association with certain physiological factors, such as lactation. Seven of the eight mobs in Figure 4.8 included cows in lactation and it is interesting to note that a mob of late-calving cows in early lactation had the highest mean plasma α-mannosidase activity, while the mob of non-lactating cows had a significantly lower mean than in all but one other mob. These observations are consistent with the possible relationship observed between seasonal variation in mean enzyme activity and milk production in a herd of Jersey cows (Figure 4.6).

Genetic factors might be expected to contribute to between-herd differences, especially between herds containing cattle of different major blood-lines, but would not be involved in seasonal or between-mob variation.

A small number of animals are sometimes recorded with very high plasma α-mannosidase activity, well above the expected range of the normal distribution curve. It is thought that such animals might be suffering from an acute disease (e.g. acute sporidesmin poisoning) which may increase the turnover of certain cell-types or cause cellular necrosis, thereby releasing lysosomal enzymes into the body fluids. It is conceivable that a heterozygous animal affected similarly, might have a plasma α-mannosidase activity within the range of normal animals and would therefore escape detection. Alternatively, animals with such high values may be homozygous for an uncommon allele which produces excessively high α-mannosidase activity. Increased release of lysosomal enzymes may occur in the presence of either excess or deficient levels of vitamins A and E, possibly due to a reduced stability of lysosomal membranes or, in the case of vitamin E deficiency, to tissue degeneration (Roels, 1969). Abnormal levels of these vitamins are unlikely to be a problem under New Zealand's pastoral farming conditions but whether or not selenium deficiency, which does occur in certain areas of New Zealand, would mimic this effect of vitamin E deficiency has not been established. Certain drugs may also affect the release of lysosomal enzymes. For example, corticosteroids are known to increase the stability of lysosomal membranes (Weissmann, 1969).

Although the accuracy and reliability of the plasma test for mannosidosis heterozygotes have been confirmed by this evaluation of the pilot test and control programme, certain limitations of the test have become obvious during this, and previous studies (Jolly *et al.*,

1973). Due to variation in plasma α-mannosidase activity with age, season and possibly sex, and to significant between-herd and even between-mob variations in activity, the reliability of the test is reduced when it is employed on small groups or single animals. In such cases an accurate estimate of the normal mean for the group cannot be obtained, and a more sophisticated supplementary test to support the plasma test would be particularly useful.

SUMMARY

An evaluation of the results of a pilot test and control programme for the mannosidosis genotype, aimed at the control of this disease in New Zealand's Angus cattle population is presented. The test employed for detecting heterozygotes was based on plasma α-mannosidase activity, with heterozygous individuals possessing approximately 37.5% of the normal activity of this enzyme in their plasma. Due to differences in plasma α-mannosidase activity previously noted between groups of cattle of differing age and sex, and to between-herd and between-mob variations in mean enzyme activity, the test is most accurate when employed on age and sex groups within a herd; with between-mob variation being considered where appropriate. The test is less reliable when employed on animals less than 12 months of age, and on small groups or single animals.

Geneology studies, confirmed the autosomal recessive nature of inheritance of the mannosidosis genotype, which was found to be carried by approximately 10% of pedigree Angus cattle in New Zealand.

CHAPTER V

LYMPHOCYTE α-MANNOSIDASE ACTIVITY - A SUPPLEMENTARY TEST FOR THE MANNOSIDOSIS GENOTYPE

INTRODUCTION

Kaback and Zeiger (1972) expressed the need for an accurate back-up test to support any mass screening programme for carriers of an inherited disease. Although their prototype testing scheme was aimed at the prevention of Tay-Sach's disease in certain North American Jewish communities this requirement for a supplementary test also applies for heterozygote detection programmes in domestic animals (Jolly et al., 1974). In Chapter IV a test for bovine mannosidosis heterozygotes, based on plasma α-mannosidase activity, was evaluated and shown to be suitable for routine use on a herd basis. The genotype of a small percentage of animals could not be accurately determined when this test was used alone on small groups of animals or on individual herd sires. In such cases an accurate alternative test is required.

Lysosomal enzymes are probably released into plasma or serum largely as a result of cellular degradation and turnover (Kaback and Zeiger, 1972). The activity of these enzymes in plasma might therefore be influenced by a variety of factors including nutrition, medication, disease and physiological status. A more reliable assessment of an individual's lysosomal enzyme complement is likely to be gained from assays on certain body tissues which are primary sources of these enzymes, and which would therefore be expected to show considerably less variation in enzyme activity than is exhibited in plasma or serum.

Enzyme assays on extracts of peripheral leucocytes have been successfully employed in the detection of heterozygotes for several

human inborn lysosomal diseases (Table 1.III), and a reduced activity of acidic α-mannosidase has been demonstrated in leucocyte extracts from cattle heterozygous for the mannosidosis genotype (Hocking et al., 1972). It was decided therefore to evaluate a test for mannosidosis heterozygotes based on leucocyte α-mannosidase activity.

Peripheral leucocytes are a mixture of cell-types, the ratios of which may vary with the health of an individual. Although lymphocytes usually constitute approximately 60% of peripheral leucocytes in normal cattle, certain infectious conditions are able to stimulate the production and release of granulocytes causing an overall increase in the number of circulating leucocytes, with a relative decrease in the percentage of lymphocytes. Should granulocytes and lymphocytes possess widely differing activities of lysosomal enzymes then the enzyme activity of mixed-leucocyte extracts might vary significantly with the relative proportions of these cell-types. Variations in the number of contaminating thrombocytes in extracts might also produce inaccuracies. It might be expected therefore that assays on relatively pure preparations of lymphocytes, granulocytes or even thrombocytes, would provide a more accurate test than assays on mixed-leucocyte extracts. The initial choice of lymphocytes was influenced by the relative abundance of these cells in bovine blood and the availability of a simple method for their separation from whole blood, as outlined in Chapter II.

DEVELOPMENT OF TECHNIQUES

1. Kinetic characterization of five lysosomal enzymes in bovine lymphocyte extracts

Prior to the evaluation of lymphocyte α-mannosidase activity as a means of detecting mannosidosis heterozygotes, the optimal assay

conditions for this enzyme in bovine lymphocyte extracts were established, and the expected reduction of acidic α-mannosidase activity in heterozygotes, in accordance with the gene dosage phenomenon, was confirmed. The kinetic characteristics of four other recognized lysosomal enzymes were also established as these enzymes were to be employed as reference parameters for α-mannosidase activity.

Materials and methods

Lymphocytes were obtained from two normal Angus bulls using the method described in Chapter II. For these preliminary studies lymphocyte pellets were sonicated in 2.0 ml 0.85% NaCl for 2 min at 3 amperes using a Dawe Soniprobe. Following centrifugation at 2,000 g x 10 min the supernatant was retained for assay. Lymphocytes were also obtained from a heterozygous bull and a calf with mannosidosis for investigation of the pH dependence of α-mannosidase activity.

Assays for α-mannosidase, β-galactosidase and total hexosaminidase activity were based on the use of fluorogenic 4-methylumbelliferone glycosides as substrates, while arylsulphatase A and α-fucosidase assays involved colour reactions using p-nitrocatechol sulphate and p-nitrophenyl-α-L-fucoside respectively. The method described by Hocking et al. (1972) was employed for α-mannosidase assays, using a 2M-sodium acetate/acetic acid buffer (pH 4.3) except when studying the pH dependence of α-mannosidase activity, in which case a range of 0.2M-Na_2HPO_4/citric acid buffer solutions was substituted. Similar methods were employed for assays of β-galactosidase and hexosaminidase activity, using 0.2M-Na_2HPO_4/citric acid buffers. Assays for α-fucosidase activity also involved the use of 0.2M-Na_2HPO_4/citric acid buffers and the method was based on that described by Zielke et al. (1972). Arylsulphatase A activity was assayed by a modification of the method of Baum et al. (1959)

where a 1M-sodium acetate/acetic acid buffer containing 0.5mM-$Na_4P_2O_7$ and 10% NaCl was employed. Assays for time sequence studies, rate curves and Lineweaver-Burk plots were performed at the optimum pH for each enzyme.

Results

The pH dependence profiles of α-mannosidase activity in lymphocyte extracts from normal, heterozygous and mannosidosis individuals are shown in Figure 5.1a. Maximum activity of α-mannosidase in normal animals occurred at pH 4.25. The mannosidosis calf showed little or no α-mannosidase activity at this pH but did have a low peak of activity between pH 5.0 and 7.0. The heterozygote possessed less than 50% of the normal activity at pH 4.25 and had an obvious but smaller peak of activity at pH 5.25.

The rate of hydrolysis of the substrate by α-mannosidase was greatest when the substrate concentration was 5mM, with inhibition of the reaction occurring at higher substrate concentrations (Figure 5.1b). The Michaelis constant (Km) for the activity of this enzyme was estimated to be 0.96mM from the Lineweaver-Burk plot presented in Figure 5.1c. A time sequence study (Figure 5.1d) showed that the reaction was still linear after a 1 hr incubation at 37°C.

The pH optimum for arylsulphatase A activity was 4.75 (Figure 5.2a) and at this pH the Km was estimated to be 1.82mM from the Lineweaver-Burk plot (Figure 5.2c). The enzyme appeared to be well saturated by a 5mM substrate concentration (Figure 5.2b) and a time sequence study (Figure 5.2d) indicated that the reaction velocity was very close to linearity after a 2 hr incubation at 37°C.

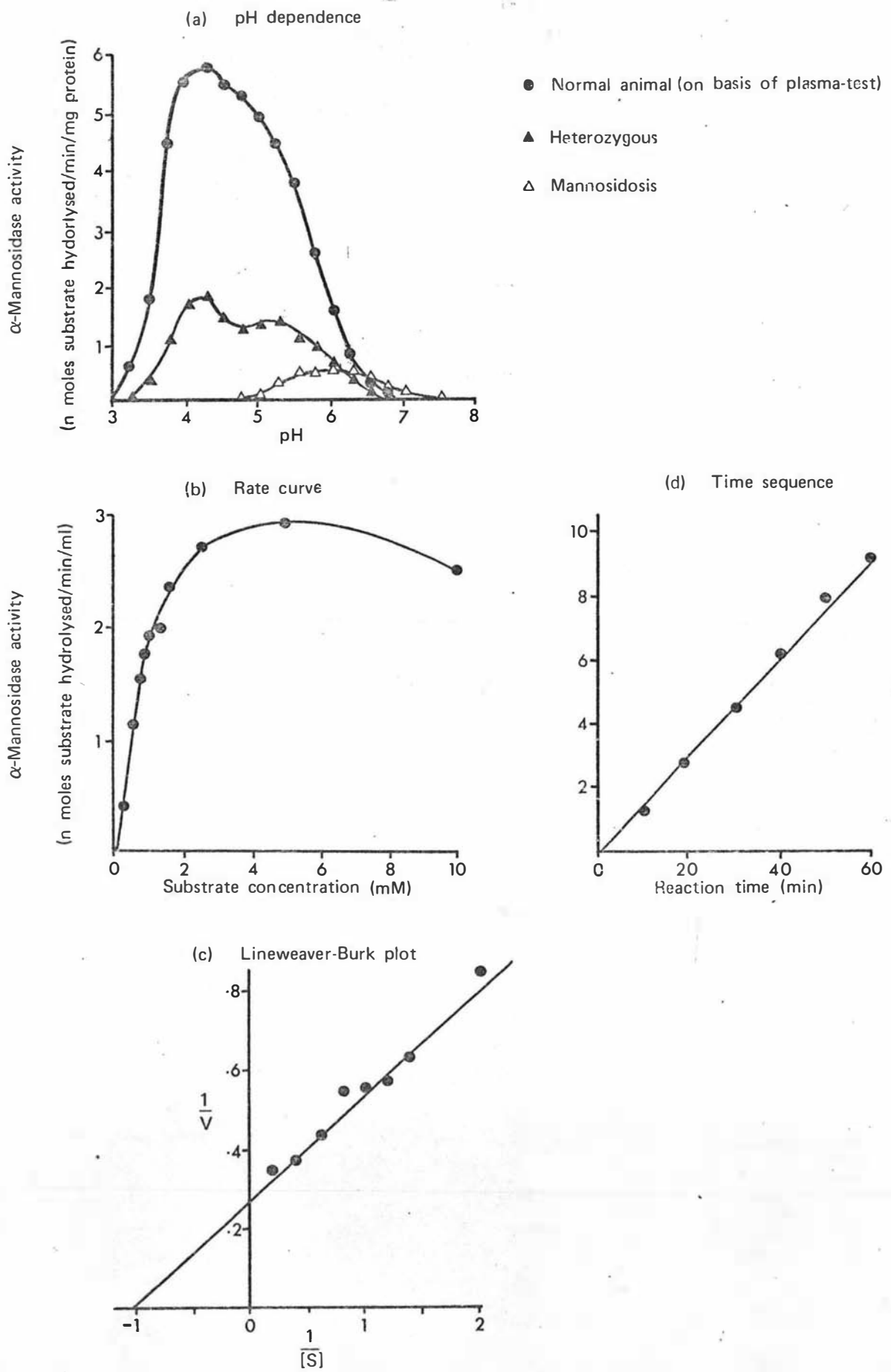


Figure 5.1 Kinetic characteristics of α-mannosidase in bovine lymphocyte extracts.

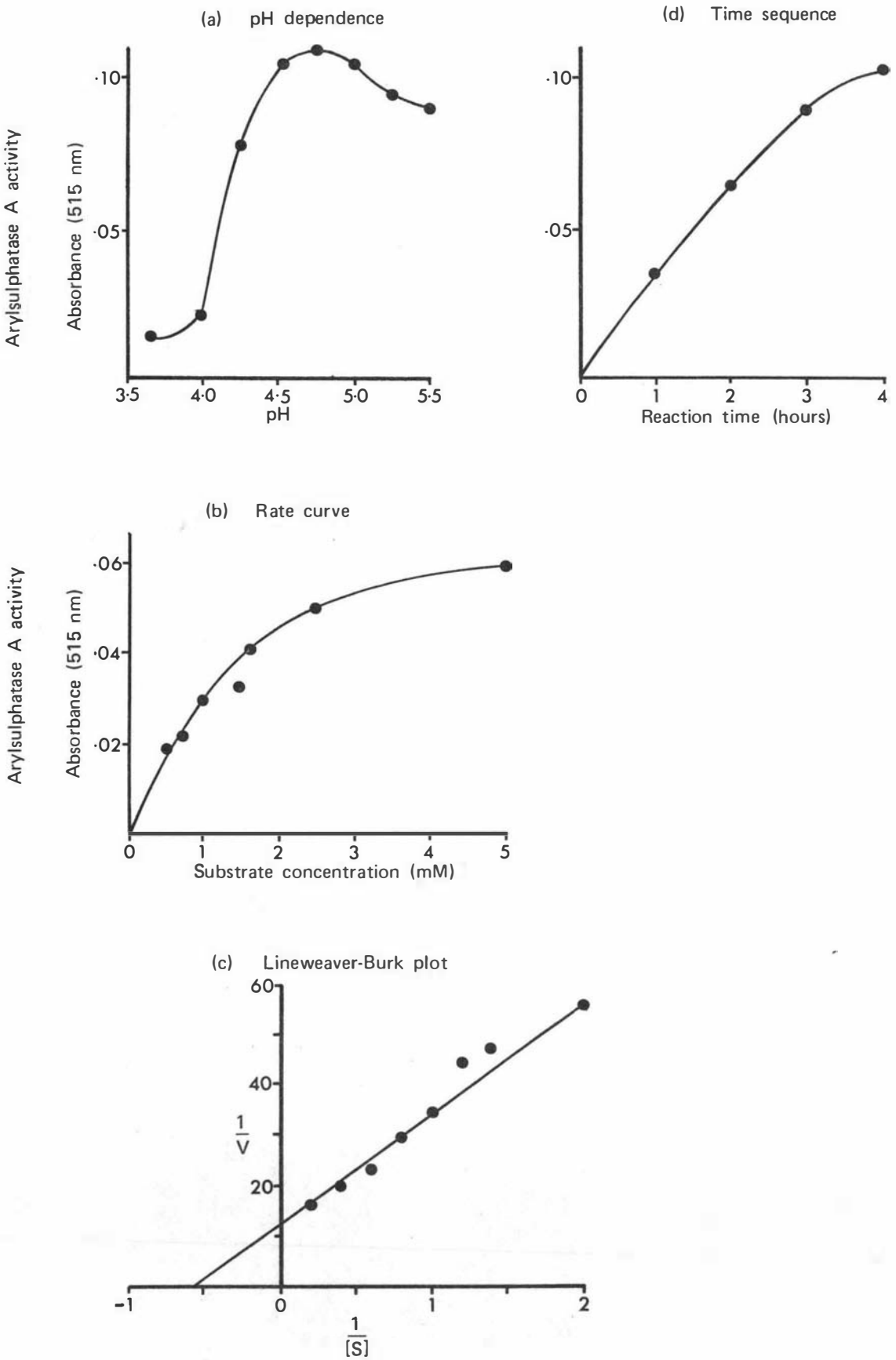


Figure 5.2 **Kinetic characteristics of arylsuphatase A in bovine lymphocyte extracts.**

α-Fucosidase showed maximum activity at pH 5.25 (Figure 5.3a) and had an estimated Km of 0.19mM, as calculated from the slope of the Lineweaver-Burk plot (Figure 5.3c). The enzyme appeared to be saturated by substrate concentrations between 1.0 and 2.0mM, above which there was slight inhibition (Figure 5.3b). The reaction velocity was not linear after 2 hr incubation at 37°C but this period of time was required to obtain sufficiently high absorbance readings.

Hexosaminidase showed maximum activity at pH 4.5 (Figure 5.4a), and had an estimated Km of 1.59mM. The enzyme was quite well saturated by a 5mM substrate concentration (Figure 5.4b) and the reaction was still linear after proceeding for 1 hr at 37°C.

A broad peak of β-galactosidase activity was measured between pH 3.5 and 5.5 (Figure 5.5a). The optimum was taken to be pH 4.5. Due to the poor solubility of the substrate, 4-methylumbelliferone-β-galacto-pyranoside monohydrate, it was not possible to construct an accurate rate curve or Lineweaver-Burk plot for this enzyme. However, a time sequence study (Figure 5.5b) indicated that the reaction remained linear after 2 hr incubation at 37°C using a 1mM substrate concentration.

Discussion and conclusions

Optimum assay conditions for each of the five lysosomal enzymes were developed from information gained in the above experiments, and are presented in Chapter II (Table 2.I).

The expected deficiency of acidic α-mannosidase activity (pH 4.3) in lymphocytes from a calf with mannosidosis was confirmed, and a heterozygous animal was found to possess less than half the normal activity of this enzyme (Figure 5.1a). The Michaelis constant (Km) estimated for acidic α-mannosidase in bovine lymphocytes was 0.96mM,

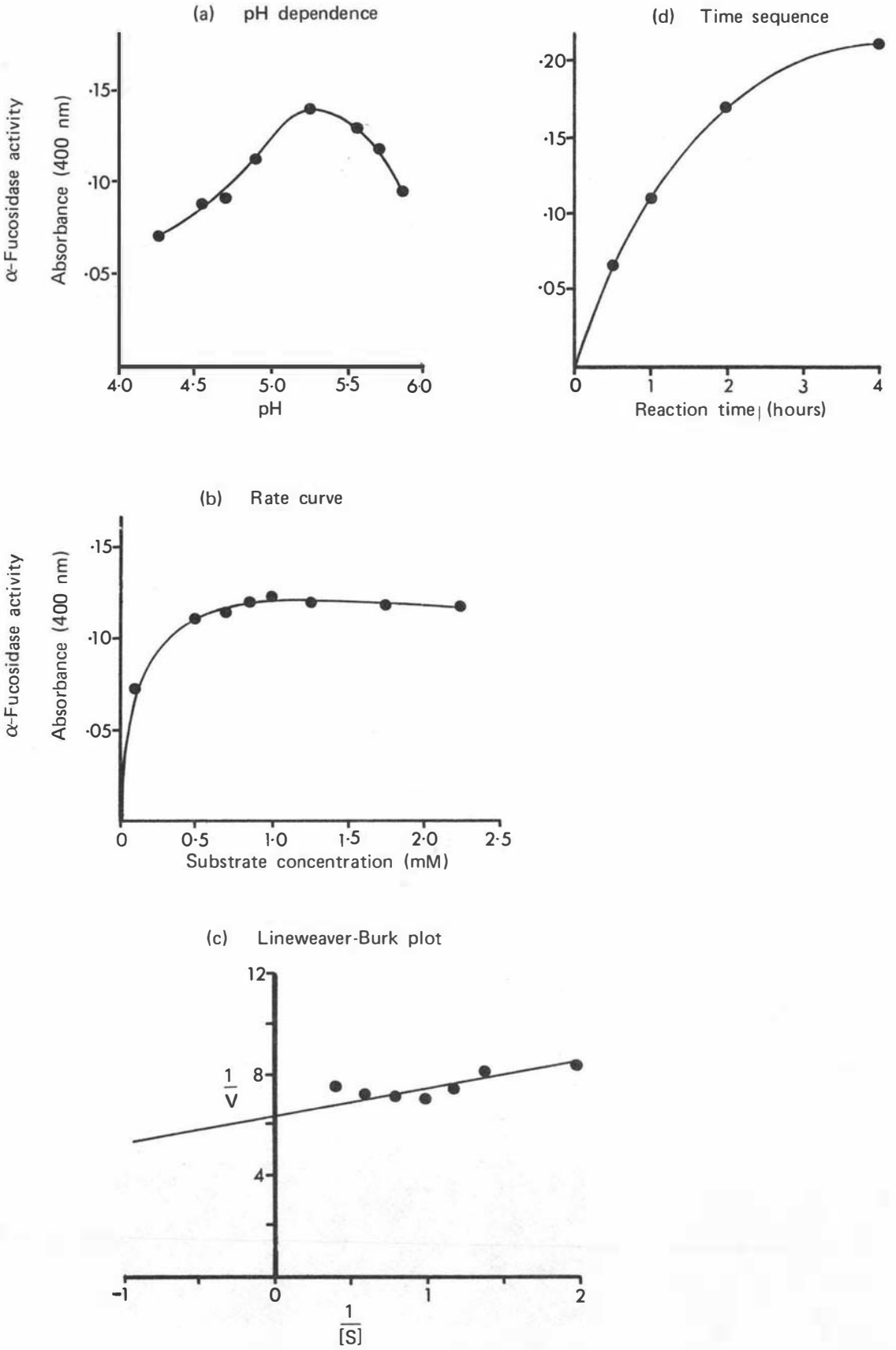


Figure 5.3 **Kinetic characteristics of α-fucosidase in bovine lymphocyte extracts.**

