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The Multiple Forms of α-D-Mannosidase in Human Plasma

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The acidic α-D-mannosidase in human plasma closely resembles liver acidic α-D-mannosidase in its affinity for concanavalin A–Sepharose, molecular weight and resolution into multiple components on DEAE-cellulose. A combination of chromatography on concanavalin A–Sepharose and gel filtration on Sephadex G-200 and Sepharose 6B suggests that four forms of intermediate α-D-mannosidase, which differ either in their molecular weight or affinity for concanavalin A, exist in human plasma. A practical classification and nomenclature for the multiple forms of intermediate α-D-mannosidase in plasma based on molecular weight and affinity for concanavalin A is proposed. Multiple forms of intermediate α-D-mannosidase were also observed by ion-exchange chromatography on DEAE-cellulose, but there was not a simple correlation between these forms and those obtained with the other separation procedures. The form of intermediate α-D-mannosidase least abundant in plasma, approx. 7% of the activity, has very similar properties to the neutral α-D-mannosidase in human liver. In contrast, the other three forms of intermediate α-D-mannosidase, which account for over 90% of the activity, do not appear to be present in liver, except perhaps in trace amounts.

Three types of α-D-mannosidase (EC 3.2.1.24) with different subcellular locations have been described in mammalian tissues. Acidic α-D-mannosidase, which has a pH optimum of 4.0–4.5, is a typical lysosomal hydrolase and is widely distributed (Snaith & Levvy, 1973). Neutral α-D-mannosidase with a pH optimum of 6.0–6.5 was first reported by Suzuki *et al.* (1969). It is located in the cytoplasmic fraction of rat liver (Marsh & Gourlay, 1971) and has been purified to homogeneity (Shoup & Touster, 1976). The third form of α-D-mannosidase, which has a pH optimum of 5.5, has been purified from a Golgi membrane fraction of rat liver (Dewald & Touster, 1973; Tulsiani *et al.*, 1977). Comparison of the properties of the three purified forms of α-D-mannosidase from rat liver indicates that they are distinct enzymes (Opheim & Touster, 1978).

Activity with a pH optimum of 5.5–6.0, intermediate between those of acid and neutral α-D-mannosidase, is also found in mammalian plasma/serum (Chéron *et al.*, 1975). It has been called intermediate α-D-mannosidase to distinguish it from the acidic and neutral α-D-mannosidases (Winchester *et al.*, 1976). Intermediate α-D-mannosidase with a pH optimum of 5.5–5.75 is the predominant form of the enzyme in human serum or plasma (Masson *et al.*, 1974; Hirani *et al.*, 1977). It differs from acidic α-D-mannosidase, which is present as a minor component in plasma, in its kinetic and physicochemical properties (Chéron *et al.*, 1975; Hultberg *et al.*, 1976; Hirani *et al.*, 1977; Kress & Miller, 1978). The two activities can be measured by a differential assay based on a difference in thermal stabilities (Winchester *et al.*, 1976; Hirani *et al.*, 1977). It has not been possible to detect neutral α-D-mannosidase in plasma by a similar assay procedure because it is less stable than the intermediate activity, which is present in excess. However, chromatography on DEAE-cellulose and concanavalin A–Sepharose has indicated that some neutral α-D-mannosidase might be present in serum (Hultberg *et al.*, 1976). There is a deficiency of acidic α-D-mannosidase in the plasma/serum and organs of patients with the lysosomal storage disease mannosidosis, but neutral and intermediate α-D-mannosidases are unaffected (Öckerman, 1967; Masson *et al.*, 1974). In contrast, an alteration in serum intermediate α-D-mannosidase activity has been reported in mucolipidosis II and III (Kress & Miller, 1978). In view of the importance of assaying the appropriate α-D-mannosidase in plasma/serum for the diagnosis of these inherited disorders, the multiple forms of the enzyme in plasma have been investigated further and compared with those in human liver.

Materials and Methods

Plasma

Venous blood from laboratory personnel was collected in tubes containing heparin. The plasma was separated on the same day by centrifugation at 1000*g* for 10min. Samples of plasma from a patient with mannosidosis were kindly provided by Dr. A. D.

Patrick of the Institute of Child Health, University of London (Guilford Street, London W.C.1, U.K.). All samples of plasma were stored at −20°C until required. Enzymic activities were stable during storage, but repeated freezing and thawing led to loss of activity.

Tissue

Post-mortem liver was stored at −20°C until required. Tissue homogenates (10%, w/v) were prepared at 4°C in the appropriate buffer for each separation procedure in a glass-walled Potter/Elvehjem homogenizer with a Teflon pestle. 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.

