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Immunopurification and characterization of human α-L-iduronidase with the use of monoclonal antibodies

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α-L-Iduronidase from human liver was purified by a three-step five-column procedure and by immunoaffinity chromatography with a monoclonal antibody raised against purified enzyme. Seven bands identified by staining with Coomassie Blue had molecular masses of 74, 65, 60, 49, 44, 18 and 13 kDa and were present in both preparations of the liver enzyme. However, relative to the immunopurification procedure, α-L-iduronidase purified by the five-column procedure was considerably enriched in the 65 kDa polypeptide band. The seven bands were identified by Western-blot analysis with two different monoclonal antibodies raised against α-L-iduronidase. The chromatographic behaviour of α-L-iduronidase on the antibody column was dependent upon the quantity of enzyme loaded. Above a particular load concentration a single peak of enzyme activity was eluted, whereas at load concentrations below the critical value α-L-iduronidase was eluted in two peaks of activity, designated form I (eluted first) and form II (eluted second). The following properties of the two forms of α-L-iduronidase were determined. (1) The two forms from liver were composed of different proportions of the same seven polypeptides. (2) When individually rechromatographed on the antibody column, each form from liver shifted to a more retarded elution position but essentially retained its chromatographic behaviour relative to the other form. (3) Forms I and II of liver α-L-iduronidase showed no difference in their activities towards disaccharide substrates derived from two glycosaminoglycan sources, heparan sulphate and dermatan sulphate. (4) The native molecular size of forms I and II of liver α-L-iduronidase was 65 kDa as determined by gel-permeation chromatography. (5) Immunoaffinity chromatography of extracts of human lung and kidney resulted in the separation of α-L-iduronidase into two forms, each with different proportions of the seven common polypeptide species. (6) Lung forms I and II were taken up readily into cultured skin fibroblasts taken from a patient with α-L-iduronidase deficiency. Liver forms I and II were not taken up to any significant extent. Lung form II gave intracellular contents of α-L-iduronidase that were more than double those of normal control fibroblasts, whereas lung form I gave contents approximately equal to normal control values. We propose that all seven polypeptides are derived from a single α-L-iduronidase gene product, and that different proportions of these polypeptides can function as a single α-L-iduronidase entity. The separation of α-L-iduronidase into two forms by immunoaffinity chromatography may result from competition between affinity and self-association equilibria.

INTRODUCTION

Deficiency of the enzyme α-L-iduronidase (α-L-iduronide iduronohydrolase, EC 3.2.1.76) in humans results in the lysosomal accumulation of heparan sulphate and dermatan sulphate fragments. The clinical presentation of this disorder varies enormously and may be the result of different mutations in the α-L-iduronidase gene [1–5]. The two extremes of what is now becoming accepted as a spectrum of clinical severity in patients affected with a single mucopolysaccharidosis type I (MPS I) had previously been described as two distinct syndromes, Hurler and Scheie (McKusick 25280) [3]. Those affected severely may have mental retardation, severe skeletal deformities, coarse hirsute facies, corneal clouding and early death, whereas those at the mild end of this clinical spectrum may have normal intelligence and lifespan with mild skeletal deformity and possible corneal clouding [4]. Biochemical characterization reveals that residual α-L-iduronidase activity is equally low in cultured skin fibroblasts taken from patients at either end of the clinical spectrum [1,5]. Differences are observed in the kinetic properties of α-L-iduronidase activity present in fibroblasts from the two extreme patient phenotypes. Intermediate phenotypes are not readily discriminated in this way [5]. In order to understand the molecular basis of the phenotype differences between patients, we have sought to obtain pure α-L-iduronidase preparations from normal human tissues. We have reported the purification of α-L-iduronidase from human liver and have identified one polypeptide with molecular mass 65 kDa after electrophoresis on SDS/polyacrylamide gels by immunoprecipitation of radiolabelled α-L-iduronidase with a monoclonal antibody raised against the purified enzyme [6]. The molecular mass of the native liver α-L-iduronidase was 65 kDa. Some catalytic properties of the liver enzyme have been investigated [7]. Other laboratories have reported the purification of human α-L-iduronidase. Rome *et al.* [8], using kidney, obtained a single species on SDS/

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polyacrylamide-gel electrophoresis with molecular mass 30 kDa and a molecular mass of 60 kDa of the native enzyme by sedimentation. Schuchman *et al.* [9] separated lung-derived α-L-iduronidase into two forms by ion-exchange chromatography. The forms differed in their uptake into α-L-iduronidase-deficient cultured skin fibroblasts and in their polypeptide composition. The molecular masses of the native forms were 85 kDa (high-uptake form) and 68 kDa (low-uptake form).

We now report a comparison of the α-L-iduronidase prepared by the earlier five-column procedure [6] against preparations by the use of monoclonal immunoaffinity chromatography. Furthermore, we compare the SDS/polyacrylamide-gel-electrophoretic patterns of immunoaffinity-purified α-L-iduronidases from human kidney and lung with that of liver. In these preparations we have obtained separation of α-L-iduronidase into two forms. The reproducibility of the band patterns found in the two forms from each source, along with immunostaining evidence, strongly support the proposal that all seven bands derive from the α-L-iduronidase gene product.

METHODS AND MATERIALS

Id1A–Affi-Gel chromatography of α-L-iduronidase

The purification of human liver α-L-iduronidase by a three-step five-column procedure and the production and characterization of the monoclonal antibodies was as described previously [6]. The Id1A and Id6H antibodies were each linked to Affi-Gel 10 by using the manufacturer's instructions (Bio-Rad Laboratories, Hornsby, N.S.W., Australia). A column (1 cm × 19 cm) of Id1A–Affi-Gel and a pre-column of foetal-calf serum linked to Affi-Gel 10 (3 ml) was equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 0.5 M-NaCl, 10% (v/v) glycerol and 0.02% NaN_3 (buffer A; all buffers were filtered through a Millipore 0.45 μm-pore-size filter). After elution from Blue A–agarose, 200 ml of enzyme (in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.75 M-NaCl) was applied through the pre-column to the Id1A–Affi-Gel column and washed with a further 200 ml of buffer A. After uncoupling of the pre-column, the Id1A–Affi-Gel column was eluted with 50 mM-sodium citrate buffer, pH 4.0, containing 2.0 M-NaCl (buffer B) over 8 h and collected in 9 ml fractions in tubes containing 1 ml of 0.5 M-Tris/HCl buffer, pH 7.5, containing 0.5 mM-dithioerythritol. Fractions containing α-L-iduronidase activity were pooled and concentrated to less than 1 ml by ultrafiltration over a YM-10 membrane (Amicon, Melbourne, Vic., Australia). Human kidney and lung α-L-iduronidases were processed as for liver, but eluted from concanavalin A–Sepharose without the Blue A–agarose step, then applied to the Id1A–Affi-Gel column. The elution from concanavalin A–Sepharose was by recycling 1.5 litres of a solution of 0.62 M-methyl α-mannoside in 50 mM-sodium dimethylglutarate buffer, pH 6.0, containing 0.5 M-NaCl and 0.1 mM-dithioerythritol for 24 h. After recycling, the solution was displaced from the column by the same buffer without added methyl α-mannoside.

