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Characterization of the Mutant α-Mannosidase in Bovine Mannosidosis

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Residual acidic α-mannosidase, varying in amount up to approx. 15% of normal values, can be measured in various organs of a calf with mannosidosis. The highest specific activity and relative proportion of residual activity were found in the liver. Chromatography on DEAE-cellulose showed that the residual activity was associated with two components, which were eluted at comparable positions with those found in normal tissues. The residual activity had a lower thermal stability and a higher K_m value for a synthetic substrate than did the normal enzyme. No differences in molecular weight or electrophoretic mobility between normal acidic α-mannosidase and the residual activity were observed by gel filtration and electrophoresis on cellulose acetate respectively. The isoelectric focusing profiles for the α-mannosidase in the normal and pathological livers were very similar. It is suggested that a mutant enzyme, resulting from a mutation in a structural gene, accounts for the residual acidic α-mannosidase in mannosidosis. The mutant enzyme, which cross-reacts with antiserum raised against normal bovine acidic α-mannosidase, is present at a decreased concentration compared with the normal enzyme. There is a correlation between the concentrations of residual activity and cross-reacting material in mannosidosis. α-Mannosidase with a pH optimum of 5.75 and which is activated by Zn^{2+} was also detected in the liver of the calf with mannosidosis. However, it is probably not a product of the defective gene because addition of Zn^{2+} indicated that it was also present in normal tissues.

The pathophysiological consequences of many genetically induced enzymopathies are well documented and relatively well understood. Evolution of knowledge on such inherited diseases of metabolism has progressed to the stage where, for a number of them, specific therapy is a reality, or at least contemplated. The logical development of therapeutic strategy may depend on understanding the enzymic defect in precise molecular terms. Some diseases may be controlled by dietary restrictions, others may need the addition of a specific cofactor, yet others may need replacement by an exogenous source of the enzyme, which must be immunologically compatible with the recipient (Desnick *et al.*, 1976*a*). A useful animal model for studying specific therapy of inborn errors of lysosomal catabolism is bovine mannosidosis, an inherited lethal lysosomal storage disease of Angus cattle (Whittem & Walker, 1957; Jolly, 1971) resulting from a deficiency of acidic α-D-mannosidase (EC 3.2.1.24) (Hocking *et al.*, 1972; Phillips *et al.*, 1974*a*). The clinical and pathological aspects of the disease, its biochemical basis and mode of inheritance have been reviewed (Jolly, 1975).

Tissues from calves with mannosidosis are deficient in acidic α-mannosidase, although some residual activity varying in amount up to 17.5% of normal values can be measured in various organs. This activity can be precipitated by antiserum raised against the normal bovine enzyme (Phillips *et al.*, 1977), and appears to account for all the cross-reacting material in the tissues of calves with mannosidosis. It is not known whether this observation reflects a decreased amount of the normal gene product with normal specific enzymic activity and antigenicity, or the presence of an altered enzyme with a decreased specific enzymic activity and a correspondingly decreased antigenicity. To clarify the situation the physicochemical, catalytic and immunological properties of acidic α-mannosidase in normal liver and in liver of a calf with mannosidosis are compared.

A preliminary report of some of these results has been presented (Burditt *et al.*, 1978).

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Materials and Methods

Tissues

The tissues from a 5-month-old female Angus calf, which had been diagnosed as suffering from mannosidosis, and from an 11-month-old normal female calf, were frozen within 1 h of death and maintained at −12°C until used. Tissue homogenates (10%, w/v) were prepared in a Potter–Elvehjem homogenizer in the appropriate buffer or in water at 4°C. The supernatants obtained after centrifugation of the homogenates in the 8 × 50ml angle rotor of an MSE High-Speed 18 centrifuge for 15min at 4°C and 37000*g* ($r_{av.}$ 107mm) or in the type-40 rotor of a Beckman model L centrifuge for 30min at 4°C and 20000*g* ($r_{av.}$ 59mm) were used.

Assay for α-mannosidase activity

α-Mannosidase was assayed by using the fluorigenic substrate 4-methylumbelliferyl α-D-mannopyranoside (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) as described previously (Phillips *et al.*, 1974*a*). As different concentrations and incubation times were used for some of the techniques, details will be given in the appropriate section. Activity measured at pH4.0 and 6.5 was termed acidic and neutral α-mannosidase respectively. One unit of activity is the amount that transforms 1 μmol of substrate/min under the specified conditions. The McIlvaine (1921) phosphate/citrate buffer system was used to investigate the pH-dependence of the α-mannosidase activity. The kinetic parameters K_m and V were calculated by the graphical method of Lineweaver & Burk (1934) using a computer program to obtain the line of best fit by the least-squares method for the regression line.

Effect of Co^{2+} and Zn^{2+} on α-mannosidase

The effects of these cations were investigated by assaying the acidic α-mannosidase in a series of solutions containing different concentrations of Co^{2+} and Zn^{2+} in 0.1M-citric acid adjusted to pH4.0 with 0.1M-sodium citrate. The pH-dependence of these effects was measured in the McIlvaine (1921) buffer system using a final cation concentration of 5mM-Co^{2+} or 1mM-Zn^{2+}.