Assay of α-D-mannosidase

The α-D-mannosidase activity in plasma, tissue supernatants or in fractions obtained by the separation procedures was assayed by using the fluorigenic substrate 4-methylumbelliferyl α-D-mannopyranoside (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) as described previously (Winchester *et al.*, 1976; Hirani *et al.*, 1977). Activities measured at pH4.2, 5.6 and 6.5 were defined as acidic, intermediate and neutral α-mannosidase activity respectively. The McIlvaine (1921) phosphate/citrate buffer system was used to investigate the pH-dependence of the activity. One unit of activity is that amount of enzyme that transforms 1μmol of substrate/min under these conditions. The effect of Co^{2+} and Zn^{2+} ions on the α-D-mannosidase activity was investigated by including 2mM-$CoSO_4$ or 2mM-$ZnSO_4$ adjusted to the correct pH in the substrate solutions, giving a final concentration of 0.5mM-Co^{2+} or -Zn^{2+} in the assay mixture. Values of K_m were determined by the graphical method of Lineweaver & Burk (1934). A computer program was used to obtain the line of best fit by the least-squares method for the regression line.

Thermal stability

The thermal stability of the α-mannosidase activity in the fractions separated on concanavalin A–Sepharose was investigated by heating the samples at 55°C for 30min. At suitable intervals of time samples were removed, cooled and the residual activity was assayed at 37°C in the usual manner (Hirani *et al.*, 1977).

Chromatography on concanavalin A–Sepharose

Plasma (1ml) or tissue supernatant (1ml), which had been diluted with 1ml of 10mM-sodium phosphate buffer, pH6.8, containing 0.5M-NaCl and 0.1mM-$MgCl_2$, -$MnCl_2$ and -$CaCl_2$, was passed through a column (6cm×2cm diam.) of concanavalin A–Sepharose [Pharmacia (G.B.) Ltd., London W.5, U.K.], which had been equilibrated in the same buffer. The column was eluted with the equilibration buffer at 4°C. Fractions (2.2ml) were collected at a flow rate of 24ml/h until the A_{280} of the effluent fell below 0.1. The material that had bound to concanavalin A–Sepharose was eluted with 30ml of 0.5M-methyl α-D-mannopyranoside [Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K.] in the equilibration buffer. Fractions of plasma obtained by other separation procedures were equilibrated in the same buffer before analysis on concanavalin A–Sepharose.

Gel filtration on Sephadex G-200

Chromatography on Sephadex G-200 (Pharmacia) was carried out by using a column (31.5cm×2.6cm diam.) of Sephadex G-200 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 (147000), bovine γ-globulin (effectively 205000) [all from Sigma (London) Chemical Co.] and Blue Dextran (Pharmacia). Fractions (2.2ml) were collected at a flow rate of 14.4ml/h. A plot of the $K_{av.}$ values of the marker proteins against the logarithms of their molecular weights was used to estimate the molecular weights of the α-D-mannosidase components.

Gel filtration on Sepharose 6B

Sepharose 6B (Pharmacia) was equilibrated in 10mM-sodium phosphate buffer, pH6.0, containing 0.1M-NaCl, and packed in a column (42.5cm×1.6cm diam.). Samples of plasma, tissue supernatant or fractions of plasma or tissue obtained by other separation procedures were applied to the column and eluted with the equilibration buffer. Fractions (2ml) were collected at a flow rate of 14.4ml/h. The following protein standards, molecular weights in parentheses (Lehmann, 1970), were also run on the column: bovine thyroglobulin (600000); apoferritin (480000); catalase (230000); bovine γ-globulin (effectively 205000); and bovine serum albumin (67000) (all from Sigma).

Ion-exchange chromatography on DEAE-cellulose

DEAE-cellulose (Whatman DE-52; Whatman Lab Sales, Maidstone, Kent, U.K.) was equilibrated in 10mM-sodium phosphate buffer, pH6.0, and packed in disposable syringes (2cm×0.9cm diam.). Plasma,

tissue supernatant and fractions obtained by other separation procedures were dialysed against the equilibration buffer before chromatography. Elution was started with the equilibration buffer and continued with a linear NaCl gradient (0–0.2M) in 100ml of the same buffer. Fractions (2.2ml) were collected at a flow rate of 60ml/h and assayed for α-D-mannosidase activity. A larger column (5cm × 2cm diam.) was also used with a NaCl gradient (0–0.2M) in 200ml of the equilibration buffer.

Concentration of fractions

A Minicon Macrosolute Concentrator (Amicon, High Wycombe, Bucks., U.K.) with a cut-off at 15000 daltons was used to concentrate some separated fractions.

Results

The multiple forms of α-D-mannosidase in human plasma and liver have been compared by analysing normal plasma and liver by several protein-separation procedures. Samples of plasma that specifically lacked either acidic or intermediate α-D-mannosidase were also analysed. These were plasma from a patient with mannosidosis, which lacks the acidic activity, and plasma that had been heated at 55°C for 1h to denature selectively the intermediate activity (hereafter called heat-denatured plasma).

