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Mannosidosis in Angus Cattle

THE ENZYMIC DEFECT

By N. C. PHILLIPS, D. ROBINSON and B. G. WINCHESTER
Department of Biochemistry, Queen Elizabeth College (University of London), Campden Hill, London W8 7AH, U.K.
and
R. D. JOLLY
Department of Veterinary Pathology and Public Health, Massey University, Palmerston North, New Zealand

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Normal calf α-mannosidase activity exists in at least three forms separable by chromatography on DEAE-cellulose and by starch-gel electrophoresis. Two components, A and B, have optimum activity between pH3.75 and 4.75, but component C has an optimum of pH6.6. Components A and B are virtually absent from the tissues of a calf with mannosidosis and the residual activity is due to component C. The acidic and neutral forms of α-mannosidase differ in their molecular weights and sensitivity to EDTA, Zn^{2+}, Co^{2+} and Mn^{2+}. An acidic α-mannosidase component (pH optimum 4.0) accounts for most of the activity in normal plasma but it is absent from the plasma of a calf with mannosidosis. Although the acidic α-mannosidase component is probably related to tissue components A and B, it can be distinguished from them by ion-exchange chromatography and gel filtration. The optimum pH of the low residual activity in the plasma from a calf with mannosidosis is pH5.5–5.75. The results support the hypothesis that Angus-cattle mannosidosis is a storage disease caused by a deficiency of lysosomal acidic α-mannosidase activity.

A lethal nervous disease in Angus cattle that morphologically resembled human lipidoses was first described in Australia by Whittem & Walker (1957). As they were unable to demonstrate the presence of stored lipid in vacuolated neurones they introduced the term 'pseudolipidosis', by which term the disease has been known until recently. The vacuoles in neurons and reticulo-endothelial cells within lymph nodes of diseased animals are lined by a single membrane and appear empty by light- or electron-microscopy (Jolly, 1971). An oligosaccharide containing mannose and glucosamine has been isolated from the lymph nodes of Angus calves with pseudolipidosis but not from normal animals (Hocking *et al.*, 1972). Oligosaccharides are also stored in the brain and excreted in the urine of diseased calves. A comparison of the activity of several lysosomal hydrolases in the tissues of normal and diseased calves revealed a marked deficiency of α-mannosidase activity (assayed at pH4.3) in diseased animals (Hocking *et al.*, 1972). The residual α-mannosidase activity in the plasma of diseased calves had a higher pH optimum than the activity in normal plasma. It had been suspected from its epidemiology that the disease was inherited. This was confirmed by measuring the α-mannosidase activity in the calves from controlled breeding experiments with cows that had previously given birth to a calf with pseudolipidosis and bulls that had sired such calves. The results were consistent with the deficiency gene being inherited as a simple autosomal recessive, with heterozygotes showing a partial deficiency of the enzyme. The disease fulfils the criteria of Hers (1965) to be classified as a lysosomal storage disease. A deficiency of α-mannosidase activity with the residual activity having a higher pH optimum than the normal activity and an accumulation of mannose-rich oligosaccharides in the tissues is also associated with the human disease, mannosidosis (Öckerman, 1967, 1969*a*; Kjellman *et al.*, 1969; Hultberg, 1970). In view of the great similarity between bovine pseudolipidosis and human mannosidosis, Hocking *et al.* (1972) proposed that the former be called mannosidosis of Angus cattle. Subsequent work has sought to compare the disease in the two species by studying the storage materials and the α-mannosidase activity in normal and diseased humans and Angus calves. It has been shown that the mannose-rich oligosaccharides isolated from the brain of calves with mannosidosis and from the urine of patients with mannosidosis are not identical (Nordén *et al.*,

1973*a*,*b*). Whether this reflects different enzyme specificities or a different basic glycoprotein structure is not yet known. Normal human liver contains three major α-mannosidase components separable by chromatography on DEAE-cellulose. Two have an acidic optimum (pH4.4) and one a neutral optimum (pH6.5). The two acidic components were absent in the two cases of mannosidosis that have been examined and the residual activity was shown to be due to the neutral component (Carroll *et al.*, 1972). Two α-mannosidase components with an acidic optimum of pH4.3 have also been separated in normal bovine liver by chromatography on DEAE-cellulose (Langley & Jevons, 1968). However, α-mannosidase activity with a neutral pH optimum has not been reported in bovine liver or any other bovine tissue. The purpose of this paper is to investigate the α-mannosidase components in normal calf tissues and to establish the nature of the enzymic defect in mannosidosis in Angus calves.