Chromatography on concanavalin A–Sepharose

A column (5.5cm × 1.3cm diam.) of concanavalin A–Sepharose [Pharmacia (G.B.), London W.5., U.K.] was equilibrated in 10mM-sodium phosphate buffer, pH6.8, containing 0.5M-NaCl, 0.1mM-$MgCl_2$, 0.1mM-$MnCl_2$ and 0.1mM-$CaCl_2$. Tissue homogenates (10%, w/v) were prepared in the same buffer and the supernatants applied to the column. Unbound material was eluted with the equilibration buffer and bound material with the same buffer containing 0.5M-methyl α-D-mannopyranoside (Phillips *et al.*, 1976). Fractions (2.2ml) were collected at a flow rate of 36ml/h and then assayed for acidic and neutral α-mannosidase activity.

Thermal stability

Tissue homogenates (10%, w/v) were prepared in 10mM-sodium phosphate buffer, pH6.8, containing 0.5M-NaCl, 0.1mM-$MgCl_2$, 0.1mM-$MnCl_2$ and 0.1mM-$CaCl_2$. The supernatants were heated for 1 h at various temperatures before being assayed for α-mannosidase at 37°C in the standard manner. The rate of denaturation of the residual acidic α-mannosidase in mannosidosis and of the normal activity was measured at 56°C in liver supernatants [10% (w/v) in water]. The stability of acidic α-mannosidase was also measured at pH4.0 and at 37°C in the presence and in the absence of substrate.

Electrophoresis on cellulose acetate

Electrophoresis of tissue supernatant (10 μl; 50%, w/v) was carried out on Cellogel (Whatman Lab Sales, Maidstone, Kent, U.K.) at pH6.5 by the method of Fluharty *et al.* (1971) for 2h at 4°C using a potential of 160V and a current of 2.5mA/band. Acidic and neutral α-mannosidase were detected after electrophoresis by placing Whatman 3MM paper soaked in 2mM-methylumbelliferyl α-D-mannopyranoside at pH4.0 or pH6.5 respectively on the gel for 30min at 37°C. The bands of enzymic activity were subsequently intensified by exposure to NH_3.

Gel filtration, isoelectric focusing and ion-exchange chromatography

Gel filtration on Sephadex G-200 (Pharmacia), isoelectric focusing using an Ampholine type 8100 column (LKB Instruments, London S.E. 20, U.K.) and chromatography on DEAE-cellulose (Whatman DE 32) were carried out as described previously (Phillips *et al.*, 1974*a*,*b*).

Protein determination

Protein was measured by the Folin method (Lowry *et al.*, 1951) with bovine serum albumin as a standard.

Serological experiments

Antiserum was raised in a rabbit against partially purified bovine kidney acidic α-mannosidase (Phillips *et al.*, 1977). Rabbit glycosidases were removed from the antiserum by passing it through a column of concanavalin A–Sepharose (Pharmacia). The im-

munoglobulins were not absorbed on the column, whereas the glycosidases were. Double diffusion of extracts of the normal liver and of the liver of the calf with mannosidosis against the antiserum was carried out as described previously (Phillips *et al.*, 1975). Single radial immunodiffusion was performed by the method of Mancini *et al.* (1965) as follows. Agar gels (1%, w/v) were prepared in antiserum that had been diluted 100-fold with phosphate-buffered saline, pH7.2 (Dulbecco's Formula; Flow Laboratories, Irvine, Scotland, U.K.). Tissue supernatants [20μl, 20% (w/v) homogenate] and a series of dilutions were placed in wells (4mm diam.) and left for diffusion to take place for 48h in a humid chamber at room temperature. Proteins that did not form an immune complex were removed by exhaustive washing in phosphate-buffered saline. The enzymically active immunoprecipitin rings were detected by incubating the gels at 37°C in a solution containing 1-naphthyl α-D-mannopyranoside (1mg/ml) (Koch–Light) and Diazo Red R.C. (2mg/ml) [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] in 0.1M-citric acid adjusted to pH4.0 with 0.2M-sodium phosphate. The ring diameters were measured with a Hyland Precision Viewer (Travenol Laboratories Inc., CA, U.S.A.). The gels were stored in 12% (v/v) acetic acid.

