Deficiency of α -Mannosidase in Angus Cattle

AN INHERITED LYSOSOMAL STORAGE DISEASE

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A disease of Angus cattle previously known as pseudolipidosis has been shown to be an inherited lysosomal storage disease, in which an oligosaccharide containing mannose and glucosamine is the storage substance. Diseased animals have a near-absolute deficiency of the lysosomal enzyme, α -mannosidase, whereas heterozygotes have a partial deficiency of this enzyme. The condition is analogous to the human disease known as mannosidosis.

A disease of Angus cattle characterized by head tremor, an aggressive tendency, ataxia, inco-ordination, failure to thrive and premature death, was described in Australia by Whittem & Walker (1957). It has since been diagnosed on numerous occasions in that country and New Zealand (various personal communications). Pathologically there is vacuolation of neurones and reticulo-endothelial cells within lymph nodes. Recognizing a similarity to the lipidoses of children, but unable to demonstrate stored lipid, Whittem & Walker (1957) introduced the name 'pseudolipidosis'. Recent work has confirmed and augmented the initial description of this disease (Jolly, 1970, 1971). It may be clinically present at birth, though more commonly it is not noted for several weeks or months. Affected animals fail to thrive, show progressive development of nervous signs and die, usually within the first 12 months of life. The vacuoles in neurones and reticulo-endothelial cells of lymph nodes appear empty under light-microscopy of frozen or paraffin-embedded sections stained by a variety of techniques. Ultrastructurally, neuronal vacuoles are lined by a single membrane and also appear empty except for a small amount of amorphous electron-dense material and some fragments of cell membranes.

The pathology and epidemiology of pseudolipidosis suggest an inherited metabolic defect possibly associated with glycoprotein or mucopolysaccharide metabolism and in which some soluble intermediary substance accumulates within the vacuoles. Experiments to determine the heritability of the disease, the nature of the stored substance, and the underlying metabolic error are described below.

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Materials and Methods

Animals

All animals used were of the Angus breed. Details of their disease status, age, sex, tissues used and the length of tissue storage at -20°C are given in Table 1. In each case where an animal is recorded as having pseudolipidosis, diagnosis was based on both clinical signs and subsequent histopathology of formalin-fixed tissues. Frozen heparinized plasma and/or freshly collected leucocytes were also used from a variety of clinically normal adult cows, but including some known to have given birth to calves with pseudolipidosis.

A herd of 12 cows was used for controlled breeding experiments. These cows, each of which had previously given birth to a calf with pseudolipidosis, were mated with one or other of two bulls that had previously sired such calves. All cows were run together on pasture on a property where the disease had never previously been diagnosed. In 2 years a total of 20 calves were born to these matings.

Chemicals and instruments

Analytical-grade reagents were used for the preparation of assay buffers. Otherwise, unless specified, all chemicals were reagent grade. The 4-methylumbelliferyl glycosides and the *p*-nitrophenyl glycosides were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and Sigma Chemical Co., St. Louis, Mo., U.S.A., respectively. Sephadex was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and ion-exchange resin from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

Fluorescence readings were recorded on a Turner model III fluorimeter with a Turner 7-60 primary

Table 1. *Animals and tissues used in enzyme assays*

For experimental details see the text.

Number of animal	Diagnosis	Age	Sex	Tissues assayed	Time frozen
1574	Normal	1½ years	M	Brain	3 years
1624	Normal	2½ years	M*	Brain, lymph node	3 years
2090	Pseudolipidosis	3 weeks	F	Brain, lymph node	1½ years
2156	Pseudolipidosis	4 months	M	Brain, lymph node	1 year
2834	Pseudolipidosis	6 weeks	F	Brain	1½ years
2860	Pseudolipidosis	8 weeks	F	Lymph node	1½ years
2873	Pseudolipidosis	3 months	F	Lymph node	1½ years
2890	Normal	3 months	F	Lymph node	1½ years
2891	Normal	3 months	F	Lymph node	1½ years
2911	Pseudolipidosis	6 months	?	Lymph node	1½ years
3380	Pseudolipidosis	10 months	M	Brain, lymph node, liver	9 months
3653	Pseudolipidosis	1 year	M*	Brain, lymph node, liver	5 months
C8/12	Normal	3 months	F	Brain, lymph node, liver	3 months
3763	Pseudolipidosis	8 weeks	F	Brain, lymph node, liver	4 months
3940	Pseudolipidosis	6 months	M	Brain, lymph node, liver	1 month
3949	Pseudolipidosis	6 months	M	Brain, lymph node, liver	1 month
3959	Normal	6 months	M	Brain, lymph node, liver	1 month

* Castrated male

filter (maximum transmission at 365nm) and a Turner 22 secondary filter (a sharp cut-off filter transmitting above 570nm). Spectrophotometric readings were determined with a Unicam SP.500 or a Hitachi model 101 u.v./visible spectrophotometer or a Unicam SP.800 recording spectrophotometer. A Technicon AutoAnalyser was used for automated plasma α -mannosidase assays.