Materials and Methods

Tissue

The tissues from a 3-week-old Angus calf, which had been diagnosed clinically and by histopathology as suffering from mannosidosis, were stored frozen at −20°C until required. The corresponding tissues from a control calf of similar age were stored under identical conditions. Frozen heparinized plasma from these two animals was also investigated. Tissue homogenates (10%, w/v) were prepared in a Potter–Elvehjem homogenizer by using a manually rotated Teflon pestle. The homogenates were prepared in water for the investigation of the pH-dependence of the α-mannosidase activity, and in the appropriate initial elution buffer for ion-exchange and gel-filtration chromatography. 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) were used in all the experiments.

Enzyme assays

α-Mannosidase activity was assayed with the fluorigenic substrate, 4-methylumbelliferyl α-D-mannopyranoside (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) by a modification of the procedure of Öckerman (1969*b*). The enzyme sample (50–200μl) was diluted to 0.5ml with phosphate–citrate buffer and incubated with 0.5ml of 1mM buffered substrate for 1 or 2h at 30°C. The reaction was stopped by the addition of 1.5ml of 0.5M-glycine–NaOH buffer, pH10.4, and the fluorescence of the liberated 4-methylumbelliferone was determined. Activity measured at pH4 and pH7 was termed acidic and neutral α-mannosidase activity respectively. The McIlvaine (1921) phosphate–citrate buffer system was used for routine assays of the acidic and neutral activities and in the investigation of the pH-dependence of the activity. One unit of activity is that amount of enzyme which transforms 1μmol of substrate/min under these conditions. The effect of EDTA and various metal ions on the α-mannosidase activity was investigated by including 1mM-EDTA, 1mM-$ZnSO_4$, 2mM-$CoSO_4$ or 1mM-$MnSO_4$ adjusted to the correct pH in the substrate solutions, giving a final concentration of 0.5 or 1mM in the assay mixture. All reagents were of A.R. grade.

Ion-exchange chromatography

Tissue supernatants (2ml) prepared in 10mM-sodium phosphate buffer, pH6.0, were analysed by ion-exchange chromatography on DEAE-cellulose (Whatman DE-32) at 4°C. The DEAE-cellulose was equilibrated in 10mM-sodium phosphate buffer, pH6.0, and packed in disposable syringes (3cm×0.9cm diam.). Elution was started with the equilibration buffer and continued with a linear NaCl gradient (0–0.3M) in 100ml of the same buffer. Fractions (1.5ml) were collected at a flow rate of 60ml/h. Samples (0.2ml) of each fraction were assayed for acidic and neutral α-mannosidase activity and the NaCl gradient was followed by measuring the conductivity of the fractions with a conductivity-measuring bridge, type MC3 [Electronic Switchgear (London) Ltd., Hitchin, Herts., U.K.]. Chromatography on CM-cellulose (Whatman CM-32) was performed under similar conditions except that 10mM-sodium phosphate, pH5.0, was used for the preparation of the samples, equilibration of the ion-exchanger and the initial elution. Enzymic fractions separated by chromatography on DEAE-cellulose were also dialysed against this buffer before re-chromatography on CM-cellulose. Plasma was dialysed against the initial elution buffer before ion-exchange chromatography.

Starch-gel electrophoresis

Starch gels were prepared in 5mM-sodium phosphate buffer, pH7.0, by the method of Smithies (1955) by using partially hydrolysed starch (Connaught Medical Laboratories, Toronto, Canada). Electrophoresis was carried out at 4°C for 4h at 300V and 45mA with 40mM-sodium phosphate buffer, pH7.0, in the electrode compartments. α-Mannosidase activity was detected on the gel surface in a manner similar to that described for other glycosidases by Robinson *et al.* (1967). Acidic and neutral α-mannosidase components were distinguished by using two substrate solutions in phosphate–citrate buffer at pH4 and 7 respectively.

Gel filtration

The molecular size of the α-mannosidase activity was investigated by using a column (34.5cm×2.5cm diam.) of Sephadex G-200 [Pharmacia (G.B.) Ltd., London W.5, U.K.] equilibrated in 10mM-sodium phosphate buffer, pH6.0, containing 0.1M-NaCl. The column was calibrated with the following standards (molecular weights in parentheses): cytochrome *c* (12400), bovine serum albumin (67000), aldolase (150000), bovine γ-globulin (effectively 205000) [all from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K.], urease (490000) (jack-bean meal; BDH Chemicals Ltd., Poole, Dorset, U.K.) and Blue Dextran [Pharmacia (G.B.) Ltd.]. Fractions (1.5ml) were collected at 14 ml/h. A plot of the elution volumes of the standards against the logarithms of their molecular weights was used to estimate the molecular weights of the α-mannosidase components.