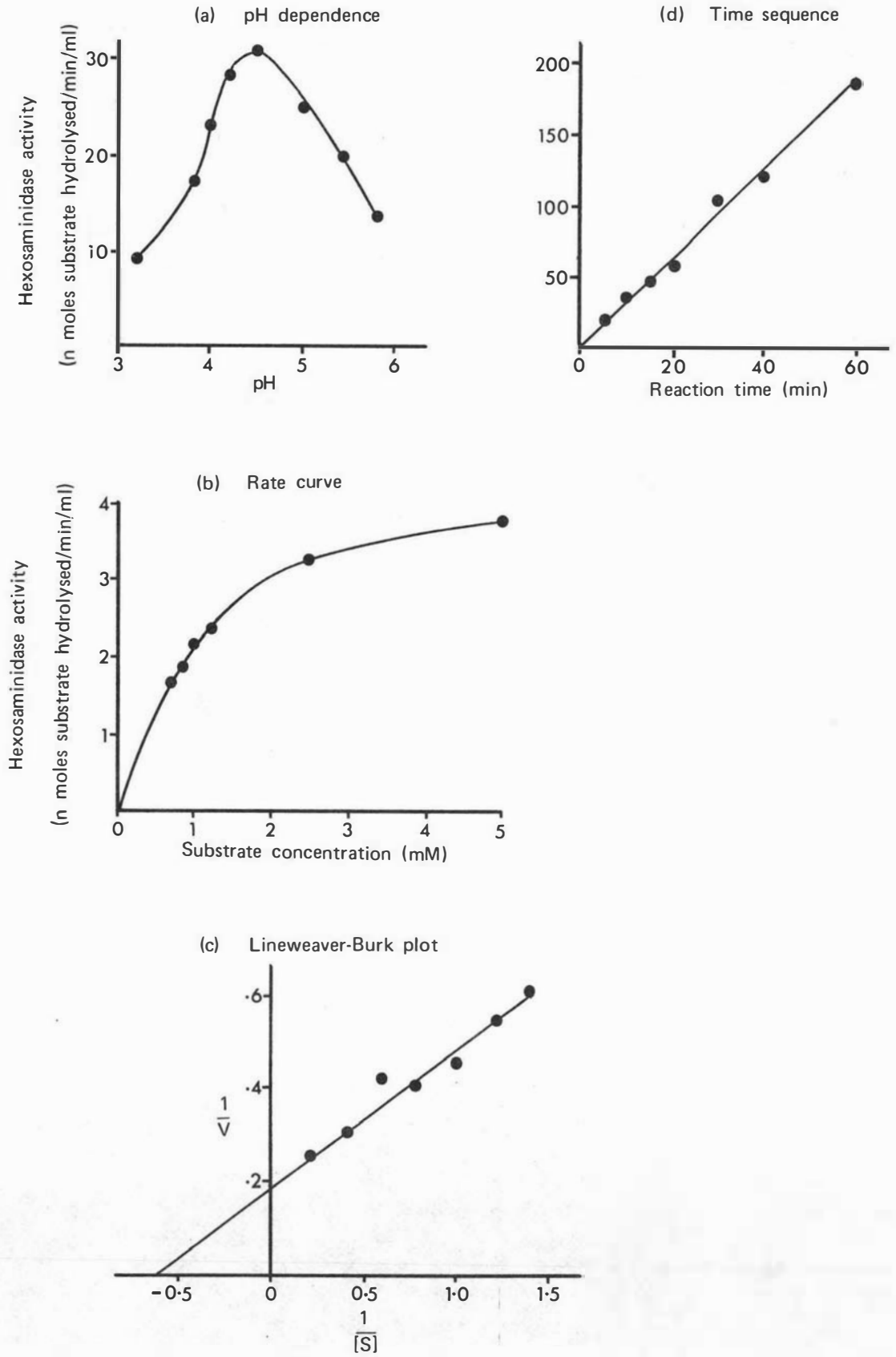


Figure 5.4 **Kinetic characteristics of total hexosaminidase in bovine lymphocyte extracts.**

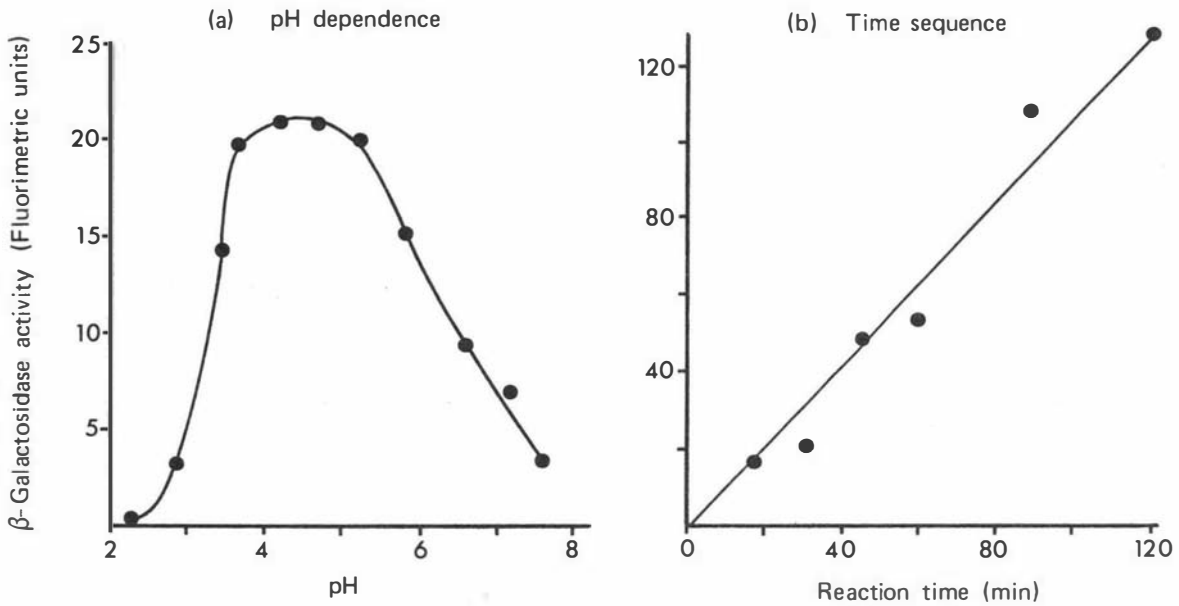


Figure 5.5 **pH dependence and time sequence studies of β-galactosidase activity in bovine lymphocyte extracts.**

slightly higher than that found in bovine plasma (0.5-0.6mM) by Winchester et al. (unpublished data).

The pH optimum of 5.25 for α-fucosidase activity in bovine lymphocytes was similar to that found in bovine plasma (Winchester et al., unpublished data), and human serum (Zielke et al., 1972). The kinetic characteristics of this enzyme in bovine lymphocyte extracts were similar to those described by Zielke et al. in human serum.

The broad peaks obtained for pH dependence of β-galactosidase and hexosaminidase activities in bovine lymphocyte extracts suggest the existence of isoenzymes with slightly differing pH optima. Hexosaminidase activity has in fact been shown to exist in two principal forms, A and B, with pH optima of approximately 4.5 in human tissues and serum (Robinson and Stirling, 1968; Barrett, 1972; Swallow et al., 1974). A further isoenzyme, hexosaminidase C, is also found in human tissues, but has a neutral pH optimum and is non-lysosomal (Dreyfus and Poenaru, 1974). At least four hexosaminidase isoenzymes, with isoelectric points in the range pH 4.0 to 6.0, have been isolated from porcine liver (Weissmann and Hinrichsen, 1969).

Three principal forms of β-galactosidase have been distinguished in human tissues, two with acidic pH optima of approximately 4.0 to 4.35 and one with a more neutral pH optimum of 5.0 to 6.5 (Robinson, 1974; Suzuki and Suzuki, 1974). Levvy et al. (1962) described a broad peak of β-galactosidase activity from pH 4.0 to 7.0 in bovine liver. Two isoenzymes with pH optima at 4.5 and 6.0 respectively were isolated from this tissue by Chytil (1965).

2. Preparation of lymphocyte extracts for enzyme assays

The value of a supplementary test depends on the ability to compare enzyme activities of bulls tested at various times. The following

experiments were designed to help establish a standard method for the preparation of lymphocyte extracts prior to enzyme assay.

(a) Release of α-mannosidase from lymphocyte pellets

The release of lysosomal enzymes from cells and lysosomes is a necessary step in the preparation of tissue extracts for enzyme assay. Lysosomes are fragile organelles and may be disrupted by hypotonic shock or rapid freezing and thawing, in addition to mechanical homogenization or sonication. This experiment was designed to compare freezing and thawing, and sonication as methods of releasing α-mannosidase activity from lymphocyte pellets.

Materials and methods

Lymphocytes were separated from 8 x 10 ml EDTA Vacutainers of blood from each of eight adult Friesian cows in the manner described in Chapter II. Samples from each animal were pooled and after the final wash, were resuspended in 7.5 ml of 0.85% NaCl containing 0.1% Triton X-100. Each cell suspension was divided into 7 x 1.0 ml aliquots and stored overnight at -12°C. After thawing, six of the identical samples from each animal were sonicated in an ice bath, for various time intervals up to 120 s at 3 amperes on a Dawe Soniprobe. One sample remained unsonicated. All samples were then centrifuged at 2,000 g x 10 min with the supernatants being retained for assay. Methods of enzyme assay were as outlined in Chapter II.

Results

The effect of sonication on protein concentration and α-mannosidase activity in lymphocyte extracts is shown in Figure 5.6. Although there was no increase in protein concentration during the initial 15 s of sonication (Figure 5.6a) a linear increase in

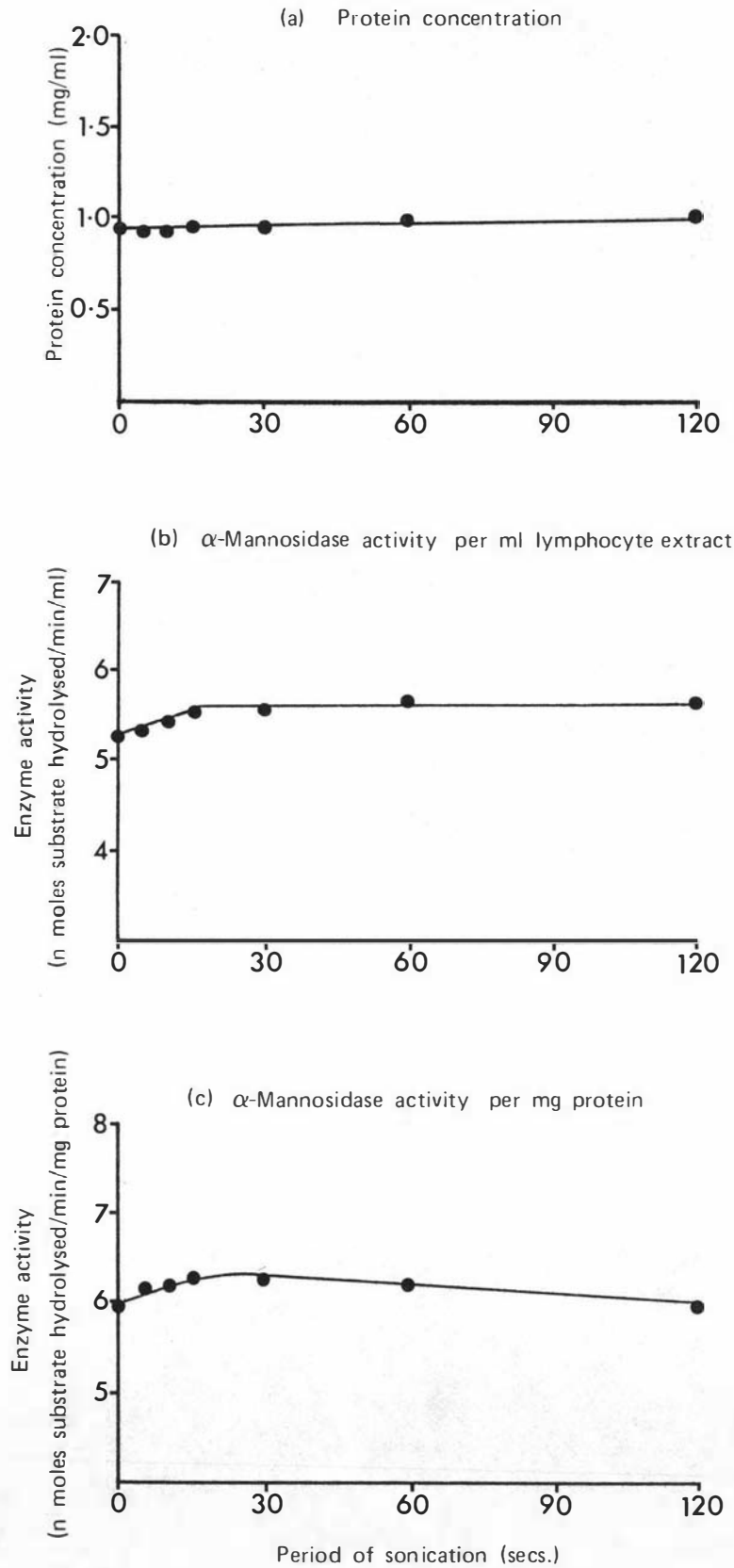


Figure 5.6 **The effect of sonication on protein concentration and α-mannosidase activity of lymphocyte extracts.**

α-mannosidase activity per ml of supernatant was observed during this period (Figure 5.6b). A gradual increase in the release of soluble protein after 60 s and 120 s sonication contributed to a decline in the activity of α-mannosidase relative to protein concentration (Figure 5.6c). Individual variation in the effect of sonication for 30 s on protein concentration and α-mannosidase activity in the eight cows is shown in Table 5.I.

Discussion and conclusions

From inspection of Figure 5.6 it was concluded that optimum release of α-mannosidase activity relative to protein occurred within 15-30 s of sonication, after which time no further increase in enzyme activity was observed. Sonication for 30 s had a negligible effect on protein concentration but liberated an extra 5.7% of α-mannosidase activity per ml of lymphocyte extract when compared with freezing and thawing (Table 5.I), and is therefore recommended.

(b) The effect of centrifugation on the removal of cellular debris

The importance of removing the relatively insoluble cellular debris from lymphocyte extracts after sonication was realized in preliminary experiments where there was flocculation of this insoluble material, creating inaccuracies in pipetting small volumes and in estimating protein concentrations.

Materials and methods

Lymphocytes were separated from 8 x 10 ml EDTA Vacutainers of blood from a six-month old Jersey calf by the method previously described, and stored as pellets at -12°C until required. On thawing,

TABLE 5.I

The effect of sonication for 30 seconds on protein concentration and α-mannosidase activity in lymphocyte extracts from eight cows

Cow no.	Protein concentration (mg/ml)			α-Mannosidase activity (nmoles substrate hydrolysed/min)					
				per ml extract			per mg protein		
	Without sonication	Sonication for 30 s	Difference	Without sonication	Sonication for 30 s	Difference	Without sonication	Sonication for 30 s	Difference
203	0.37	0.45	+0.08	2.25	2.39	+0.14	6.08	5.31	-0.77
83	0.37	0.35	-0.02	2.57	2.79	+0.22	6.95	7.79	+1.02
92	0.85	0.83	-0.02	2.81	2.76	-0.05	3.31	3.33	+0.02
164	1.42	1.41	-0.01	2.87	3.08	+0.21	2.02	2.18	+0.16
98	0.65	0.64	-0.01	5.65	6.03	+0.38	8.69	9.42	+0.73
18	2.76	2.76	0.0	5.82	6.16	+0.34	2.11	2.23	+0.12
114	0.74	0.69	-0.05	8.69	9.90	+1.21	11.74	14.35	+2.61
23	0.56	0.60	+0.04	8.14	7.82	-0.32	14.54	13.03	-1.51
Average	0.965	0.966	+0.001 (+0.1%)	4.85	5.12	+0.27 (+5.7%)	6.93	7.23	+0.30 (+4.3%)

1.5 ml of 0.85% saline containing 0.1% Triton X-100 was added to each pellet prior to sonication for 30 s. All samples were pooled prior to centrifugation of 4 x 1.0 ml aliquots for 5, 10, 15 and 20 min respectively. Estimations of protein concentration and assays for α-mannosidase and hexosaminidase activities were performed on the clear supernatants, and on an uncentrifuged sample, as outlined in Chapter II.

Results

As shown in Figures 5.7a, b and c, both the protein concentration and enzyme activity in the supernatant were constant after 5 min centrifugation at 2,000 g. Protein concentration decreased by 60% on centrifugation while α-mannosidase activity per ml of lymphocyte extract remained relatively constant and hexosaminidase activity showed a 7.3% increase.

Discussion and conclusions

Centrifugation of lymphocyte extracts at 2,000 g for 5 min effectively removed cellular debris from suspension without causing a notable loss in activity of either α-mannosidase or hexosaminidase (Figure 5.7b). The activity of hexosaminidase per ml of lymphocyte extract actually showed an increase after centrifugation, possibly due to the removal of some inhibitory factor. The specific activities of both enzymes (per mg of protein) were predictably elevated by the removal of protein, becoming constant after 5 min centrifugation at 2,000 g (Figure 5.7c). Problems associated with the flocculation of insoluble protein material may therefore be overcome, without loss of enzyme activity, by centrifugation of lymphocyte extracts for 5 min at 2,000 g after sonication.

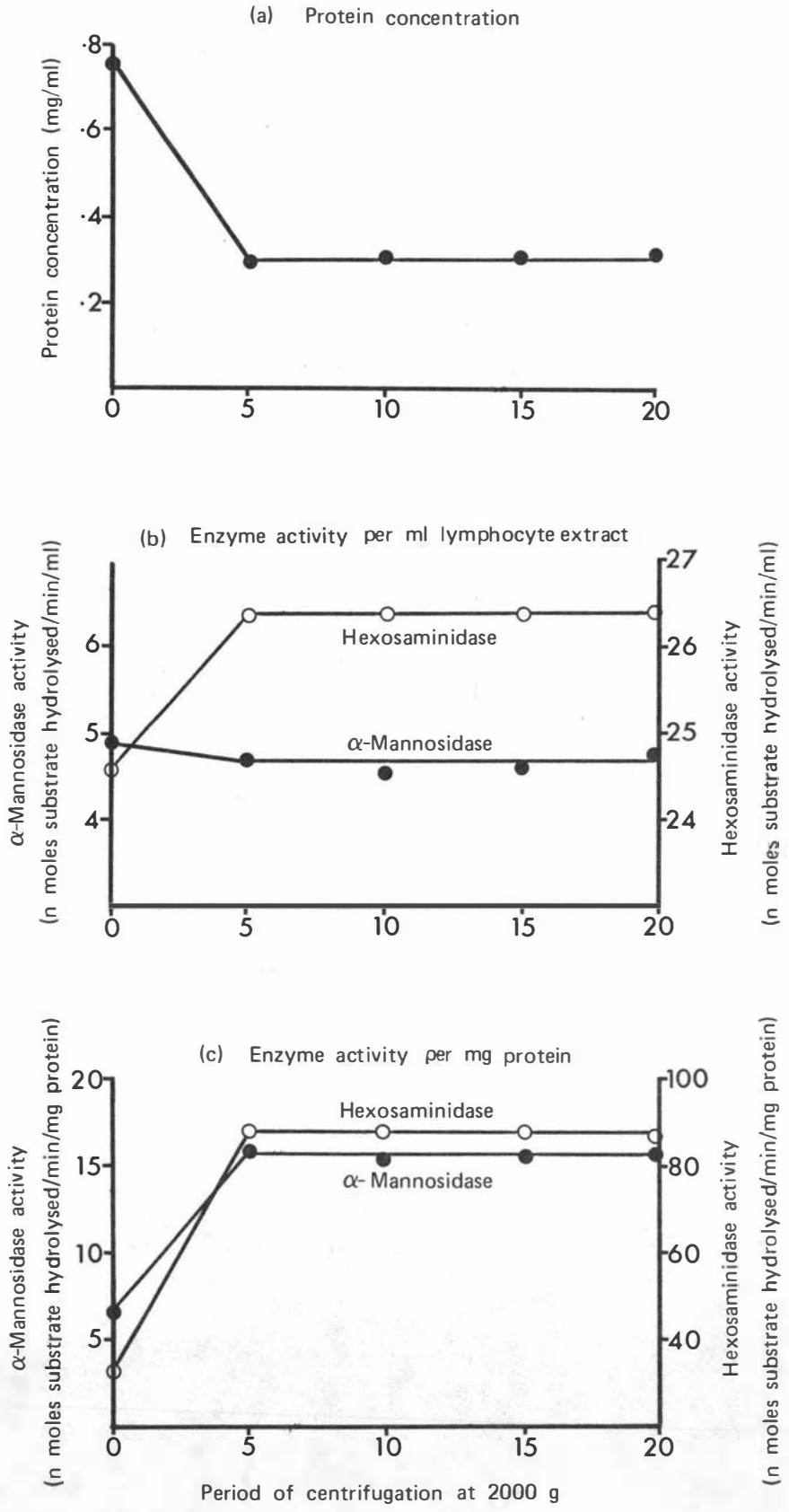


Figure 5.7 **The effect of centrifugation of lymphocyte extracts on protein concentration and the activities of α-mannosidase and total hexosaminidase.**

(c) The effect of Triton X-100 on protein concentration and α-mannosidase activity of lymphocyte extracts

The nonionic detergent Triton X-100 is often included when diluting samples for enzyme assays, to minimize the loss of protein due to adsorption and denaturation on surfaces, and to facilitate the release of any membrane-bound enzyme activity (Barrett, 1972). The following experiment was designed to investigate the effect of this detergent on the activity of acidic α-mannosidase (pH 4.3) and the soluble protein concentration of lymphocyte extracts.

Materials and methods

Two x 10 ml EDTA Vacutainers of blood were collected from each of seven Angus cows, and lymphocytes were separated by the method described in Chapter II. After the final wash, lymphocytes from each animal were resuspended in 2.5 ml of 0.85% NaCl. Duplicate 1.0 ml aliquots of each sample were centrifuged at 160 g x 5 min and stored at -12°C. On thawing, 1.0 ml of 0.85% NaCl was added to one duplicate sample and 1.0 ml of 0.85% NaCl containing 0.1% Triton X-100 added to the other. After sonication for 30 s all samples were centrifuged at 2,000 g x 5 min and the supernatants retained for assay. Protein estimations and assays for α-mannosidase activity were as previously described.

Results

As shown in Table 5.II the addition of 0.1% Triton X-100 to lymphocyte extracts caused an average increase of 110% in the protein concentration of extracts and 25% increase in α-mannosidase activity per ml. The specific activity of α-mannosidase (per mg protein) was reduced by 43% due to the greater elevation in protein concentration relative to enzyme activity.

TABLE 5.II

The effect of Triton X-100 on protein concentration and α-mannosidase activity in lymphocyte extracts from seven cows

Cow no.	Protein concentration (mg/ml)			α-Mannosidase activity (nmoles substrate hydrolysed/min)					
				per ml extract			per mg protein		
	Saline	Saline + Triton X-100	% increase	Saline	Saline + Triton X-100	% increase	Saline	Saline + Triton X-100	% decrease
59	0.15	0.32	113%	2.64	3.16	20%	17.60	9.88	44%
71	0.21	0.46	119%	2.89	3.86	34%	13.78	8.39	39%
33	0.20	0.48	140%	4.17	5.11	23%	20.86	10.65	49%
48	0.15	0.31	107%	2.52	2.79	11%	16.79	8.99	46%
35	0.31	0.64	106%	2.19	2.68	22%	7.18	4.19	42%
7	0.12	0.25	108%	1.33	1.77	33%	11.57	7.08	39%
18	0.25	0.50	100%	3.11	3.98	28%	12.44	7.97	36%
Average	0.20	0.42	110%	2.69	3.36	25%	14.32	8.16	43%

Discussion and conclusions

The inclusion of 0.1% Triton X-100 in the saline diluent for lymphocyte pellets increased the level of α-mannosidase activity in extracts, probably through the release of membrane-bound enzyme that would otherwise have been removed with the cellular debris. The protein concentration of extracts was also increased in the presence of Triton X-100, thereby allowing accurate protein estimations to be performed on a smaller volume. Because of these practical advantages and the theoretical advantage of reducing protein adsorption and denaturation, it is recommended that 0.1% Triton X-100 should be included routinely during the preparation of lymphocyte extracts.

FACTORS AFFECTING THE ACTIVITY OF LYSOSOMAL ENZYMES IN BOVINE LYMPHOCYTES

1. Stability of five lysosomal enzymes in bovine lymphocytes

Problems associated with the transport of samples from different parts of the country often preclude the separation and freezing of lymphocytes on the day of collection. The effect of varying the period between blood collection and separation of lymphocytes on the activity of five lysosomal enzymes was investigated in the following experiment.

Materials and methods

Six x 10 ml EDTA Vacutainers of blood were collected from each of 13 adult Angus cows. Lymphocytes were separated from two Vacutainers from each animal at 1, 24 and 48 hr after collection. Prior to lymphocyte separation the samples were retained at room-temperature, but lymphocyte pellets were stored at -12°C until required for assay. Methods used for enzyme assays and protein estimations were as described in Chapter II.

Results

The mean activity of each of five lysosomal enzymes at 1, 24 and 48 hr after blood collection is shown in Figure 5.8 with 5% fiducial limits about each mean. α-Mannosidase activity showed a linear increase over the 48 hr period although this increase was not statistically significant ($p > 0.05$). The activity of hexosaminidase remained essentially unchanged but a significant reduction in activity was observed for β-galactosidase ($p < 0.05$), arylsulphatase A ($p < 0.01$) and α-fucosidase ($p < 0.01$) after 48 hr. Only α-fucosidase showed a significant reduction in mean activity after 24 hr.