Methods

Determination of neutral carbohydrate. This was done by the phenol- H_2SO_4 method of Dubois *et al.* (1956).

Determination of hexosamine. Hexosamine was determined by a modification of the method of Blix (1948). In some cases interfering neutral and acidic material was removed by ion-exchange treatment by the method of Boas (1953). A sample (0.5ml) of the unknown or standard hexosamine solution containing up to 130 μg of hexosamine hydrochloride was adjusted to pH 9–10 with solid Na_2CO_3 . Acetylacetone reagent (0.75ml of redistilled acetylacetone in 25ml of 0.625M- Na_2CO_3 , renewed daily) was added to bring the volume to 1.0ml. The solution was kept at 89–92°C in a water bath for 45 min, was cooled and then 5.0ml of 95% (v/v) ethanol was added, followed by 0.5ml of Ehrlich's reagent (1.6g of analytical-grade *p*-dimethylaminobenzaldehyde dissolved in 30ml of conc. HCl and 30ml of 95%

ethanol, renewed weekly). After 1–2h at room temperature, the E_{530} was read against a reagent blank lacking hexosamine.

Determination of the hexose/hexosamine ratio. The hexose/hexosamine ratio was calculated by subtracting the hexosamine content of the test solution from the total amount of reducing sugar as determined by Nelson's (1944) modification of the method of Somoygi (1952). It was assumed that hexose and hexosamine were the only reducing groups present.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (BDH Chemicals Ltd., Poole, Dorset, U.K.) as a standard.

Paper chromatography of monosaccharides. Paper chromatography was done, after hydrolysis of the test material (0.5M- H_2SO_4 , 12h, 95–100°C), on Whatman no. 1 paper with the following solvents: butanol-pyridine-water (6:4:3, by vol.); ethyl acetate-pyridine-water (2:1:2, by vol.); ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.). Sugar spots were located by the AgNO_3 -alkaline ethanol method of Trevelyan *et al.* (1950).

Amino acid analysis. Amino acid analyses were done, after hydrolysis of the test material (6M-HCl, 12h, 100–105°C), on a Beckman model 120C amino acid analyser by the standard methods for protein hydrolysates.

Extraction and characterization of storage material. Frozen lymph nodes from a calf with pseudolipidosis

were homogenized in 5 vol. of chilled 10% (w/v) trichloroacetic acid and were centrifuged at 5°C for 15 min. The pellet was re-extracted with trichloroacetic acid and re-centrifuged. The combined supernatants were filtered to remove excess of lipid and the trichloroacetic acid was extracted six times with equal volumes of diethyl ether. The resultant solution was neutralized with dilute aq. NH_3 , freeze-dried and the solid residue was redissolved in a smaller volume of distilled deionized water. Portions of this solution were loaded on a column of Sephadex G-25 (86.5 cm $\times$ 1.6 cm) and eluted with distilled deionized water. Sequential samples (approx. 1.0 ml) of eluate were collected, their u.v. spectra recorded, and the hexosamine and neutral-carbohydrate contents determined. Lymph nodes from a normal calf were similarly extracted and analysed.

Subsequently, gel filtration was repeated with extracts of lymph nodes from two calves with pseudolipidosis, and the abnormal carbohydrate-containing fractions were pooled and freeze-dried. This material was used for paper chromatography, amino acid analysis and the hexose/hexosamine ratio determination. A portion of this material was mixed with an equal volume of 4 M-formic acid and loaded on a cation-exchange-resin column (AG 50 W, X8, H^+ form, 200–400 mesh; 15 cm $\times$ 1.5 cm). Fractions eluted with 2 M-formic acid and then with 0.05 M-tris buffer, pH 8.5, were analysed for neutral carbohydrate. Those fractions of the formic acid eluate that contained carbohydrate were pooled, freeze-dried and subjected to amino acid analysis.

Tissue extraction for lysosomal enzyme assay. Frozen tissues from calves with pseudolipidosis and their normal controls were homogenized in 10 vol. of chilled 1 M-NaCl. The homogenate was allowed to settle and then was sonicated for 2 min with an MSE 100 W sonicator. In most cases the homogenate was centrifuged at 3000g for 10 min and the supernatant stored at -12°C until used for enzyme assays. In some cases the homogenate was stored at -12°C and thawed and centrifuged immediately before use. Where necessary, dilutions of the various extracts were made in 1 M-NaCl.