Results

Evidence for acidic α-mannosidase activity in the tissues of a calf with mannosidosis

α-Mannosidase activity was measured at pH4.0 in various organs of the Angus calf with mannosidosis and in the corresponding organs of a control calf, matched for breed, age and sex (Table 1). The proportion of residual acidic α-mannosidase in the organs of the calf with mannosidosis relative to the activity in the corresponding normal organ varied from organ to organ, as has been observed previously (Phillips *et al.*, 1977). The highest relative proportion and specific activity were found in the liver. There is a small peak of activity, which can be augmented by the addition of Zn^{2+}, with a pH optimum of 4.0–4.2 in the activity–pH profile for α-mannosidase in the liver of the calf with mannosidosis (Figs. 1*b* and 7). This suggests that an acidic α-mannosidase component and not components with a higher pH optimum accounts for the residual acidic α-mannosidase in mannosidosis. The acidic α-mannosidase activity in normal bovine tissues can be separated from the neutral α-mannosidase and resolved into two forms, A and B, by chromatography on DEAE-cellulose (Phillips *et al.*, 1974*a*). Comparison of the elution profiles on DEAE-cellulose for the activity in the normal liver and in the liver of the calf with mannosidosis showed that two small peaks of activity

Table 1. *Residual acidic α-mannosidase and cross-reacting material in the tissues of a calf with mannosidosis as a percentage of those in the corresponding tissues of a normal calf*

α-Mannosidase (munits/mg of protein) was assayed at pH 4.0 in supernatants from homogenates (10%, w/v) in water with a substrate concentration of 5 mM. The values for plasma (munits/ml), lymphocytes and neutrophils are based on measurements made on numerous other animals. Cross-reacting material was measured by radial immunodiffusion.

	Residual activity (%)	Cross-reacting material (%)
Liver	15	20
Brain	13	—
Pancreas	12	—
Spleen	4	8
Lymph nodes	5	—
Kidney	3.5	8
Plasma	<3	—
Lymphocytes	<2	—
Neutrophils	<2	—

corresponding to peaks A and B in the normal liver were present in the liver of the calf with mannosidosis (Fig. 2). Peak B was eluted at a slightly higher NaCl concentration than normal in the sample from the calf with mannosidosis. The activity in peaks A and B from both samples had an acidic pH optimum, but there was a second maximum of activity at pH5.75 for peak B in the liver from the calf with mannosidosis. Therefore α-mannosidase components with an acidic pH optimum account for the residual acidic activity in mannosidosis.

Comparison of the acidic α-mannosidase in the liver of the calf with mannosidosis with the actiity in a normal calf liver

(*a*) *Thermal stability in tissue extracts.* Extracts of the livers of the calf with mannosidosis and of the control calf were heated for 1h at a range of temperatures before being assayed at 37°C for acidic α-mannosidase activity (Fig. 3). It can be seen that, whereas the normal acidic α-mannosidase was stable up to at least 55°C, the activity in the liver of the calf with mannosidosis was unstable above 40°C. The neutral α-mannosidase, which was assayed at pH6.5, from normal and affected animals was also unstable above 40°C.

The rate of denaturation of the acidic α-mannosidase in both livers was measured at 55°C (Fig. 4). The denaturation of the residual acidic α-mannosidase was essentially first order at this temperature.

As the residual acidic α-mannosidase was unstable at relatively low temperatures its stability was also investigated at 37°C and pH4.0, the conditions of the standard assay. In the absence of substrate there

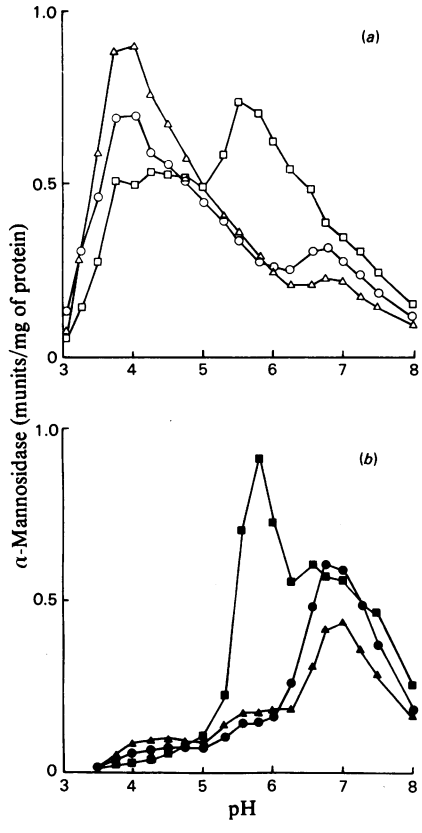

Fig. 1. *pH-dependence of the α-mannosidase activity in the control liver and in the liver of the calf with mannosidosis in the presence and in the absence of Co^{2+} and Zn^{2+}*

Tissue homogenates (10%, w/v) were assayed for 1 h at 37°C with a substrate concentration of 4.5 mM in the presence and in the absence of Co^{2+} (5 mM) and Zn^{2+} (1 mM). (*a*) Normal tissue: ○, no ions; □, Co^{2+}; △, Zn^{2+}. (*b*) Mannosidosis: ●, no ions; ■, Co^{2+}; ▲, Zn^{2+}.

was appreciable (46%) loss of activity over 1 h, but in the presence of substrate no activity was lost. In contrast, the activity in the normal liver was stable under these conditions both in the presence and in the absence of substrate.

(*b*) *Effect of Co^{2+} and Zn^{2+}.* Zn^{2+} (1 mM) activated the acidic α-mannosidase in the normal liver and in the liver from the calf with mannosidosis (Fig. 1). The maximum effect was at pH 4.0, the pH optimum of the activity in the normal tissue. Although the neutral α-mannosidase in both tissues was inhibited by Zn^{2+}, there was also activation between pH 5.0 and 6.0, especially in the liver from the calf with mannosidosis.