Id6H–Affi-Gel chromatography

Enzyme eluted from Id1A–Affi-Gel and dialysed against buffer A was concentrated to 10 ml by ultrafiltration and run on to the Id6H–Affi-Gel column (1 cm × 12 cm) equilibrated in buffer A at 4 °C. The column was clamped off for 16 h. A wash with buffer A was collected in ten 10 ml fractions of 10 min duration and was immediately followed by elution with buffer B and the collection of 7 ml fractions of 20 min duration each. Fractions were collected into 1 ml of 0.6 M-Tris/HCl buffer, pH 7.5, containing 0.1 mM-dithioerythritol.

SDS/polyacrylamide-gel electrophoresis

Samples for electrophoresis were prepared by co-precipitation with sodium deoxycholate as described previously [10], except that the acetone wash contained 1% (v/v) triethylamine. All samples were boiled for 2 min in sample buffer [11] containing 30 mM-dithioerythritol. Discontinuous gels (12%) were run according to the method of Laemmli [11]. Gels were fixed for 1 h in 40% (v/v) methanol/10% (v/v) acetic acid, then stained with colloidal Coomassie Blue 3GA (Gradipore, Pyrmont, N.S.W., Australia). Molecular masses of polypeptides were calculated from plots of relative mobility versus log(molecular mass) of the following standard proteins (Pharmacia, North Ryde, N.S.W., Australia): phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

Uptake into α-L-iduronidase-deficient cultured skin fibroblasts

Human skin fibroblasts from a patient with a clinically severe form of mucopolysaccharidosis type I were grown to confluency in 25 cm^2 flanks in BME medium (Flow Laboratories, Melbourne, Vic., Australia) containing 10% (v/v) foetal-calf serum (Gibco, Glen Waverly, Vic., Australia). Medium was removed and the cell layer was rinsed once with serum-free medium. The appropriate fraction from a single preparation of purified α-L-iduronidase in 0.5 ml was added to 2 ml of foetal-calf serum (which had been heat-treated at 50 °C for 20 min) before filter sterilization. The protein mixture was then added to 18 ml of sterile medium, mixed and divided into four 5 ml aliquots and added to the rinsed cell layers. At the indicated times the cells from each flask were harvested by trypsin release, washed (three times) in phosphate-buffered saline and pelleted [12]. The pellets were homogenized in 0.05 ml of 0.1% (v/v) Triton X-100 by repeated (six times) freeze–thawing and assayed for α-L-iduronidase activity.

Superose 12 f.p.l.c.

Gel-permeation chromatography was on a Superose 12 column (10 mm × 300 mm; Pharmacia) in 50 mM-sodium citrate buffer, pH 5.0, containing 0.5 M-NaCl at 25 °C. Samples of filtered α-L-iduronidase (200 μl) were injected, chromatographed at 0.5 ml/min, and collected in 0.5 ml fractions, which were assayed for α-L-iduronidase activity.

Enzyme assays

Unless otherwise stated, α-L-iduronidase activity was determined by the fluorogenic assay method described in ref. [6]. Enzyme units are expressed as μmol of product formed/min. Radioactive substrates α-L-iduronosyl-(α1→4)-anhydro[1-^{3}H]mannitol 6-sulphate and α-L-iduronosyl-(α1→3)-anhydro[1-^{3}H]talitol 4-sulphate were used to determine α-L-iduronidase activity towards

heparan sulphate- and dermatan sulphate-derived disaccharides (respectively) as previously described [7]. Both substrates were used at concentrations of 40 μM and incubations were for 30 min at 37 °C so that the product concentration did not exceed 20% of initial substrate concentrations. The reaction rate was linear over the 30 min period. Protein assay was by a modification of the Lowry procedure [13], with bovine serum albumin as standard.

Western blotting

Purified or partially purified α-L-iduronidase was electrophoresed in 10% polyacrylamide (as for the SDS/polyacrylamide-gel electrophoresis method). Nitrocellulose was soaked in 20 mM-Tris/HCl buffer, pH 7, containing 0.25 M-NaCl (buffer C), then placed in direct contact with the polyacrylamide gel, and any air bubbles were removed from the interface. The gel/nitrocellulose was sandwiched between Whatman filter paper and porous pads, then placed in an electro-blotting apparatus (Transphor; Hoefer, San Francisco, CA, U.S.A.). Electrophoretic transfer was towards the anode at 70 V and 800 mA for 1 h with water cooling. After the transfer, the nitrocellulose paper was immunostained. For immunostaining, residual protein-binding sites were blocked by incubating the nitrocellulose membrane overnight at 4 °C in buffer C containing 1% (w/v) ovalbumin or 1% (w/v) bovine serum albumin, 0.02% (v/v) Tween 20 and 3 mM-NaN_3. The membrane was then rinsed in buffer C, then placed in culture supernatant containing monoclonal antibody (Id1A or Id17A) for 4 h at 25 °C. The membrane was then washed three times in 100 ml of buffer C and incubated for 1 h at room temperature with a 1:200 dilution of a horseradish-peroxidase-conjugated sheep anti-(mouse immunoglobulin) antibody (Silenus, Melbourne, Vic., Australia) in buffer C containing 1% (w/v) ovalbumin. The membrane was then washed three times in 100 ml of buffer C and developed in 100 ml of colour-development reagent containing 60 mg of 4-chloro-1-naphthol and 60 μl of 30% (v/v) H_2O_2 dissolved in 100 ml of buffer C (as described by the manufacturer, Bio-Rad Laboratories).

RESULTS

Immunoaffinity purification of α-L-iduronidase

The most effective of a range of eluents, which maximized recovery and stability, was 50 mM-sodium citrate buffer, pH 4.0, containing 2.0 M-NaCl. Recovery was most effective when elution was over at least 8 h. Shorter elution times required greater elution volumes, resulting in loss of activity, probably by dilution [6]. All fractions contained 0.01 mM-dithioerythritol and 0.01% (v/v) Trition X-100 to decrease enzyme inactivation and to minimize adsorption. Fractions containing activity were pooled, concentrated and adjusted to pH 7.0 for long-term storage. Immunopurification of α-L-iduronidase from the Blue A–agarose eluate was at least 4 times more efficient than the five-column procedure (Table 1). The immunopurification step routinely attained 100% yield when the column was loaded with 10 units or more of α-L-iduronidase.