Results and Discussion

α-Mannosidase activity in the tissues of normal calves and those with mannosidosis

The pH-dependence of the α-mannosidase activity in the tissues of a normal Angus calf and of an Angus calf with mannosidosis was compared (Fig. 1). In normal tissues there is a pH optimum between pH3.75 and 4.75 and another at pH6.6, suggesting that both acidic and neutral α-mannosidase activities occur in bovine tissues. The total α-mannosidase activity and the relative contributions of the acidic and neutral activities to this activity varied considerably among the tissues. For example, as shown in Fig. 1, the acidic activity is low in the liver but is greater than the neutral activity in the lymph nodes.

In the mannosidosis tissues the acidic activity is considerably decreased or virtually absent and the majority of the residual activity has a neutral optimum of pH6.6. The loss of the activity with an acidic pH optimum would explain the observation by Hocking *et al.* (1972) that α-mannosidase activity assayed at pH4.3 was almost absent from the brain, liver and lymph nodes of calves with mannosidosis. Acidic activity is also absent from the plasma of the calf with mannosidosis, but the residual activity has an optimum of pH5.5–5.75 rather than of pH6.6 as in the tissues. There is no neutral activity in the plasma of either the calf with mannosidosis or the normal calf.

Acidic α-mannosidase activity has been described in the reproductive tract of the bull (Conchie & Mann, 1957), in cow's milk (Mellors & Harwalkar, 1968), and in bovine liver (Langley & Jevons, 1968) and serum (Courtois & Mangeot, 1972). Although there are no previous reports of neutral α-mannosidase activity in bovine tissues, it has been found in several other mammalian species (Suzuki *et al.*, 1970; Marsh & Gourlay, 1971; Bullock & Winchester, 1973). In mannosidosis of Angus cattle there is a marked decrease in the acidic α-mannosidase activity, and the residual activity has an optimum of pH6.6 in the tissues and of pH5.5–5.75 in plasma. To emphasize the difference between the tissues of a normal calf and a calf with mannosidosis, many of the following experiments have been performed with extracts of the kidney, a tissue in which the normal activity of acidic α-mannosidase is high.

Ion-exchange chromatography of α-mannosidase activity

To investigate whether distinct acidic and neutral enzymic components existed in normal tissues and whether specific acid components were absent in mannosidosis, the activity in all the tissues of a normal calf and of a calf with mannosidosis was analysed by ion-exchange chromatography on DEAE-cellulose (Fig. 2). The column eluates were assayed at pH4 and 7 to distinguish most clearly between possible acidic and neutral components. This ensured that one type of activity did not contribute to the assay of the other. Although the absolute amounts of activity and the relative proportions of the peaks varied from tissue to tissue, two peaks of acidic components (A and B) and one neutral (C) were found in all the normal tissues (Fig. 2). There was also some indication of minor components between peaks B and C in the elution profiles. The pH optima of the activity in the separated peaks were pH4.25–4.5 for components A and B and pH6.5–6.75 for component C. In some instances the activity–pH curves for components A and B appeared to have two maxima at about pH4 and 5. The unadsorbed acidic material from peak A was analysed further by chromatography on CM-cellulose (Fig. 3*a*). It was adsorbed on the CM-cellulose at pH5.0 and eluted as a single peak by a NaCl gradient. Thus there are three major enzymic components, two with acidic pH optima and one neutral in normal bovine tissues.

Only the neutral component C was found in comparable amounts in the mannosidosis tissues. The decrease in acidic activity indicated by the activity–pH curves was shown to be due to the virtual absence of both the acidic components A and B. The residual activity is due to the neutral component C and vestigial amounts of components A and B. A similar situation has been reported in the liver of a human patient with mannosidosis (Carroll *et al.*, 1972).