Plasma collection. Blood was collected in vacuum tubes containing heparin and the plasma removed within 2 h of collection. It was then stored at -12°C until assayed.

Leucocyte collection. Blood from each animal was collected in three 7.0 ml vacuum tubes containing solid EDTA. After centrifugation, buffy coats were removed with a Pasteur pipette and pooled. A second centrifugation was necessary to separate the leucocytes from erythrocytes that had been carried over at the first separation. The white layer was again removed and suspended for 5 min in 7.0 ml of 0.075 M-KCl at 37°C , to lyse any erythrocytes remaining. The cells were finally washed in 0.85% NaCl, and

after centrifugation and removal of the supernatant were stored as a pellet at -12°C . Before enzyme assays, the cells were thawed and sonicated for 2 min in 1.0 ml of 0.85% NaCl with an MSE 100 W sonicator.

Lysosomal enzyme assays. Lysosomal glycosidases were assayed by using synthetic 4-methylumbelliferyl glycosides or *p*-nitrophenyl glycosides.

For α -mannosidase assays, 20 μl of 2 M-sodium acetate buffer (pH 4.30), 50 μl of 10 mM-4-methylumbelliferyl α -D-mannopyranoside and 10 μl of undiluted tissue extract were mixed in Parafilm-sealed tubes and were incubated in a shaking water bath at 36 – 38°C for 0 and 20 min (or 30 min for the leucocyte extracts). Samples were run in duplicate. The reaction was stopped by the addition of 2.0 ml of 0.2 M-glycine buffer (pH 10.0). Fluorescence was measured and the release of 4-methylumbelliferone determined by reference to a standard curve. For investigation of the effect of pH on α -mannosidase activity, 1.5 M-sodium citrate buffers of appropriate pH values were substituted for the acetate buffer.

β -Acetylglucosaminidase and β -glucuronidase were assayed by the same basic technique by using the appropriate 4-methylumbelliferyl glycosides. But in the glucuronidase assay only 5 mM substrate solution was used: the buffers used in these assays were 1.5 M-sodium citrate (pH 4.50) and 2 M-sodium acetate (pH 4.70) for β -acetylglucosaminidase and β -glucuronidase respectively.

For β -galactosidase assays on lymph-node extracts, 20 μl of 2 M-sodium acetate buffer (pH 4.10), 100 μl of 15 mM-*p*-nitrophenyl β -D-galactopyranoside and 40 μl of undiluted tissue extract were mixed in Parafilm-sealed test tubes and were incubated in a shaking water bath at 36 – 38°C for 0, 1 and 2½ h. The reaction was stopped by the addition of 1.0 ml of 5% (w/v) trichloroacetic acid and the protein suspension was centrifuged. The clear supernatant was removed, neutralized with 0.30 ml of 1 M-NaOH, and 2.0 ml of 1.0 M-glycine- Na_2CO_3 buffer (pH 10.0) was added. The E_{400} was determined and plotted against time. From the mean gradient of these plots the rate of release of *p*-nitrophenol was determined by reference to a standard curve.

For assay of brain and liver extracts, volumes of tissue extract and buffer, and lengths of incubation were varied to allow for variations in enzyme activity.

Other lysosomal glycosidases were assayed by the same basic technique by using the appropriate *p*-nitrophenyl glycoside. Buffers added to the different assay mixtures were 1.5 M-sodium citrate (pH 4.50) for β -glucosidase, 2.0 M-sodium acetate (pH 4.50) for α -galactosidase, 1.5 M-sodium citrate (pH 4.00) for α -glucosidase and 2.0 M-sodium acetate (pH 5.30) for α -fucosidase.

Cathepsin D was assayed in tissue extracts by a modification of the method of Schwabe & Kalnitsky

(1966). Acid-denatured haemoglobin was made up as a 2% solution in 0.2M-sodium formate buffer (pH 3.50). A portion of this solution (0.20ml) and 0.02–0.10ml of tissue extract were mixed in Parafilm-sealed tubes and incubated in a shaking water bath at 36–38°C for 0 and 30min. The reaction was stopped by the addition of 1.0ml of 5% trichloroacetic acid solution, and after centrifugation the enzyme activity was determined by measuring the acid-soluble peptides by the method of Lowry *et al.* (1951).