SDS/polyacrylamide-gel electrophoresis of Id1A–Affi-Gel eluates

The pattern on SDS/polyacrylamide-gel electrophoresis obtained with α-L-iduronidase prepared by the five-column procedure is compared with that obtained by immunopurification on Id1A–Affi-Gel in Fig. 1(*a*). Although bands other than the 65 kDa polypeptide were present in α-L-iduronidase prepared by the five-column procedure shown in Fig. 1(*a*), their amounts were very low, whereas the enzyme product of immunopurification, as seen in Fig. 1(*a*), clearly contains seven bands with molecular masses of 74, 65, 60, 49, 44, 18 and 13 kDa. Upon staining with silver (not shown) the bands at 18 and 13 kDa assumed intensities of staining equal to, if not greater than, those of the higher-molecular-mass species.

N-Terminal amino acid sequence data (P. R. Clements & J. J. Hopwood, unpublished work) reveal that the 65 kDa and 60 kDa species have identical sequences up to 25 residues, and that the 49 kDa and 44 kDa species share a common *N*-terminal sequence that is different from that of the 65 kDa and 60 kDa species. The 13 kDa peptide appears to contain two different *N*-terminal sequences, which are both different from those of the 65 kDa and 60 kDa species and the 49 kDa and 44 kDa species.

Western blotting and partial epitope characterization

Liver α-L-iduronidase that had been subjected to SDS/polyacrylamide-gel electrophoresis was electroblotted on to nitrocellulose membrane, and immunostained with Id1A monoclonal antibody. Id1A detected polypeptides of molecular masses 74, 65, 49 and 44 kDa

Table 1. Purification of liver α-L-iduronidase

These values shown for method 2 are from a separate preparation from those shown for method 1, and, for comparison, have been corrected to correspond with the method-1 values. For most preparations using method 2, more than 10 units were applied to Id1A–Affi-Gel by combining two or more eluates from step 2 of method 1. This routinely gave recoveries of 100%.

	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Method 1. Five-column procedure				
1. High-speed supernatant	8.1	0.0029	1	100
2. Concanavalin A–Sepharose/Blue A–agarose	7.8	0.111	382	96
3. CM-Sepharose/Bio-Gel HT	1.9	0.874	3000	23
4. Cu^{2+}-chelating Sepharose	1.5	50	172000	18
Method 2. Immunopurification after step 2 above				
1. Id1A–Affi-Gel	6.5	50	172000	80

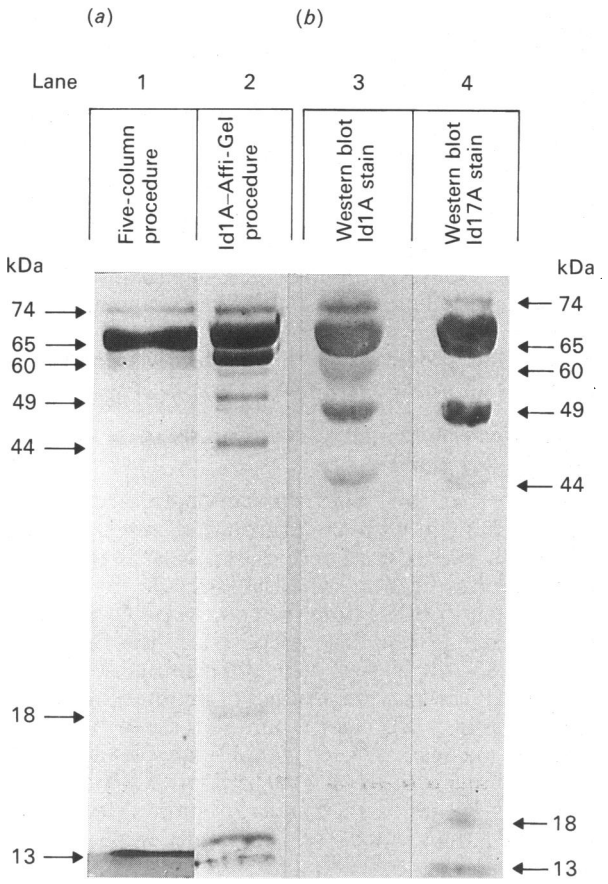


Fig. 1. Comparison of α-L-iduronidase from preparations by methods 1 and 2 shown in Table 1 and immunostaining of Western blots

(*a*) Comparison of polypeptides in liver α-L-iduronidase purified by the five-column procedure (method 1) and by Id1A–Affi-Gel immunoaffinity chromatography (method 2) by SDS/polyacrylamide-gel-electrophoretic (12% acrylamide) analysis. Molecular mass is indicated as determined from a plot of distances migrated of standard proteins against the logarithms of the molecular masses. Lane 1, five-column procedure, gel from ref. [6], 0.25 unit loaded; lane 2, ID1A–Affi-Gel eluate, 0.5 unit loaded. (*b*) Western blot of α-L-iduronidase prepared by method 2 and transferred from the SDS/polyacrylamide gel (10% acrylamide) on to nitrocellulose. Each lane had 1.0 unit of α-L-iduronidase loaded. Detection was by the monoclonal antibodies Id1A (lane 3) and Id17A (lane 4) followed by horseradish-peroxidase-conjugated sheep anti-(mouse immunoglobulin) antibody.

(Fig. 1b). Id1A reacted weakly with the 60 kDa species, but did not react with the 18 kDa and 13 kDa species. A Western blot was immunostained with another monoclonal antibody, Id17A, which reacts with a different epitope from that recognized by Id1A. This was demonstrated by the reaction of Id17A with the 18 kDa and 13 kDa species present in blots of Id1A-immunopurified preparations of α-L-iduronidase. Id1A and Id17A displayed a common reactivity on immunoblots with the 74 kDa, 65 kDa and 49 kDa bands, but Id17A reacted very weakly with the 44 kDa band and did not detect the 60 kDa species.