Chromatography on concanavalin A–Sepharose

The α-D-mannosidase activity in normal plasma was analysed by chromatography on concanavalin A–Sepharose (Fig. 1*a*). Whereas all the acidic α-D-mannosidase appeared to bind to the column, the intermediate α-D-mannosidase was recovered in both the unadsorbed and adsorbed fractions. When the activity in these two fractions was rechromatographed on the column, the activity originally in the unadsorbed fraction was again obtained in the unadsorbed fraction and vice versa for the activity originally in the adsorbed fraction. This showed that the separation of the intermediate activity into two forms was not due to overloading the column. Chromatography of the plasma from the patient with mannosidosis on concanavalin A–Sepharose confirmed that two forms of intermediate α-D-mannosidase differing in their affinity for the lectin existed in plasma (Fig. 1*b*). Approx. 30% of the intermediate activity was recovered in the unadsorbed fraction and 70% in the adsorbed fraction. The elution profile for the activity in the heat-denatured plasma showed that the α-D-mannosidase activity at pH4.0 in normal plasma was not due to the intermediate α-D-mannosidase but to acidic α-D-mannosidase, which binds to concanavalin A.

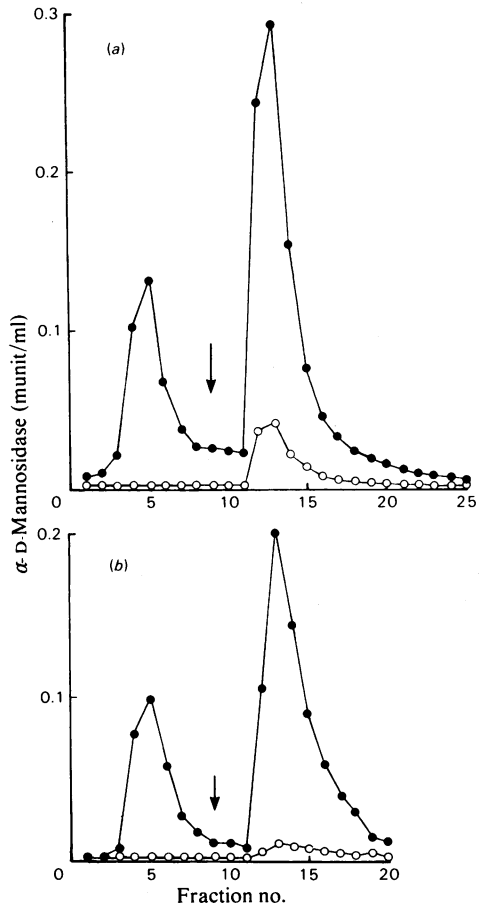

Fig. 1. *Chromatography on concanavalin A–Sepharose of (a) normal human plasma and (b) plasma from a patient with mannosidosis*

Plasma (1ml) was applied to the column in 10mM-sodium phosphate buffer, pH6.8, containing 0.5M-NaCl, 0.1mM-$MgCl_2$, 0.1mM-$CaCl_2$ and 0.1mM-$MnCl_2$, which was also used to elute the material that did not bind to the column. Elution was continued from fraction 9 with the same buffer containing in addition 0.5M-methyl α-D-mannopyranoside. The fractions were assayed for α-D-mannosidase at pH4.2 (○) and pH5.6 (●).

The properties of the α-D-mannosidase activity in the unadsorbed and adsorbed fractions obtained by chromatography of normal plasma on concanavalin A–Sepharose were compared. The activity–pH profiles showed that the activity in the unadsorbed fraction had a pH optimum of 5.75–6.0 and contained virtually no activity at pH4.0 (Fig. 2). In contrast, the adsorbed fraction contained both activity with a pH optimum of 5.75–6.0 and acidic α-D-mannosidase with a pH optimum of 4.25. The presence of the

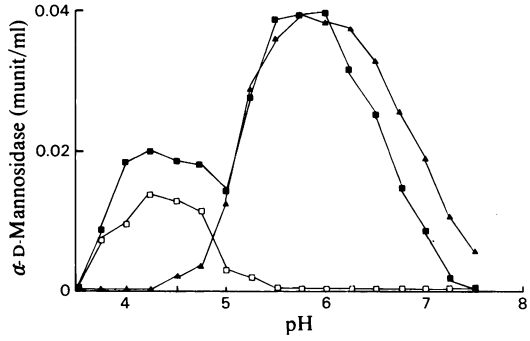

Fig. 2. *pH-dependence of the α-D-mannosidase activity in the fractions obtained by chromatography on concanavalin A–Sepharose*

▲, α-D-Mannosidase activity that did not bind to concanavalin A–Sepharose. Activity that did bind to concanavalin A–Sepharose before (■) and after (□) heating at 55°C for 30min.

acidic activity in the adsorbed fraction was confirmed by heating the material for 30min at 55°C, at which temperature the intermediate activity is denatured. The residual activity after heating had a pH optimum of 4.25 (Fig. 2). No activity remained in the unadsorbed fraction when it was heated under these conditions, indicating the absence of acidic activity. The activity in both fractions was stimulated by Co^{2+} (0.5mM), with maximal effect at pH5.5 and pH5.25 for the unadsorbed and adsorbed fractions respectively. Zn^{2+} (0.5mM) also stimulated the intermediate activity in both fractions with maximal effect at pH5.25. The acidic α-D-mannosidase in the adsorbed fraction was also activated by the Zn^{2+}.