Automated plasma α -mannosidase assays. Automated α -mannosidase assays were done in a Technicon AutoAnalyser. Substrate solution (0.47 mM-4-methylumbelliferyl α -D-mannopyranoside in 37 mM-sodium acetate buffer, pH 4.3) was mixed with plasma with air segmentation at flow rates of 1.5 ml/min and 0.05 ml/min respectively. Incubation was at 40°C for approx. 12 min and the solution was then mixed with 0.5M-glycine buffer (pH 10.0; flow rate 2.50 ml/min). The fluorescence of the solution was determined with a flow-through cell on a Turner III fluorimeter by using cell holder 4 and slit-width setting 3. Testing was at the rate of 40 samples/h.

Results

Controlled breeding experiment

Of the 20 calves born to the 12 cows over 2 years, six were either born with pseudolipidosis or developed

clinical signs within 3 months. In each case diagnosis was confirmed *post mortem* by histopathological examination of formalin-fixed tissues. Affected calves were of both sexes and were sired by both bulls.

Extraction and characterization of storage material

Extraction of lymph nodes from a calf with pseudolipidosis in 10% trichloroacetic acid, followed by gel filtration on Sephadex G-25, and analysis of the eluate for hexosamine and neutral carbohydrate resulted in the elution pattern shown in Fig. 1. The large central peak of neutral carbohydrate (or uronic acid) and hexosamine (elution volume 87–103 ml) did not occur with a similarly treated extract of lymph nodes from a normal calf. Hydrolysates of material eluted in this region gave two spots on paper chromatography with all three solvents used. These spots co-chromatographed with mannose and glucosamine. Determination of the hexose/hexosamine ratio in hydrolysates gave a ratio of 3:1. Results of amino acid analysis of this material are shown in Table 2.

When the material isolated by gel filtration was further treated on an AG-50 cation-exchange column, more than 90% of the carbohydrate-containing material was unretarded at the lower pH used. However, a significant amount of carbohydrate-containing material was retarded as shown by subsequent elution with tris buffer, pH 8.5. The amino acid content of the unretarded fraction is shown in

Fig. 1. Sephadex G-25 elution patterns of the trichloroacetic acid extract of lymph nodes from an animal with pseudolipidosis

For experimental details see the text. $\blacktriangle$, E_{490} in the phenol – H_2SO_4 test; $\blacksquare$, hexosamine content of eluate expressed as μ g of glucosamine hydrochloride/ml; $\bullet$, E_{200} of fractions. The first 60 ml of eluate contained no reactive material.

Table 2. *Amino acid analyses of oligosaccharide material*

For experimental details see the text. N.D. is not determined; N is negligible.

Amino acid	Content (mmol/mol of glucosamine)	
	Sephadex-treated material	Sephadex- and AG 50-resin-treated material
Lysine	8	N.D.
Histidine	N	N.D.
Arginine	6	N.D.
Aspartic acid/asparagine	51	3
Threonine	11	N
Serine	26	18
Glutamic acid/glutamine	230	30
Proline	23	N
Glycine	32	4
Alanine	24	4
Cysteine and cysteic acid	62	6
Valine	15	N
Methionine	N	N
Isoleucine	N	N
Leucine	N	N
Tyrosine	N	N
Phenylalanine	N	N
Galactosamine	4	N
Ammonia	757	N.D.
Total amino acids	488	65

Table 2 beside the values for the material before ion-exchange treatment.

The elution volume of the abnormal central peak in the Sephadex elution pattern when compared with the elution volume of haemoglobin and NaCl (not shown) suggests that the abnormal material would probably have a molecular weight of approx. 1000–2000. This observation, with the analyses recorded above, indicates that the material extracted from the lymph nodes of calves with pseudolipidosis is an oligosaccharide containing mannose and glucosamine in the ratio of 3:1 and with a very low content of amino acids.

Lysosomal enzyme activities in normal and pseudolipidosis calves

The isolation of a mannose-containing oligosaccharide from calves with pseudolipidosis, combined with the known pathology and epidemiology of the disease, suggested that this was a lysosomal storage disease caused by an enzymic defect in glycoprotein catabolism. Consequently nine lysosomal enzymes, including eight glycosidases and cathepsin D, were assayed in brain, liver and lymph

node from calves with pseudolipidosis and from normal controls (Table 1). The results of these assays are shown in Table 3. There was an almost complete absence of α -mannosidase from all three tissues in all calves with pseudolipidosis, under the assay conditions used. In this group all other enzyme activities assayed were higher than in the controls, although in some cases the degree of elevation was relatively small. It was greatest for β -glucuronidase, in which up to a sixfold elevation was recorded.