Both Id1A and Id17A monoclonal antibodies bind purified α-L-iduronidase as determined by e.l.i.s.a. and immunoprecipitate active enzyme by the method described in ref. [6]. In preliminary experiments the common expression of the Id1A and Id17A epitopes on α-L-iduronidase was demonstrated by a two-site e.l.i.s.a. Briefly, Id1A or Id17A was bound to a solid phase and used to absorb α-L-iduronidase, and then this complex was probed with a biotin-conjugated Id1A antibody and developed with a streptavidin–biotin–horseradish peroxidase complex detection reagent (Amersham, Sydney, N.S.W., Australia). In this system Id1A–biotin only reacted with α-L-iduronidase bound to Id17A (as opposed to that bound by Id1A), indicating (*a*) that only one Id1A epitope is expressed on the native α-L-iduronidase, (*b*) that the Id1A and Id17A epitopes are co-expressed on the α-L-iduronidase molecule and (*c*) that Id1A and Id17A recognize distinct epitopes. The removal of *N*-linked carbohydrate residues from α-L-iduronidase with glycopeptidase F failed to affect the binding of both Id1A and Id17A, as demonstrated in Western-blot experiments (P. McCourt & D. Brooks, unpublished work).

Two forms of α-L-iduronidase

The chromatographic behaviour of α-L-iduronidase activity on Id1A–Affi-Gel was dependent upon the amount of enzyme loaded on to the column. When 17 units of enzyme were applied the elution profile was as shown in Fig. 2(*a*), with a sharp front of activity being eluted after application of 2 column volumes of eluent and with a tailing of activity at the trailing edge of the peak. However, separation of α-L-iduronidase into two forms (I and II) by immunoaffinity chromatography was observed when the Id1A–Affi-Gel column was loaded with 3.5 units, as shown in Fig. 2(*b*). SDS/polyacrylamide-gel electrophoresis of fractions across the elution profile of the Id1A–Affi-Gel column are shown in Fig. 2(*c*). A progression from a set of lower-molecular-mass bands in form I with bands at 60 kDa and 44 kDa predominating to a set with bands in form II at 65 kDa and 49 kDa predominating can be seen. All seven bands are present across the gel, but the relative intensity of each band varies. The earlier-eluted form I has apparently a lower affinity than form II for Id1A–Affi-Gel. Chromatography of forms I and II individually on Superose 12 resulted in the elution of activity in identical volumes that corresponded to both forms having a molecular mass of 65 kDa as determined by a plot of log(molecular mass) of standard proteins against $K_{av.}$ values. This is consistent with previous molecular-mass values obtained for the native enzyme from human liver but with the caution that these values are highly variable subject to solvent conditions of pH and ionic strength [6].

Activity of forms I and II towards disaccharide substrates

Forms I and II showed no difference in their activities towards the two substrates α-L-iduronosyl-(α1→4)-anhydro[1-^{3}H]mannitol 6-sulphate and α-L-iduronosyl-(α1→3)-anhydro[1-^{3}H]talitol 4-sulphate, which are derived from, and retain some structural features of, the two natural substrates heparan sulphate and dermatan sulphate respectively (Table 2).

Repeat Id1A–Affi-Gel immunoaffinity chromatography

Fractions pooled to yield forms I and II (Fig. 3*a*) were rechromatographed separately on IdIA–Affi-Gel (Fig.

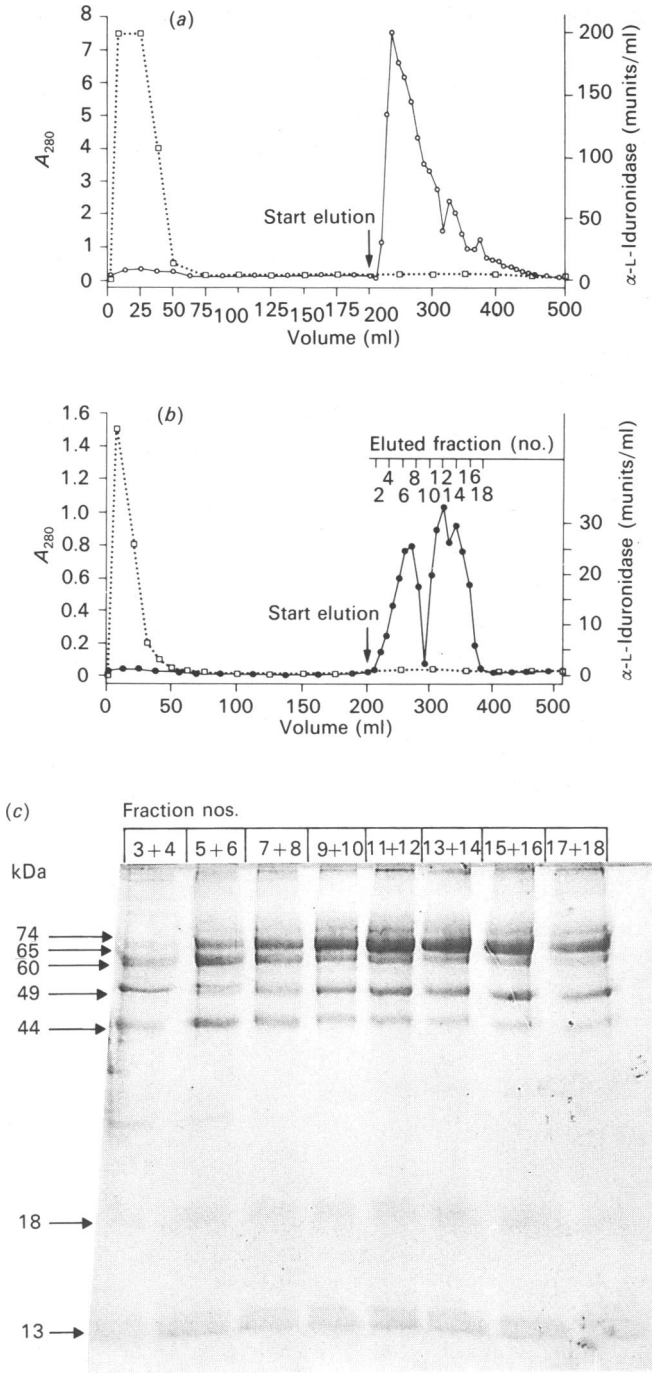


Fig. 2. Id1A–Affi-Gel chromatography of different amounts of α-L-iduronidase

(*a*) Id1A–Affi-Gel chromatography of 17 units of liver α-L-iduronidase. Protein (□ · · · □) was determined as A_{280} and α-L-iduronidase activity (○—○) is given in munits/ml. (*b*) Separation of α-L-iduronidase into two forms by Id1A immunoaffinity chromatography. Chromatography of 3.5 units of liver α-L-iduronidase on Id1A–Affi-Gel was as described in the Methods and materials section. Form I appears in fractions 3–10, and form II in fractions 11–18. Protein (□ · · · □) was determined as A_{280} and α-L-iduronidase activity (●—●) is given in munits/ml. (*c*) Fractions across the Id1A–Affi-Gel elution profile from (*b*) were combined in pairs and the total material was subjected to SDS/polyacrylamide-gel electrophoresis. Paired fraction numbers are indicated above the lanes and molecular mass of polypeptides is indicated on the side of the gel. The amount of α-L-iduronidase loaded on to the gel was as follows: fraction 3+4, 0.12 unit; fraction 5+6, 0.33 unit; fraction 7+8, 0.05 unit; fraction 9+10, 0.19 unit; fraction 11+12, 0.48 unit; fraction 13+14, 0.59 unit; fraction 15+16, 0.53 unit; fraction 17+18, 0.23 unit.