For the analysis of plasma, assays were performed at pH4 and at pH5.5, the pH optimum of the

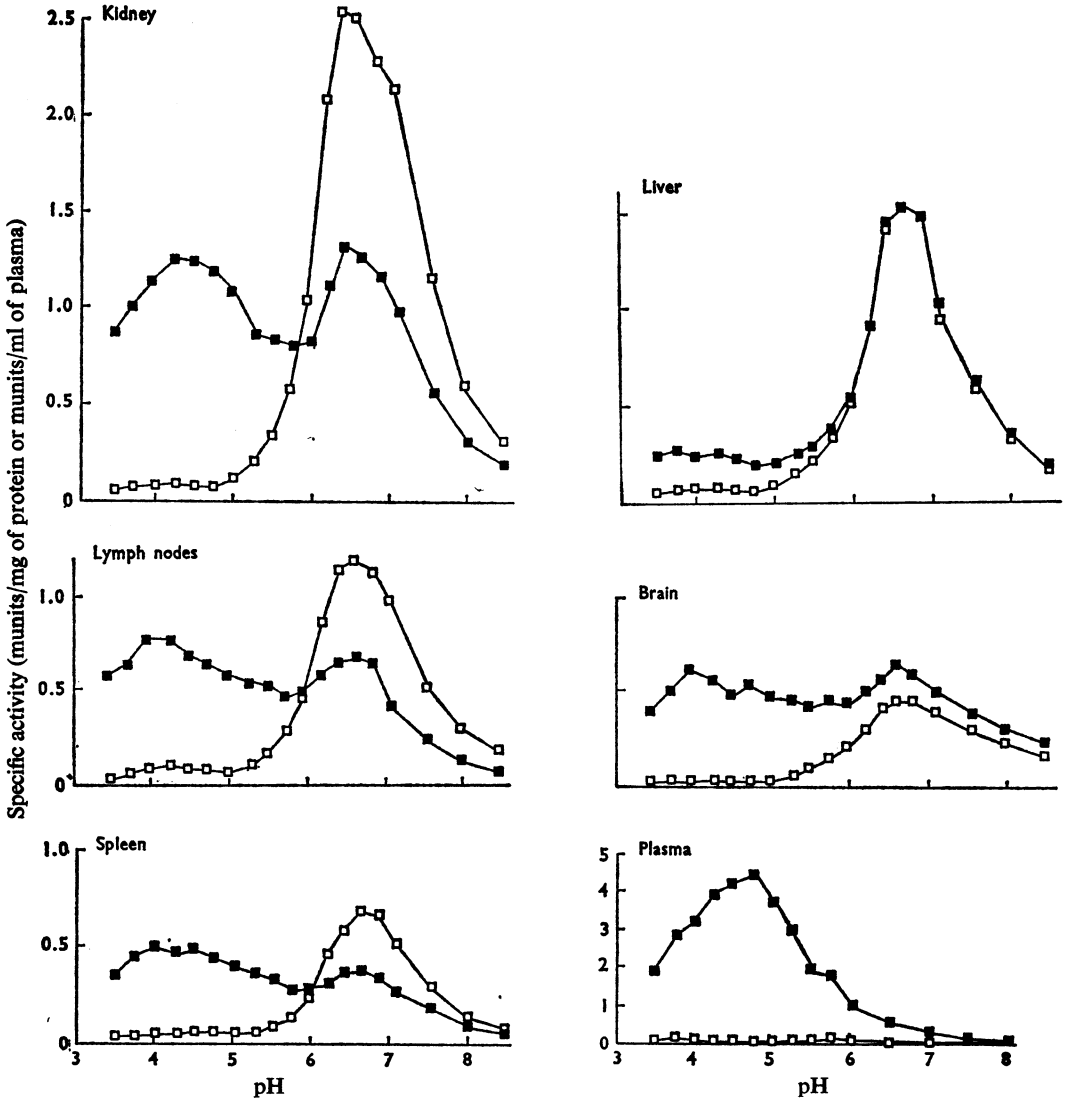

Fig. 1. *pH-dependence of α-mannosidase activity in the tissues of a normal calf and of a calf with mannosidosis*

The supernatants (0.1 ml) from homogenates (10%, w/v) of the tissues of a normal and a diseased calf were assayed for α-mannosidase activity at a series of pH values. Plasma (50 μl) was assayed directly. ■, Normal calf; □, calf with mannosidosis.

residual activity in the plasma of calves with mannosidosis. The α-mannosidase activity in normal calf plasma was not adsorbed on DEAE-cellulose at pH 6.0. Therefore the material from the unadsorbed peak, which had higher activity at pH 4.0 than at 5.5, was analysed on CM-cellulose at pH 5.0 (Fig. 3*b*). Two peaks were resolved, both of which contained material more active at pH 4.0 than at pH 5.5. The elution position of the larger component did not correspond to that of either tissue component A or B, but the smaller component was eluted in a position corresponding to tissue component A. Thus the predominant acidic component in plasma is different from the two tissue acidic components A and B. The small amount of activity in the plasma of a calf with mannosidosis was also unadsorbed on DEAE-cellulose at pH 6.0, but the breakthrough peak contained material with higher activity at pH 5.5