The acidic α-mannosidase activity was assayed at pH 4.0 at a series of concentrations of Zn^{2+} from 0.5 mM to 5.0 mM (Fig. 5). Increasing the concentration of Zn^{2+} beyond 1 mM did not produce any further activation, which was consistently greater for the normal enzyme than for the residual activity in mannosidosis.

In contrast Co^{2+} (5 mM) inhibited the acidic α-mannosidase in the control liver and in the liver from the calf with mannosidosis (Fig. 1). The neutral α-mannosidase was activated by the Co^{2+}, with maximal effect at pH 5.8 and not at pH 6.7, the pH optimum of the neutral activity.

The inhibition by Co^{2+} of the acidic α-mannosidase at pH 4.0 was found to increase somewhat with the concentration of Co^{2+} over the range 0.5–5.0 mM (Fig. 5). The degree of inhibition was slightly greater for the residual activity in mannosidosis, but appeared to approach a maximum value of approx. 50% at 5.0 mM-Co^{2+}.

(*c*) *Effect of substrate concentration.* The values of K_m for the normal acidic α-mannosidase and the

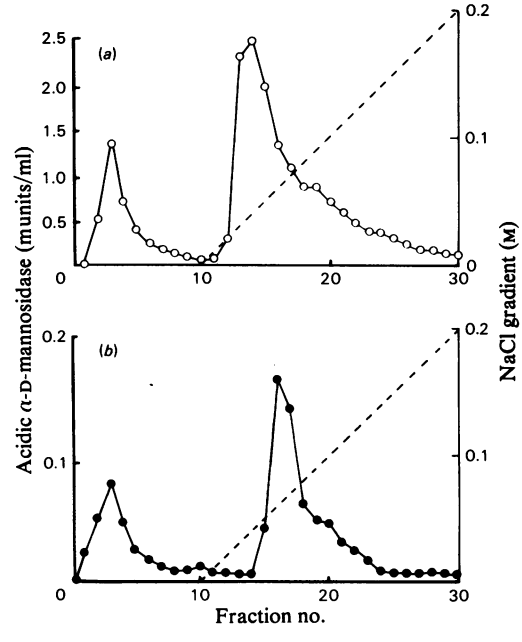

Fig. 2. *Chromatography on DEAE-cellulose of the acidic α-D-mannosidase in (a) the control liver and in (b) the liver of the calf with mannosidosis*

Tissue supernatants (1 ml; 50%, w/v) were applied to a column (2 ml bed volume) equilibrated in 10 mM-sodium phosphate buffer, pH 6.0. Material was eluted with a linear NaCl gradient (0–0.2 M) and fractions (2.2 ml) were collected. ----, NaCl gradient; ○, normal activity at pH 4.0; ●, mannosidosis activity at pH 4.0.

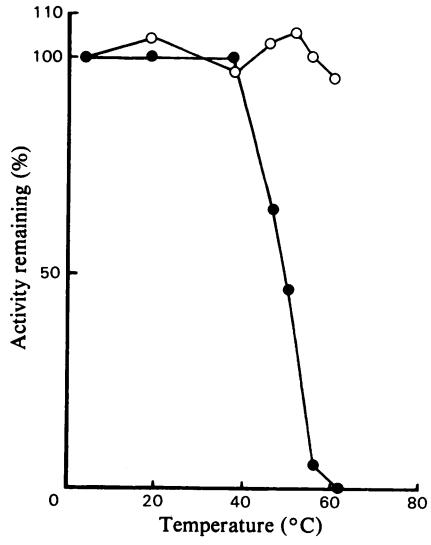

Fig. 3. *Thermal stability of the acidic α-D-mannosidase in the control liver and in the liver of the calf with mannosidosis*
Tissue supernatants were preincubated for 1 h at different temperatures before being assayed at 37°C for α-D-mannosidase at pH4.0. ○, Control; ●, mannosidosis.

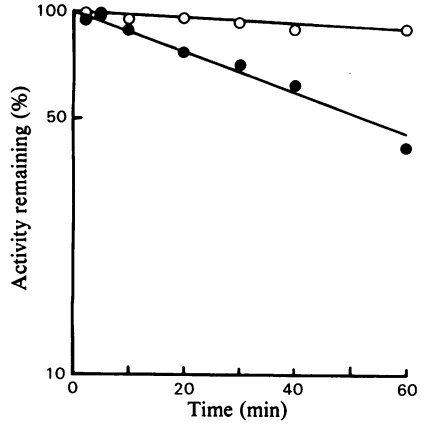

Fig. 4. *Thermal denaturation at 55°C of the acidic α-D-mannosidase in the control liver and in the liver of the calf with mannosidosis*
Tissue supernatants were incubated at 55°C and at suitable intervals of time samples were removed and assayed for acidic α-D-mannosidase at 37°C. ○, Control; ●, mannosidosis.

residual acidic mannosidase in mannosidosis were measured at pH4.0 in the presence and absence of 1 mM-Zn^{2+} (Table 2). The residual activity in mannosidosis had a greater value of K_m than the normal enzyme. Zn^{2+} decreased the values of K_m for the normal and residual activity.