3*b*) and each peak of α-L-iduronidase activity was eluted later than in the first chromatography. The amount of enzyme activity re-applied to Id1A–Affi-Gel was decreased to 25% (form I) and 15% (form II) of the original activities, as a result of the process of concentration and dialysis required to restore each pooled eluted form to the conditions (buffer A) necessary for binding to Id1A–Affi-Gel. These losses are consistent with the findings of previous reports of the physical properties of α-L-iduronidase [6,8,9], where adsorption on surfaces and losses on dialysis were described.

SDS/polyacrylamide-gel electrophoresis of the eluate from the initial separation into forms I and II of 8.5 units of α-L-iduronidase on Id1A–Affi-Gel is shown in Fig. 4(*a*). Alongside these are shown the eluates that resulted from rechromatography of each individual form I and form II on Id1A–Affi-Gel. The pattern of form I, where the 60 kDa and 44 kDa bands predominate, was retained after rechromatography and the relative intensity of staining of the bands was similar. In form II first eluate the 65 kDa and 49 kDa bands appear to predominate, and after rechromatography this becomes even more obvious.

Id6H–Affi-Gel chromatography of α-L-iduronidase

A third monoclonal antibody (designated Id6H) was raised against a different epitope from that recognized by Id1A (as determined by e.l.i.s.a.; D. A. Brooks, unpublished work). The optimum conditions for Id6H–Affi-Gel chromatography of α-L-iduronidase preparations were determined in a similar series of experiments to those described above for Id1A–Affi-Gel. The conditions selected as optimal were not compatible with stability of α-L-iduronidase, so buffer A was used to apply and wash the Id1A–Affi-Gel, and buffer B was used for elution. These conditions, although resulting in a lower loading than optimal, were necessary to obtain pure preparations of eluted enzyme. The time required for maximum binding to Id6H–Affi-Gel was 16 h, and the applied enzyme was concentrated in order to occupy a single column volume (10 ml), in contrast with the loading of Id1A with 200 ml of enzyme solution.

Forms I and II from the experiment shown in Fig. 4(*a*) were rechromatographed on a column of Id6H–Affi-Gel. Id6H–Affi-Gel did not discriminate between the affinity of forms I and II of liver α-L-iduronidase, both of which, when separately applied to Id6H–Affi-Gel, were eluted from the column in sharp peaks with the same elution volumes. The pattern of bands in form II that were eluted from the Id6H–Affi-Gel column (Fig. 4*a*, lane 5), although faint, was the same as the pattern from the Id1A–Affi-Gel column. Insufficient form I material bound to obtain stained bands on a gel.

Id1A–Affi-Gel chromatography of different tissue extracts

Extracts of liver, lung and kidney were processed as for liver (see the Methods and materials section) but were eluted from concanavalin A–Sepharose without recycling over Blue A–agarose. Each concanavalin A–Sepharose

Table 2. Activities of liver α-L-iduronidase forms I and II towards substrates derived from heparan sulphate and dermatan sulphate

Radiolabelled substrates derived from heparan sulphate, namely α-L-iduronosyl-(α1→4)-anhydro[1-^{3}H]mannitol 6-sulphate (IdoAanM6S), and from dermatan sulphate, namely α-L-iduronosyl-(α1→3)-anhydro[1-^{3}H]talitol 4-sulphate (IdoAanT4S), were incubated with enzyme from peak fractions shown in Fig. 2(*b*), which represent form I (fraction 8) and form II (fraction 13). The incubation conditions are as described in the Methods and materials section. Abbreviations: HS, heparan sulphate; DS, dermatan sulphate.

Fraction (from Fig. 2*b*)		α-L-Iduronidase activity (munits/ml) IdoAanM6S (HS-derived)	IdoAanT4S (DS-derived)	Ratio HS/DS
8	Form I	51.7	19.3	2.7
13	Form II	130.0	51.5	2.5
	Ratio form II/I	2.5	2.7	

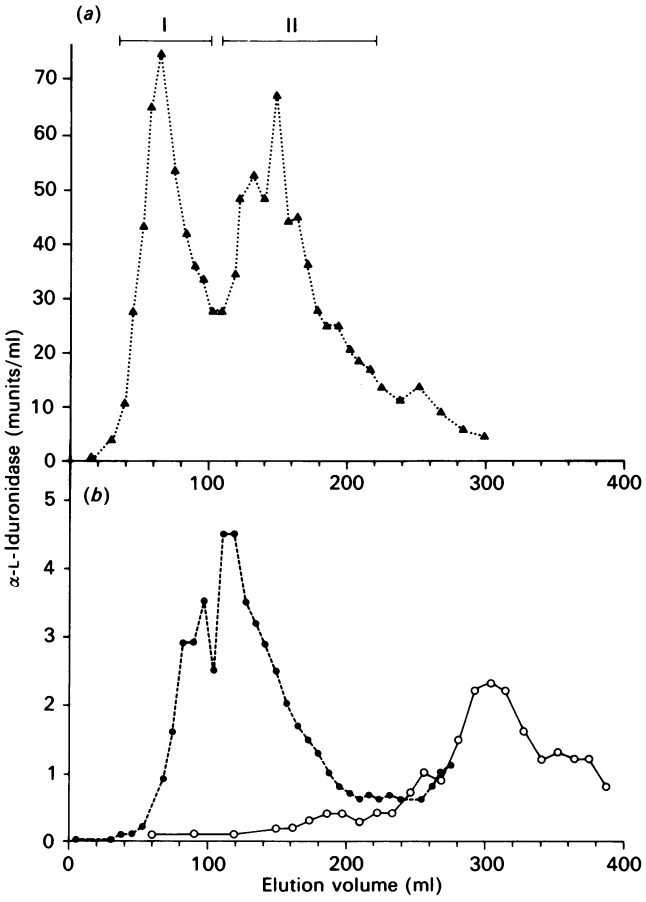


Fig. 3. Id1A–Affi-Gel chromatography and rechromatography of α-L-iduronidase

(*a*) First run, elution profile only (▲···▲). The Id1A column was loaded with 8.5 units of liver α-L-iduronidase and chromatography was as described in the Methods and materials section. The peaks were pooled separately as shown and a portion of each was retained for SDS/polyacrylamide-gel electrophoresis. (*b*) Second run, elution profile only (pool I, 1.0 unit, ●---●; pool II, 0.6 unit, ○—○). Each pool was concentrated separately and dialysed into buffer A for rechromatography on Id1A–Affi-Gel. The rechromatography eluates were subjected to SDS/polyacrylamide-gel electrophoresis as shown in lanes 2 and 4 in Fig. 4(*a*). Form II was also rechromatographed on Id6H–Affi-Gel and the pattern on SDS/polyacrylamide-gel electrophoresis is shown in lane 5 in Fig. 4(*a*).