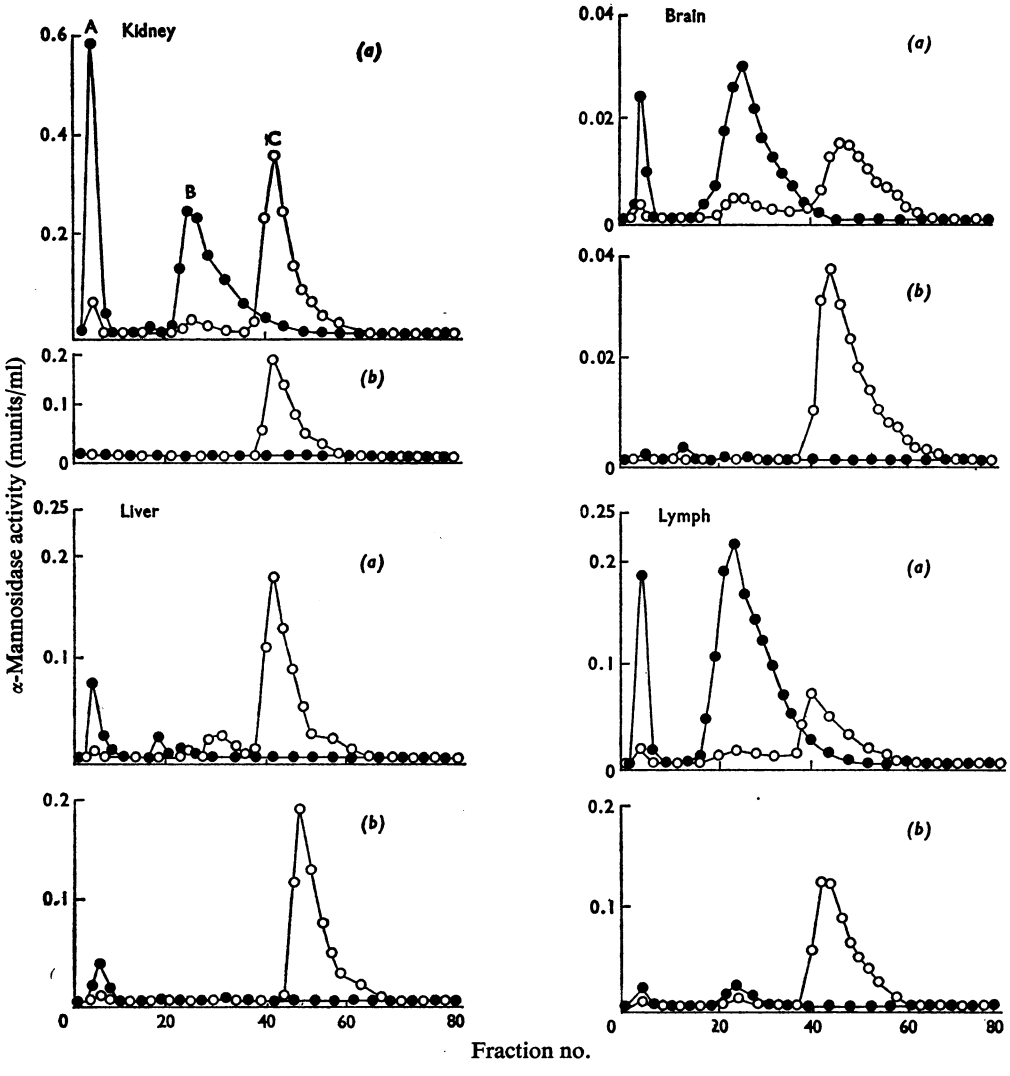

Fig. 2. *Chromatography on DEAE-cellulose of α-mannosidase activity in tissues of a normal calf and of a calf with mannosidosis*

Supernatants (2.0ml) from homogenates (10%, w/v) of the tissues of a normal and diseased calf were applied to the column. Samples (0.2ml) from the fractions were assayed for acidic and neutral α-mannosidase activity. For each tissue elution pattern (*a*) is for the normal animal and (*b*) for the calf with mannosidosis. ●, α-Mannosidase activity measured at pH4; ○, α-mannosidase activity measured at pH7.0. Discussion of peaks A, B and C is in the text.

than pH4.0. When plasma from a calf with mannosidosis was analysed on CM-cellulose, no peaks were detected in the eluate, presumably because of dilution of the low activity in the sample, so it was not possible to determine whether a distinct component with a pH optimum of 5.5 accounted for the residual activity in this plasma.