Discussion and conclusions

Blood samples were kept at room temperature and not exposed to light prior to separation of lymphocytes in an attempt to simulate the conditions likely to be encountered by samples in transit to the laboratory.

A significant reduction in the activity of β-galactosidase, arylsulphatase A and α-fucosidase was observed in lymphocytes separated after 48 hr (Figure 5.8). Hexosaminidase and α-mannosidase activity did not alter significantly, although a linear increase in the activity of α-mannosidase suggests that this trend might have been significant had a greater number of animals been included in the experiment. In tissues undergoing regressive changes certain lysosomal enzymes have been observed to retain their original activity for some time, and in fact may even show a significant increase in activity (Taft, 1960; de Duve, 1963). The apparent increase in α-mannosidase activity observed in lymphocytes after storage at room temperature might therefore be a genuine phenomenon, and warrants further investigation. This enzyme has already been shown to be very stable in bovine plasma at room temperature (Jolly et al., 1973).

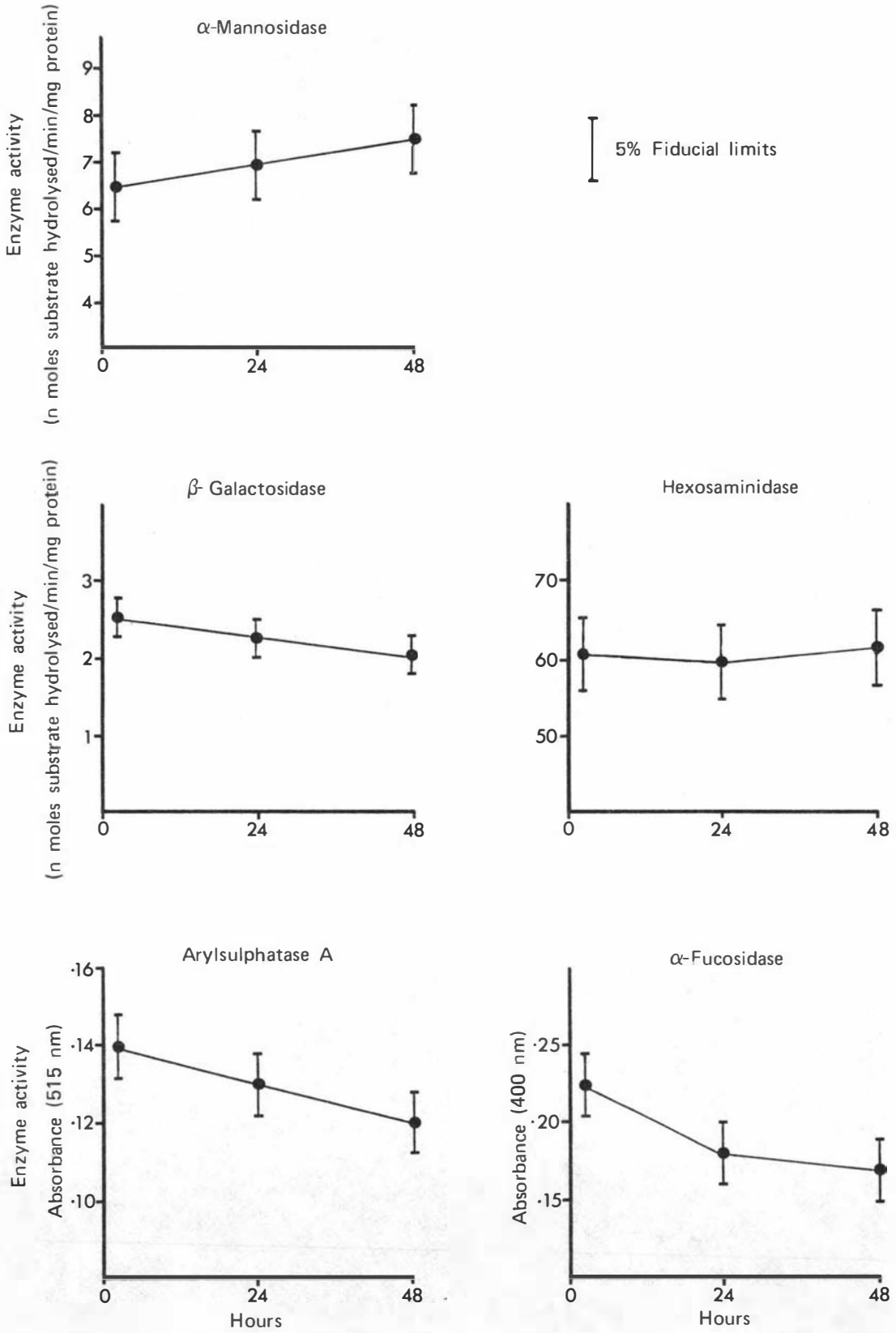


Figure 5.8 **Variation in activity of five lysosomal enzymes relative to the time interval between collection of blood and separation of lymphocytes.**

The significant reduction in activity of β-galactosidase, arylsulphatase A and α-fucosidase may be due to the relative instability of these enzymes.

The results of this experiment suggest that lymphocytes should preferably be separated from whole blood within a few hours of collection to avoid any alterations in activity of lysosomal enzymes. In many instances however it is not possible for blood samples to reach the laboratory on the day of collection. It was concluded therefore that the period between blood collection and lymphocyte separation should be standardized to a time interval which will allow the majority of samples to reach the laboratory. Twenty-four hours was selected as a suitable interval as most samples should be received within this period, and in the above results only α-fucosidase showed a significant alteration in activity to lymphocytes stored for this period of time.

2. In vivo stimulation of lymphocytes by Strain 19 Brucella abortus vaccine

When peripheral lymphocytes are stimulated *in vitro* by the addition of phytohaemagglutinin they become morphologically similar to primitive blast-type cells with more abundant cytoplasm than is normally found in mature lymphocytes. An increase in the activity of lysosomal enzymes in these transformed lymphocytes would not be surprising and has in fact been reported by Hirschhorn *et al.* (1969) and Nadler and Egan (1970).

Whether or not there is a similar transformation of peripheral lymphocytes *in vivo* in response to an antigenic stimulus, such as Strain 19 *Brucella abortus* vaccine, has not been established but must be considered as a factor which might affect the activity of lysosomal enzymes within lymphocytes. In the following experiment the effect of an antigenic stimulus on the activity of five lysosomal enzymes in peripheral lymphocytes was investigated.

Materials and methods

Six Friesian calves less than six months of age were injected subcutaneously with 2.0 ml of Strain 19 Brucella abortus vaccine*. Six similar calves acted as unvaccinated controls. Lymphocytes were collected from each calf 3 days prior to the injection of antigen, again on the day of injection, and every third day until 12 days after injection. Samples were stored at -12°C until thawed and prepared for protein estimations, and assays for the activity of five lysosomal enzymes: α-mannosidase, hexosaminidase, β-galactosidase, α-fucosidase and arylsulphatase A. Assay methods were as outlined in Chapter II.

Results

The mean activities of each enzyme in lymphocytes from control and vaccinated groups of calves up to 12 days after vaccination are presented in Figure 5.9. None of the five enzymes showed a significant alteration in mean activity in lymphocyte extracts from unvaccinated control calves over the period of sampling. In lymphocyte extracts from vaccinated calves only α-mannosidase showed a significant variation in mean activity. After vaccination the mean α-mannosidase activity in vaccinated calves increased to reach a peak at six days, after which time there was a gradual decline in activity. The post-vaccination activity of α-mannosidase was significantly elevated in the 6th, 9th and 12th day lymphocyte samples as shown by 5% fiducial limits about each mean. Fiducial limits are not shown about mean activities for other enzymes as no significant variations were recorded.

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* Tasman Vaccine Laboratories, Upper Hutt, New Zealand.

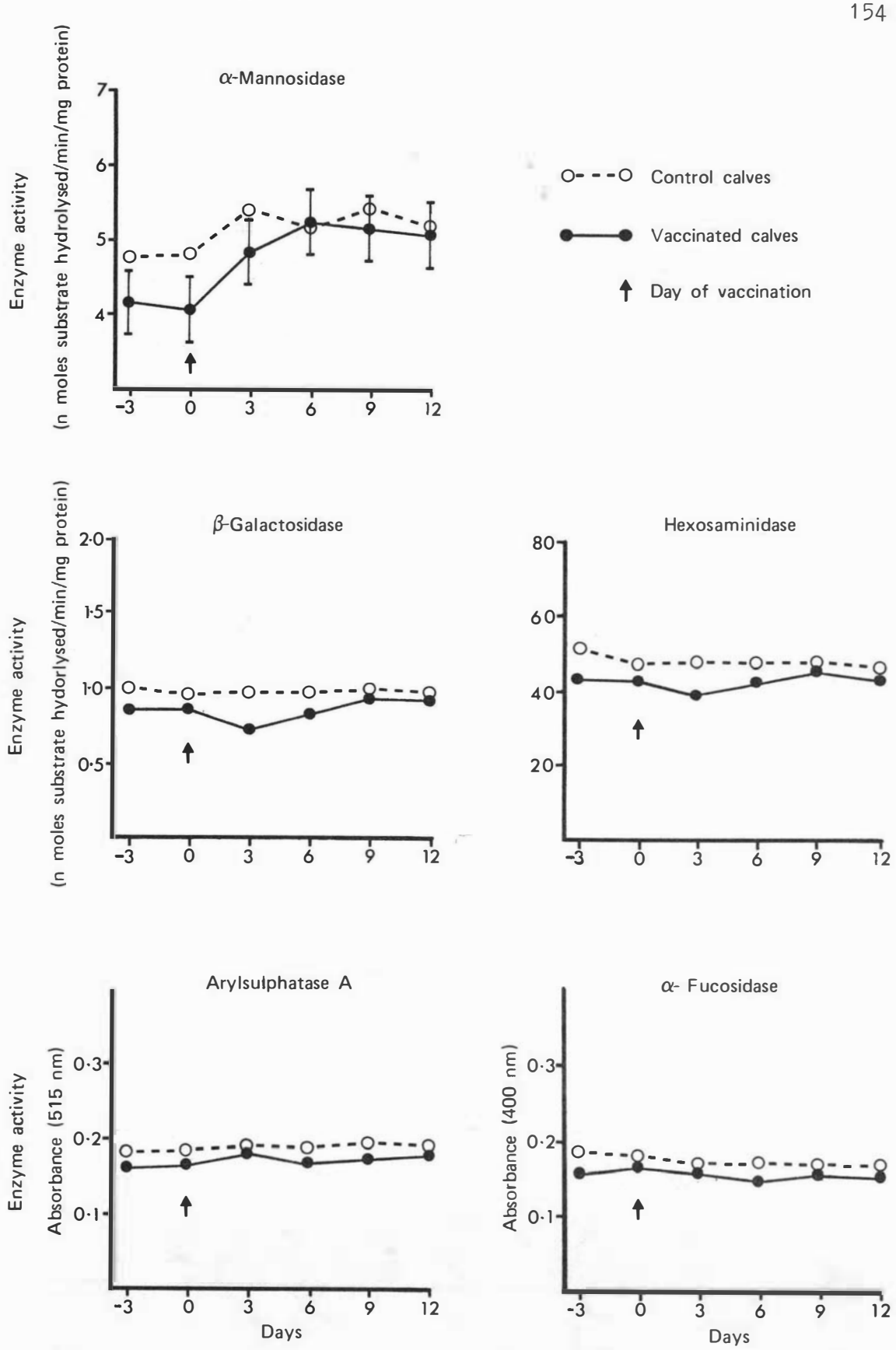


Figure 5.9 **The effect of an antigenic stimulus (Strain 19 Brucella antigen) on the activity of five lysosomal enzymes in bovine lymphocytes.**

Discussion and conclusions

A significant elevation in the mean activity of α-mannosidase in lymphocyte extracts from six calves after injection with Strain 19 *Brucella abortus* vaccine indicates that there may be transformation of peripheral lymphocytes *in vivo* in response to an antigen. Four other lysosomal enzymes however showed no significant variation in activity after vaccination. The reasons for these inconsistent results is not clear. However, the increase in α-mannosidase activity in response to the *Brucella* antigen suggests that exposure of animals to other antigens, either through disease or vaccination, could result in a similar increase, thereby introducing a possible source of error in the use of lymphocyte α-mannosidase activity as a test for the mannosidosis genotype. The increase in α-mannosidase activity may be of sufficient magnitude to elevate a heterozygous animal into the low-normal range on the basis of lymphocyte extract α-mannosidase activity. This is unlikely to be a serious problem in a mass screening programme but it is recommended that animals obviously suffering from an infectious disease should be noted and their lymphocyte test regarded with caution, especially if it falls within the low-normal range. Calves are not routinely tested for mannosidosis before six months of age, when *Brucella* vaccination is usually performed, therefore the use of this vaccine is unlikely to be a problem.

LYMPHOCYTE α-MANNOSIDASE ACTIVITY IN NORMAL AND HETEROZYGOUS CATTLE

The level of α-mannosidase activity in a lymphocyte extract will depend largely on the number of lymphocytes contributing to the sample. This number would be expected to show considerable variation between samples and it is essential therefore that enzyme activity is "normalized"

by relating it to some parameter which is strongly correlated with the original number of cells. The most logical procedure would be to express enzyme activity in terms of the actual number of cells present, but methods of estimating cell-numbers are unlikely to be sufficiently precise for this purpose. A commonly employed parameter is the soluble protein concentration of leucocyte extracts, which should be directly proportional to the number of cells. Harzer (1973) considered this method inaccurate due to the presence of inconsistent amounts of contaminating protein, and favoured a method whereby the activity of the specific lysosomal enzyme was related to the mean relative activity of three or four other lysosomal enzymes (reference enzymes). Various other authors have also supported the use of a ratio between the deficient enzyme and a closely related lysosomal enzyme. The most notable example is in the detection of heterozygotes for human G_{M2}-gangliosidosis Type I (Tay-Sach's disease) where considerable success has been achieved in using a ratio between hexosaminidase A and B in leucocytes and serum (O'Brien et al., 1971, 1972). Similar methods have been employed in detecting heterozygotes for human Type II glycogenosis, using the ratio between acid and neutral α-glucosidase (Koster et al., 1974), and fucosidosis, where a ratio between α-fucosidase and α-mannosidase was employed (Matsuda et al., 1973).

In this chapter the value of lymphocyte extract α-mannosidase activity in the detection of mannosidosis heterozygotes is evaluated, and a comparison between the use of protein concentration and four reference enzymes as parameters for normalization of α-mannosidase activity is presented.

1. α-Mannosidase activity relative to protein concentration of lymphocyte extracts

Materials and methods

Animals included in this evaluation were 147 Angus bulls over 18 months of age from numerous herds throughout New Zealand. An attempt was made to include as many heterozygous bulls as possible, therefore the selection of animals cannot be regarded as random. Blood was collected by the author where possible or by practising veterinarians, all samples being submitted to this laboratory for lymphocyte separation within 24 hr of collection. Two x 10 ml Vacutainers containing EDTA as anticoagulant were collected from each bull. In most cases an additional sample of blood was, or had previously been collected into 7 ml heparinized Vacutainers from which plasma, separated on the day of collection and stored at -12°C, was used for assays of plasma α-mannosidase activity. More than seven progeny of 28 of the above bulls were tested by the plasma test as part of a mannosidosis control scheme being run by the Ministry of Agriculture and Fisheries and the New Zealand Angus Association, and these results were available for comparison with the results of their sires. To be regarded as normal on the basis of a progeny test, a sire must have produced at least seven normal calves but no heterozygotes from normal dams.

Methods employed for lymphocyte separation, α-mannosidase assays and protein estimations were as presented in Chapter II. Assays were performed in batches of 20-30 samples with a standard lymphocyte extract being included in each batch to guard against day to day variations in technique.

Results

There was a relatively strong correlation ($r = 0.73$, $p < 0.01$) between α-mannosidase activity and protein concentration in lymphocyte extracts from 102 presumed normal bulls. The activities of α-mannosidase per mg of protein in lymphocyte extracts from 147 bulls are presented in Figure 5.10. Superimposed on this figure is additional information regarding the genotype of each bull according to the routine plasma test, plasma tests of progeny, or information that a bull was an obligate heterozygote by having sired a calf with mannosidosis (see key). There appeared to be two distinct populations which corresponded more or less with the diagnosis of bulls as normal or heterozygous on the basis of the other tests. Skewness estimation (Snedecor and Cochran, 1967) confirmed the apparent positive skewness in the distribution of lymphocyte extract α-mannosidase activities of normal bulls ($g_1 = 0.57 \pm 0.22$). This skewness was corrected by logarithmic transformation of values ($g_1 = 0.01 \pm 0.22$). All bulls with lymphocyte extract α-mannosidase activities below 3.0 units were either heterozygous or equivocal on other tests or, in one case, had not been tested by other methods. The latter bull did however possess a heterozygous parent. The highest activity recorded in a heterozygous bull was 4.2 units, but on reinvestigation of this obligate heterozygote a value of 3.1 units was obtained. Both tests are included in the results. All bulls with values greater than 4.5 units were either normal or equivocal on the basis of other tests or had not been tested by other methods.

The area between 3.0 and 4.5 units of activity included eight presumed normal bulls, five heterozygotes (two obligate) and one bull which had not been tested by other methods but which possessed a heterozygous parent. One bull with an equivocal plasma test but judged normal on its progeny test, had an initial lymphocyte extract α-mannosidase

Key to Figures 5.10, 5.11, 5.12 and 5.13:

- ● Bulls considered normal with regard to the mannosidosis genotype on the basis of plasma tests and/or tests of progeny.
- ○ Bulls considered heterozygous for the mannosidosis genotype on the basis of plasma tests and/or tests of progeny.
- ⊙ Bulls without information on genotype from other tests but with one heterozygous parent.
- ▒ Bulls without additional information on genotype
- n Normal on basis of plasma test.
- c Heterozygous on basis of plasma test.
- e Equivocal plasma test.
- p Diagnosis of genotype based on plasma tests of progeny.
- o Obligate heterozygotes.
- - Duplicate samples

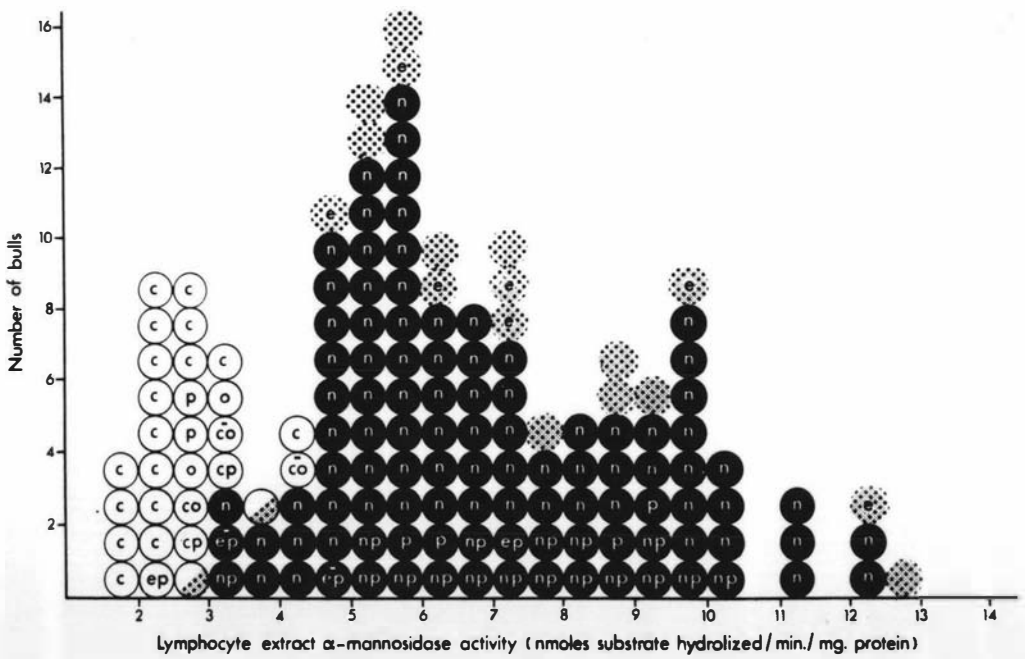


Figure 5.10 Distribution of lymphocyte extract α-mannosidase activities per mg protein of 147 individual bulls. Information concerning known mannosidosis genotype is superimposed.

activity of 3.2 units, but showed a retest of 4.8 units. Once again the results of both tests are included in Figure 5.10. Of ten bulls whose plasma test had produced an equivocal result only one fell between 3.0 and 4.5 units of activity on the lymphocyte test. Eight of these bulls had values greater than 4.5 and one less than 3.0 units.

An estimate of the area of overlap between the normal and heterozygous populations in Figure 5.10 was obtained by placing confidence limits on each population mean after logarithmic transformation of values, i.e. $\bar{x} \pm t_{0.01}\ s_{\bar{x}}$ (Snedecor and Cochran, 1967). At the 99% level of confidence the theoretical area of overlap extended from 3.0 to 5.4 units of activity. The mean activity of α-mannosidase in lymphocyte extracts from bulls diagnosed as heterozygous on the basis of this test plus either a plasma test or progeny test was 2.63 units, 38% of the mean of those judged normal on similar grounds (6.98 units).

As most bulls included in this investigation were from different properties and many of the plasma tests performed at different times, a study of the relationship between plasma and lymphocyte extract α-mannosidase activities could not include all the above animals. A relatively low correlation coefficient ($r = 0.2$, $p < 0.05$) was estimated for this relationship in seventy normal bulls where a valid study could be made.

Discussion and conclusions

The separation of 147 bulls into heterozygous and normal populations on the basis of their lymphocyte extract α-mannosidase activities was in close agreement with diagnoses based on information from plasma tests and/or tests of progeny (Figure 5.10). Approximately 10% of bulls were included in a range where the highest values of the heterozygous population overlapped with the lowest normal values. Two of the bulls in this

area were resampled and the lymphocyte test repeated, with apparent clarification of their status. A theoretical area of overlap was estimated to extend from 3.0 units to 5.4 units of activity at the 99% confidence level, but the accuracy of this estimate can only be established after a greater number of heterozygous individuals have been tested. If the overlap area did prove to be as wide as this estimate would suggest then the lymphocyte test would be of limited use as approximately 30% of animals would be expected to produce an equivocal result.

Due to a low correlation between plasma and lymphocyte extract α-mannosidase activities it is likely that the genotype of an animal with an equivocal plasma test may be resolved by the supplementary test based on lymphocyte extract α-mannosidase activity. In fact, only one of the ten bulls in Figure 5.10 with an equivocal plasma test had a lymphocyte test within the apparent area of overlap between normal and heterozygous populations. The conservative criteria employed in the diagnosis of normality on the original plasma test is demonstrated by the fact that eight of the ten equivocal bulls appeared to be normal on the lymphocyte test.

The mean α-mannosidase activity in lymphocyte extracts of bulls finally diagnosed as heterozygous was 38% of the mean of those diagnosed as normal. This is in close accordance with the situation in plasma where the mean α-mannosidase activity of heterozygotes is 37.5% of the normal mean (Chapter IV, page 103).

In spite of an overlap between normal and heterozygous populations it is concluded that a test for bovine mannosidosis heterozygotes, based on the estimation of α-mannosidase activity per mg of protein in lymphocyte extracts is a useful adjunct to the routine plasma test, and should help resolve most problem cases. There is however a need to test a

greater number of heterozygous animals so that a more accurate estimation of the region of overlap can be obtained.

2. α-Mannosidase activity relative to the mean activity of four lysosomal reference enzymes

Materials and methods

Assays for the activity of β-galactosidase, hexosaminidase, α-fucosidase and arylsulphatase A were performed on lymphocyte extracts from 115 of the bulls whose lymphocyte extract α-mannosidase activities are presented in Figure 5.10, and whose status with regard to the mannosidosis genotype had been clearly determined on the basis of plasma tests and/or tests of progeny. Methods employed for enzyme assays and protein estimations are described in Chapter II.