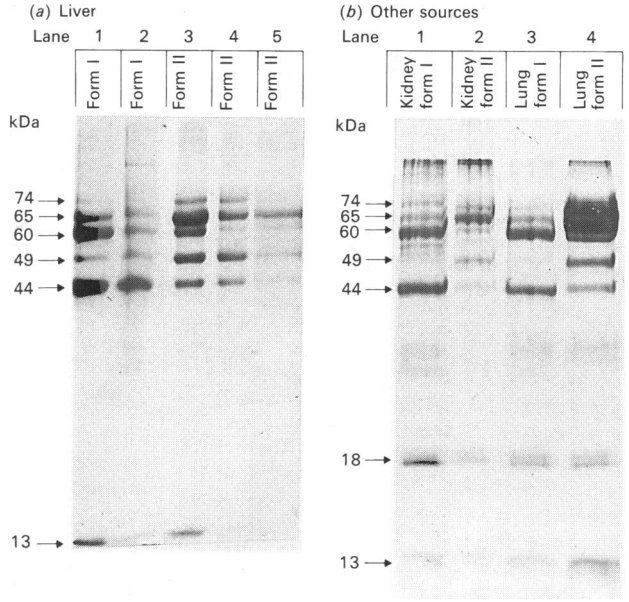


Fig. 4. SDS/polyacrylamide-gel electrophoresis of forms I and II of α-L-iduronidase from liver, kidney and lung with rechromatography of the liver forms

(*a*) SDS/polyacrylamide-gel electrophoresis of liver α-L-iduronidase eluted as form I (0.5 unit) (lane 1) and form II (0.5 unit) (lane 3) from Id1A–Affi-Gel as shown in Fig. 3(*a*); rechromatography of form I on Id1A–Affi-Gel (0.3 unit) (lane 2); rechromatography of form II on Id1A–Affi-Gel (0.15 unit) (lane 4) and on Id6H–Affi-Gel (0.05 unit) (lane 5). Molecular mass is indicated as determined from a plot of distances migrated of standard proteins against the logarithms of their molecular masses. (*b*) SDS/polyacrylamide-gel electrophoresis of α-L-iduronidase from other tissues. Kidney form I (0.5 unit) (lane 1); kidney form II (0.5 unit) (lane 2); lung form I (0.5 unit) (lane 3); lung form II (0.5 unit) (lane 4).

eluate was applied to the Id1A–Affi-Gel column and eluted under the standard conditions described in the Methods and materials section. Comparison of the separated forms by SDS/polyacrylamide-gel electrophoresis (Fig. 4*b*) shows similar trends in the patterns of the low-affinity and high-affinity forms (I and II respectively), with a lower-molecular-mass set of bands present in form I in each case. In form I of kidney, liver and lung the 60 kDa and 44 kDa species are predominant. Form II of lung, however, contains a 74 kDa species that is present in similar amounts to the 65 kDa, 60 kDa and 49 kDa species. A double band at approx. 200 kDa can be seen in Fig. 4(*b*) in both kidney and lung.

Uptake into MPS I fibroblasts

α-L-Iduronidase forms I and II from liver and lung were tested for uptake into α-L-iduronidase-deficient cultured skin fibroblasts (MPS I cells) by their addition to the cells in culture medium and assay of the intracellular activity after an appropriate time in culture. Form II from lung was the most effective at correcting the deficiency of α-L-iduronidase and resulted in an intracellular enzyme activity that, after 2 days in culture, was double that found in normal skin fibroblasts cultured in the absence of added α-L-iduronidase (Fig. 5). The addition of form I from lung gave 90% of normal values after 20 h in culture, but forms I and II from liver were taken up by the MPS I cells to a very small extent, which was not maintained.

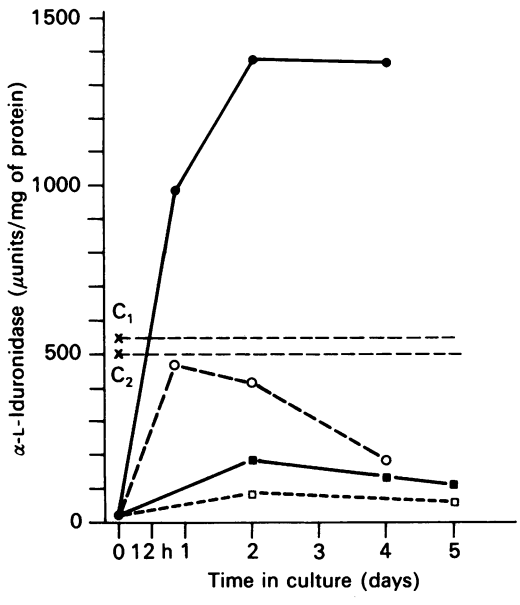


Fig. 5. Behaviour of liver and lung forms I and II of α-L-iduronidase when added to the culture medium of skin fibroblasts from a patient with severe α-L-iduronidase deficiency