To relate the multiple forms of α-D-mannosidase in plasma to those in liver, an extract of normal human liver was fractionated on the column of concanavalin A–Sepharose (Fig. 3). It has been shown previously that the acidic α-D-mannosidase in liver binds to concanavalin A, whereas the neutral α-D-mannosidase does not (Phillips *et al.*, 1976). The α-D-mannosidase in the liver fraction that did not bind to the column had a pH optimum of 6.25, compared with a pH optimum of 5.75–6.0 for the activity in the corresponding plasma fraction. It was stimulated by Co^{2+} (0.5mM) over the whole pH range but with greatest effect at pH5.5. Although Zn^{2+} (0.5mM) stimulated the activity over the range of pH4.25–6.25, it had a slight but reproducible inhibitory effect at higher pH values.

The values of K_m for the α-D-mannosidase activity in the fractions that did not bind to concanavalin A were 0.05mM at pH6.5 in liver and 4.23mM at pH5.6 in plasma. The value of K_m at pH5.6 for the liver activity was also 0.05mM. These observations suggest that the activity is not identical in the fractions from the two tissues.

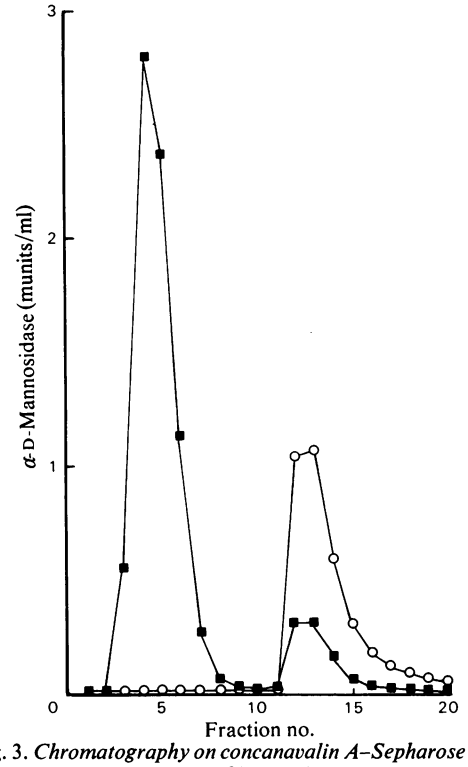

Fig. 3. *Chromatography on concanavalin A–Sepharose of an extract of human liver*

The supernatant (1ml) from an homogenate (20%, w/v) of human liver was applied to and eluted from the column under the same conditions as for Fig. 1. α-D-Mannosidase was assayed at pH4.2 (○) and at pH6.5 (■).

The activity in liver fraction that did bind to concanavalin A consisted predominantly of acidic α-D-mannosidase, which had a pH optimum of 4.25 and was activated and inhibited by Zn^{2+} and Co^{2+} respectively. However, the activity between pH5 and 6 was activated slightly by both Zn^{2+} and Co^{2+}, suggesting the presence of small amounts of the intermediate α-D-mannosidase in the liver.

Gel filtration on Sephadex G-200

Chromatography of normal human plasma on Sephadex G-200 showed that the acidic and intermediate α-D-mannosidases had different molecular weights (Fig. 4*a*). The acidic activity was present as a single peak with a mol.wt. of approx. 300000. A single peak of acidic activity was also obtained when the heat-denatured plasma was analysed. In contrast, the intermediate activity was resolved into two peaks, one in the void volume and the other corresponding to an apparent mol.wt. of 145000–160000. The

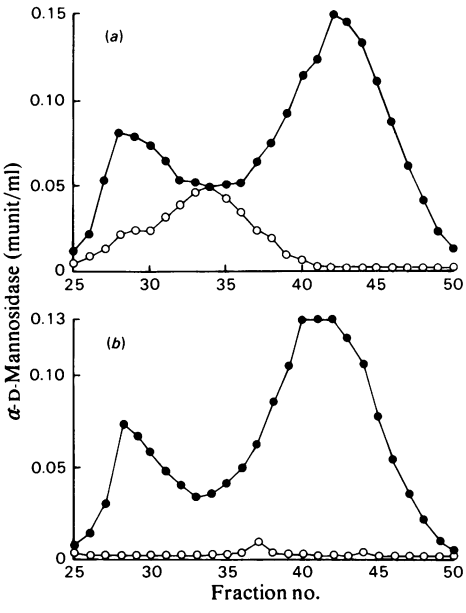

Fig. 4. *Gel filtration on Sephadex G-200 of (a) normal human plasma and (b) plasma from a patient with mannosidosis*

The details of chromatography are given in the Materials and Methods section. The fractions were assayed for α-D-mannosidase at pH4.2 (○) and at pH5.6 (●).