For the lymphocyte extract from each animal a lysosomal reference index (Li) was calculated using the following formula:

$$Li = \frac{1}{4}\left\{\frac{E_{Ai}}{\bar{E}_A} + \frac{E_{Fi}}{\bar{E}_F} + \frac{E_{Gi}}{\bar{E}_G} + \frac{E_{Hi}}{\bar{E}_H}\right\}$$

E_{Ai}, E_{Fi}, E_{Gi} and E_{Hi} represent the activities of arylsulphatase A, α-fucosidase, β-galactosidase and hexosaminidase respectively per ml of lymphocyte extract from an individual bull, and $\bar{E}_A$, $\bar{E}_F$, $\bar{E}_G$ and $\bar{E}_H$ represent the overall mean activities for each of these four enzymes. The lysosomal reference index (Li) has no units and will vary on either side of 1.0 according to the number of lymphocytes in each sample and their enzyme activity. A second lysosomal reference index (Li') was calculated using the same formula but with enzyme activity expressed relative to the protein concentration of lymphocyte extracts.

Results

Correlation coefficients for the relationships between α-mannosidase activity and the activities of four other lysosomal enzymes plus Li and Li' are presented in Table 5.III, and compared with the relationship between α-mannosidase activity and protein concentration. The strongest correlation ($r = 0.77$) was found between α-mannosidase and hexosaminidase activity per ml of extract, while α-fucosidase ($r = 0.62$), β-galactosidase ($r = 0.59$) and arylsulphatase A ($r = 0.53$) were also significantly correlated with α-mannosidase activity. The correlation between α-mannosidase activity per ml of lymphocyte extract and the lysosomal reference index (Li) was the same as that between α-mannosidase activity and protein concentration ($r = 0.73$). When enzyme activity was expressed per mg of protein all but one of the corresponding relationships still showed significant but weaker correlations. Hexosaminidase activity again showed the strongest correlation with α-mannosidase activity ($r = 0.51$).

The distribution of α-mannosidase activities per ml of lymphocyte extract relative to Li for the 115 bulls is shown in Figure 5.11. Superimposed on this figure is additional information regarding the genotype of each bull based on a test of his plasma or plasma tests of his progeny. Once again there appeared to be two populations of bulls corresponding more or less with diagnoses based on other tests, but the apparent overlap between normal and heterozygous populations was greater than that observed in Figure 5.10. Four presumed heterozygous bulls had values within one standard deviation of the mean value for the normal population, while one bull with a normal progeny test appeared to lie within the heterozygous population.

The distribution of α-mannosidase activities per ml of lymphocyte extract relative to hexosaminidase activity for the same bulls is shown

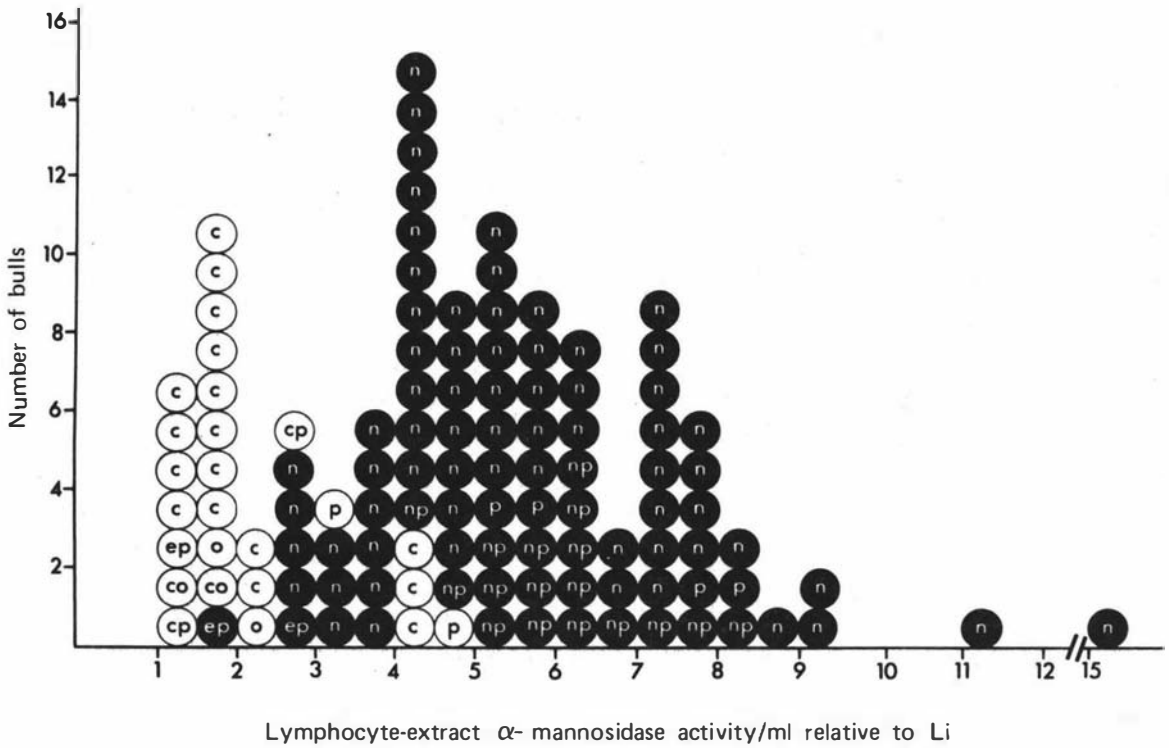


Figure 5.11 **Distribution of α-mannosidase activities per ml of lymphocyte extracts in relation to the mean relative activity of four other lysosomal reference enzymes per ml of extract (Li) from 115 individual bulls. Information concerning known mannosidosis genotype is superimposed.**
(See Figure 5.10 for key)

TABLE 5.III

Relationship between α-mannosidase activity and various reference parameters in bovine lymphocyte extracts

Reference parameter	Correlation coefficient (r) for relationship with α-mannosidase activity		
		Enzyme activity expressed/ml of extract	Enzyme activity expressed/mg protein
Arylsulphatase A	-	0.53 ($p < 0.01$)	0.29 ($p < 0.01$)
α-Fucosidase	-	0.62 ($p < 0.01$)	0.15 ($p > 0.05$)
β-Galactosidase	-	0.59 ($p < 0.01$)	0.39 ($p < 0.01$)
Hexosaminidase	-	0.77 ($p < 0.01$)	0.51 ($p < 0.01$)
Lysosomal index Li	-	0.73 ($p < 0.01$)	-
" " Li'	-	-	0.29 ($p < 0.01$)
Protein concentration	0.73 ($p < 0.01$)	-	-

in Figure 5.12. Hexosaminidase activity has been expressed as the ratio $E_H/\bar{E}_H$ rather than in actual units of activity so that values would be of a similar magnitude to those in Figures 5.10 and 5.11. Values for the normal population extended further into the heterozygous population than was previously observed, and two bulls with heterozygous plasma tests had values well above the mean for the normal population.

When lymphocyte extract α-mannosidase activities per mg of protein were expressed relative to Li' the distribution of values for the 115 bulls (Figure 5.13) showed a greater overlap between normal and heterozygous populations than when α-mannosidase activities were expressed in terms of protein concentration alone (Figure 5.10).

Discussion and conclusions

The correlation coefficient estimated for the relationship between α-mannosidase activity per ml of lymphocyte extract and the lysosomal reference index (Li) ($r = 0.73$) was identical to that obtained for the relationship between α-mannosidase activity and protein concentration in lymphocyte extracts. Theoretically therefore the use of Li as a reference parameter offers no advantage over the use of protein concentration. In fact, the overlap obtained between heterozygous and normal populations was greater when Li was employed as the parameter, as this resulted in four presumed heterozygotes being included within the normal population (Figure 5.11).

The activity of α-mannosidase in lymphocyte extracts was more highly correlated with hexosaminidase activity than with any of the other three lysosomal enzymes or with either Li or protein concentration (Table 5.III). It might be expected therefore that hexosaminidase activity would be a reliable reference parameter, but the expression of α-mannosidase activity per ml of extract relative to $E_H/\bar{E}_H$ (Figure 5.12) appeared to be even

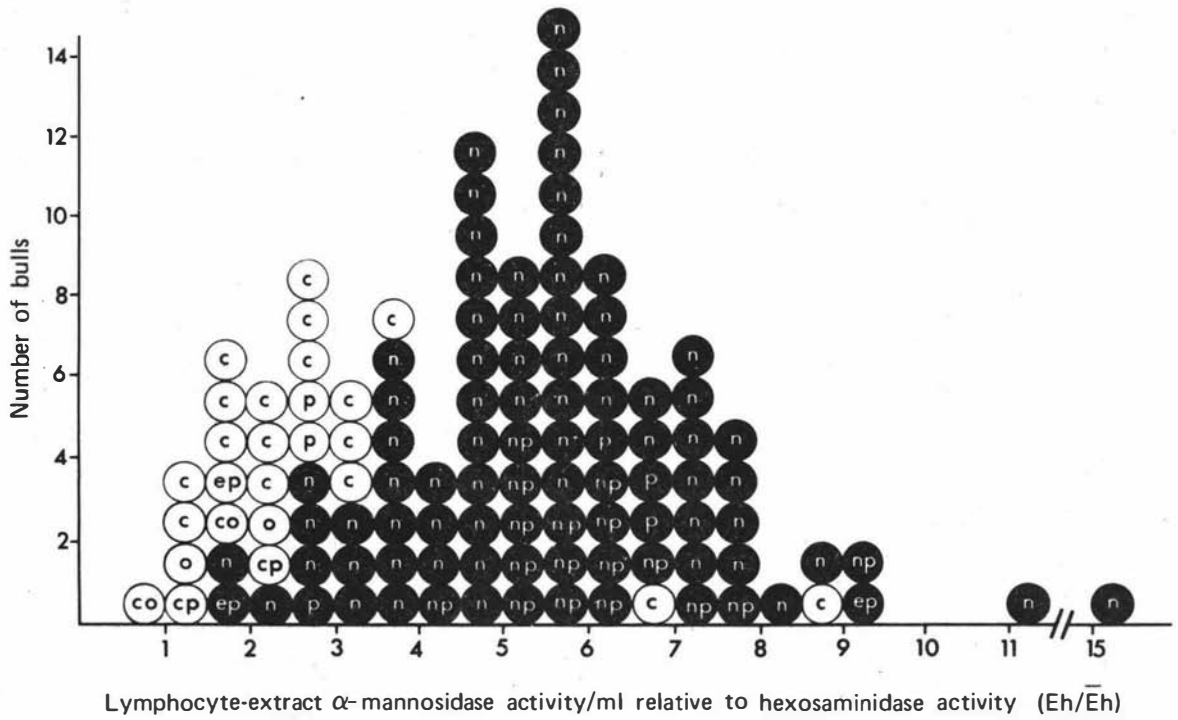


Figure 5.12 Distribution of α-mannosidase activities per ml of lymphocyte extract in relation to the mean relative activity of hexosaminidase per ml of extract (Eh/Ēh) from 115 individual bulls. (See Figure 5.10 for key)

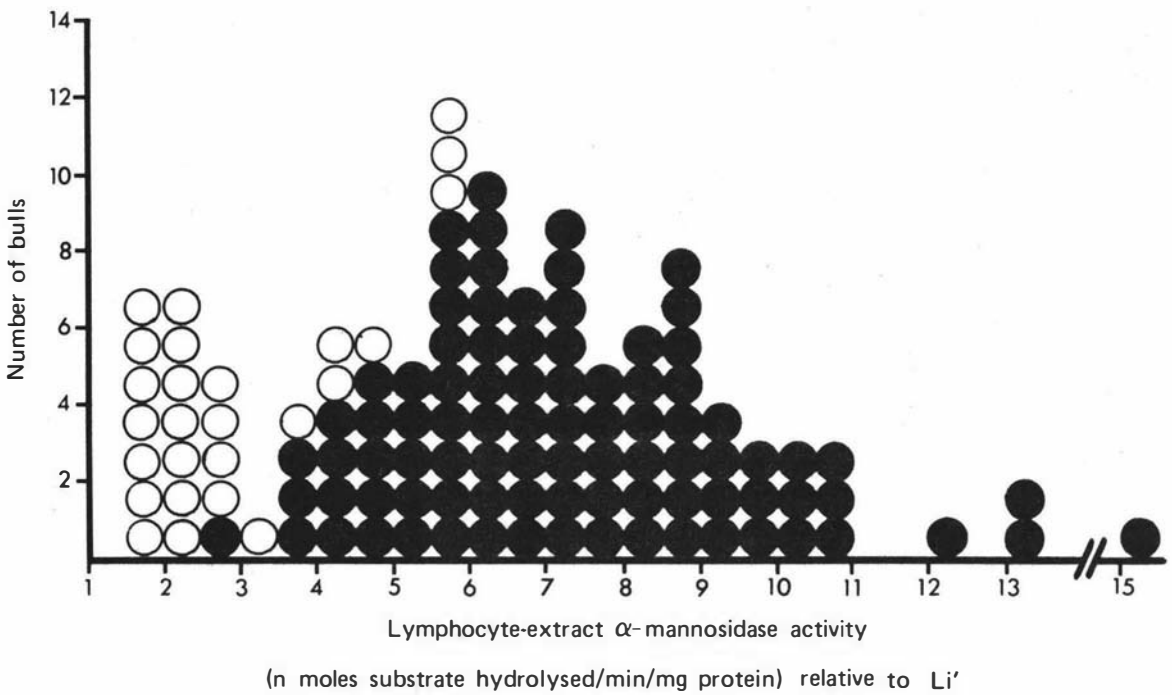


Figure 5.13 **Distribution of α-mannosidase activities per mg protein in relation to the mean relative activity of four lysosomal reference enzymes per mg protein (Li') in lymphocyte extracts from 115 individual bulls.** (See Figure 5.10 for key)

less effective in separating heterozygous bulls from the normal population than when enzyme activity was expressed relative to Li.

The strong correlations between α-mannosidase activity per ml of extract and the activity of the four reference enzymes were obviously due largely to the number of contributing lymphocytes, but even after the individual enzyme activities were divided by the protein concentration of lymphocyte extracts, significant correlations still existed between α-mannosidase and the activities of arylsulphatase A, β-galactosidase, hexosaminidase and the reference index Li'. Expression of α-mannosidase activity per mg of protein in relation to Li' (Figure 5.13) however, offered no advantage over the use of protein concentration alone as a parameter.

It was concluded from this experiment that the use of lysosomal reference enzymes as parameters for the activity of α-mannosidase in lymphocyte extracts was less reliable in separating heterozygotes from the normal population than when α-mannosidase activity was expressed in terms of protein concentration.

GENERAL DISCUSSION AND CONCLUSIONS

In a series of preliminary experiments the kinetic characteristics of α-mannosidase and four other lysosomal enzymes in bovine lymphocyte extracts were determined. The near absolute deficiency of acidic α-mannosidase activity (pH 3.75-4.5) described in tissues and body fluids of calves with mannosidosis (Hocking et al., 1972; Phillips et al., 1974b) was demonstrated in a lymphocyte extract from a mannosidosis calf. A heterozygous bull was shown to possess less than half the normal activity of this enzyme in its lymphocytes (Figure 5.1a). Further experiments were designed to establish a standard method for the prepara-

tion of lymphocyte extracts prior to enzyme assays so that comparisons could be made between enzyme activities of samples tested on different occasions. Information from these experiments was applied in the development of optimum assay conditions and methods of preparing extracts as presented in Chapter II.

The preparation of lymphocyte extracts for assay involves many steps at which variation in technique may alter the relative activity of α-mannosidase or of other lysosomal enzymes. It is important therefore that as many procedures as possible are performed by the same operator using a standard method. Even the initial centrifugation of blood samples prior to removal of the buffy coat is a possible source of variation. A decrease in the size of leucocytes, with apparent loss of cytoplasm, has been reported after centrifugation at 1,400 g (Dunne et al., 1970), therefore any variation in the speed at which samples are centrifuged may result in an alteration in the enzyme activity of cell extracts. The separation of pure populations of lymphocytes may be facilitated by the immediate mixing of collected blood with EDTA anticoagulant. In samples collected slowly or incompletely mixed with the anticoagulant, aggregates of platelets may develop and may interfere with the separation of lymphocytes. For this reason it is advisable to collect blood from the jugular vein of cattle, rather than from the middle coccygeal blood vessels, and so ensure the collection of a free-flowing sample. A significant reduction in the activity of β-galactosidase, arylsulphatase A and α-fucosidase was observed in lymphocytes which were not separated from whole blood until 48 hr after collection, but there was an apparent increase in α-mannosidase activity during this period (Figure 5.8). Whereas samples obtained locally may be processed within a few hours of collection, it may take up to 24 hr for samples from other parts of the country to reach the laboratory. It

is important therefore that the time interval between the collection of blood and separation of lymphocytes is standardized to say 24 hr.

The reliability of a test for heterozygosity, based on the assay of enzyme activity in body tissues or fluids, depends on the recognition of environmental, physiological or pathological factors which might alter the activity of an enzyme, and interfere with the gene dosage relationship between enzyme activity in normal and heterozygous individuals. The activity of α-mannosidase in bovine plasma, for example, varies with age and possibly sex, and significant seasonal, between-herd and even between-mob variations have been demonstrated, thereby placing certain limitations on the application of the plasma test for mannosidosis heterozygotes. α-Mannosidase activity might however be expected to show less variation in lymphocyte extracts than in plasma as lymphocytes are a primary source of the enzyme, whereas plasma probably depends largely upon the leakage or secretion of enzyme from tissues and the turnover of body cells.

A possible source of variation in the activity of lysosomal enzymes in lymphocyte extracts is the transformation of lymphocytes *in vivo* in response to an antigenic stimulus. The mean α-mannosidase activity in lymphocyte extracts from six calves was significantly elevated 6 days after injection with Strain 19 *Brucella abortus* vaccine (Figure 5.9). Although this vaccine is only employed in female calves less than six months of age, this finding does suggest that certain infectious agents might produce a similar reaction, and lymphocyte tests of any animal suffering from an infectious disease should therefore be interpreted with caution.

The number of normal calves tested was not sufficient to allow statistical investigation of possible variation in lymphocyte extract

α-mannosidase activity with age, nor were sufficient numbers of animals tested from individual properties to indicate the presence of between-herd differences. The possibility of between-sex differences in the activity of α-mannosidase in lymphocyte extracts should not be discounted. Although approximately 50 normal female cattle have so far been tested by this method, a valid comparison cannot be made between their mean value and that of the bulls. The animals were from a variety of properties and were tested in several different batches, therefore the possible interference of between-herd differences and day to day variations in technique could not be eliminated. It is desirable that experiments be specifically designed to investigate the possible influence of age, sex and environment on the activity of α-mannosidase in lymphocytes.

The separation of 147 bulls into normal and heterozygous populations according to α-mannosidase activity per mg of protein in lymphocyte extracts was in close agreement with diagnoses based on plasma tests and/or tests of progeny. There was no advantage in relating α-mannosidase activity to the activity of hexosaminidase, or to lysosomal reference indexes representing the mean relative activity of four lysosomal enzymes. In fact, the separation of normal and heterozygous populations was less efficient when reference enzymes were employed as parameters for α-mannosidase activity.

Human G_{M2}-gangliosidosis Type II (Sandhoff's disease) is caused by a deficiency of both hexosaminidase A and B (O'Brien et al., 1971). By relating total hexosaminidase activity in leucocyte extracts to the mean relative activity of three lysosomal reference enzymes Harzer (1973) claimed that 18 presumed heterozygotes for this disease could be distinguished from 30 presumed normal individuals. The heterozygous group included five obligate heterozygotes and 13 members of the family of

affected children, while the normal population included another 13 family members plus 17 controls. Harzer presented his results as reciprocal values, thereby exaggerating the separation between presumed normal and heterozygous groups, and it is unlikely that a clear separation would have existed had the results not been expressed in this manner. Although Harzer criticized the use of protein concentration as a parameter for enzyme activity in leucocyte extracts, he supplied no evidence to suggest that the activity of hexosaminidase was more strongly correlated with the mean relative activity of the reference enzymes than with protein concentration.

Masson et al. (1974) recommended the use of a single reference enzyme in the detection of heterozygotes for human mannosidosis. The activity of acidic α-mannosidase in serum and leucocytes did not differentiate between heterozygotes and normal controls, but when the results were expressed as a ratio of hexosaminidase to α-mannosidase activity, in either serum or leucocyte extracts, there was no overlap between the two populations. Similarly, Matsuda et al. (1973) were unable to separate two obligate heterozygotes for the fucosidosis genotype from eight controls on the basis of α-fucosidase activity per mg protein in leucocyte extracts. However, when the ratio of α-fucosidase to α-mannosidase activity per mg protein was calculated the heterozygotes had values intermediate between those of the normal controls and a child with fucosidosis. Further support for the use of a reference enzyme was provided by Koster et al. (1974) who claimed that Type II glycogenosis heterozygotes could be more clearly distinguished from diseased homozygotes and normal controls if acid α-glucosidase activity was expressed relative to the activity of neutral α-glucosidase rather than in terms of the protein concentration of leucocyte extracts. None of these authors however demonstrated a positive correlation between the activity

of the deficient enzyme and their chosen reference enzyme. Similar results might also have been obtained by relating enzyme activity to some independently variable "nonsense" parameter such as body weight or blood-glucose levels. Admittedly the numbers of subjects involved in their investigations have generally been too small for useful statistical analyses, but the necessity for choosing a parameter which does not vary independently of the enzyme in question should have been discussed.

A method for detecting Type II glycogenosis heterozygotes, based on assays of acid α-glucosidase activity per mg of protein in extracts of lymphocytes stimulated by phytohaemagglutinin (PHA), was described by Hirschhorn et al. (1969). Prior to stimulation with PHA the heterozygotes could not be differentiated from controls, but after stimulation there was a relatively clear separation between the lymphocyte extract acid α-glucosidase activities of heterozygotes and controls. This separation was even more distinct when changes in acid α-glucosidase activity, induced by stimulation of lymphocytes, were related to changes in the activity of acid phosphatase. The response of acid α-glucosidase activity in stimulated lymphocytes from heterozygotes was less than that of acid phosphatase, whereas in control individuals both enzymes showed a similar increase in activity. Similar success in heterozygote detection has been obtained for lysosomal acid phosphatase deficiency (Nadler and Egan, 1970). In this case acid phosphatase activity showed a relative lack of stimulation in lymphocytes from heterozygotes when compared to the activity of acid α-glucosidase. The reason for a lack of response of the partially deficient enzyme to stimulation by PHA is not known, but Hirschhorn et al. (1969) suggested that it may be due to altered rates of synthesis, activation or degradation of the enzyme. The lack of a sufficient number of subjects in both studies precludes full assessment of the value of this technique in heterozygote detection, but it is an interesting concept which warrants further investigation.

It was concluded from the investigations presented in this chapter that a supplementary test for the mannosidosis genotype, based on the activity of α-mannosidase in lymphocyte extracts, would be a useful adjunct to the plasma test when testing single animals, and would resolve the genotype of most animals with an equivocal plasma test. Approximately 10% of such animals would however be expected to have an equivocal lymphocyte test and the availability of a further supplementary test for use in conjunction with the lymphocyte test would be of value. The test appeared to be most reliable when enzyme activity was expressed relative to the protein concentration of lymphocyte extracts rather than in terms of the relative activity of lysosomal reference enzymes. Further evaluation of the lymphocyte test is necessary to accurately establish the limits of the region of overlap between heterozygous and normal populations.

SUMMARY

Optimum assay conditions were established for five lysosomal enzymes in bovine lymphocyte extracts. These included α-mannosidase, arylsulphatase A, α-fucosidase, β-galactosidase and total hexosaminidase. A standard method for preparing lymphocyte extracts, prior to enzyme assay, was developed as a result of information gained from experiments described in this chapter.

The reliability of lymphocyte α-mannosidase activity in the detection of heterozygotes for the mannosidosis genotype was evaluated on samples obtained from mature bulls, most of which were also tested by the plasma test and/or plasma tests of their progeny. The relative merits of using protein concentration and the relative activity of lysosomal reference enzymes to correct for the original number of lymphocytes in each sample were investigated, and protein concentration was shown to be the most useful parameter.

The mean value of α-mannosidase activity in lymphocyte extracts from heterozygous bulls was 38% of the mean value for normal bulls.

The lymphocyte test is recommended for use in situations where the reliability of the plasma test is reduced, and in retesting animals whose plasma test has produced an equivocal result.