Starch-gel electrophoresis

The existence of multiple forms of α-mannosidase in normal bovine tissues was confirmed by starch-gel electrophoresis at pH7.0. It was possible to differentiate between the acidic and neutral activities by using two substrate solutions, buffered at pH4 and

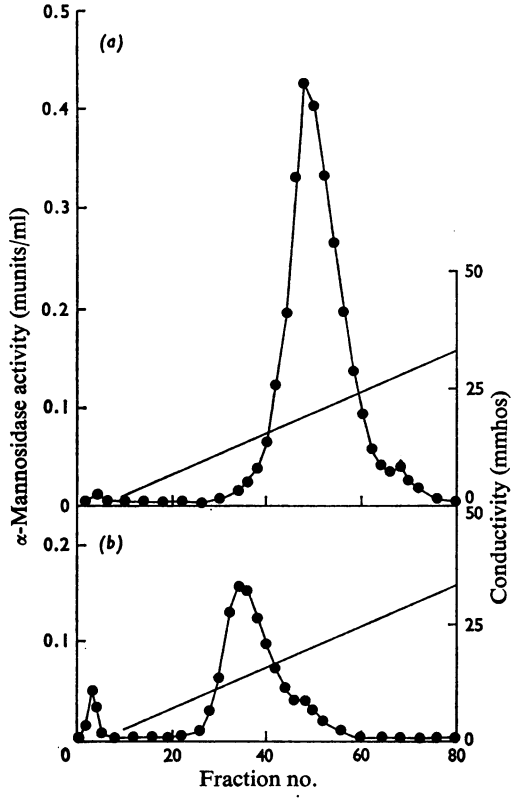

Fig. 3. *Chromatography on CM-cellulose of (a) α-mannosidase peak A from DEAE-cellulose chromatography and (b) normal calf plasma α-mannosidase activity*

A column (3cm×0.9cm diam.) of CM-cellulose was equilibrated in 10mM-sodium phosphate, pH5.0. Elution was started with the equilibration buffer and continued with a linear 0–0.3M-NaCl gradient in the same buffer. Fractions (1.5ml) were collected at 60ml/h. (*a*) α-Mannosidase peak A from the chromatography of a normal calf kidney on DEAE-cellulose. (*b*) Normal calf plasma (0.3ml) was dialysed against the initial elution buffer and diluted to 0.5ml before application to the column. ●, α-Mannosidase activity measured at pH4; ——, conductivity.

pH7 respectively, to detect the enzymic activity on the gel surface (Fig. 4). One neutral and two acidic bands were observed in preparations from all the normal tissues, but the acidic bands were almost absent in preparations from the tissues of the calf with mannosidosis. Component C from DEAE-cellulose was shown to correspond to the fast-moving neutral band and components A and B to the slower- and faster-moving acidic bands respectively. The width of band B was consistent with the existence of minor acidic components between components B

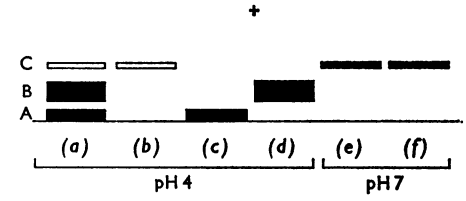

Fig. 4. *Starch-gel electrophoresis of α-mannosidase activity in normal and diseased calf kidney*

The activity in samples (*a*)–(*d*) was detected with substrate buffered at pH4 and in samples (*e*) and (*f*) with substrate buffered at pH7. (*a*) and (*e*), supernatant from homogenate (10%, w/v) of normal kidney; (*b*) and (*f*), supernatant from homogenate (10%, w/v) of diseased kidney; (*c*) and (*d*), peaks A and B respectively from chromatography on DEAE-cellulose of preparation from normal kidney.

and C in the DEAE-cellulose elution profile. Normal plasma only showed a single band near the origin, with a slightly lower mobility than the tissue A band. Three bands of α-mannosidase activity, two with acidic and one with neutral activity, have also been demonstrated in human tissue extracts by electrophoresis on cellulose acetate (Poenaru & Dreyfus, 1973).