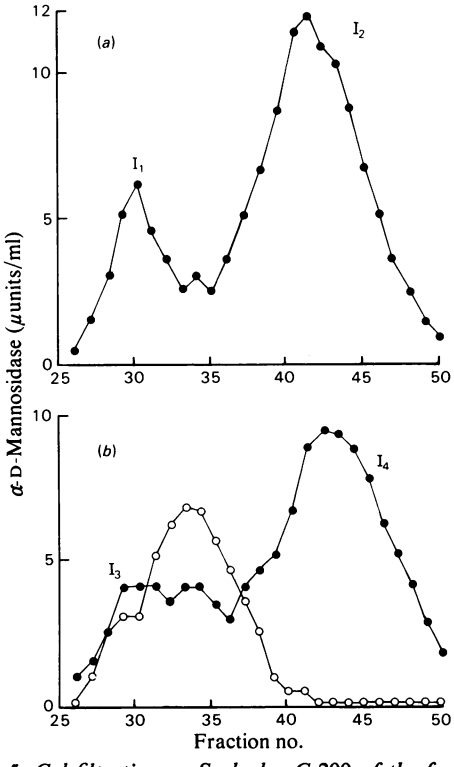

Fig. 5. *Gel filtration on Sephadex G-200 of the fractions obtained from plasma after chromatography on concanavalin A–Sepharose*

The fractions were assayed for α-D-mannosidase at pH4.2 (○) and at pH5.6 (●). (*a*) Material that did not bind to concanavalin A–Sepharose. (*b*) Material that did bind to concanavalin A–Sepharose.

activity in both peaks had a pH optimum of 5.75–6.0 and was activated by Co^{2+} and Zn^{2+}. The proportions of the intermediate activity in the two peaks were obtained from the elution profile for the plasma from the patient with mannosidosis (Fig. 4*b*). Approx. 23% of the recovered activity was in the peak excluded from the gel and 77% in the peak of activity retarded by the column. It was also possible, in the absence of the acidic activity, to obtain a better estimate of the molecular weight of the intermediate activity retarded by the column. A value of approx. 130000 was calculated.

The two fractions obtained when plasma is fractionated on concanavalin A–Sepharose were also analysed on Sephadex G-200 (Fig. 5). Intermediate activity with both the higher and lower molecular weights was present in both fractions. The complementary experiment was also performed. The two peaks of activity obtained when normal plasma is subjected to gel filtration were analysed by chromatography on concanavalin A–Sepharose. This confirmed that both the high- and low-molecular-weight forms of intermediate α-D-mannosidase consisted of activity that did, and activity that did not, bind to the lectin. These observations suggest that at least four forms of intermediate α-D-mannosidase occur in human plasma (I_1–I_4 in Fig. 5).

Gel filtration on Sepharose 6B

The nature of the intermediate α-D-mannosidase excluded from Sephadex G-200 was investigated further by chromatography of normal plasma on Sepharose 6B (Fig. 6*a*). The acidic α-D-mannosidase was again recovered as a single peak of activity. The intermediate α-D-mannosidase was also recovered as a single broad peak of activity, suggesting initially that there was a continuous gradation in size of the intermediate α-D-mannosidase. However, when the materials in the descending and ascending limbs of the peak were rechromatographed separately on the Sepharose 6B column, two discrete peaks of intermediate activity were resolved (Figs. 6*b* and 6*c*). The elution volume for the peak of the activity with the higher molecular weight was between those for the standards thyroglobulin (600000) and apoferritin (480000). This experiment showed that plasma intermediate α-D-mannosidase exists in forms with an apparent mol.wt. of either 480000–600000 or 130000.

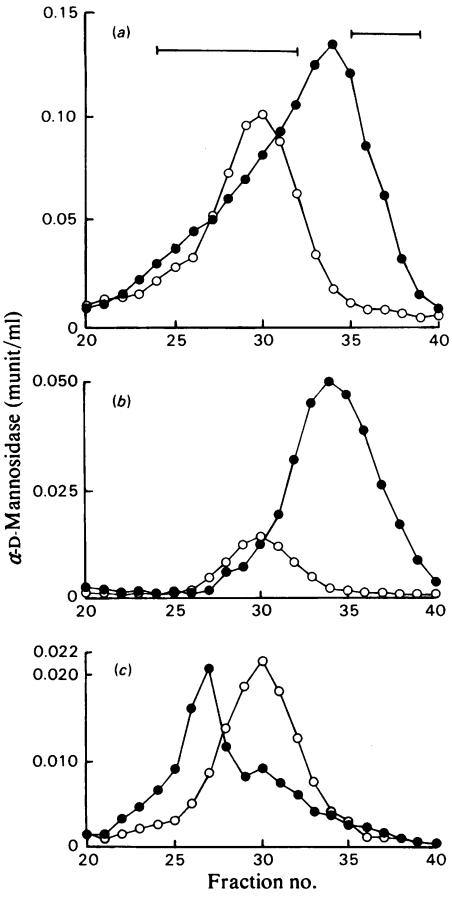

Fig. 6. *Gel filtration of human plasma on Sepharose 6B*
Normal human plasma was chromatographed on Sepharose 6B. The fractions (2.2ml) were assayed for α-D-mannosidase at pH4.2 (○) and at pH5.6 (●). The material in fractions 35–39 and in fractions 24–32 was pooled, concentrated and re-chromatographed on the column. (*a*) Normal human plasma, (*b*) fractions 35–39 and (*c*) fractions 24–32.