CHAPTER VI

PRELIMINARY INVESTIGATIONS INTO THE DEVELOPMENT OF OTHER SUPPLEMENTARY TESTS FOR THE MANNOSIDOSIS GENOTYPE

INTRODUCTION

Although the lymphocyte test for mannosidosis heterozygotes appears capable of resolving the genotype of many animals with equivocal plasma tests, there is an overlap between values exhibited by normal and heterozygous populations. It was decided therefore that other possible tests should be investigated to see if they offered further advantages. Enzyme assays on cultured fibroblasts from skin have been successfully employed in detecting heterozygotes for several inborn lysosomal disorders of man (Table 1.IV). For economic reasons this method is likely to be impractical for routine use in domestic animals but it was considered worthy of investigation since it might have value in testing individual valuable cattle for the mannosidosis genotype. Assays of α-mannosidase activity in granulocytes and thrombocytes were also considered as possible alternative tests.

MATERIALS AND METHODS

All animals employed in these experiments were of the Angus breed. Skin fibroblasts were cultured from eight two-year old female cattle who were clearly heterozygous for the mannosidosis genotype on the basis of their plasma α-mannosidase activity, and from eight normal controls of the same age. Fibroblasts were also cultured from skin biopsies obtained from a six-month old calf with mannosidosis. Standard conditions of culture were employed for all samples.

Granulocyte extracts were prepared from 52 mixed-aged male and female cattle from several herds. Lymphocyte extracts and plasma were also obtained from each of these animals. Thrombocyte extracts were prepared from 38 of the above cattle.

Details of the methods employed in cell culture, preparation of granulocyte, lymphocyte and thrombocyte extracts, and assays for α-mannosidase activity are presented in Chapter II.

RESULTS

1. α-Mannosidase activity in cultured skin fibroblasts

The pH dependence of α-mannosidase activity in cultured fibroblasts from normal, heterozygous and mannosidosis cattle is illustrated in Figure 6.1. Optimum activity in cells from the normal animal occurred at pH 4.0, but smaller peaks of activity were apparent at pH 5.0-5.5 and pH 6.5-7.0. Acidic α-mannosidase activity (pH 4.0) was lower in the heterozygous animal and the peak of intermediate activity (pH 5.0-5.5) was more obvious. In cells from the mannosidosis calf only a small peak of acidic α-mannosidase activity was noted but intermediate and neutral activities did not appear to be reduced. A comparison between acidic α-mannosidase activities in cultured fibroblasts from eight presumed heterozygotes, eight normal control cattle and a calf with mannosidosis is shown in Figure 6.2. There was a significant difference ($t = 2.92$; d.f. = 14; $p < 0.05$) between the mean values for heterozygous and normal populations but individual values showed considerable overlap. The level of α-mannosidase activity in the mannosidosis calf was clearly lower than in any of the heterozygous animals.

2. α-Mannosidase activity in granulocyte extracts

The pH dependence of α-mannosidase activity in granulocyte extracts from normal, heterozygous and mannosidosis cattle is presented in

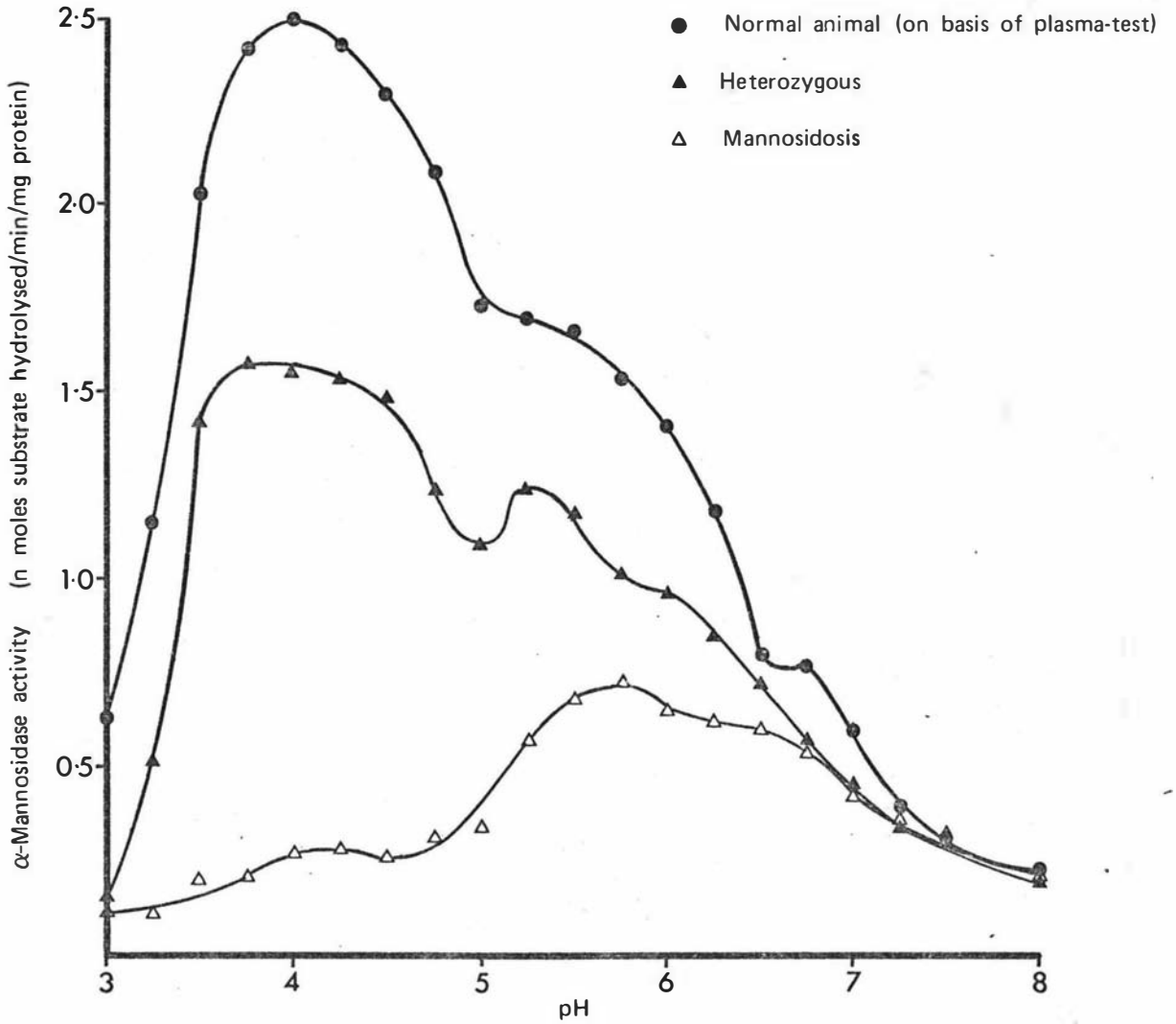


Figure 6.1 **pH dependence of α-mannosidase activity in cultured bovine fibroblasts.**

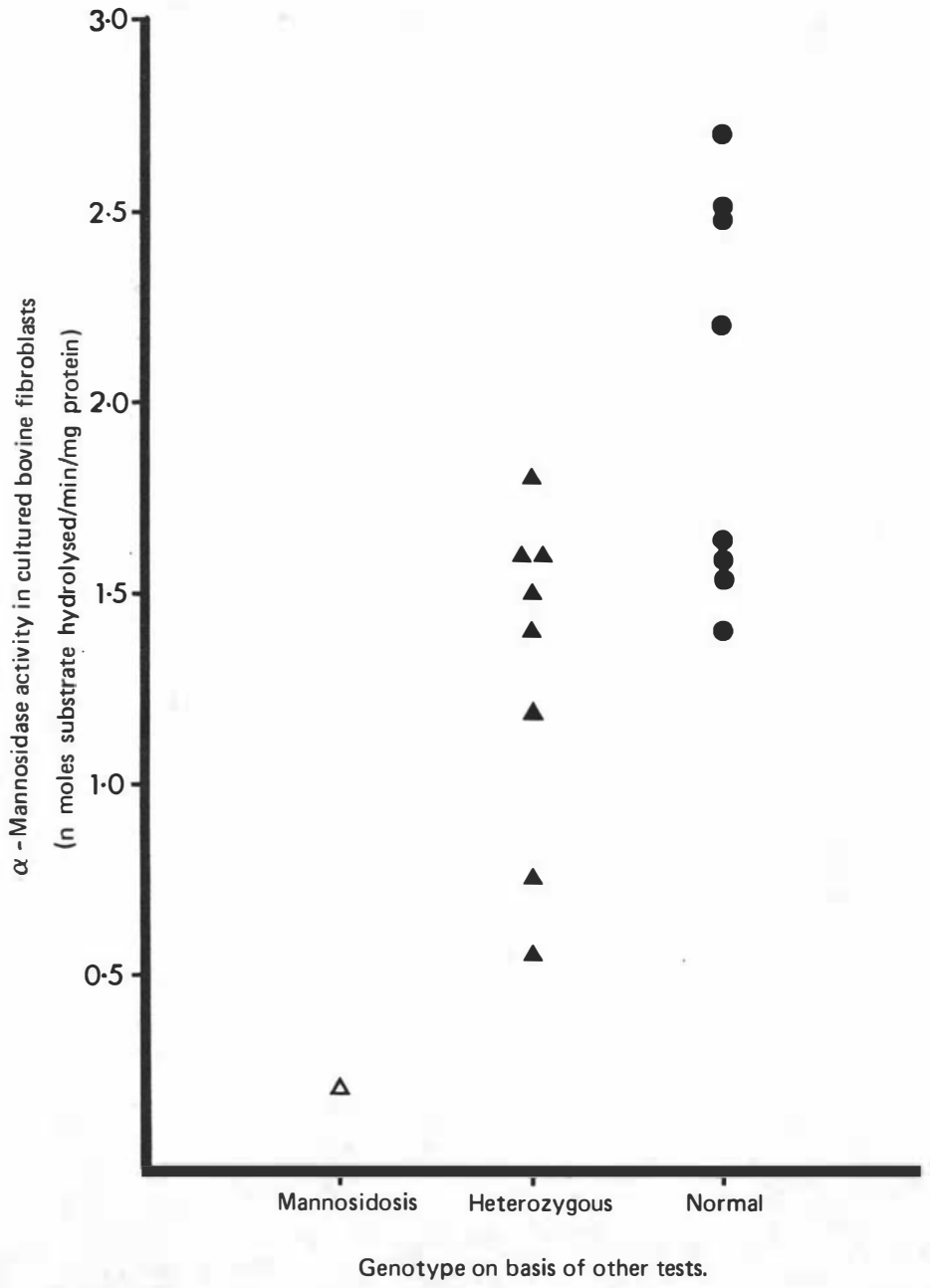


Figure 6.2 **α - Mannosidase activity in cultured bovine fibroblasts**

Figure 6.3. Optimum activity was recorded at pH 4.0 in both normal and heterozygous individuals, but with lesser activity in the latter animal. Levels of α-mannosidase activity assayed in the mannosidosis calf were negligible, even at more neutral pH values (5.5-7.0).

The distribution of acidic α-mannosidase activities of granulocyte extracts from 52 mixed-aged male and female cattle is shown in Figure 6.4. The separation of animals into different populations was in complete agreement with diagnoses of genotype based on plasma tests and lymphocyte tests. There was no overlap in values between 26 presumed heterozygotes and 26 normal controls. The mean α-mannosidase activity in granulocyte extracts from the heterozygous cattle was 3.4 units (nmoles substrate hydrolysed/min/mg protein), 33.4% of the mean value for the normal controls (10.7 units). There was no apparent influence of either age or sex on the activity of α-mannosidase in granulocyte extracts.

3. α-Mannosidase activity in thrombocyte extracts

The distribution of acidic α-mannosidase activities of thrombocyte extracts from 38 mixed-aged male and female cattle is shown in Figure 6.5. Two separate populations are evident but normal animals and presumed heterozygotes are included within each population.

Despite the considerable overlap in individual values, the mean value for the presumed heterozygotes (1.6 units) was significantly lower than that for the normal animals (2.1 units) ($t = 3.97$, d.f. = 36, $p < 0.01$).

DISCUSSION

It has been claimed that heterozygotes for inborn lysosomal diseases may be more reliably differentiated from normal individuals on the basis of enzyme assays on cultured skin fibroblasts than on serum or leucocytes, due to the uniformity of the cell population (Kaback and Howell, 1970,

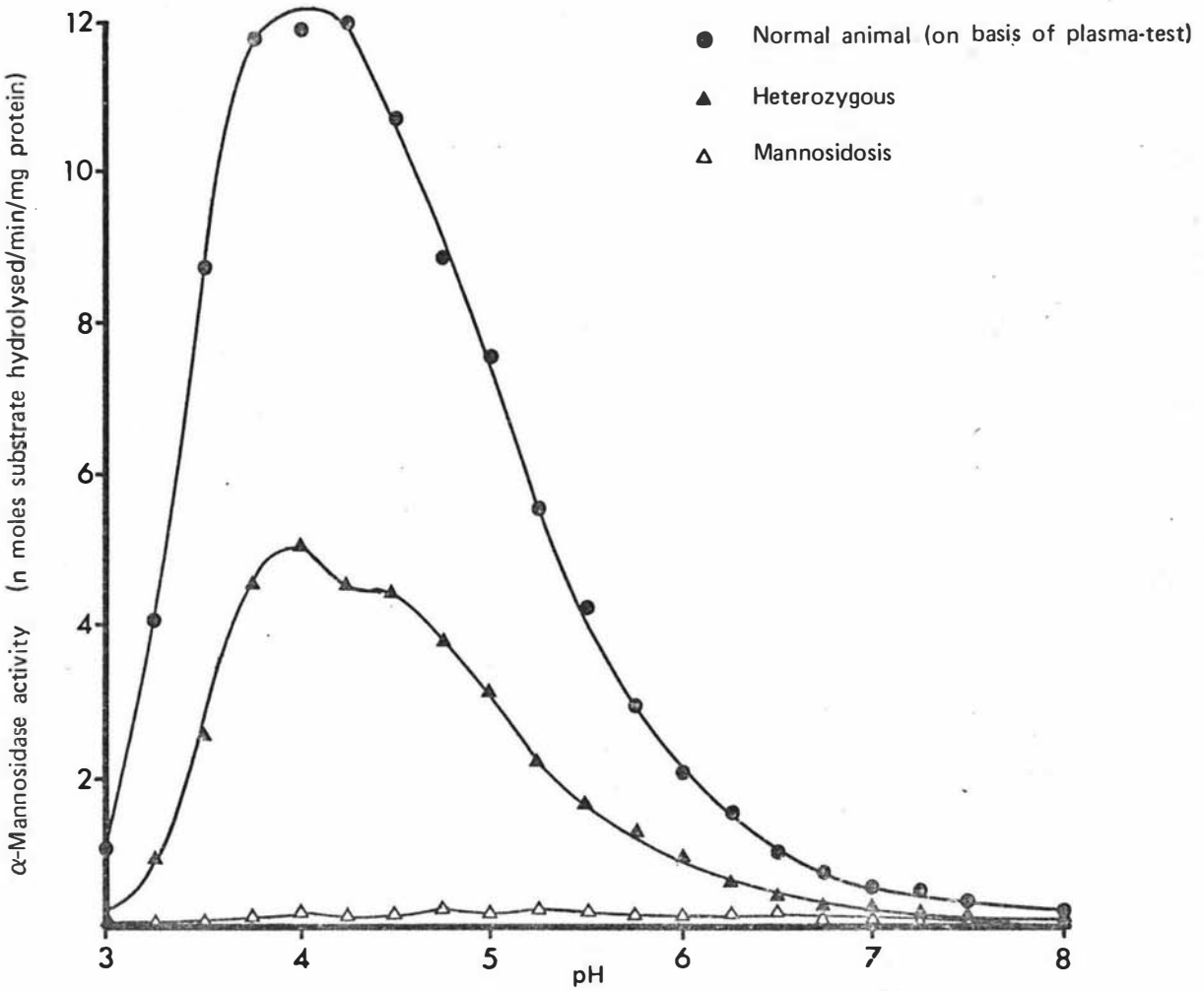


Figure 6.3 pH dependence of α-mannosidase activity in bovine granulocyte extracts.

Key to Figures 6.4 and 6.5:

● Animals considered normal with regard to the mannosidosis genotype on the basis of plasma tests and lymphocyte tests.

○ Animals considered heterozygous for the mannosidosis genotype on the basis of plasma tests and lymphocyte tests.

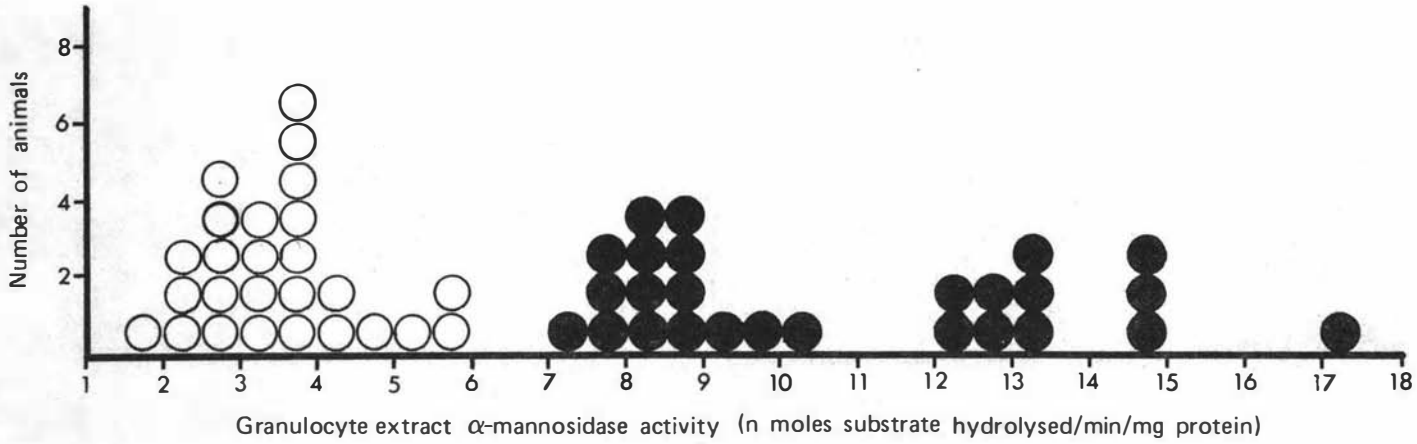


Figure 6.4 Distribution of α-mannosidase activities in granulocyte extracts from 52 mixed-aged male and female cattle.

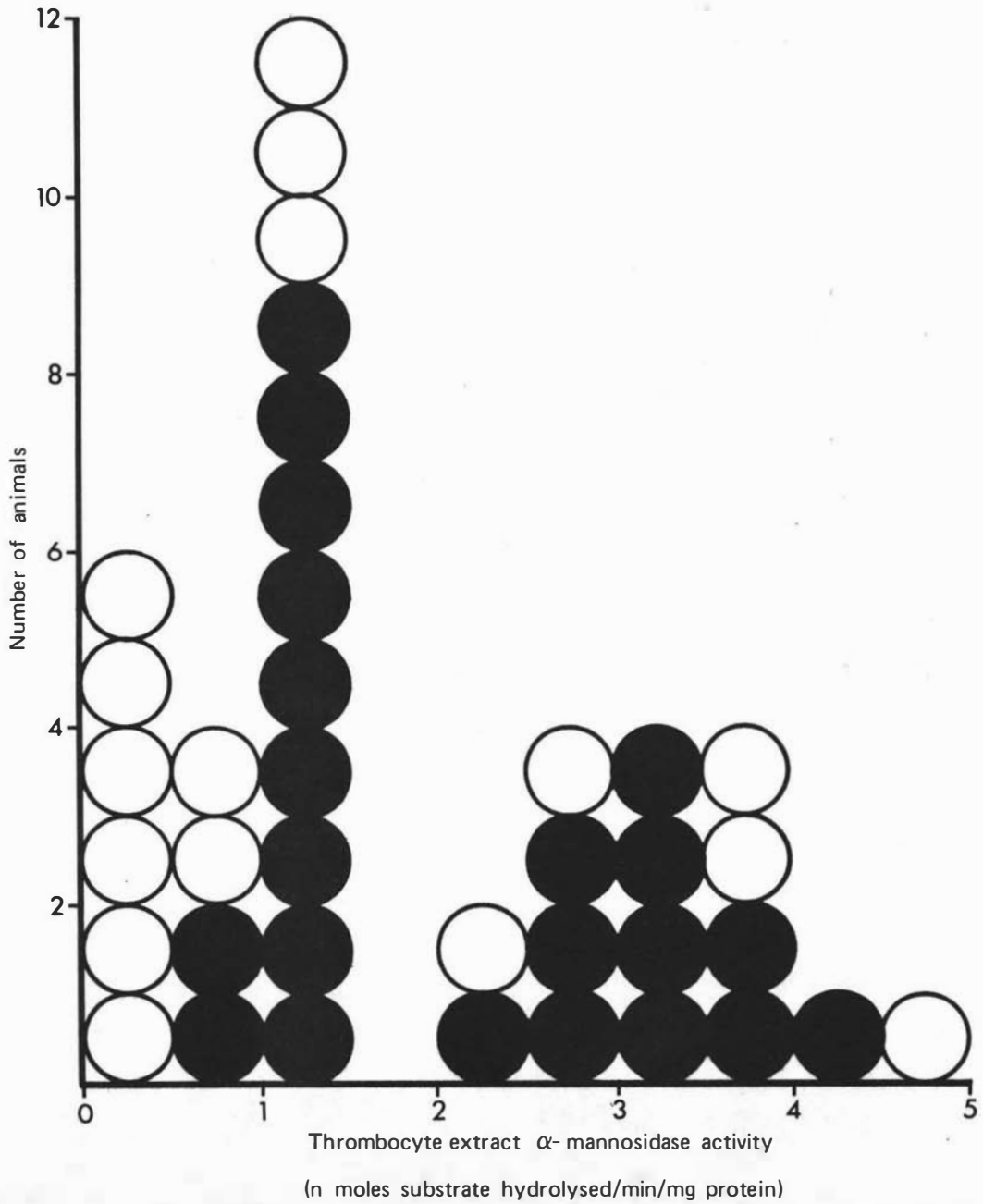


Figure 6.5 Distribution of α-mannosidase activities in thrombocyte extracts from 38 mixed-aged male and female cattle. (See Figure 6.4 for key)

1973; Beutler et al., 1971). Such claims however have been based on small numbers of individual tests and may be ill-founded. The activity of lysosomal enzymes in cultured fibroblasts has been shown to vary in relation to the growth curve of the culture, and considerable differences in enzyme activity have been observed between various cell-groups and even between individual cells in a culture (Galjaard et al., 1974). Some of this variation persists even when cells are cultured under standardized conditions (Milunsky et al., 1972).

The activity of α-mannosidase in cultured skin fibroblasts could not accurately distinguish between eight presumed heterozygotes for bovine mannosidosis and eight normal controls. In spite of a significant difference ($p < 0.05$) between the mean values there was considerable overlap in the range of individual α-mannosidase activities of heterozygous and normal populations (Figure 6.2). Although the number of animals included in this experiment was small, it appears that the difference in mean acidic α-mannosidase activity between normal and heterozygous populations of cattle is not as great in cultured skin fibroblasts as in lymphocyte extracts, granulocyte extracts or plasma. This was only a preliminary experiment designed to assess the possible value of assays on cultured fibroblasts in detecting mannosidosis heterozygotes and it appears that further evaluation of this test is not warranted, especially if a more practical and accurate alternative method is available.

In contrast to the results obtained with cultured fibroblasts, assays of α-mannosidase activity in granulocyte extracts differentiated between presumed mannosidosis heterozygotes and normal controls. The mean α-mannosidase activity in granulocyte extracts from 26 heterozygotes was 33.4% of the mean for 26 normal controls, this being in close agreement with relative values recorded in plasma and lymphocytes (Chapters IV

and V respectively). No overlap occurred between the two populations (Figure 6.4). Excluded from Figure 6.4 were the results obtained from 16 adult cows which had α-mannosidase activities more than twice as high as the mean value for the other cattle. All 16 samples were assayed on the same day and a standard sample routinely included, did not vary significantly from previous assays. The source of this variation is not known but is thought most likely to be due to some unappreciated variable in the method of preparing extracts rather than an increase in enzyme activity of these particular granulocytes per se. The occurrence of a technical error during enzyme assays or protein estimations cannot be discounted but is considered unlikely. Further experiments are necessary to establish the nature of this variation and to ensure that it will not interfere with a test for mannosidosis heterozygotes based on α-mannosidase activity in granulocyte extracts.