(*d*) *Chromatography on concanavalin A–Sepharose.* Both the acidic α-mannosidase in the normal liver and in the liver from the calf with mannosidosis bound to concanavalin A–Sepharose. As the neutral α-mannosidase did not bind to the lectin it was thus possible to compare the acidic activities more clearly in the absence of any contaminating neutral activity. The activity–pH profiles for the bound material from the two livers were not identical (Fig. 6). Any apparent differences owing to different concentrations of acidic α-mannosidase were eliminated by normalizing the data on a percentage maximum basis.

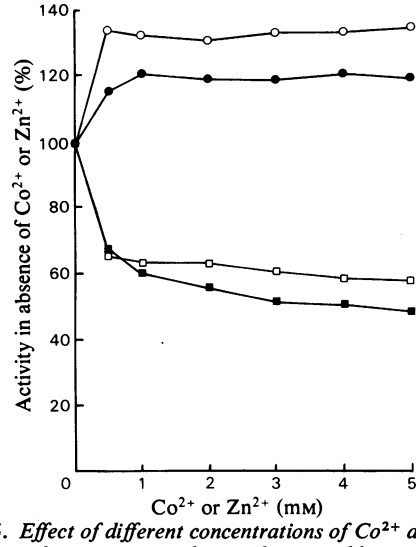

Fig. 5. *Effect of different concentrations of Co^{2+} and Zn^{2+} on the acidic α-D-mannosidase in the control liver and in the liver of the calf with mannosidosis*
The activity was measured at pH4.0 with a substrate concentration of 1 mM in the presence of Co^{2+} (□, control; ■, mannosidosis) and Zn^{2+} (○, control; ●, mannosidosis).

Table 2. *Values of K_m for the hydrolysis of 4-methylumbelliferyl α-D-mannopyranoside by the acidic α-mannosidase in a normal liver and in the liver from a calf with mannosidosis*
α-Mannosidase was assayed at pH4.0 at a series of substrate concentrations in the presence and absence of Zn^{2+} (1 mM). Values are means ± S.D. for the numbers of separate determinations in parentheses.

	K_m (mM)	
	No Zn^{2+}	Zn^{2+} (1 mM)
Normal	0.57 ± 0.05 (3)	0.45 (2)
Mannosidosis	1.09 ± 0.37 (4)	0.66 (2)

Although the two activities have the same pH optimum, the profile for the residual activity is shifted towards neutrality compared with that of the normal activity and there is relatively more activity present between pH5.25 and 7.0. Addition of 1mM-Co^{2+} or 1mM-Zn^{2+} confirmed that both acidic α-mannosidases were inhibited and activated respectively by these two cations and that the inhibition by Co^{2+} was greater for the residual activity in mannosidosis. The maximal effects for these cations occurred at a slightly higher pH for the residual activity than for normal α-mannosidase. The activation by Zn^{2+} of activity between pH5.0 and 6.0 that had been observed in whole tissue extracts was also seen in the absence of the neutral activity, especially in the activity from the calf with mannosidosis (Fig. 7). However, the activation by Co^{2+}, maximal at pH5.75–6.0, was absent, presumably because of the removal of the neutral α-mannosidase.

(*e*) *Isoelectric focusing.* Two major peaks of acidic α-mannosidase isoelectric between pH5.5 and 6.0 and between pH7.8 and 8.2 with a marked shoulder at pH7.3 were observed in the isoelectric-focusing profile for the normal liver (Fig. 8*a*). The activity in these peaks had a pH optimum of 4.25 and probably corresponded to peaks B and A respectively separable by chromatography on DEAE-cellulose. Both peaks showed microheterogeneity. Corresponding peaks were also present in decreased concentrations in the isoelectric-focusing profile for the residual acidic α-mannosidase in the liver from the calf with mannosidosis (Fig. 8*b*). However, the predominant component in the activity isoelectric above neutrality, had a pI value of pH7.2–7.3.

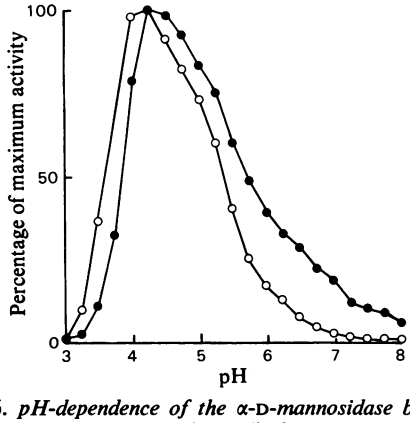

Fig. 6. *pH-dependence of the α-D-mannosidase bound to concanavalin A–Sepharose*
The pH-dependence of the material that was bound to concanavalin A–Sepharose and eluted by 0.5M-methyl α-D-mannopyranoside was measured. The data are normalized on a percentage basis. ○, Control liver; ●, mannosidosis liver.