Chromatography on Sepharose 6B of the fractions from plasma separated on concanavalin A–Sepharose yielded broad and non-uniformly shaped peaks of intermediate activity (Figs. 7*a* and 7*b*). This again indicated that the high- and low-molecular-weight forms of intermediate α-D-mannosidase were present in both fractions separated on concanavalin A–Sepharose. The corresponding fractions from liver were also analysed on Sepharose 6B. Comparison of the elution profiles for the activity from plasma and liver that did not bind to concanavalin A (Figs. 7*a* and 7*c*) showed that the neutral α-D-mannosidase in liver had a similar molecular weight to the shoulder of intermediate activity, presumably α-D-mannosidase I_1, in plasma. There was no activity in liver corresponding to the major plasma component in this fraction, α-D-mannosidase I_2. The acidic α-D-mannosidase in the fractions from plasma and liver that did bind to concanavalin A–Sepharose had the same elution volume (Figs. 7*b* and 7*d*). However, there was no activity in the liver fraction corresponding to α-D-mannosidase I_3 in plasma and only a trace of activity corresponding to the major intermediate component in plasma, α-D-mannosidase I_4.

It is concluded from the gel-filtration and lectin-binding studies that plasma intermediate α-D-mannosidase can be separated into four forms. Two have a molecular weight of approx. 130000, but only one of them binds to concanavalin A–Sepharose. The other two have a higher molecular weight (500000–600000), with again only one of the forms binding to the lectin.

Ion-exchange chromatography on DEAE-cellulose

Four peaks of α-D-mannosidase activity were resolved when normal plasma was analysed by ion-exchange chromatography on DEAE-cellulose (Fig. 8*a*). The material in all the peaks had greater activity at pH5.6 than at pH4.2. A similar profile was obtained by Hultberg *et al.* (1976) and in accordance with their data, the four peaks were called A, B_1, B_2 and C. The corresponding profiles for the samples of heat-denatured plasma and plasma from patients with mannosidosis showed that the intermediate α-D-mannosidase was associated with peaks A, B and C but the acidic α-D-mannosidase was only associated with peaks A and B.

The activity in the peaks separated by ion-exchange chromatography was analysed by chromatography on concanavalin A–Sepharose and Sephadex G-200. The acidic α-D-mannosidase in the peaks A and B bound to the concanavalin A–Sepharose and had the same apparent molecular weight. The majority of the intermediate activity in the peaks was bound to concanavalin A, but there was always some unbound activity, highest in peak C. Whereas the intermediate activity in peak A was predominantly the low-molecular-weight form, approx. 30% of the intermediate activity in peak B_2 was the higher-molecular-weight form. It was not possible to analyse the activity in peaks B_1 and C by gel filtration because of the low recovery of activity achieved. Thus the peaks of activity obtained by ion-exchange chromatography were heterogeneous when analysed by the other separation procedures.

When an extract of human liver was chromatographed on DEAE-cellulose, three major peaks of activity were also obtained, A, B_1+B_2 and C. The activity in peaks A and B_1+B_2 has an acidic pH optimum and the activity in peak C a neutral pH optimum (Carroll *et al.*, 1972; Chester *et al.*, 1975).