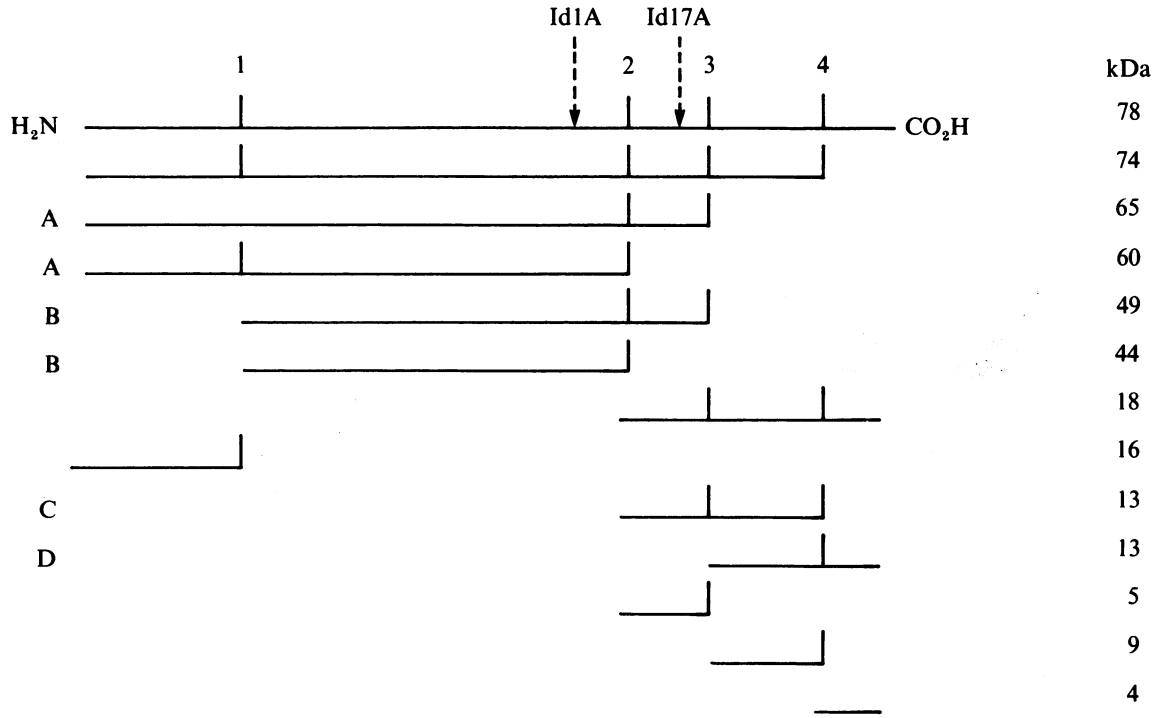
Activities of α-L-iduronidase are shown in skin fibroblasts from two normal controls, C_1 and C_2 (×---) and in α-L-iduronidase-deficient cells after the indicated times in culture in the presence of liver form I (0.56 unit) (□--□) liver form II (0.57 unit) (■—■), lung form I (1.09 units) (○--○) and lung form II (0.95 unit) (●—●).

DISCUSSION

The purification of human liver α-L-iduronidase by monoclonal immunoaffinity chromatography enhanced the yields over the previously published five-column procedure from 16% to 80%. α-L-Iduronidase produced by the immunoaffinity procedure contained seven polypeptides as determined by SDS/polyacrylamide-gel electrophoresis. The following evidence established that none of the seven polypeptides was a contaminant, but that all represent processed forms of α-L-iduronidase. Firstly, the enzyme resulting from immunopurification with the use of a monospecific antibody should, under the stringent washing conditions used, be free of all but the most tightly bound, and therefore specifically interacting, proteins. Secondly, the pattern of seven bands was reproduced in many α-L-iduronidase preparations, with only minor variations in intensity of staining of each polypeptide band. Thirdly, the 65 kDa band is the most intense in preparations of α-L-iduronidase from the five-column procedure, but the other six bands were present in minor amounts that varied from one preparative batch to the next. Fourthly, all seven bands were identified by Western-blot analysis with the monoclonal antibodies Id1A and Id17A, which recognize different epitopes on α-L-iduronidase, and this provided definitive confirmation that the bands were from α-L-iduronidase. Fifthly, the seven-band pattern, with minor variations, was repeated in α-L-iduronidase immunoaffinity-purified from other human tissues, namely kidney and lung. Sixthly, the immunoaffinity procedure allowed separation of all three tissue sources of α-L-iduronidase into two forms with different populations of the seven bands. In all cases the low-affinity form I contained a set of low-molecular-mass bands, whereas the high-affinity form II contained a set of bands with higher molecular-mass values. Finally, the bands in form II were also repeated after rechromatography with a third monoclonal antibody, Id6H. These observations support a case for the seven bands having all arisen from a single precursor α-L-iduronidase molecule.

The separation of α-L-iduronidase into two forms was dependent upon the amount of enzyme units loaded on to the column. The separation may therefore occur as a result of competing binding equilibria involving interaction with self [6] and with the monoclonal-antibody column. In order to establish a basis for the two forms, several properties were investigated. Fractions across the Id1A–Affi-Gel elution profile, in which the two forms were clearly separated, show a gradual progression from the polypeptides with lower molecular mass (i.e. 60 kDa and 44 kDa) at the start of the profile towards the polypeptides with higher molecular mass (i.e. 65 kDa and 49 kDa) at the later-eluted stages of the profile. Although this progression does not explain the physical separation of the two forms, it is suggestive of a complex compositional arrangement within the forms. There may not be homogeneity within a form, but the profile suggests that a high content of the lower-molecular-mass polypeptides may account for the binding properties of form I to Id1A–Affi-Gel, and similarly for form II with the higher-molecular-mass components.

A native molecular mass of 65 kDa for both form I and form II allows us to speculate that the different



Scheme 1. Proposed sites of proteolytic processing of α-L-iduronidase

The proposed 78 kDa precursor may be processed by different proteolytic events at sites 1, 2, 3 and 4. A single clip at site 2 on the 78 kDa precursor produces the 60 kDa and 18 kDa polypeptides. The 18 kDa component can be clipped at site 3 to produce a 13 kDa polypeptide with an Id17A epitope and a 5 kDa component, which may be lost from the system. Proteolysis at site 3 on the 78 kDa precursor would yield the 65 kDa component containing both Id1A and Id17A epitopes and the 13 kDa polypeptides without the Id17A epitope. The proposed 78 kDa precursor may also be processed at site 4 to produce 74 kDa and 4 kDa fragments. Proteolysis of the 74 kDa polypeptide at site 2 would produce a 60 kDa polypeptide and a 14 kDa component with the Id17A epitope. Proteolysis of 74 kDa at site 3 would yield a 65 kDa polypeptide by removal of a 9 kDa fragment without the Id17A epitope, which may be unstable and removed from the system. The 65 kDa and 60 kDa components may be clipped at site 1 to produce a 16 kDa plus a 49 kDa associated system and a 16 kDa plus a 44 kDa associated system respectively. The 16 kDa component may be unstable and is removed or may not be separated from the 18 kDa fraction on SDS/polyacrylamide gel electrophoresis.