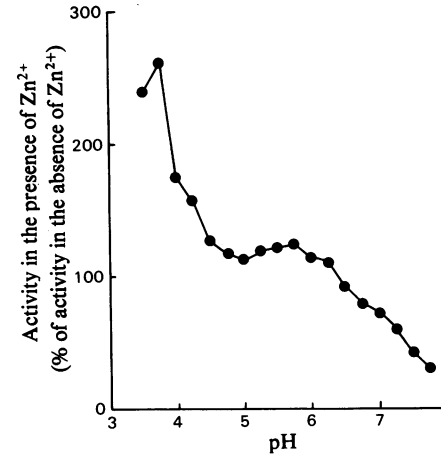

Fig. 7. *Effect of Zn^{2+} on concanavalin A–Sepharose-bound α-mannosidase activity from calf with mannosidosis*
The pH-dependence of the α-mannosidase that was bound to concanavalin A–Sepharose was measured in the presence and in the absence of Zn^{2+} (1mM). ●, Activity in presence of Zn^{2+} as a percentage of activity in the absence of Zn^{2+}.

(*f*) *Electrophoresis on cellulose acetate.* The normal acidic enzyme and the residual acidic activity in mannosidosis were not separable by electrophoresis on cellulose acetate at pH6.5.

(*g*) *Gel filtration on Sephadex G-200.* No apparent difference in molecular weight between the normal acidic enzyme and the residual acidic activity was observed by gel filtration.

(*h*) *Immunological properties.* Double diffusion (Ouchterlony) of extracts of the normal liver and of the liver of the calf with mannosidosis against the antiserum raised against normal bovine kidney acidic α-mannosidase resulted in the formation of single immunoprecipitin lines with α-mannosidase activity for both samples (Fig. 9).

The activity in the immunoprecipitin line for the sample from the calf with mannosidosis took up to 12h to appear compared with 1–2h for the normal activity. No spur formation occurred when the two liver samples were placed in adjacent wells. However, the line for the mannosidosis sample appeared to be continuous with the trailing edge rather than the middle of the precipitin line for the normal enzyme, suggesting that a lower concentration of immunologically identical material was present. Post-staining for protein did not reveal any further immunoprecipitin lines even when higher concentrations of tissue extracts were used. Thus the residual acidic α-mannosidase in mannosidosis cross-reacts with antiserum raised against the normal enzyme and

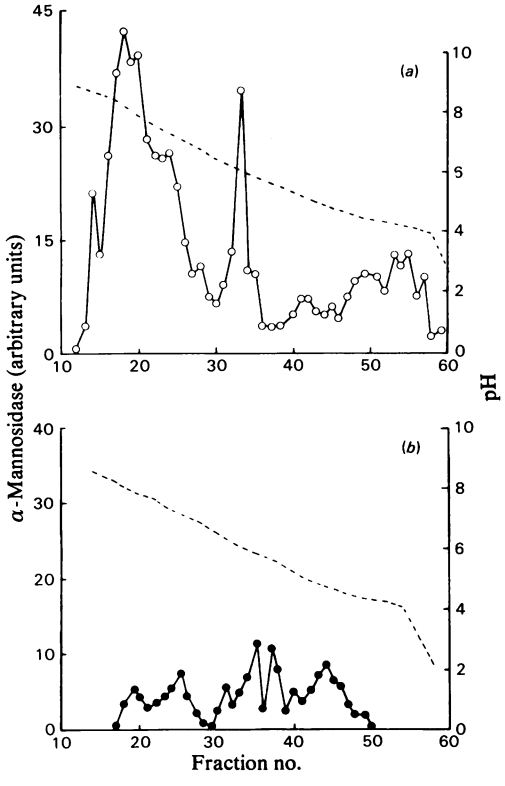

Fig. 8. *Isoelectric focusing of the acidic α-D-mannosidase in the control liver and in the liver of the calf with mannosidosis*

Samples (1 ml) of tissue supernatants [10 and 20% (w/v) respectively] of (*a*) the control liver and of (*b*) the mannosidosis liver were focused for 60h at 400V and 0.5mA over the range of pH3–10. ○, Control; ●, mannosidosis.

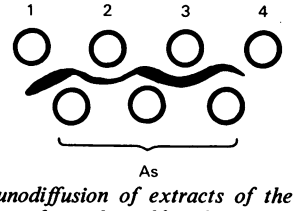

Fig. 9. *Immunodiffusion of extracts of the control liver and of the liver from the calf with mannosidosis against anti- (bovine kidney acidic α-D-mannosidase) serum*

The precipitin lines were stained for α-D-mannosidase with 1-naphthyl α-D-mannopyranoside. As, antiserum; 1 and 3, normal liver; 2 and 4, liver from calf with mannosidosis.

appears to account for all of the cross-reacting material present.

The concentration of cross-reacting acidic α-mannosidase in the liver of the calf with mannosidosis was measured by single radial immunodiffusion. Fig. 10 shows that the concentration of cross-reacting material was directly proportional to the concentration of protein for both the normal and the pathological liver, but that the concentration of cross-reacting material in mannosidosis was only approx. 20% of that in the normal tissue. The relative concentrations of cross-reacting material in the kidney and spleen of the calf with mannosidosis were also measured (Table 1). There is a correlation between the concentration of residual acidic α-mannosidase and cross-reacting material.