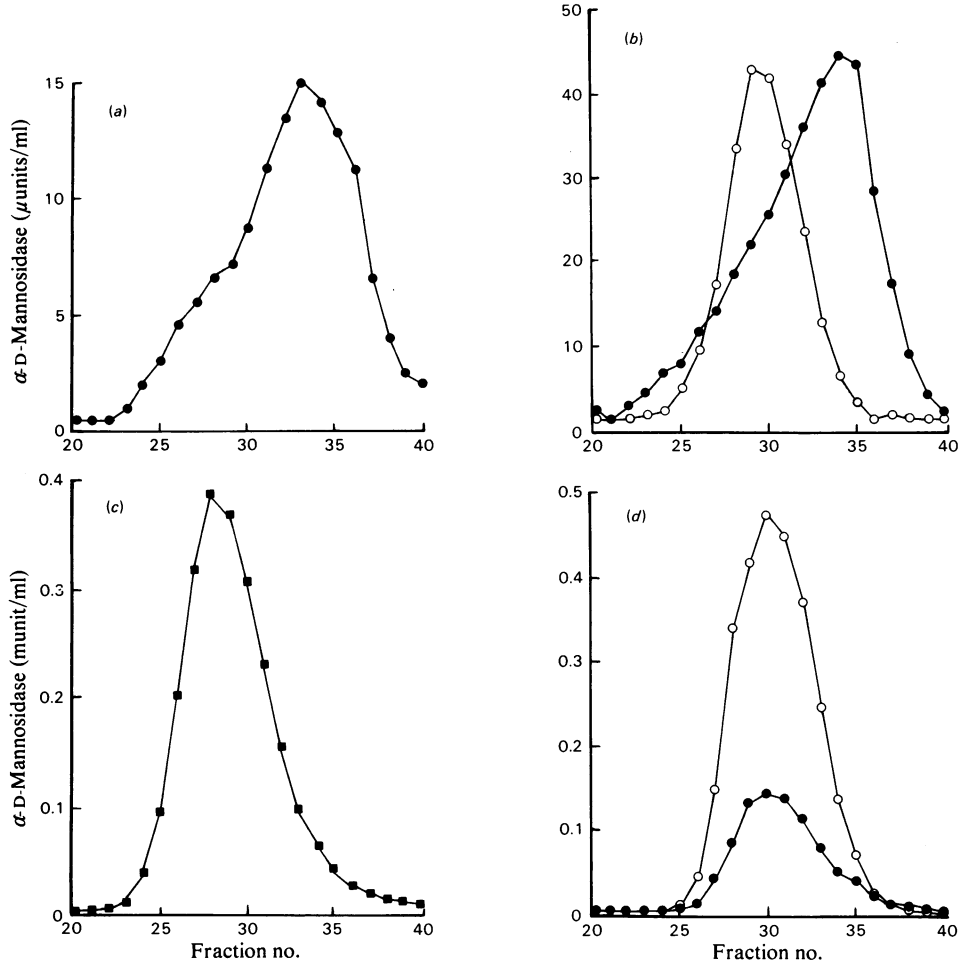

Fig. 7. *Gel filtration on Sepharose 6B of the fractions obtained from plasma and liver by chromatography on concanavalin A–Sepharose*

Material from plasma that (*a*) did not bind and (*b*) did bind to concanavalin A–Sepharose. Material from liver that (*c*) did not bind and (*d*) did bind to concanavalin A-Sepharose. α-D-Mannosidase was assayed at pH 4.2 (○), at pH 5.6 (●) and at pH 6.5 (■).

The intermediate activity in plasma associated with peaks A and B appears to be absent from the liver. However, some of the intermediate activity in plasma was eluted in a volume comparable with that for the neutral α-D-mannosidase, peak C, in liver. Gel filtration (Fig. 7) had suggested that intermediate α-D-mannosidase with a similar molecular weight to neutral α-D-mannosidase was present in the plasma fraction that did not bind to concanavalin A. Therefore this fraction and the corresponding fraction from liver were analysed on DEAE-cellulose (Figs. 8*b* and 8*c*). Virtually all the activity in the liver fraction was recovered as peak C, but activity was recovered in peaks A, B_1+B_2 and C for the plasma fraction. These experiments suggest that a small proportion of the intermediate activity in plasma is identical with the neutral α-D-mannosidase in peak C in liver, but that the intermediate activity in peaks A and B is due to components essentially absent from liver.

Discussion

The chromatographic behaviour of the acidic α-D-mannosidase in plasma is very similar to that of the acidic α-D-mannosidase in organs. Acidic α-D-mannosidase from both sources binds to concanavalin A–Sepharose (Phillips *et al.*, 1976), has a similar

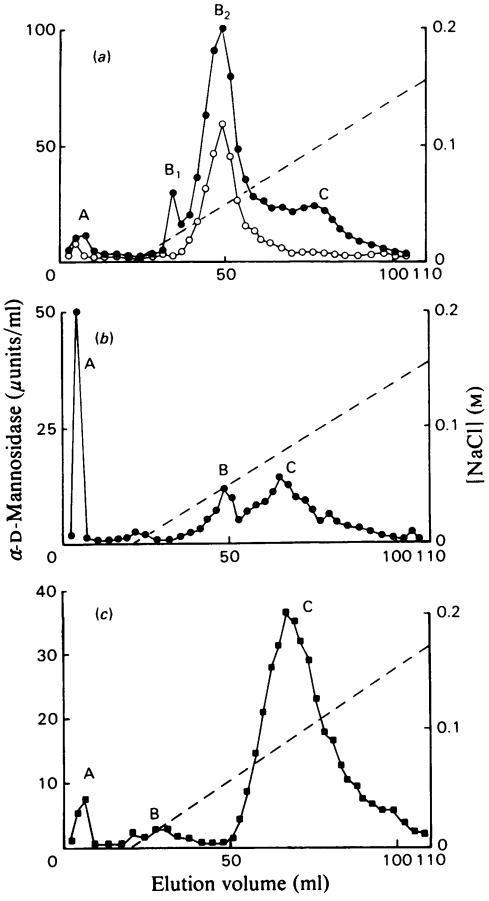

Fig. 8. *Chromatography on DEAE-cellulose*
The details of chromatography are given in the Materials and Methods section. α-D-Mannosidase was assayed at pH4.2 (○), pH5.6 (●) and pH6.5 (■). (*a*) Normal human plasma; (*b*) material from plasma that did not bind to concanavalin A; (*c*) material from liver that did not bind to concanavalin A. ----, NaCl gradient.

apparent molecular weight (Phillips *et al.*, 1974) and can be resolved into three peaks, A, B_1 and B_2 by ion-exchange chromatography on DEAE-cellulose (Chester *et al.*, 1975). The resolution between peaks B_1 and B_2 was never complete and the relative proportions of activity in all three peaks were variable. Peak B from liver was eluted at a slightly lower salt concentration than the corresponding peak in plasma. A similar observation has been made by Willcox & Renwick (1977).