Plasma or serum from two calves with pseudolipidosis and one normal calf were assayed for α -mannosidase activity by using citrate buffers at a range of pH values. The results shown in Fig. 2 indicate an absolute deficiency of α -mannosidase, activity for plasma and serum from calves with pseudolipidosis in the pH range 3.75–4.75, whereas there was considerable activity in the plasma of the control calf. In the buffer pH range 4.75–6.50, α -mannosidase activity was observed in samples from both normal and diseased calves. This suggests the presence of a second enzyme with α -mannosidase activity. Assays with lymph-node extracts of normal animals have indicated the presence of only the lower pH peak of α -mannosidase activity.

Table 3. *Lysosomal enzyme activities in tissue extracts*

For experimental details see the text. All glycosidase activities are expressed as nmol of substrate hydrolysed/min per mg of protein. Cathepsin D activity is expressed as ΔE_{750} in Folin reaction/min per mg of protein.

Enzyme assayed	Animals with pseudolipidosis												Control animals						
	2090	2156	2860	2873	2911	3380	3653	3763	3940	3949	2834	Mean	1624	2890	2891	3959	C8/12	1578	Mean
Lymph-node extract																			
α -Mannosidase	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	3.8	2.6	1.9	3.0	—	2.8
β -Acetylglucosaminidase	312	272	222	208	286	270	267	250	166	250	—	250	94	81	93	70	125	—	93
β -Glucuronidase	23.6	27.9	19.0	21.1	28.7	25.9	66.0	28.4	17.5	45.0	—	30.3	4.2	4.5	6.8	4.3	5.1	—	5.0
β -Galactosidase	4.5	4.7	3.7	3.8	3.4	3.9	4.7	4.5	3.4	3.9	—	4.1	1.6	2.2	2.0	1.7	2.7	—	2.0
α -Galactosidase	1.27	1.76	1.56	1.42	1.11	1.07	0.61	1.65	0.94	0.97	—	1.24	0.75	0.86	1.17	0.86	1.04	—	0.94
β -Glucosidase	0.70	0.44	0.57	0.69	0.62	0.60	0.74	1.05	0.62	0.72	—	0.68	0.28	0.54	0.52	0.45	0.79	—	0.52
α -Glucosidase	1.55	1.38	1.30	1.70	1.50	1.43	1.30	1.3	—	—	—	1.43	0.41	0.58	0.57	—	0.57	—	0.53
α -Fucosidase	—	—	—	6.5	5.8	—	7.6	8.8	—	—	—	7.2	—	—	2.0	—	2.9	—	2.5
Cathepsin D	0.059	0.058	0.052	0.056	0.045	0.056	0.058	0.064	0.050	0.054	—	0.055	0.044	0.044	0.042	0.040	0.056	—	0.045
Brain extract																			
α -Mannosidase	<0.03	<0.04	—	—	—	<0.05	<0.04	<0.04	<0.05	<0.04	<0.04	<0.05	0.90	—	—	1.33	1.00	0.94	1.04
β -Acetylglucosaminidase	27.9	29.6	—	—	—	41.4	29.6	28.8	24.1	25.3	28.2	29.4	14.0	—	—	14.3	12.5	11.3	13
β -Glucuronidase	1.47	1.88	—	—	—	2.90	2.42	1.12	1.81	1.77	1.73	1.89	0.47	—	—	0.35	0.52	0.32	0.42
β -Galactosidase	1.3	1.9	—	—	—	3.3	2.4	1.2	2.3	1.7	1.6	2.0	0.67	—	—	0.67	0.57	0.53	0.61
α -Galactosidase	0.24	0.29	—	—	—	0.39	0.35	0.26	0.34	0.33	0.32	0.32	0.19	—	—	0.27	0.15	0.18	0.20
β -Glucosidase	0.77	0.83	—	—	—	0.97	0.74	0.86	0.93	1.20	0.86	0.90	0.46	—	—	1.12	0.68	0.58	0.71
α -Glucosidase	1.1	1.3	—	—	—	2.2	1.9	—	—	—	—	1.6	—	—	—	—	—	0.41	0.41
α -Fucosidase	1.1	1.3	—	—	—	1.4	1.4	—	—	—	—	1.3	—	—	—	—	0.32	0.32	0.32
Cathepsin D	0.018	0.021	—	—	—	0.031	0.023	0.019	0.021	0.021	0.019	0.022	0.014	—	—	0.012	0.015	0.013	0.013
Liver extract																			
α -Mannosidase	—	—	—	—	—	0.02	0.02	0.02	0.02	0.02	—	0.02	—	—	—	1.8	2.0	—	1.9
β -Acetylglucosaminidase	—	—	—	—	—	73	104	103	94	76	—	90	—	—	—	48	44	—	46
β -Glucuronidase	—	—	—	—	—	18.9	38.4	25.0	19.2	17.6	—	23.8	—	—	—	5.2	5.9	—	5.6
β -Galactosidase	—	—	—	—	—	3.5	4.1	3.1	3.0	3.7	—	3.5	—	—	—	2.1	1.9	—	2.0
α -Galactosidase	—	—	—	—	—	0.35	0.45	0.28	0.32	0.38	—	0.36	—	—	—	0.32	0.28	—	0.30
β -Glucosidase	—	—	—	—	—	22.4	18.5	20.3	26.8	11.4	—	19.9	—	—	—	16.3	16.4	—	16.4
Cathepsin D	—	—	—	—	—	0.043	0.051	0.039	0.041	0.042	—	0.043	—	—	—	0.023	0.031	—	0.027