Although the number of animals was small, there appeared to be two separate populations with granulocyte extract α-mannosidase activities within the normal range (Figure 6.4). This phenomenon could represent the presence of two or more normal alleles for α-mannosidase activity as discussed in previous chapters, though further work is needed to test this hypothesis.

The pH dependence curve for α-mannosidase activity in cultured skin fibroblasts (Figure 6.1) showed a relatively low acidic activity (pH 4.0), even in the normal animal, but prominent peaks of intermediate (pH 5.0-5.5) and neutral activity (pH 6.5-7.0) were noted. Studies of pH dependence of α-mannosidase activity in granulocyte extracts from a calf with mannosidosis revealed an apparent lack of intermediate and neutral forms of α-mannosidase in addition to the expected deficiency of the acidic form (Figure 6.3). As these isoenzymes are not deficient in other tissues from calves with mannosidosis (Phillips et al., 1974b;

Winchester et al., unpublished data) it may be assumed that they are absent from granulocytes of normal and heterozygous cattle. A slight "shoulder" is present in the pH dependence curve for α-mannosidase activity in granulocytes from the heterozygous individual, but this is at a lower pH than would be expected if it represented the form with an intermediate pH optimum. Granulocytes are relatively short-lived cells with a specific function and apparently do not require these isoenzymes. Further studies on this observation could possibly suggest the function of the intermediate and neutral forms of α-mannosidase in other tissues.

The distribution of α-mannosidase activities in thrombocyte extracts from 38 cattle showed two distinct populations without overlap, but normal controls and presumed heterozygotes were scattered throughout both populations. The mean thrombocyte extract α-mannosidase activity for the normal animals was 2.12 units and that for the heterozygotes was 1.57 units. Although these mean values were significantly different ($p < 0.01$) the possible use of α-mannosidase assays on thrombocyte extracts in testing for mannosidosis heterozygotes is not encouraging. The major problem associated with the use of thrombocytes is the difficulty of obtaining consistently good preparations. If a blood sample is not mixed immediately with an anticoagulant on collection, aggregates of thrombocytes develop which often contain leucocytes and erythrocytes. In such cases the separation of a pure population of thrombocytes is not possible.

The mean activities of acidic α-mannosidase in extracts of normal bovine granulocytes, lymphocytes and thrombocytes are 10.7, 6.98 and 2.1 units respectively. It is conceivable therefore that the enzyme activity of leucocyte extracts may vary according to the relative proportion of lymphocytes, granulocytes and thrombocytes included in each sample. Assays on pure populations of granulocytes and/or lymphocytes would

therefore be expected to provide a more reliable indication of an individual's lysosomal enzyme complement than would assays on mixed-leucocyte extracts. It is important that thrombocytes are eliminated from extracts. Due to their relatively low α-mannosidase activity, large numbers of contaminating thrombocytes may cause an apparent reduction in the activity of α-mannosidase relative to protein concentration in lymphocyte or granulocyte extracts.

It was concluded that assays for α-mannosidase activity in cultured skin fibroblasts and thrombocyte extracts were unlikely to prove successful in the detection of mannosidosis heterozygotes. In contrast, the development of a test based on α-mannosidase activity in extracts of granulocytes appeared very encouraging. Further evaluation of the granulocyte test is recommended to establish the distribution of values for normal and heterozygous populations. The concurrent use of supplementary tests based on α-mannosidase activity in lymphocyte extracts and granulocyte extracts could considerably increase the probability of accurately determining an animal's status with regard to the mannosidosis genotype.

SUMMARY

Preliminary investigations into the development of alternative tests for detecting mannosidosis heterozygotes are presented. A test based on the activity of acidic α-mannosidase in granulocyte extracts appears very promising, but assays of α-mannosidase activity in cultured skin fibroblasts and thrombocyte extracts could not accurately distinguish between normal and heterozygous animals. Granulocytes have a higher mean acidic α-mannosidase activity than lymphocytes, fibroblasts or thrombocytes but do not appear to possess the isoenzymes with intermediate and neutral pH optima.

CHAPTER VII

ENZYME REPLACEMENT THERAPY - AN "EXPERIMENT OF NATURE" IN A CHIMERIC MANNOSIDOSIS CALF

INTRODUCTION

Recent developments in prenatal diagnosis and in the detection of heterozygotes have improved the opportunity to offer constructive genetic counselling and to assert some degree of control of inborn errors of lysosomal catabolism in man. Despite this progress affected children will continue to be born, and will require treatment. Correction of the underlying genetic anomaly does not appear to be an immediate reality, therefore any attempt at specific therapy must involve an alternative means of degrading the stored material, either by replacement of the defective enzyme or by activation of the product of the defective gene. Attempts at enzyme replacement therapy have varied from the infusion of patients with normal plasma or purified enzyme preparations, to the transplantation of organs or tissues which would be expected to provide a continuous endogenous supply of the required enzyme. With few exceptions, most attempts at therapy have been discouraging.

An opportunity to investigate the value in enzyme replacement therapy of a transplant of normal tissue was provided by a naturally occurring chimeric mannosidosis calf in which, as a result of fused placental blood circulations, lymphocytes from a normal co-twin had become established as a fully acceptable transplant. This "experiment of nature" is described in this chapter.

MATERIALS AND METHODS

A castrated male Angus calf was presented for examination at 9 months of age showing clinical signs of mannosidosis. Its sire was an obligate heterozygote and its dam had previously been diagnosed as heterozygous on the basis of her plasma α-mannosidase activity. The activity of acidic α-mannosidase in the plasma of the calf was almost completely absent, as would be expected in an animal with mannosidosis, but the activity of this enzyme in lymphocytes was within the low-normal range. A suggestion that the calf might be a blood-cell chimera was supported by information that it was a twin and although the twin was not available for examination, it was known to be female and clinically normal. Tissues for further biochemical and histopathological examinations were collected after euthanasia at approximately 14 months of age when the disease was clinically advanced.

Control tissues for enzyme assays were obtained from a normal Angus calf and an Angus calf with mannosidosis, both of which were approximately 12 months of age. Tissues from these two animals plus thirty-five other mannosidosis calves up to 18 months of age were used as controls in histopathological studies.

Chromosome preparations for karyotype studies were prepared from peripheral lymphocytes stimulated by phytohaemagglutinin and incubated for 48 hr at 37°C. Colchicine was added 2.5 hr prior to harvesting to block mitotic division at metaphase in actively dividing cells. Cells were fixed in an acetic acid-ethanol solution (3:1) and stained with Giemsa.

Methods for assay of α-mannosidase activity and estimation of protein concentration were as described in Chapter II. Sections for histopathological studies were prepared from paraffin and resin-embedded tissues by the procedures outlined in Chapter II.

RESULTS

1. Identification of the calf as a placental chimera

Karyotypes from 200 peripheral lymphocytes stimulated by phytohaemagglutinin were examined. Although the calf was a male, 77% of its lymphocytes carried the female (XX) genotype, and 23% the male (XY) genotype, providing strong evidence that it was a placental chimera possessing lymphocytes from its female co-twin. The normal complement of 60 chromosomes was observed in cells of both genotypes.

2. α-Mannosidase activity in various tissues

Profiles of the pH dependence of α-mannosidase activity in various tissues from the chimera, the mannosidosis control and the normal control calf are shown in Figure 7.1. The activity of acidic α-mannosidase (pH 3.75-4.5) in lymphocyte and lymph node extracts from the chimera was considerably greater than that measured in the mannosidosis control, and could be placed in the low-normal range on the basis of lymphocyte α-mannosidase activity (Chapter V). In the brain, liver, kidney and plasma of the chimera the acidic α-mannosidase activity closely resembled that shown by the mannosidosis control. α-Mannosidase activity assayed at higher pH values in all calves represents the activity of isoenzymes which are thought to be non-lysosomal and which are not deficient in mannosidosis.

3. Histopathological studies

When compared with the mannosidosis control calves, the chimera showed a considerable reduction in the degree of vacuolation of free macrophages and reticuloendothelial cells in lymph nodes (Figures 7.2a and b, 7.3a and b) and of Kupffer cells (Figure 7.4a and b). Vacuolation of the exocrine epithelium of the pancreas (Figures 7.5a and b, 7.6a and b) and lacrimal gland (Figure 7.7a and b) was also much less severe

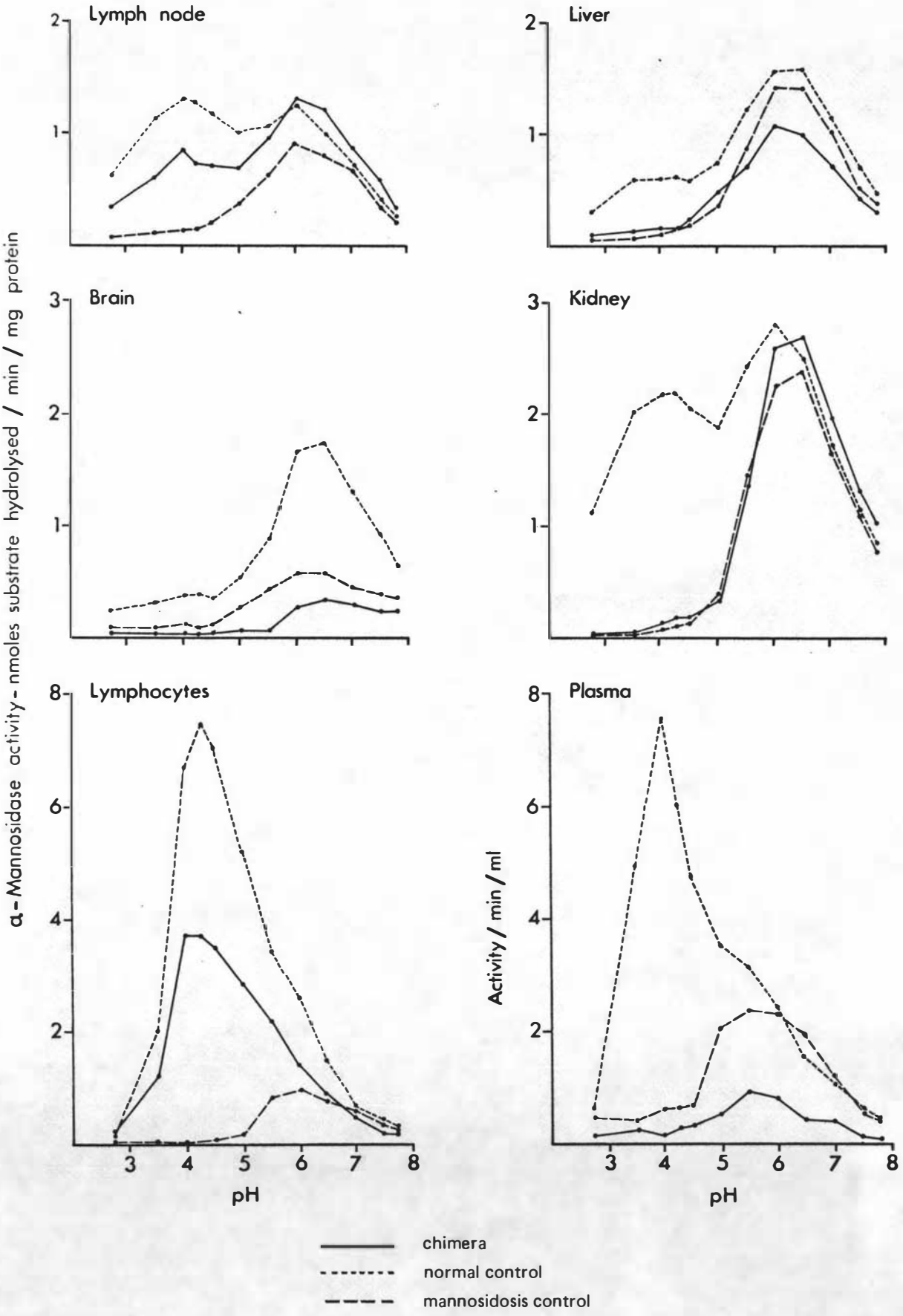
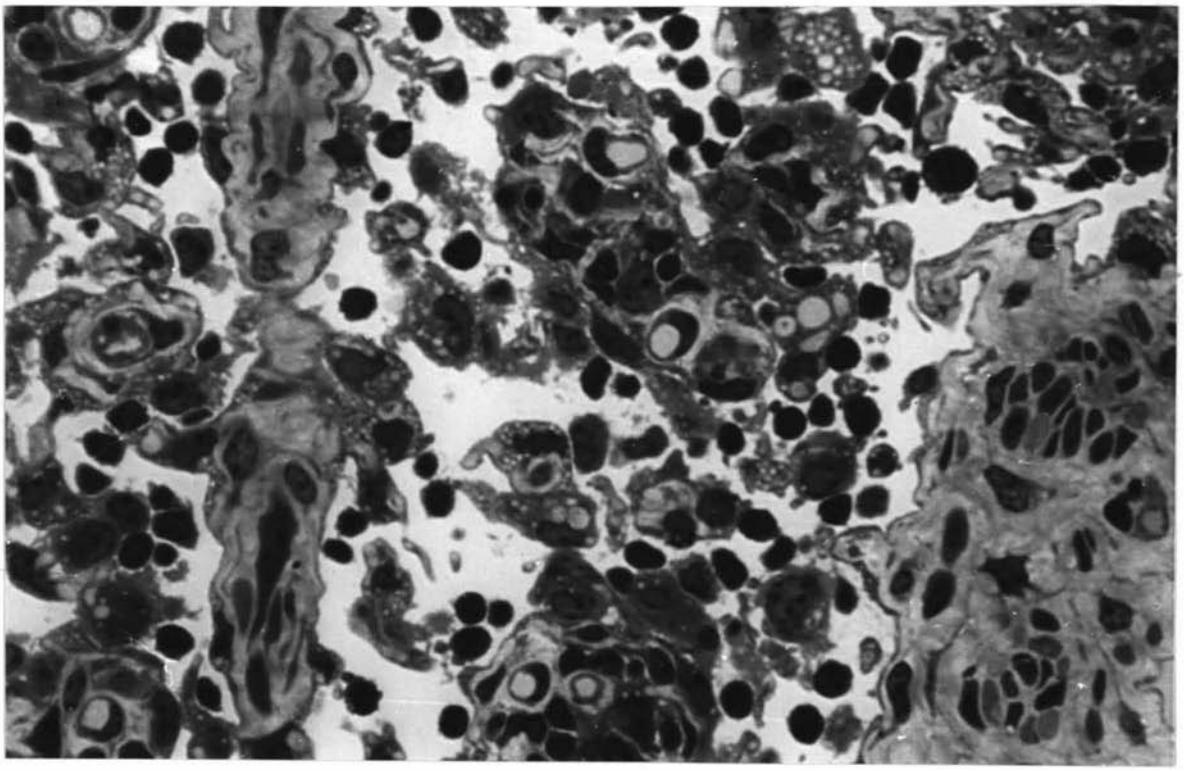


Figure 7.1 pH dependence of α-mannosidase activity in various tissues.

Figure 7.2a: Medullary area of a lymph node from the chimeric calf showing relatively mild vacuolation of reticuloendothelial cells and free macrophages. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 720)

Figure 7.2b: Medullary area of a lymph node from a calf with mannosidosis showing highly vacuolated reticulo-endothelial cells and free macrophages. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 720)



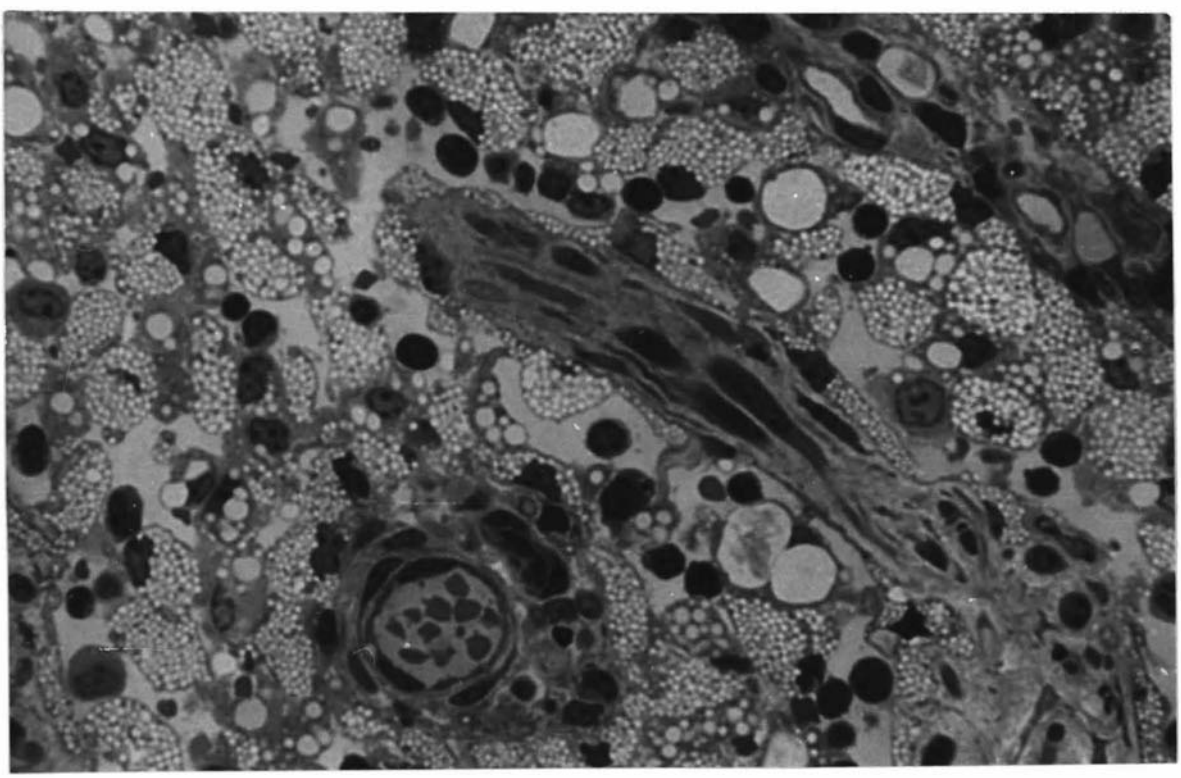
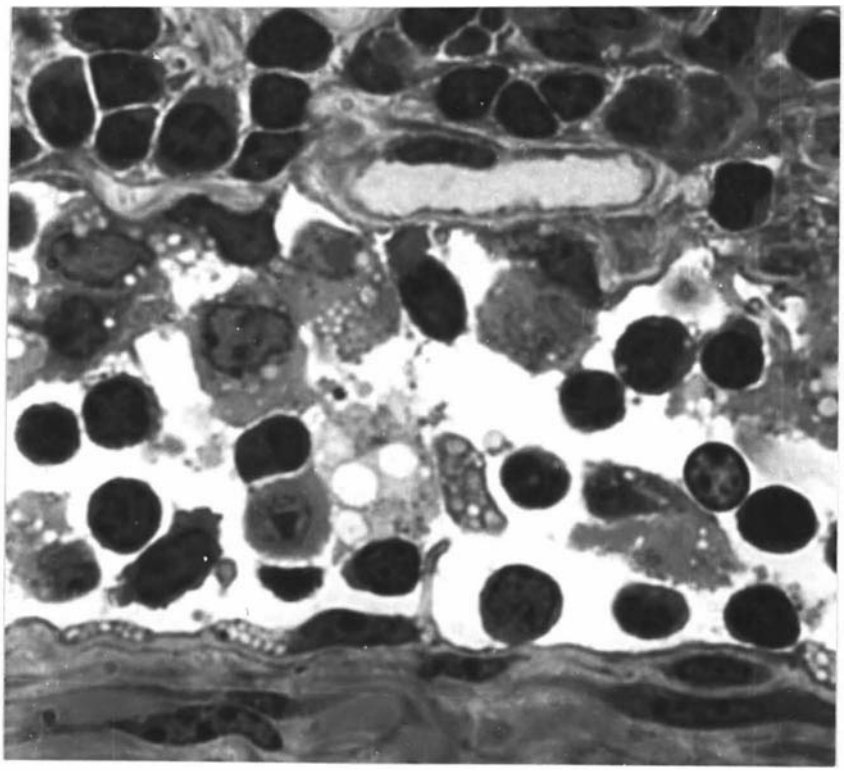


Figure 7.3a: Medullary area of a lymph node from the chimeric calf showing relatively mild vacuolation of reticuloendothelial cells and free macrophages. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 2,000)

Figure 7.3b: Medullary area of a lymph node from a calf with mannosidosis showing highly vacuolated reticulo-endothelial cells and free macrophages. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 2,000)



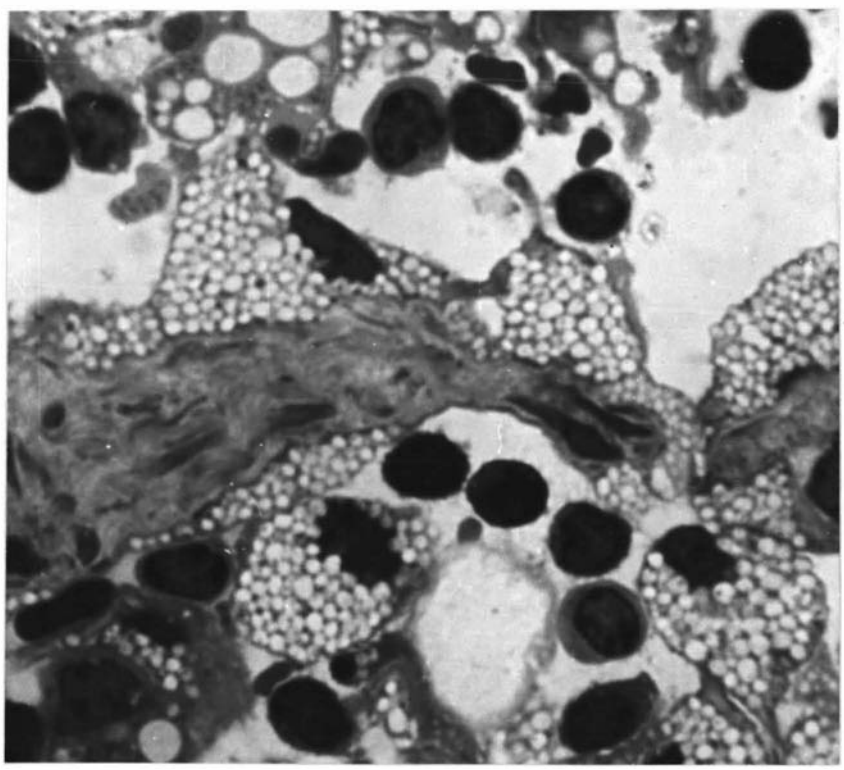
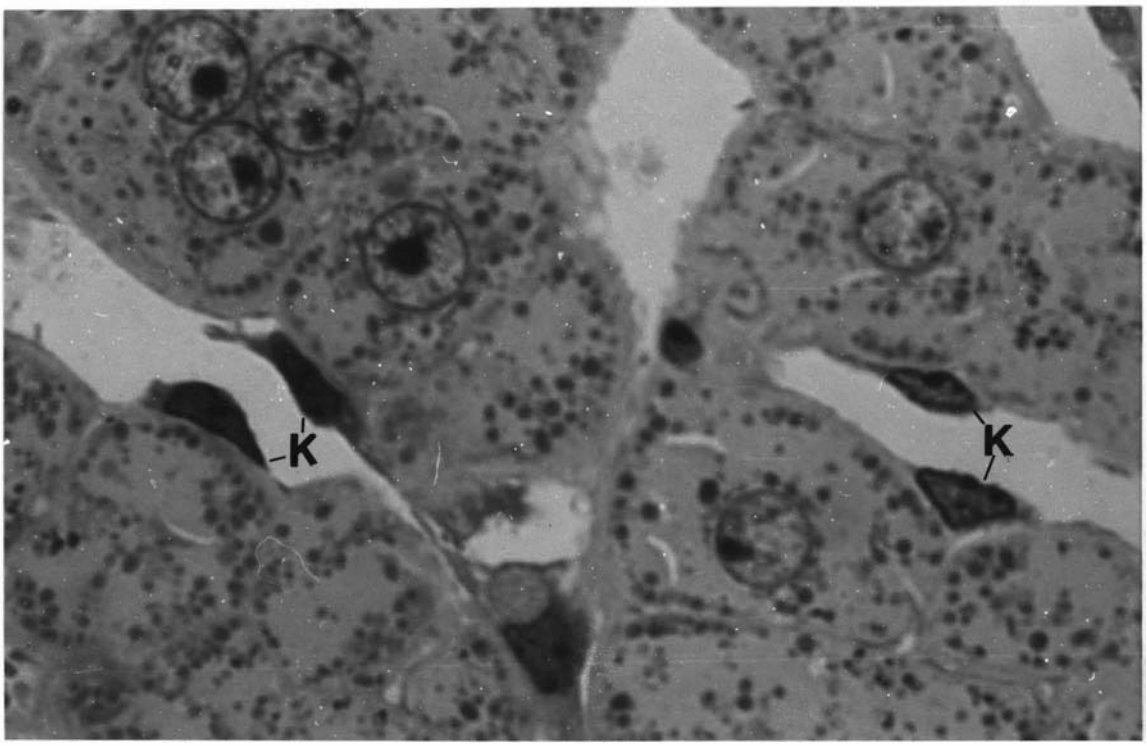