A combination of chromatography on concanavalin A–Sepharose and gel filtration on Sephadex G-200 or Sepharose 6B suggests that four forms of intermediate α-D-mannosidase occur in normal human plasma in addition to the acidic α-D-mannosidase components. Four peaks of intermediate α-D-mannosidase were also observed after chromatography on DEAE-cellulose. However, it was not possible to correlate these peaks of activity with the four forms described above because the activity in the peaks was heterogeneous when analysed by the other separation procedures. Although none of the separation procedures gave complete resolution of the multiple forms, chromatography on concanavalin A did yield discrete, albeit heterogeneous, peaks of activity. Therefore it is proposed to base a definition and nomenclature for the multiple forms of intermediate α-D-mannosidase in plasma on this technique (Fig. 5 and Table 1). The material in plasma that is unadsorbed on concanavalin A–Sepharose contains intermediate α-D-mannosidase with mol.wts. of approx. 500000–600000 and of 130000–140000. These are designated α-D-mannosidase I_1 and I_2 respectively. The material in plasma that does bind to concanavalin A also contains intermediate α-D-mannosidase of these two molecular weights. These forms are designated α-D-mannosidase I_3 and I_4 respectively. It was possible to calculate the proportions of the intermediate activity in each of these forms from Figs. 1(*b*), 4(*b*) and 5(*a*) (see Table 1), if it was assumed that the proportions were the same in normal plasma and in plasma from a patient with mannosidosis. The intermediate activity in several homozygotes and heterozygotes for mannosidosis has been shown to be in the range for controls (Hirani *et al.*, 1977).

The relationship, if any, between the intermediate α-D-mannosidase forms with high and low molecular weights is not apparent. Aggregation of the smaller forms to produce large forms could be an explanation. However, when either the high- or low-molecular-weight fraction was rechromatographed on Sephadex G-200, virtually all the activity was recovered in fractions corresponding to the original molecular weight. Therefore the high- or low-molecular-weight forms are not readily interconvertible. Similarly rechromatography showed that the forms that bound to concanavalin A were not readily converted to non-binding forms and vice versa. These observations do not preclude the presence of common protein subunits, perhaps one with a high affinity for concanavalin A or another with a tendency to aggregate. The relative proportions of these and other subunits could determine the physiological form of the enzyme. The full explanation of these observations must await the purification and structural analysis of the enzyme.

It is necessary to relate our definition of the multiple forms of plasma intermediate α-D-mannosidase to the previous classification of Hultberg *et al.* (1976) (see Table 1). They suggest that tissue-like neutral α-D-mannosidase accounts for the plasma

Table 1. *Classification of multiple forms of α-D-mannosidase in human plasma*

Form	Intermediate α-D-mannosidase (%)	Chromatography on concanavalin A–Sepharose	10^{-3} × Approx. mol.wt. by gel filtration	Chromatography on DEAE-cellulose	Nomenclature of Hultberg *et al.* (1976)
Acidic α-D-mannosidase	—	Adsorbed	300	Peaks A, B_1 and B_2	A, B_1 and B_2
α-D-Mannosidase I_1	7	Unadsorbed	500–600	Largely peak C	Neutral form
α-D-Mannosidase I_2	23	Unadsorbed	130–140	and some peaks A and B	—
α-D-Mannosidase I_3	16	Adsorbed	500–600	Largely peak B and some peak C	S
α-D-Mannosidase I_4	54	Adsorbed	130–140	Trace of peak A	S

intermediate α-D-mannosidase that does not bind to concanavalin A–Sepharose. However, α-D-mannosidase I_1, which has a similar molecular weight and chromatographic properties to the neutral α-D-mannosidase in liver, only accounts for approx. 23% of the intermediate activity that does not bind to concanavalin A–Sepharose. α-D-Mannosidase I_2, which also does not bind to concanavalin A but which has a lower molecular weight than neutral and acidic α-D-mannosidase, accounts for the remainder. The intermediate α-D-mannosidase S of Hultberg *et al.* (1976) corresponds to α-D-mannosidase I_3 and I_4 of our definition.

The relationship of the intermediate and neutral α-D-mannosidase in plasma to the multiple forms of non-lysosomal α-D-mannosidase isolated from or described in several mammalian tissues is not understood (Tulsiani *et al.*, 1977; Shoup & Touster, 1976; Snaith, 1977; Burditt *et al.*, 1978).

The three forms of intermediate α-D-mannosidase most abundant in plasma, I_2, I_3 and I_4, do not appear to be present in liver except in trace amounts, which could originate from the blood in the liver sample. However, plasma intermediate α-D-mannosidase I_1 and the cytosolic neutral α-D-mannosidase are probably the same enzyme or very closely related forms.

Our definition of the multiple forms of intermediate α-D-mannosidase does not necessarily imply protein homogeneity. It is an operational definition. Microheterogeneity due to small differences in charge, which might be detected by ion-exchange chromatography, would not be observed by chromatography on concanavalin A–Sepharose or by gel filtration. This definition does, however, provide a clear experimental basis for describing the changes in these forms under different physiological conditions and in disease.

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