Fig. 2. Variations of α -mannosidase activity with pH in serum and plasma from one control animal and two calves with pseudolipidosis

For experimental details see the text. ■, Control calf serum; ○, serum from calf A with pseudolipidosis; ▲, plasma from calf B with pseudolipidosis.

α -Mannosidase activity in heterozygotes

α -Mannosidase activity was measured in plasma and leucocytes collected from ten mature cows that were presumed to be heterozygotes for the genotype under investigation, in that each had previously given birth to a calf that developed pseudolipidosis. In addition, plasma and leucocytes were assayed for β -acetylglucosaminidase and β -glucuronidase. Similar assays were performed on the same tissues from ten mature cows from an Angus herd in which the disease had never been known to occur. All assays were done at the same time and the results are shown in Table 4. With the exception of two animals (439 and 456), α -mannosidase activity in the control tissues was considerably higher than that of the presumed heterozygotes. Relatively little difference was noted between the two groups for the other enzymes assayed. No significant β -glucuronidase activity was found in plasma from either group with the technique used, so results are not shown.

α -Mannosidase activities in a herd of mature Angus cows

Heparinized plasma samples from 28 mature cows in a herd where a case of pseudolipidosis had previously been diagnosed were assayed for α -mannosidase activity by using the automated method. The results (Fig. 3) demonstrate a separation of the herd into two populations on the basis of the activity of this enzyme in plasma.

Table 4. Glycosidase activities in plasma and leucocyte extracts of control animals and presumed heterozygotes

For experimental details see the text. Enzyme activities are expressed as nmol of substrate hydrolysed/min per ml of plasma, or nmol of substrate/min per mg of protein in leucocyte extracts.																													
		Control animals										Presumed heterozygotes																	
		439	456	533	537	548	562	587	596	597	635	Mean	3	4	5	9	10	11	12	13	16	17	Mean						
Enzyme assayed																													
Heparinized plasma																													
α -Mannosidase	7.8	7.3	23.4	22.5	20.2	20.8	18.7	22.5	19.0	19.6	18.2	6.2	6.0	6.8	6.9	5.4	5.4	1.3	1.9	6.0	5.4	5.13							
β -Acetylglucosaminidase	35	58	44	44	44	47	32	41	39	50	51	44.1	34	41	33	43	37	48	30	18	21	44	34.9						
Leucocyte extracts																													
α -Mannosidase	0.52	0.53	0.92	0.94	3.8	3.5	3.2	3.3	3.0	4.0	2.4	0.77	0.45	0.58	0.72	0.38	0.67	2.1	—	0.58	0.33	0.73							
β -Acetylglucosaminidase	4.6	6.1	5.3	4.5	9.7	4.2	4.9	6.3	7.8	15.5	6.9	8.9	5.5	7.6	9.6	4.5	8.9	10.5	—	5.4	7.1	7.6							
β -Glucuronidase	0.29	0.27	0.29	0.40	0.35	0.30	0.19	0.32	0.42	0.60	0.34	0.37	0.30	0.40	0.33	0.28	0.46	0.44	—	0.37	0.29	0.36

Fig. 3. *Relative plasma α -mannosidase activities in a herd of mature Angus cows*

For experimental details see the text.