Discussion

Characterization of the residual acidic α-mannosidase in the liver of a calf with mannosidosis suggests that a mutant enzyme is present in mannosidosis. The evidence for this conclusion is the detection by enzyme separation procedures of α-mannosidase components with an acidic pH optimum, but with a lower stability and different kinetic properties from the normal enzyme. Neutral α-mannosidase or a structurally unrelated acidic α-mannosidase does not account for the residual activity in mannosidosis, because the residual activity cross-reacts with antiserum raised against normal acidic α-mannosidase. It has been shown previously that this antiserum did not cross-react with neutral α-mannosidase and that it reacted with normal bovine acidic α-mannosidase to give a single active immunoprecipitin band in immunodiffusion (Phillips *et al.*, 1977).

The differences between the properties of the mutant and normal enzymes are consistent with a mutation in a structural gene for acidic α-mannosidase

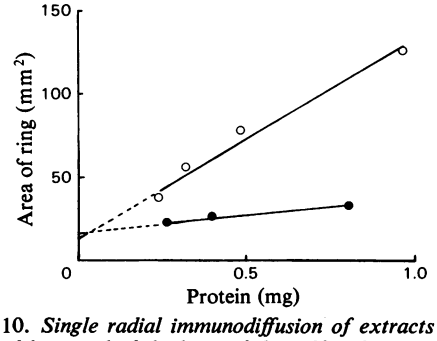

Fig. 10. *Single radial immunodiffusion of extracts of the control liver and of the liver of the calf with mannosidosis against antiserum*

Details of the immunodiffusion and detection of the enzymically active precipitin bands are given in the Materials and Methods section. The intercepts on the ordinate correspond to the well area. ○, Control; ●, mannosidosis.

in mannosidosis. Such a mutation would result in a mutant protein with a different tertiary or quaternary structure from the normal gene product. The greater susceptibility of this altered structure to proteolysis by endogenous enzymes and/or to irreversible denaturation might explain the decreased stability of the mutant enzyme. Similarly, the difference in kinetic properties might be due to the change in structure affecting the active site. However, the mutation does not appear to affect the inhibition by Co^{2+} or activation by Zn^{2+} appreciably. The degree of inhibition or activation by Co^{2+} and Zn^{2+} respectively is comparable in the mutant and normal enzymes. Increasing the concentration of Zn^{2+} beyond 1 mM did not produce any further increase in the activity of either enzyme, suggesting that a defect in the Zn^{2+}-binding site is not responsible for the increase in K_m.

The mutation does not affect all the antigenic sites on the enzyme because the mutant enzyme cross-reacts with antiserum raised against the normal enzyme. The concentration of cross-reacting material measured by radial immunodiffusion was much lower in the liver and other organs of the calf with mannosidosis than in the corresponding control organs (Table 1 and Fig. 10). The absence of enzymically inactive cross-reacting material and the correlation between the concentrations of residual acidic α-mannosidase and cross-reacting material suggest that the mutant enzyme accounts for all the cross-reacting material in mannosidosis and that it has a similar avidity to the normal enzyme for antibodies against the normal enzyme. A similar conclusion was drawn from immunoprecipitation techniques (Phillips *et al.*, 1977) and there is a good agreement between the concentrations of cross-reacting material measured by antibody consumption and radial immunodiffusion.

The apparent decrease in the concentration of cross-reacting enzymic molecules in mannosidosis could be due to the greater instability of the mutant enzyme. Alternatively the mutation could lead directly to a lower concentration of cross-reacting molecules. A mutation in a regulatory gene resulting in a lower concentration of the fully cross-reacting normal enzyme through a decreased rate of synthesis is very unlikely. This is because the residual acidic α-mannosidase in mannosidosis has different enzymic and physicochemical properties from the normal enzyme. Therefore it is concluded that there is a lower concentration of a cross-reacting mutant enzyme in mannosidosis. Human acidic α-mannosidase is a heteromultimeric enzyme (Marinkovic & Marinkovic, 1976). In view of the great similarity between human and bovine α-mannosidase (Phillips *et al.*, 1974*a*,*b*), it is probable that the bovine enzyme is also multimeric. Thus a mutation in one of the subunits is a possible interpretation of the serological experiments.

A defect in the post-translational processing of the enzyme by proteolysis, glycosylation, de-glycosylation or phosphorylation cannot be entirely excluded. The elution profiles for chromatography on DEAE-cellulose were very similar for the normal and mutant enzymes, but peak B was eluted at a slightly higher salt concentration in mannosidosis. Isoelectric focusing indicated an apparent decrease in pI for mutant α-mannosidase-A. These differences could arise from a single amino acid substitution or altered conformation in the mutant enzyme. They were insufficient to permit separation of the normal and mutant enzymes by electrophoresis. Gel filtration and chromatography on concanavalin A–Sepharose indicated no gross changes in molecular weight and glycosylation respectively. Further, any post-translational modification would have to be detrimental specifically to α-mannosidase, because the activities of eight other lysosomal hydrolases have been found to be increased or in the normal range in several tissues of a calf with mannosidosis (Hocking *et al.*, 1972).