polypeptides comprising forms I and II are derived from the same parent polypeptide, which is cleaved in different ways to produce fragments that remain associated in the active enzyme molecule. One model (see Scheme 1) can be constructed to provide a mechanism for the production of seven polypeptides from a single precursor α-L-iduronidase. This working model takes into account the *N*-terminal amino acid sequence data showing the same (A) sequence for 65 kDa and 60 kDa polypeptides, which is different from the same (B) sequence observed for 49 and 44 kDa polypeptides, which in turn are different from the two sequences (C and D) found with the 13 kDa polypeptides. The model also needs to account for the presence of one Id1A epitope on 74 kDa, 65 kDa, 60 kDa, 49 kDa and 44 kDa polypeptides, and one Id17A epitope on 74 kDa, 65 kDa, 49 kDa, 18 kDa and 13 kDa polypeptides. The model proposes that there may be a short-lived 78 kDa precursor form of α-L-iduronidase, which may be similar to the 76 kDa precursor observed in cultured skin fibroblasts [14]. As illustrated in Scheme 1, the 78 kDa precursor may be processed at four different proteolytic cleavage sites. *N*-Terminal amino acid analysis and further monoclonal-antibody epitope mapping will be useful in the determination of which, if any, of these proposals is correct.

Lysosomal enzymes undergo a series of processing steps on their way to the lysosome. Trimming of the glycosyl moieties occurs in the Golgi apparatus where, finally, phosphorylation occurs, before exocytotic transfer to the primary lysosome. Enzymes then remain active with half-lives of weeks in the lysosome, during which time they may accumulate a series of proteolytic clips and oligosaccharide trimmings while remaining active and intact. Some of this processing may be required for some aspects of functional control of the enzyme, whereas other processing may be part of the turnover event of the enzyme itself. At some point, the number of cleavages may result in the enzyme being directed towards final degradation. Thus what is defined as a mature lysosomal enzyme may be a mixture of differently cleaved forms that together form a non-homogeneous array of active enzyme species. The variation between tissue types may reflect different populations of proteinases in different cell types. Gupta *et al.* have demonstrated that in different cultured cell types a precursor protein is processed to give different maturation products [15]. Hasilik & Neufeld [16] have used polyclonal antibodies to examine the processing of several lysosomal enzymes in cultured human skin fibroblasts. Each enzyme appears to produce a series of proteolytic fragments during its lifetime as active enzyme. Similarly, for α-L-iduronidase,

Myerowitz & Neufeld [14] applied this technique using polyclonal antibodies raised against the human kidney enzyme [8]. Conversion in human fibroblasts was from a 76 kDa species, which appeared to be the precursor form, into a 72 kDa species and then a 66 kDa species over 24 h and 48 h chase periods. These species are similar to those that we observed in immunoaffinity-purified preparations in the high-molecular-mass range, and it may be that the lower-molecular-mass forms (49 and 44 kDa) will be observed if the maturation experiments are continued for longer chase periods.

Forms I and II from liver showed no difference in their activities towards disaccharide substrates derived from the natural substrates heparan sulphate and dermatan sulphate. This counters the possibility that the two forms had arisen to catalyse separately the degradation of the two glycosaminoglycans, which have a wide but unequal tissue distribution. Both glycosaminoglycans accumulate in the tissues and urine of all known patients with α-L-iduronidase deficiency. A proposal [17] of two forms of α-L-iduronidase acting on different substrates has not been substantiated. The possibility remains that complexes may form within the lysosome to bring together one group of enzymes that degrade only dermatan sulphate, and another group that degrade only heparan sulphate. Complex formations of this type would improve the efficiency of breakdown of the glycosaminoglycan fragments by removing the products of each enzyme as they form. We have observed significant product and substrate inhibition in kinetic studies *in vitro* with purified lysosomal enzymes involved in glycosaminoglycan degradation [2,18,19], and this effect would be relieved by the formation of multi-enzyme complexes.

The differences that we observed in the uptake properties of the enzyme from different tissues may relate to glycosyl moieties. Sando & Neufeld showed that cells deficient in α-L-iduronidase could be 'corrected' for that deficiency by co-culture with normal cells [20]. Investigation of this phenomenon has led to the understanding of cellular uptake processes and the biogenesis of lysosomes via the mannose 6-phosphate receptors [21]. The phosphorylated mannose chains may not be present on all enzyme molecules, however, and conversely some tissue types such as liver do not require the mannose 6-phosphate receptors for assembly of lysosomal enzymes [21]. Therefore it is not surprising that α-L-iduronidase from lung is taken up by cultured skin fibroblasts differently from that from liver.

A difference in uptake has been shown for the two forms isolated from human lung [9]. However, the lung forms isolated from human lung [9]. However, the lung forms described by Schuchman *et al.* [9] showed some similarities in polypeptide composition with our 75 kDa, 65 kDa and 40/45 kDa bands, whereas the high-uptake form showed greater intensity in the high-molecular-mass bands at 72/75 kDa and 67 kDa, with a trace at 94 kDa. Bands at 43/48 kDa and 37 kDa were also present. Although not identical with the patterns that we observe from immunopurified α-L-iduronidase (lacking the lower-molecular-mass bands that we observe at 18 kDa and 13 kDa), the Schuchman *et al.* [9] data have greater similarity than the single 30 kDa polypeptide reported for α-L-iduronidase purified from human kidney [8]. Although our five-column procedure may have preferentially purified the 65 kDa species at the expense of the other six, the purification procedure used by Schuchman *et al.* [9] may favour high-molecular-mass species, including a 94 kDa species that we have not observed.

Multiple forms of lysosomal enzymes, which have presented unique problems to enzymologists, are being observed with increasing frequency [2,22]. Our experience with enzymes purified from human liver has been that, whereas α-L-iduronidase presents with seven polypeptides, iduronate 2-sulphatase preparations contain two forms with several bands (J. Bielicki, P. Clements & J. Hopwood, unpublished work), and glucosamine 6-sulphatase can be resolved into two major forms comprising one and two polypeptides respectively, three in all [23]. Sulphamate sulphohydrolase, however, has been purified as single polypeptide species [19], and galactosamine 4-sulphatase contains two polypeptides linked by a disulphide bridge [24]. The latter is the only other enzyme to have been purified in high yield by us by the use of monoclonal immunoaffinity chromatography, and it may be that this technique will allow the identification of more polypeptide species in preparations of glucosamine 6-sulphatase, sulphamate sulphohydrolase and iduronate 2-sulphatase. α-L-Iduronidase remains as an extreme example of multiple polypeptides in the lysosomal enzymes, and its purification and establishment of purity represent the challenge that this group of enzymes presents to the protein chemist. We suggest that the challenge is met when monoclonal antibodies can be used to resolve the dilemma.

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