Figure 7.4a: Liver from the chimeric calf. None of the Kupffer cells (K) in this field contain clear vacuoles. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,760)

Figure 7.4b: Liver from a calf with mannosidosis. The cytoplasm of Kupffer cells (K) is packed with clear vacuoles. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 1,760)


K
K

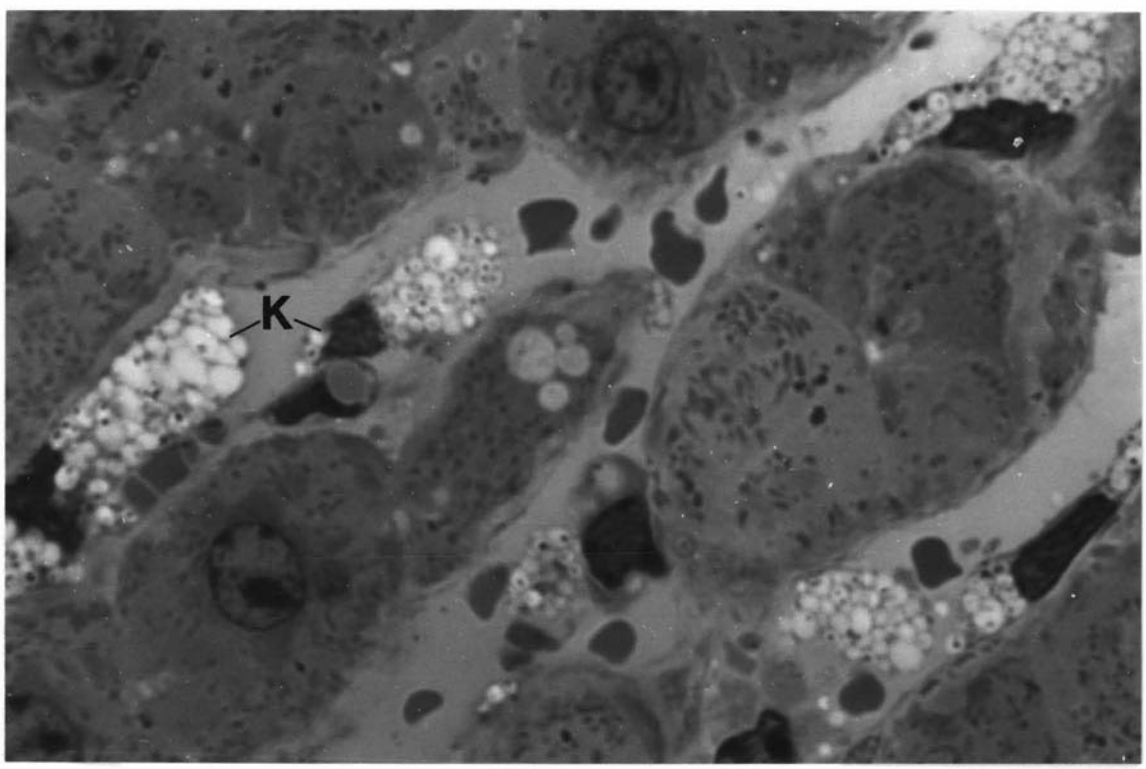
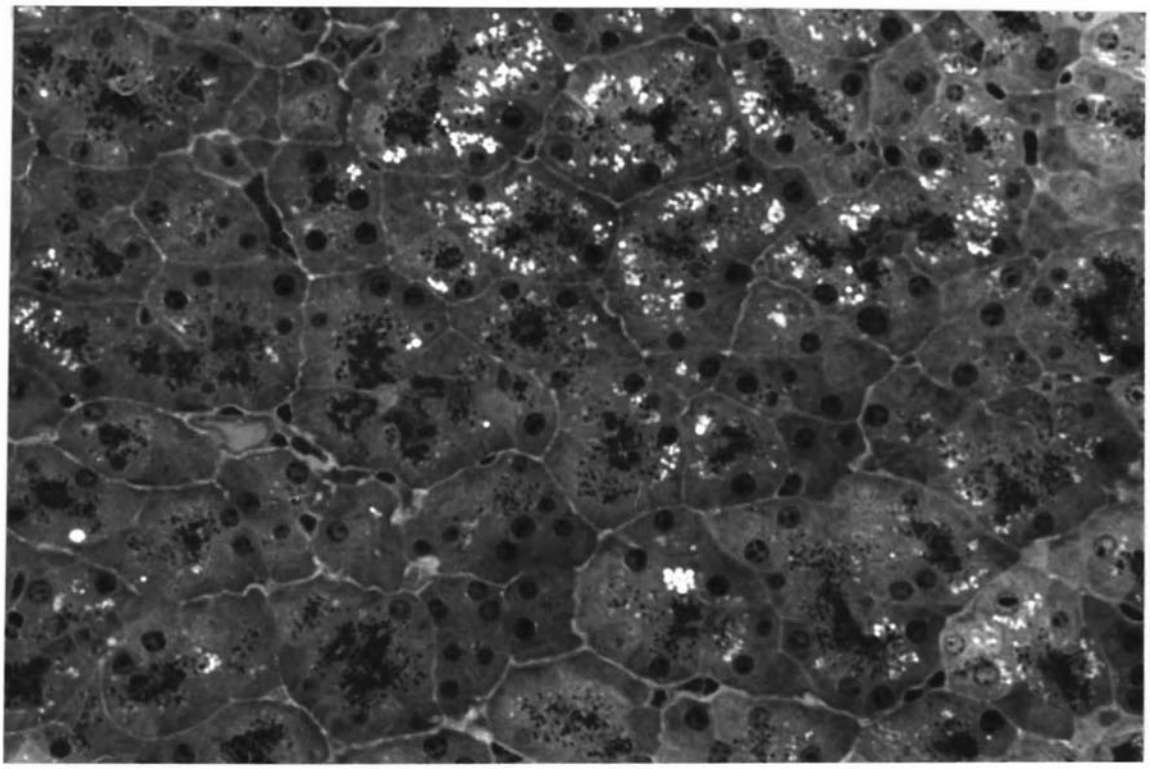

K

Figure 7.5a: Pancreas from the chimeric calf showing relatively mild vacuolation of exocrine epithelial cells. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 400)

Figure 7.5b: Pancreas from a calf with mannosidosis showing severe vacuolation of exocrine epithelial cells. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 400)



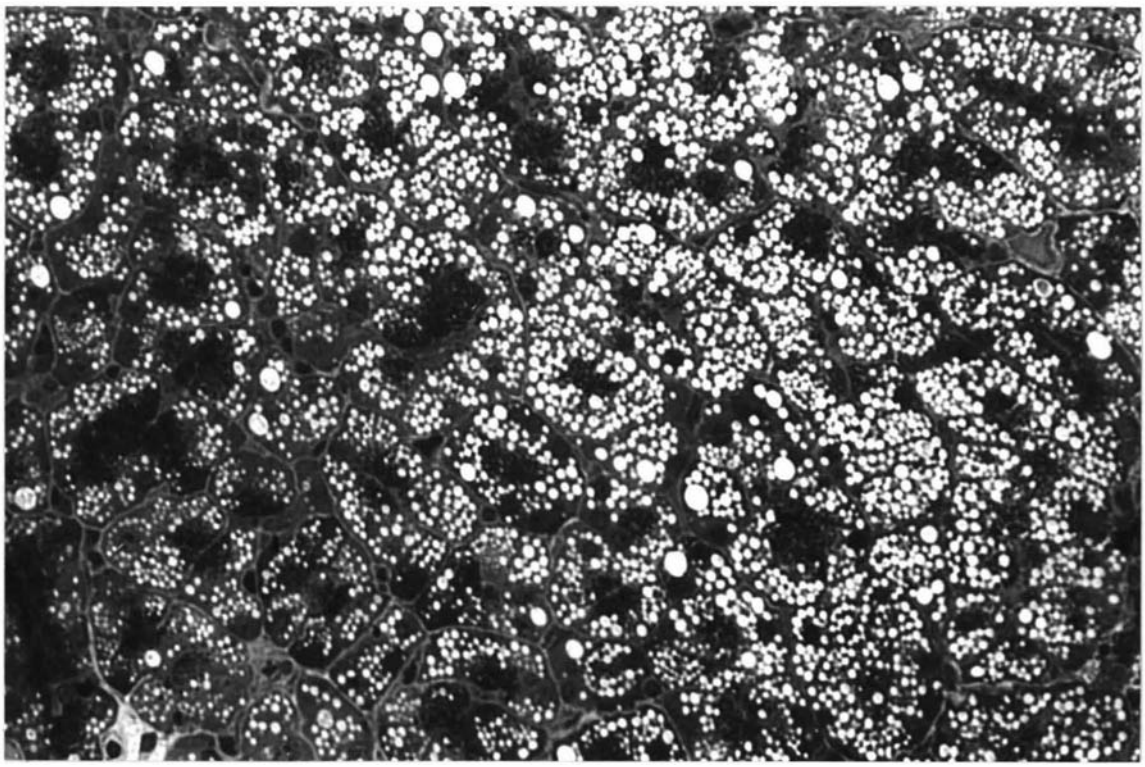
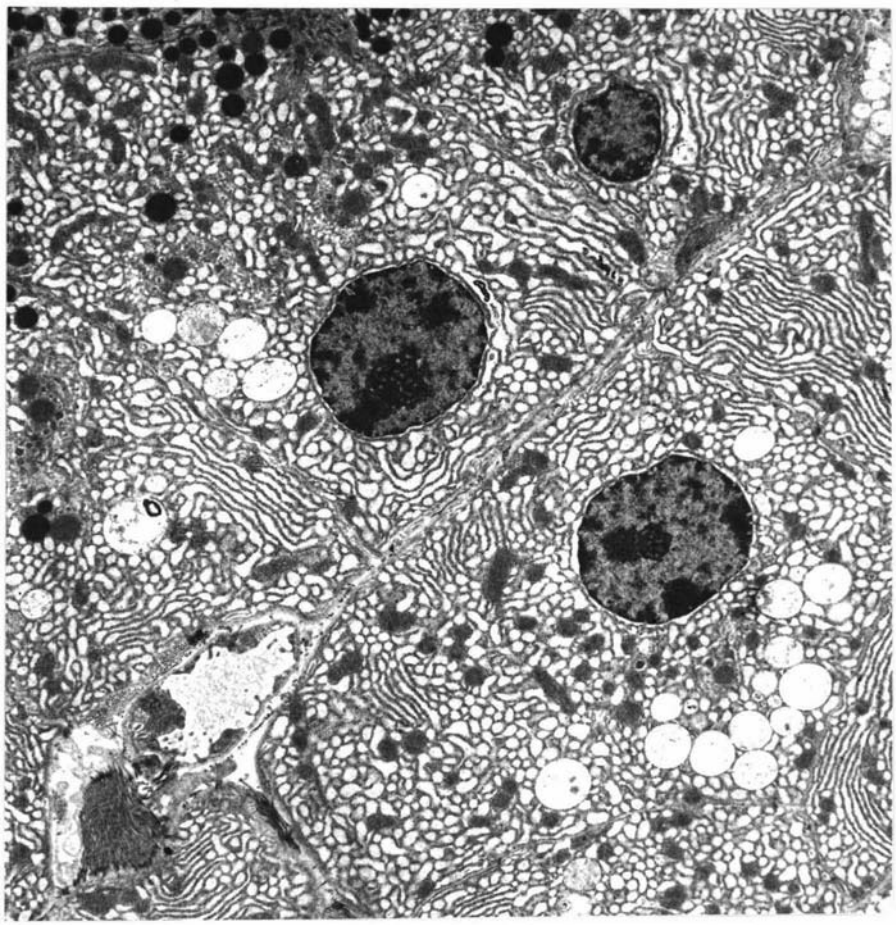


Figure 7.6a: Pancreatic exocrine epithelial cells from the chimeric calf containing a small number of relatively clear vacuoles less than 2 μm in diameter. (E.M. x 4,700)

Figure 7.6b: Pancreatic exocrine epithelial cells from a calf with mannosidosis containing a larger number of vacuoles up to 4.5 μm in diameter. (E.M. x 4,700)



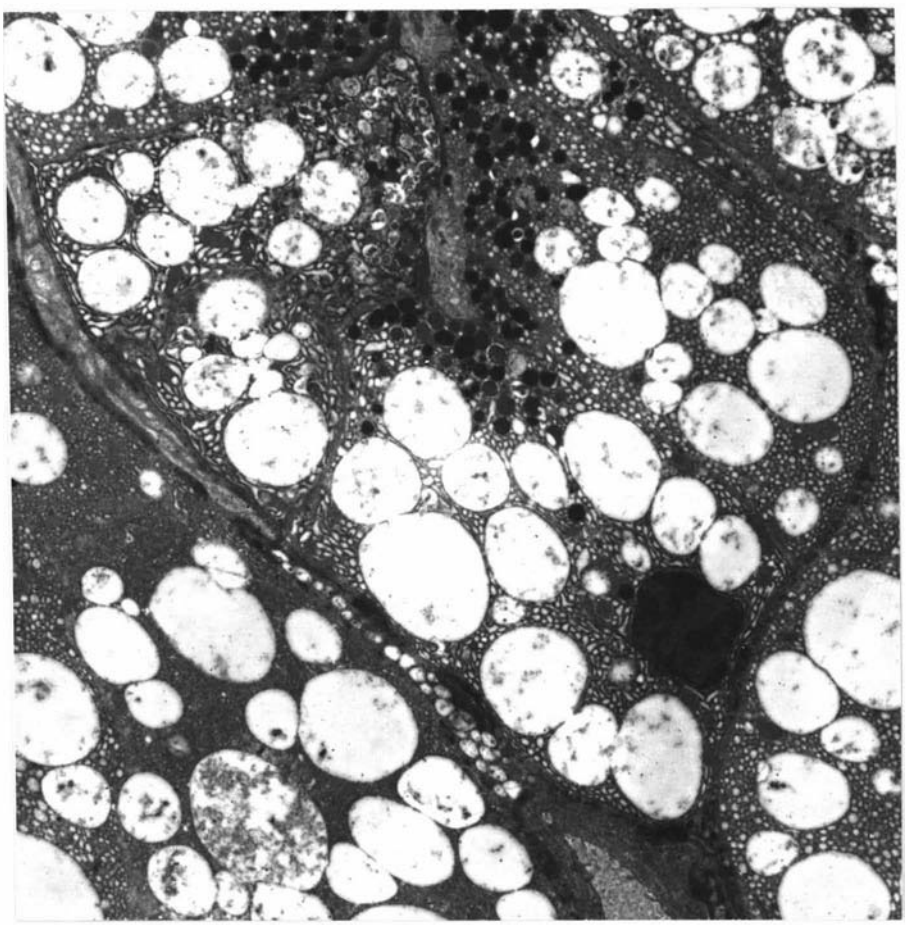
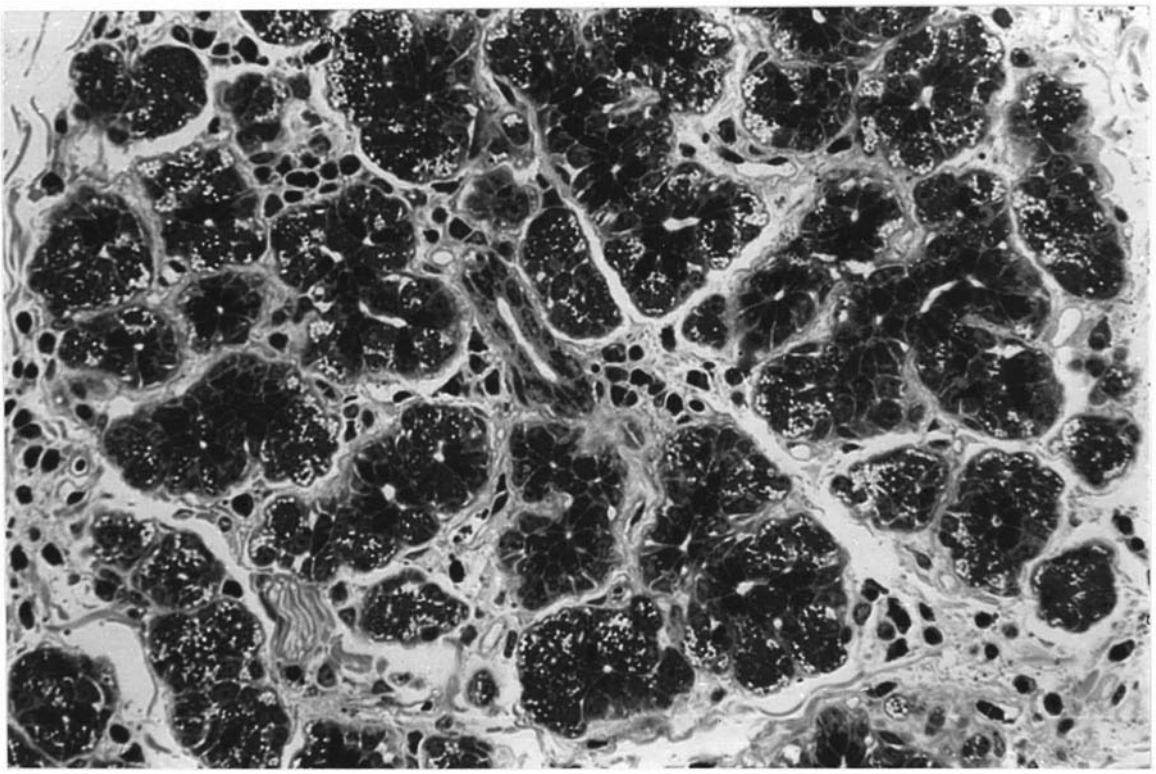
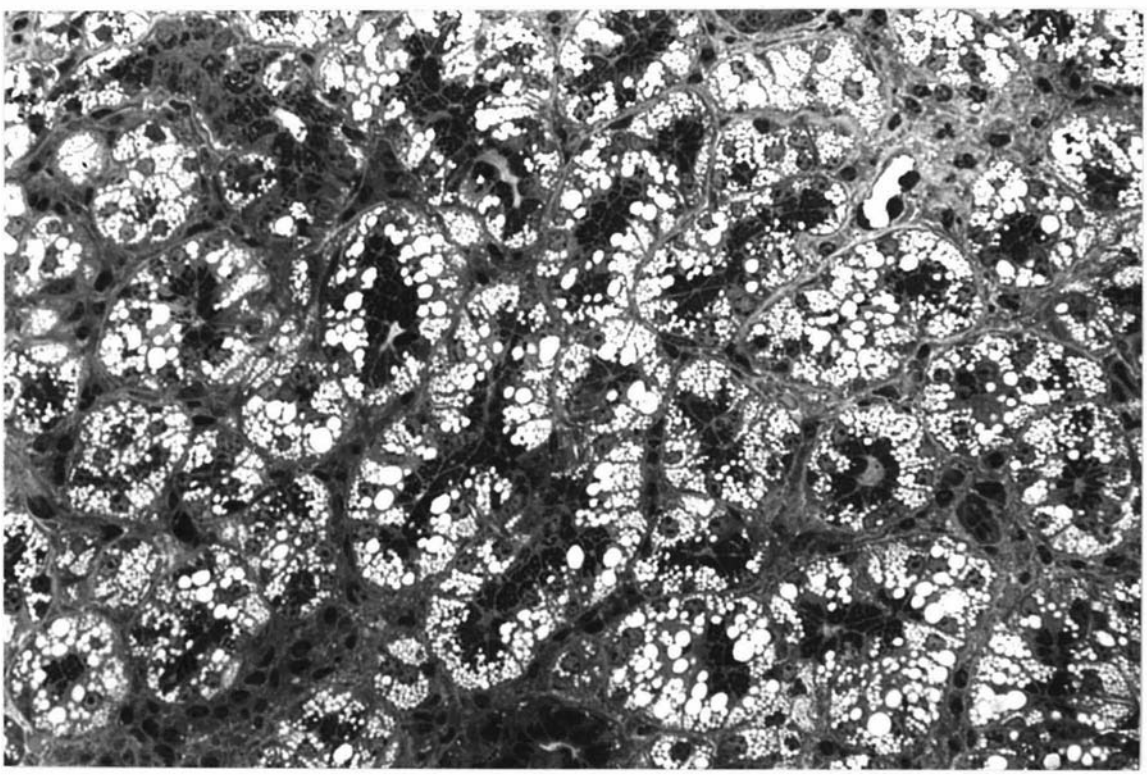


Figure 7.7a: Lacrimal gland from the chimeric calf showing relatively mild vacuolation of exocrine epithelial cells. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 400)

Figure 7.7b: Lacrimal gland from a calf with mannosidosis showing severe vacuolation of exocrine epithelial cells. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 400)





in the chimera. Although vacuoles were present in the above tissues of the chimera they were smaller in size and less abundant. However, the vacuolation of neurones in the brain and spinal cord of the chimera appeared to be just as severe as in positive control calves with mannosidosis (Figure 7.8a and b).

DISCUSSION

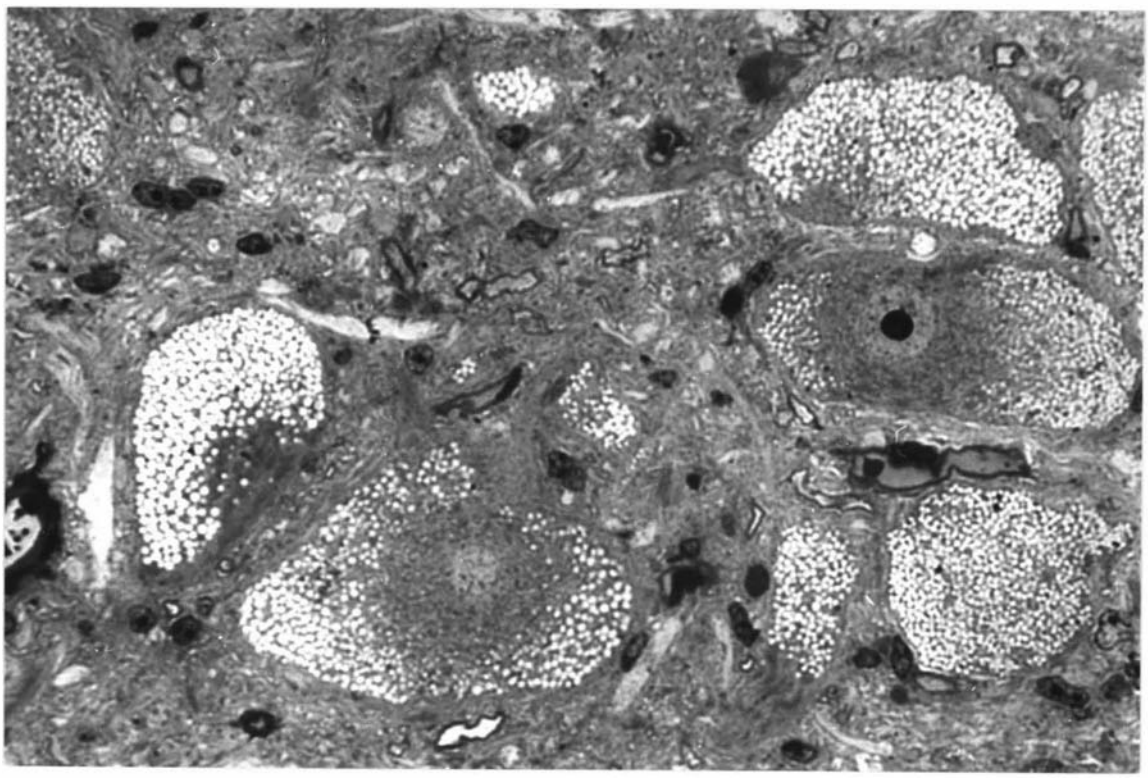
Blood-cell chimerism is a well recognized phenomenon in cattle. Although natural twinning rates are low in this species, anastomosis of chorionic blood vessels of the placentae of twins occurs in approximately 90% of twin pregnancies (Williams et al., 1963), allowing the transfer of various blood cell-types, including lymphocytes, which may become implanted in the co-twin and produce their own cell-lines (Fechheimer, 1970). The fortuitous discovery of a chimeric mannosidosis calf which possessed a population of normal lymphocytes provided a rare opportunity to investigate some aspects of enzyme replacement therapy for an inborn lysosomal disease. Unfortunately the twin was not available for study, but it was concluded on the basis of acidic α-mannosidase activity in peripheral lymphocytes and lymph nodes from the chimera that the co-twin was probably normal with regard to the mannosidosis genotype.