Gel filtration

Gel filtration on Sephadex G-200 of a normal kidney extract demonstrated that the acidic and neutral α-mannosidase activities had different molecular sizes (Fig. 5*a*). The corresponding elution profile (Fig. 5*b*) for the activity in the kidney of the calf with mannosidosis showed that the residual neutral activity had the same molecular weight as the neutral activity in the normal tissues, 490000–560000. In contrast with the apparently homogeneous peak of neutral activity, the peak of acidic activity in the normal tissue appeared to be heterogeneous. Discernible maxima corresponded to molecular weights of 275000 to 390000, but there were also small amounts of activity at higher and lower molecular weights. Chromatography on DEAE-cellulose of material from the mid-points of the ascending and descending limbs of the acidic gel-filtration peak (materials I and II in Fig. 5*a*) showed that the ratio of component A to component B was greater on the descending limb. This suggested that the molecular weight of component A might be less than that of B. Gel filtration of the individual components A and B purified by ion-exchange chromatography supported this suggestion. Although the peaks still looked heterogeneous the activity maxima for components A and B corresponded to molecular weights of 280000 and 340000 respectively.

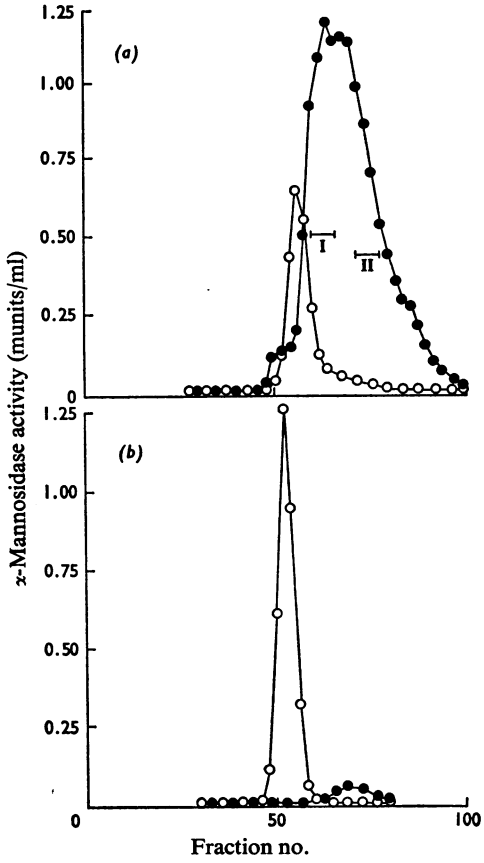

Fig. 5. *Gel filtration on Sephadex G-200 of the α-mannosidase activity in a normal and a diseased calf kidney*

Supernatants (2ml) from homogenates (20%, w/v) of (*a*) normal calf kidney and (*b*) kidney from calf with mannosidosis were applied to the column (34.5cm× 2.5cm diam.). Fractions (1.5ml) were collected at 14ml/h. Samples (0.2ml) from fractions were assayed for enzymic activity. ●, α-Mannosidase activity measured at pH4; ○, α-mannosidase activity measured at pH7. Fractions I and II are discussed in the text.

Most of the activity in normal plasma was excluded from the Sephadex matrix, indicating a large molecular weight, but there was a small peak in the position corresponding to component A. Both gel filtration and ion-exchange chromatography show that component A is a minor component of normal plasma and that the major plasma component is different from the two tissue acidic components, A and B.

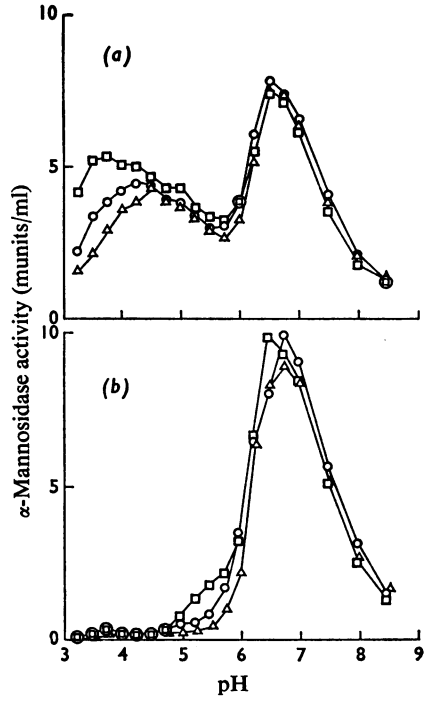

Fig. 6. *Effect of EDTA and Zn^{2+} on the α-mannosidase activity in normal calf kidney and in kidney of a calf with mannosidosis*

Samples (50μl) of the supernatants from a normal kidney (*a*) and a kidney from a calf with mannosidosis (*b*) were assayed at a series of pH values in the presence and absence of 0.5mM-EDTA or -$ZnSO_4$. ○, Control; □, $ZnSO_4$; △, EDTA.