Thus the decreased concentration of acidic α-mannosidase in bovine mannosidosis is probably due to a decreased concentration of a mutant enzyme with a lower stability and affinity for substrate than the normal enzyme, but with a comparable avidity for antibodies raised against the normal enzyme. These observations are consistent with a mis-sense mutation in a structural gene for acidic α-mannosidase. A mutation in a structural gene has also been postulated for human mannosidosis on the basis of the altered kinetic properties and stability of the residual acidic α-mannosidase (Beaudet & Nichols, 1976; Desnick *et al.*, 1976*b*). Cross-reacting material was detected in cultured fibroblasts from a patient by using heterologous antiserum (Mersmann & Buddecke, 1977).

The properties of the normal and mutant bovine acidic α-mannosidases could be compared more clearly when the neutral α-mannosidase had been removed by chromatography on concanavalin A–Sepharose. Although the two enzymes had the same pH optimum, the activity–pH profile for the mutant enzyme was shifted towards neutrality and there was relatively more activity between pH 5.0 and 7.0. The activation by Zn^{2+} of activity in this pH range was also very clearly seen in the residual activity in mannosidosis (Fig. 7). It is suggested that these observations are due to the presence in bovine tissues of a third type of α-mannosidase, which is activated by Zn^{2+} and has a pH optimum intermediate between the acidic and neutral α-mannosidases. In mannosidosis tissues, in the absence of most of the acidic activity, it can be detected in the activity–pH profile in the presence and absence of Zn^{2+}. It is probably present in normal tissues, but is concealed by the high concentration of acidic α-mannosidase. Further

evidence for the existence of this intermediate α-mannosidase came from ion-exchange chromatography on DEAE-cellulose. The activity in peak B from the liver of the calf with mannosidosis had a second pH optimum at pH 5.75 and was markedly activated by Zn^{2+} at this pH. This was not due to the neutral activity, which was eluted as peak C, and suggests that this third type of α-mannosidase has a similar chromatographic behaviour to α-mannosidase B. The presence in bovine tissues of a third minor type of α-mannosidase with an intermediate pH optimum has been suggested previously on the basis of metal ion effects (Phillips *et al.*, 1974*a*). Its relationship to the major bovine plasma α-mannosidase component, which has a pH optimum of 5.5, is not known (Winchester *et al.*, 1976). A distinct α-mannosidase component with a pH optimum of 5.3 has been isolated from a fraction of rat Golgi membranes (Tulsiani *et al.*, 1977), and Snaith (1977) has also detected α-mannosidase activity with a pH optimum of 5.2 in rat liver. Therefore the third type of α-mannosidase detected in the liver of the calf with mannosidosis is almost certainly not a product of the defective gene. α-Mannosidase activity with a pH optimum of 5.5 was observed in the liver of the original patient with mannosidosis (Hultberg, 1970).

The presence in bovine mannosidosis of a cross-reacting mutant enzyme enhances the prospects of experimental enzyme replacement in this animal model by decreasing the chance of immunological complications. In a previously reported experiment of Nature involving a chimaeric calf with mannosidosis, there was a natural transplant of normal lymphocytes early in foetal development (Jolly *et al.*, 1976). Subsequently there was a considerable decrease in the storage oligosaccharides and the extent of lesions in visceral organs. Despite this and evidence indicating a decrease of storage material in the brain, the neurological course of the disease was essentially unaltered, again emphasizing the problem of the blood/brain barrier.

Stabilization or activation of the mutant enzyme *in vivo* is an alternative therapeutic strategy. As the mutant enzyme–immunoglobulin complex is active and stable, administration of antiserum raised against the normal enzyme might stabilize the mutant enzyme. Whether such an immune complex would reach the appropriate sites is not known. Activation of the mutant enzyme by the addition of Zn^{2+} (Hultberg & Masson, 1975) or Co^{2+} (Desnick *et al.*, 1976*b*) has been suggested as a potential form of therapy in human mannosidosis. In the bovine model, although Zn^{2+} does increase the activity *in vitro* of both the mutant and normal acidic α-mannosidase, it has been concluded that the mutation does not affect the Zn^{2+}-binding site significantly. Therefore it is unlikely that increasing the concentration of Zn^{2+} in tissues would stabilize the enzyme or increase the activity sufficiently *in vivo*, and experiments with $ZnSO_4$ medication tend to confirm this (R. D. Jolly, unpublished work). Nevertheless any increase in activity may be worthwhile and if this can be shown then administration of zinc salts could be an ancillary treatment. It should be remembered though that zinc salts can be highly toxic. Further investigation of the stability, metabolic fate and activity towards natural substrates of the mutant enzyme will be necessary to understand the pathogenesis of bovine mannosidosis and to devise therapeutic strategies.

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