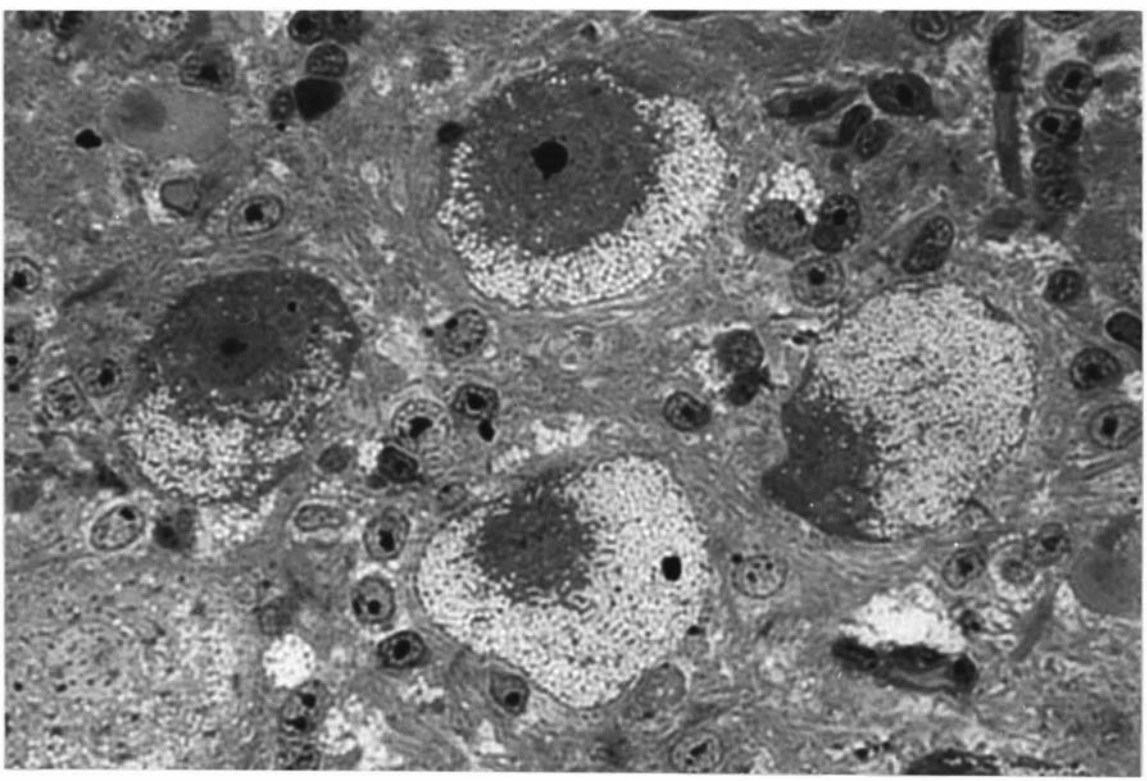
There is no doubt that α-mannosidase from the population of normal cells influenced the pathology of the disease. Although the neurones in the brain of the chimera appeared to be just as severely vacuolated as in mannosidosis control calves, relatively mild vacuolation was observed in Kupffer cells, reticuloendothelial cells and free macrophages in lymph nodes, and exocrine epithelial cells in the pancreas and lacrimal glands of the chimera.

Estimations of the level of storage product (expressed as neutral sugar and hexosamine) were performed in this laboratory on homogenates

Figure 7.8a: Medulla of the brain of the chimeric calf showing severe vacuolation of neurones. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 550)

Figure 7.8b: Medulla of the brain of a calf with mannosidosis showing severe vacuolation of neurones. (Epoxy resin embedded, toluidine blue-basic fuchsin, x 750)





of lymph nodes, liver and brain from the chimera, and compared with the relative levels in five mannosidosis calves and two normal controls (Table 7.I). The reduced severity of pathological lesions in lymph nodes and liver of the chimeric calf was consistent with a considerable reduction in the level of storage material in these tissues. The level of hexosamine and neutral sugar in the brain of the chimera was also much less than that measured in other mannosidosis calves despite the severity of neuronal lesions observed in this calf.

The colonization of lymph nodes by normal cells must have been significant as the activity of acidic α-mannosidase in this tissue approached that expected to be shown by a normal animal (Figure 7.1). Most phagocytic cells in the lymph nodes contained a few small vacuoles, suggesting that they did not originate from the normal co-twin. It is thought likely that these cells gained some enzyme either by endocytosis or by selective uptake of enzyme which had leaked into the surrounding medium from normal lymphocytes. This ability of cells to selectively imbibe lysosomal enzymes from their surrounding medium has been demonstrated in cultured skin fibroblasts from patients with certain mucopolysaccharide storage diseases (Hickman *et al.*, 1974). Although the levels of acidic α-mannosidase activity assayed in the plasma, liver, kidney and brain of the chimera were negligible (Figure 7.1), it is possible that small amounts of enzyme were released into the blood stream and tissue fluids and were taken up in sufficient quantities by macrophages, reticuloendothelial cells and exocrine epithelial cells to reduce the storage of oligosaccharides and the degree of vacuolation. *In vitro* studies on cultured skin fibroblasts from patients with various mucopolysaccharidoses have shown that the deficient enzyme can be taken up from the culture medium by the defective cells (Fratantoni *et al.*, 1968; Neufeld and Cantz, 1971; Di Ferrante *et al.*, 1973) and that only a small fraction of the normal enzymic complement need be absorbed to correct the

TABLE 7.I

Quantitative estimation of the level of storage product, expressed as hexosamine and neutral sugar, in tissue homogenates from the chimeric calf and from normal and mannosidosis controls. These estimations were performed by Miss C. E. Murphy, B.Sc. and are included for completeness with her permission.

	Hexosamine (mg/gm wet tissue)			Neutral carbohydrate (mg/gm wet tissue)		
Calves	Brain	Lymph node	Liver	Brain	Lymph node	Liver
Mannosidosis 1	0.5	3.0	2.8	1.1	4.8	24.4
" 2	0.6	2.4	2.8	1.2	4.0	14.5
" 3	0.6	3.6	1.8	1.7	4.6	12.3
" 4	0.5	5.2	1.8	1.1	7.1	11.9
" 5	0.6	1.5	2.1	1.2	3.4	16.3
Chimera	0.2	0.4	0.8	0.6	1.4	18.1
(duplicate or	0.2	0.5	0.6	0.6	1.3	18.8
triplicate samples)	0.2			0.6		
Normal 1	0.1	0.4	0.4	0.3	0.8	10.8
" 2	0.1	0.3	0.3	0.3	0.9	20.3

defect (Bach et al., 1972; O'Brien et al., 1973). The population of normal lymphocytes in the chimeric calf might therefore be expected to supply sufficient acidic α-mannosidase to induce at least partial mitigation of the disease.

Estimations of the amount of storage product in tissues might be expected to provide a more reliable indication of the therapeutic value of the lymphocyte transplant in the chimeric calf than would a subjective assessment of the severity of neuronal lesions. The blood-brain barrier is considered to be a major obstacle to enzyme replacement therapy but a reduction in the level of storage material in the brain of the chimera suggests that some α-mannosidase could have traversed this barrier and catabolized stored oligosaccharides. It may not be necessary however for the enzyme to actually cross the blood-brain barrier in order to reduce the level of storage material in the brain. If storage products are being catabolized elsewhere in the body then a negative gradient may be established, and providing these compounds are relatively soluble and of low molecular weight, then their escape from sites of storage may be accelerated. Significant levels of the storage oligosaccharides are excreted in the urine of calves with mannosidosis (Norden et al., 1973c) suggesting that there is some release from storage sites. It is quite conceivable therefore that degradation of these oligosaccharides in tissues or organs containing the necessary enzyme would be followed by increased release from tissues not directly affected by the replaced enzyme. A similar hypothesis was favoured by Desnick (1974) with regard to beneficial effects of kidney transplants in patients with Fabry's disease.

The concept of enzyme replacement therapy is based on the assumption that the accumulation of storage material within secondary lysosomes is a reversible process. The histological lesions of mannosidosis are

already well advanced when an affected calf is born, and in a study on human foetuses with Tay-Sach's disease, lesions were obvious by the end of the third month of gestation (Adachi et al., 1974). The question arises therefore as to whether or not the administration of exogenous enzyme once lesions have developed is likely to be of any value. Unfortunately these studies on the chimeric calf do not allow any claims to be made in this respect as the "transplant" of normal tissue was obtained prenatally and therefore probably acted largely in limiting the development of lesions rather than in curing them. In spite of an apparent reduction in the level of storage material in the brain, the calf showed typical clinical signs of mannosidosis, although these were less severe than a proportion of other cases seen. Failure to significantly alter the eventual clinical course of the disease, even under these favourable transplant conditions, is discouraging and suggests that tissue or organ transplants are unlikely to prove successful in curing storage diseases where there is severe neurological involvement.

Attempts at enzyme replacement therapy by transfusions of normal leucocytes have met with only moderate success in human medicine. Knudson et al. (1971) reported dramatic but transient clinical and biochemical improvement in a child with Hunter's syndrome (Type II mucopolysaccharidosis) after leucocyte transfusions. Brady et al. (1973) reported a temporary reduction in plasma ceramidetrihexoside levels in a patient with Fabry's disease after infusion with a leucocyte suspension. A patient with Sanfilippo syndrome (Subtype B) was infused with normal HL-A identical leucocytes by Moser et al. (1974) in an attempt to promote the survival and reproduction of these cells, thereby providing a continuous source of the enzyme. In this case a transient increase in the urinary ratio of small glycosaminoglycans to large glycosaminoglycans was related to the leucocyte infusion but few or no donor cells persisted for more than six weeks, and there was no long-term improvement in the

clinical or biochemical manifestations of the disease. Moser et al. suggested that transplants of normal bone-marrow into affected children may prove more successful, but O'Brien (personal communication) is not optimistic about the success of this method of therapy due to the considerable problems associated with rejection of bone-marrow transplants, even with perfect histocompatibility matches. Limited success in enzyme replacement therapy has been obtained by organ transplantation. So far only renal transplants in patients with Fabry's disease have produced encouraging results (Desnick et al., 1971, 1973; Philippart, 1973).

An encouraging aspect of the "lymphocyte transplant" in the chimeric mannosidosis calf was the considerable reduction in the severity of lesions and the level of storage oligosaccharides in visceral tissues, and if this disease had been purely visceral then the therapy might have been reasonably effective. Transplants of normal tissues or organs might therefore prove to be an effective method of therapy in the few inborn lysosomal disorders which do not exhibit severe neurological involvement.

SUMMARY

Acidic α-mannosidase supplied to a calf with mannosidosis by means of a natural transplant of lymphocytes, and possibly other cells, was able to reduce the severity of lesions in lymph nodes, pancreas, lacrimal glands and liver. Despite a reduction in the amount of storage material in the brain the vacuolation of neurones was just as severe as that observed in positive control calves with mannosidosis, and the clinical course of the disease was not significantly altered. It is postulated that if the disease had been a purely visceral inborn lysosomal disorder then the "therapy" would have been relatively effective.

CHAPTER VIII

GENERAL DISCUSSION

Mannosidosis of Angus calves is characterized clinically by ataxia, incoordination and tremors (Jolly, 1970), and must therefore be differentiated from such diseases as familial ataxia of Angus calves (Barlow et al., 1968), cerebellar atrophy caused by certain intrauterine virus infections, meningitis, encephalitis, certain plant intoxications and other cerebellar lesions including medulloblastoma (Jolly and Alley, 1969). In some cases however the nervous signs of mannosidosis are minimal and affected calves show mainly an ill-thrift syndrome.

The histopathological features of mannosidosis, described in Chapter III, are distinctive but not necessarily pathognomonic for this disease. Widespread vacuolation of neurones, pancreatic exocrine cells and macrophages within lymph nodes is also a feature of intoxications by ingestion of the plants Swainsona spp., Astragalus spp. and Oxytropis spp. (see page 35). Somewhat similar lesions, particularly of the central nervous system, may be seen in sections of paraffin embedded tissues from Friesian calves with G_{M1}-gangliosidosis (Donnelly et al., 1973a) and Shorthorn cattle with Type II glycogen storage disease (Richards, unpublished data; Jolly, personal communication).

The demonstration of a deficiency of acidic α-mannosidase in tissues and plasma of calves with mannosidosis (Hocking et al., 1972) provided a simple method for the specific diagnosis of this disease. Due to a gene dosage relationship for α-mannosidase activity, heterozygotes may also be differentiated from normal animals in as much as they have reduced activity of α-mannosidase in their tissues and body fluids. The development of a test for mannosidosis heterozygotes based on α-mannosidase activity in plasma (Hocking et al., 1972; Jolly et al., 1973)

and evidence that there was an abnormally high gene frequency for mannosidosis in New Zealand's Angus cattle population, led to the initiation of a pilot test and control programme for the mannosidosis genotype. An analysis of the results of this scheme is presented in Chapter IV. The genotype was found to be widespread throughout New Zealand, being carried by at least 10% of Angus cattle, and is considered to be of economic importance to the New Zealand beef industry (Jolly et al., 1974).

The plasma test for mannosidosis heterozygotes was extensively evaluated during the pilot scheme and the importance of restricting its use to homogeneous groups of animals within herds must be emphasized. Although the genotype of most animals could be accurately determined by this test it was apparent that a more sophisticated back-up test would be required for situations where small groups of animals or individual bulls were to be tested. The need for a supplementary test was expressed by Kaback and Zeiger (1972) in relation to a mass screening programme for the Tay-Sach's genotype in Ashkenazi Jewish populations, and was restated by Jolly et al. (1974) in their recommended requirements for a similar testing scheme in domestic animals. Consequently, a test for mannosidosis heterozygotes, based on α-mannosidase activity in lymphocyte extracts, was developed and evaluated (Chapter V). Although this test appeared capable of resolving the genotype of most cattle whose plasma test result was equivocal, the overlap in values between normal and heterozygous populations was greater than had been hoped. Further possible tests were therefore investigated (Chapter VI), the most promising of which was based on α-mannosidase activity in granulocyte extracts. Granulocytes may be separated from the same blood samples as lymphocytes with little extra effort, and both tests are currently being employed on animals referred to this laboratory with equivocal plasma tests. The correlation between α-mannosidase activity in lymphocyte and granulocyte

extracts has not been determined as the number of animals so far tested by both methods is too small, but the likelihood of obtaining an equivocal result from both tests on an animal appears to be less than if either test is employed alone. On occasions a retest using fresh samples may be necessary. The most reliable method of determining an animal's status with regard to the mannosidosis genotype is to test a number of its progeny. A statistically reliable ($p < 0.01$) progeny test for normality should include tests on at least seven progeny, therefore this method is essentially limited to mature bulls, but it should be employed whenever possible if there is any doubt concerning the genotype of such animals.

The control of mannosidosis in New Zealand does not require that all Angus cattle be tested and heterozygotes culled. If the genotype is eradicated from bull-producing herds then heterozygous bulls will no longer be sold to commercial beef producers and further losses due to mannosidosis should not occur, no matter how many heterozygous cows remain in their herds. It is recommended therefore that all bull-producing herds be screened for the mannosidosis genotype using the routine plasma test, supported where necessary, by the lymphocyte and granulocyte tests. Heterozygous bulls should be culled, and although it is desirable that heterozygous cows be treated similarly, such a policy could cause serious economic loss to a breeder whose herd has a high prevalence of carriers, and could cause the loss of valuable genetic material which may be the result of several years of selective breeding. Heterozygous cows may either be included in commercial herds and mated to non-carrier bulls or, if they are of sufficient merit, they may even be retained in the stud herd and have all subsequent progeny tested for the mannosidosis genotype. A national testing programme for the mannosidosis genotype has now been introduced by the Ministry of Agriculture and Fisheries in conjunction with the New Zealand Angus Association with the aim of eradicating the disease from the bull-

producing herds of New Zealand. This is the first time such a scheme has been introduced for the control of an inherited disease in domestic animals.

In spite of the recommendation of Jolly et al. (1974) (see page 51) the national mannosidosis testing programme currently in operation has not made provision for the proper genetic counselling of breeders. At present the results and interpretations of a herd test are returned to the breeder with no instruction as to how his particular problem, if any, should be handled in order to minimize economic loss and wastage of valuable genes. During the pilot testing programme, pedigrees were obtained for each animal tested and these proved valuable in deciding the status of a number of animals with equivocal test results. In the current scheme however, pedigrees are not requested and the percentage of animals listed as "equivocal" is therefore greater than necessary.

In domestic animals the gene frequency for an inherited disease may be rapidly reduced by the recognition and culling of heterozygous individuals, but such is not the case for similar diseases in man. The prototype screening programme for inborn lysosomal diseases in human medicine is a programme initiated in 1971 for the prevention of Tay-Sach's disease in Ashkenazi Jewish populations (Kaback and Zeiger, 1972; Kaback et al., 1974). The intention of this scheme is to identify heterozygous couples and offer them the opportunity of producing only unaffected children, by means of prenatal diagnosis and abortion of foetuses with the disease. Although this scheme will reduce the birth of diseased children, it will not reduce the gene frequency for the disease and in fact would be expected to cause a slight relative increase in the gene frequency, as heterozygous parents may be encouraged to have larger families than previously, and their children will bear a two-thirds chance of being heterozygous.

It is desirable that an effort is made to slightly overcall heterozygotes or equivocals in any screening programme, in either human or veterinary medicine, so that heterozygotes do not escape detection. During the pilot testing scheme for the mannosidosis genotype a small percentage of cattle designated heterozygous were almost certainly normal, but only one animal designated normal has subsequently proven to be heterozygous. A bull whose plasma α-mannosidase activity appeared to lie within the low-normal range has since sired several heterozygous progeny from normal cows. On the basis of information concerning the distribution of plasma α-mannosidase activities in normal and heterozygous populations the probability of this bull being heterozygous was considered to be less than 1%. Occasional errors of this type must be expected as the designation of genotype can only be based on probability. Extreme variations about the mean could possibly be caused by extraneous non-genetic factors, such as concurrent disease, or could be due to the possession of alleles which code for abnormally high α-mannosidase activity as discussed in previous chapters. It is difficult to allow for such factors in a mass screening programme.

The mean level of α-mannosidase activity in plasma and lymphocytes of mannosidosis heterozygotes was approximately 38% of the mean for normal animals. This is lower than would be expected from a simple gene dosage relationship. Similar observations have been reported in two inherited enzyme deficiency disorders of man, homocystinuria (Goldstein et al., 1973) and orotic aciduria (Fallon et al., 1964), where the respective mean levels of enzyme activity in heterozygotes were 17% and 21% of the normal mean. With structural gene mutations the level of enzyme activity in heterozygous individuals might be expected to be approximately 50% of normal because of the presence of one normal gene in each diploid cell. Fallon et al. suggested therefore that the unusual gene dosage relationship in orotic aciduria could be caused by

a mutation in a regulator gene, leading to significant repression of structural gene activity. It is the intention of this laboratory to further investigate the gene dosage relationship in bovine mannosidosis.

The recognition of zymogen granules within storage vacuoles in the exocrine pancreas of calves with mannosidosis (Figure 3.15) was interpreted as evidence that crinophagy could be involved in contributing glycoprotein substrates to the lysosomal system, at least in this tissue. The process of crinophagy is a relatively new concept, thought to be involved in the regulation of cellular secretions (Farquhar, 1969; de Duve, 1969), and has been suggested as playing an important role in the overloading of lysosomes of secretory cells in certain storage disorders (Van Hoof, 1973a and b; Daems et al., 1973). Further investigation of crinophagy in tissues of calves with mannosidosis may produce a greater understanding of its mechanisms and occurrence.

The mannosidosis genotype is carried by approximately 10% of Angus cattle in New Zealand and therefore it can be estimated that some 3,000 calves with mannosidosis should be born each year. Considerably fewer cases than this are recognized. Calving records from a herd of heterozygous cattle at Massey University have revealed that neonatal mortality is significantly ($p < 0.01$) higher in calves with mannosidosis than in normal or heterozygous calves (Jolly and Thompson, 1976), and this may be one reason why less than the expected number of cases are diagnosed.

The treatment of inborn lysosomal diseases by replacement of the deficient enzyme will not become economically feasible in domestic animals, but recent attempts at this form of therapy in similar diseases of man have created interest in the use of animal models to help resolve the many unanswered questions that have arisen. Several groups of investigators have been able to correct the defect in cultured skin fibroblasts from human patients with various storage disorders by

administration of the deficient enzyme to the culture medium, but the value of such *in vitro* experiments is limited, and the availability of animal models is desirable. Although cattle can hardly be regarded as ideal experimental animals, the high gene frequency for bovine mannosidosis in New Zealand and the availability of affected calves make this disease a valuable research model.

The chance discovery of a chimeric mannosidosis calf possessing a population of normal lymphocytes provided some interesting information regarding the value in enzyme replacement therapy of a transplant of normal tissue. Although this natural transplant was unable to significantly mitigate the neurological manifestations of mannosidosis, the severity of lesions in visceral tissues was reduced (see Chapter VII), suggesting that had the disease not involved the central nervous system then "therapy" may have been relatively successful. The results of this investigation, and that of Moser *et al*. (1974), who infused HL-A identical lymphocytes into a patient with Sanfilippo syndrome, suggest however that the administration of normal leucocyte suspensions to patients is likely to be of little or no value in the treatment of storage diseases with severe neuronal involvement. An experimental Angus herd, consisting entirely of mannosidosis heterozygotes has been set up at Massey University with the aim of producing mannosidosis calves for research into various aspects of enzyme replacement therapy.

Bovine mannosidosis may be further exploited as a model for the evaluation of methods of heterozygote detection. Most claims of success in the identification of heterozygotes for human inborn lysosomal disorders have been based on a small number of tests, and statistical analysis of results has seldom been possible. Due to the abnormally high gene frequency for mannosidosis in New Zealand's Angus cattle population, and the availability of reliable methods for detecting

heterozygous animals, new tests may be evaluated on a large number of individuals of known genotype. It is proposed to employ this model in an investigation of the test for heterozygotes based on enzyme assays on lymphocytes stimulated by phytohaemagglutinin. This method has been used with apparent success in the detection of heterozygotes for human Type II glycogenosis (Hirschhorn et al., 1969) and acid phosphatase deficiency (Nadler and Egan, 1970), but its mechanisms are not understood.

Thus the detailed study of bovine mannosidosis has led to the initiation of the first screening programme for heterozygotes of an inherited disease in domestic animals. This programme will lead to the control of mannosidosis in the bull-producing herds of New Zealand, thereby reducing the number of affected calves born to a minimal level. Furthermore, these studies have established a basis for the exploitation of bovine mannosidosis as a model for research into various aspects of closely related diseases of man, and for investigating a number of basic cellular mechanisms including the process of crinophagy, and the lysosomal catabolism of glycoproteins.

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