Discussion

The results reported in the present paper and in a previous publication (Jolly, 1971) fulfil the requirements for pseudolipidosis to be classified as an inherited lysosomal storage disease. Lysosomes are concerned with the intracellular degradation of polymeric material and for this they are equipped with a wide range of hydrolytic enzymes (Tappel, 1969; Barrett, 1969; Patel & Tappel, 1970). Should one of these enzymes be deficient, then the particular reaction it catalyses would not take place, with consequent accumulation of the substrate within lysosomes. The concept and principles of disease caused by inborn errors of lysosomal catabolism due to specific enzyme deficiencies were first clearly stated by Hers (1965), who described Pompe's disease, a form of glycogen-storage disease due to a deficiency of α -glucosidase, in these terms. Since then a number of inherited diseases of humans have been shown to belong to this group (Brady, 1966; Brady *et al.*, 1967; Van Hoof & Hers, 1968; O'Brien *et al.*, 1971; Öckerman, 1967, 1969; Cummings, 1971) and are discussed in relation to the theory by Hers & Van Hoof (1969). For a disease to be classified as an inherited lysosomal storage disease the following criteria must be satisfied: (i) the disease should be inherited; (ii) there should be storage of some oligomeric or polymeric material, though this need not be homogeneous; (iii) this material should, at least initially, be stored within lysosomes; (iv) there should be a congenital deficiency of one of the normal lysosomal enzymes; (v) the deficient enzyme should be capable of degrading the storage material.

That pseudolipidosis was an inherited disease had been suspected from its epidemiology (Whittem & Walker, 1957; Jolly 1970; R. D. Jolly, unpublished work). Further support for this aetiology is obtained

from the results of experimental mating of cows that had previously had affected calves, to bulls that had sired such calves. Out of 20 calves, six developed the disease, closely approximating the expected 25% incidence if the disease was inherited as a simple autosomal recessive. Only calves born to matings directly under our supervision were considered.

Pathologically, pseudolipidosis is characterized by the presence of vacuoles in neurones and reticular cells of lymph nodes, and these are lined by a single membrane (Jolly, 1971; R. D. Jolly, unpublished work). Although not proved to be of lysosomal origin, their structure is compatible with this derivation and is similar to that of vacuoles in other lysosomal storage diseases where the stored product is soluble and extracted from the tissues in the course of preparation for microscopy (Hers & Van Hoof, 1969). So far, despite the use of a number of staining techniques, no specific substance has been demonstrated within these vacuoles by light- or electron-microscopy (Whittem & Walker, 1957; Jolly, 1971). This is to be expected if the substance stored within them was the mannose-glucosamine oligosaccharide extracted from lymph nodes of calves with pseudolipidosis. Being water-soluble it would be expected to be lost during the various manipulations of tissues in preparation for microscopy. Further support for this hypothesis comes from the crucial experiment in which α -mannosidase, a lysosomal enzyme associated with the catabolism of heterosaccharide units of glycoproteins, was shown to be deficient in tissues from calves with pseudolipidosis. The origin of the storage material is easily explained as the product of partial degradation of glycoproteins. The more complex heterosaccharide units of glycoproteins contain a mannose-glucosamine core in approximately the same proportions as that shown in the extracted material, and this core is linked at the reducing end to the polypeptide chain through a glycosylamine link to asparagine (Spiro, 1970). There are typically one or more side chains containing *N*-acetylglucosamine, galactose, sialic acid and/or fucose attached to this core (Spiro, 1970; Jamieson *et al.*, 1971; Thomas & Winzler, 1971). According to present theories, the lysosomal degradation of glycoprotein-heterosaccharide units occurs stepwise from the non-reducing end (Tappel, 1969). Several of the glycosidases involved, including α -mannosidase, have been shown to be specific for the non-reducing terminal glycosidic links (Bosman & Hemsworth, 1971). Therefore on theoretical grounds a storage product containing mannose and glucosamine should result from a deficiency of α -mannosidase. This product would correspond to the heterosaccharide core region with the *N*-acetylglucosamine, galactose, sialic acid/fucose side chains removed and with only mannose present as the non-reducing terminal residue(s).

The presence of only 1% amino acid associated with the oligosaccharide after ion-exchange treatment is indicative that the protein-heterosaccharide link can be hydrolysed without complete hydrolysis of the heterosaccharide. The method used for the isolation of oligosaccharide relatively free of amino acids relied on protonation of free amino groups at a low pH value and subsequent retention of amino group-containing material on cation-exchange resin. Elution of the resin with formic acid removed most of the carbohydrate-containing materials, but subsequent elution with tris buffer at pH 8.5 gave a smaller carbohydrate peak that was not further analysed. This was most probably oligosaccharide material still linked to residual amino acids.

The amino acid analyses given in Table 2 are at variance with the predictions resulting from current theories of glycoprotein structure, in which asparagine is believed to be the main heterosaccharide polypeptide link (Spiro, 1970). Aspartic acid should therefore be the main amino acid after hydrolysis of the storage oligosaccharide. The observation that glutamic acid was the main amino acid may indicate that it, or glutamine, may be involved in this linkage. There is no chemical evidence to support this suggestion, but it is difficult to explain the high residual content of glutamic acid otherwise.