Effect of Zn^{2+} and EDTA on calf kidney α-mannosidase activity

Mammalian acidic α-mannosidase activity has been shown to be Zn^{2+}-dependent and to be inactivated by EDTA (Snaith & Levvy, 1969; Bullock & Winchester, 1973). The neutral activity in rat liver is also affected by EDTA and various metal ions (Marsh & Gourlay, 1971). Therefore the effect of Zn^{2+} and EDTA on the acidic and neutral activities in normal kidney and in the kidney of a calf with mannosidosis was investigated (Fig. 6). Zn^{2+} (0.5mM) had a slight inhibitory effect on the neutral activity but increased the acidic activity and lowered the pH optimum to 3.75. In contrast EDTA increased the acidic optimum to pH4.5 and decreased the activity around pH3.75. An explanation of these observations could be that there is a form of α-mannosidase activity, optimal at pH3.75, which is activated by Zn^{2+} and inhibited by EDTA. There is also activity

optimal at pH4.5–5.0, which is unaffected by Zn^{2+} or EDTA. The apparent pH optimum in tissue homogenates would depend on the relative proportions of these two forms present. Support for this explanation comes from the different values of the pH optimum of the acidic activity in the various tissues and from the pH curves for the separated components A and B, which often showed two maxima at pH3.75–4.0 and 4.5–5.0.

Fig. 6(*b*) shows that the decreased acidic activity in the kidney of the calf with mannosidosis is not caused by a deficiency of Zn^{2+} or the presence of an inhibitory ion. However, Zn^{2+} did produce a significant increase in the activity around pH5.5, the optimum pH of the residual activity in the plasma of the calf with mannosidosis. Thus it is possible that the decrease of the acidic activity in the tissues of the calf with mannosidosis also results in residual potential activity optimal at pH5.5. An alternative explanation is that this activity, optimal at pH5.5, is a normal minor constituent of tissues but its presence is obscured by the large amounts of neutral and acidic activity. The residual activity in the plasma of the calf with mannosidosis was enhanced by the presence of Zn^{2+}.

Confirmation that EDTA had a selective inactivating effect on the acidic activity was obtained by preincubating a normal kidney extract with 1mM-EDTA for 20min at 37°C. Subsequent assays at various pH values showed that the activity at pH4.0 was decreased by 58%, but the activity at pH5.0–5.5 by only 5%. The effect of the EDTA on the individual components was investigated by chromatographing a normal kidney extract on DEAE-cellulose and assaying the eluate at pH4, 5.5 and 7 in the presence and absence of 1mM-EDTA. In the presence of EDTA the activity at pH4 in peaks A and B was decreased by 81 and 80% respectively and the activity at pH7 in peak C by 53%. However, the activity at pH5.5 in peaks A and B was only decreased by 12 and 19% respectively. Thus the EDTA-insensitive α-mannosidase activity is associated with both the acidic components A and B.

A marked distinction between the acidic and neutral activities was found when a normal kidney homogenate was assayed in the presence of 1mM-Co^{2+} or -Mn^{2+}. The neutral activity was increased by 40 and 50% and the acidic activity decreased by 25 and 27% by Co^{2+} and Mn^{2+} respectively. These results, together with the less dramatic effects of Zn^{2+} and EDTA, suggest that neutral α-mannosidase activity may also be a metalloenzyme. Co^{2+} and Mn^{2+} are also most effective in stabilizing rat liver neutral α-mannosidase activity (Marsh & Gourlay, 1971). Courtois & Mangeot (1972) have shown that although Co^{2+} has a remarkable activating effect on human serum α-mannosidase it has virtually no effect on bovine serum α-mannosidase. This is consistent with the observation that neutral α-mannosidase activity is absent from bovine plasma.

It has been shown that there is a deficiency of the two major acidic α-mannosidase components, A and B, in the tissues of an Angus calf with mannosidosis. Although a different acidic component accounts for most of the activity in plasma, it is also absent in mannosidosis. Therefore it is probable that this plasma component and tissue components A and B are all related and under the same genetic control. The neutral tissue component C, which is not decreased in mannosidosis, has different properties from components A and B. It is unlikely that it is under the same genetic control. The storage of mannose-containing oligosaccharides, coupled with the loss of acid α-mannosidase activity, supports the hypothesis that Angus cattle mannosidosis is a lysosomal storage disease. The presence of the remaining neutral α-mannosidase may reflect its possible non-lysosomal origin, as demonstrated in rat tissues by Marsh & Gourlay (1971).

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