The two peaks of α -mannosidase activity at different pH values (Fig. 2) suggest the presence of two enzymes. Second enzymes with α -mannosidase activity have also been demonstrated in tissues of the rabbit and rat (pH optimum 6.6) by Suzuki *et al.* (1970), and in the ovine testicle (pH optimum 6.8) by B. G. Winchester (personal communication). It has always been assumed that the enzyme in Angus calves with pH optimum 4.3 is a lysosomal enzyme, as the pH optimum is close to those reported for lysosomal α -mannosidase in other species. There is no information to suggest the localization of the second enzyme.

The increase in activity of other lysosomal enzymes that accompanied the deficiency of α -mannosidase in tissues from calves with pseudolipidosis (Table 3) is in accord with the results published for other lysosomal storage diseases (Van Hoof & Hers, 1968; Öckerman, 1967; Öckerman & Kohlin, 1968).

The effect of the phenomenon of gene dosage on enzyme activities is well recognized in many inborn errors of metabolism, with heterozygotes having tissue activities of the enzyme in question between the normal and that displayed by the homozygous diseased individuals (Aebi, 1967). The large mean difference in plasma and leucocyte extract α -mannosidase activities between the ten cows believed to be heterozygotes and the ten control cows indicates that a gene-dosage effect operates in α -mannosidase deficiency of Angus cattle. If the gene-dosage effect is as pronounced as it appears, then any population in

which heterozygote individuals occur should be readily divisible into two groups on the basis of plasma α -mannosidase activity. The results of plasma α -mannosidase assays with animals from a number of herds indicate that it is possible to divide animals of the same age and sex within a herd into two groups on the basis of the plasma enzyme activity. The results for mature cows in one such herd are shown in Fig. 3. Further, a study of genealogies indicates that the enzyme activities occur in a manner consistent with a deficiency gene being inherited as a simple autosomal recessive (R. D. Jolly, unpublished work). In the control group (Table 4) two cows (439 and 456) had α -mannosidase activities similar to those of the heterozygote group. Although a wide range of normal activity can be expected, this anomaly can best be explained on the basis that they were themselves heterozygotes. Such we believe to be the case, as subsequent testing of 91 females in this herd revealed seven animals with plasma enzyme activities distinctly lower than the remainder. Of these, two were the daughters of cows 439 and 456, and two others were closely related.

The relative mean activities of plasma and leucocyte-extract α -mannosidase in presumed heterozygotes shown in both Table 4 and Fig. 3 are below the values expected from a simple 'proportional' gene-dosage effect. Where this operates, the heterozygotes have tissue enzyme activity midway between normal and that of the diseased individuals, although of course there will be individual variation from the mean in any particular case (Aebi, 1967; Beutler *et al.*, 1966). In α -mannosidase deficiency of the degree shown to occur in Angus calves, heterozygotes would be expected to have mean tissue activities 50% of normal. That our observations of a lower than 50% enzyme activity are valid is supported by analysis of results in 29 other instances where a histogram of plasma α -mannosidase activity for an age-and-sex group within a herd has shown a clearcut separation into two groups. In these populations, the enzyme activity in the presumed heterozygote group has consistently been below 50% and with an average of 40% of the normal mean (R. D. Jolly, unpublished work). Although an activity below 50% normal was shown in leucocyte extracts from heterozygotes (Table 4) it remains to be seen whether this difference is generally reflected in other tissues.

It is clear that three populations of animals exist within the Angus breed depending on their plasma (and presumably other tissue) α -mannosidase activity measured at pH 4.3: (i) normal animals with relatively high enzyme activity; (ii) animals with pseudolipidosis, who have negligible tissue activity of this enzyme; although not recorded above, diseased calves have been shown to have negligible plasma α -mannosidase activity as would be expected from the results in Fig. 2; (iii) animals with a partial deficiency

of α -mannosidase, which are presumed to be heterozygotes. These findings may be of use in the development of a screening test for the elimination of the mutant gene from Angus herds.

Many of the observations recorded above and by Jolly (1971) are similar to those recorded for a partial α -mannosidase deficiency in a child (Öckerman, 1967, 1969, and personal communication; Kjellman *et al.*, 1969). In view of the term 'mannosidosis' introduced for the human disease, and which is in line with the terminology currently used for other lysosomal storage diseases, it is proposed that the name 'mannosidosis of Angus cattle' replace the present misleading name of 'pseudolipidosis'.

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