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THE COMMON IDENTITY OF FIVE GLYCOSIDASES IN HUMAN LIVER

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Summary

An enzyme has been isolated from human liver by DEAE-cellulose chromatography and has been shown by competitive substrate inhibition to be capable of hydrolysing synthetic β-D-galactosides, β-D-glucosides, β-D-fucosides, β-D-xylosides, and α-L-arabinosides. Another form of α-L-arabinosidase activity elutes with the major β-D-galactosidase component on DEAE-chromatography, but has a different identity on the basis of its stability at 4°C.

Liver samples from patients with Gaucher's disease are deficient in β-D-fucosidase as well as β-D-glucosidase activity.

Introduction

The enzymes responsible for the catabolism of carbohydrate-containing compounds have been studied extensively. These glycosidases (EC 3.2.1.-) generally occur in a soluble form after tissue homogenisation and enzymic activity is frequently present in more than one molecular form. Consequently the purification of these enzymes often presents considerable problems.

β-D-Galactosidase has been shown to exist in up to four forms depending on the source of the enzyme and the method of purification. Frequently at least one of these forms has been associated with another glycosidase activity, leading to the suggestion that some of these enzymes have multiple specificities. In non-mammalian sources the common identity has been proposed for β-D-galactosidase, β-D-glucosidase, β-D-fucosidase, and α-L-arabinosidase in almond emulsin on the basis of inhibition by aldono-(1-5)-lactones [1]. In barley, using the same techniques, it was suggested that the β-D-galactosidase is associated with α-L-arabinosidase activity, and that a separate β-D-glucosidase is associated with β-D-fucosidase [1]. In the limpet, *Patella vulgata*, β-D-glucosidase, β-D-fucosidase, and α-L-arabinosidase were thought to have a common identity, but separate from β-D-galactosidase [1], again based on inhibition studies using lactones. However, in the marine gastropod, *Turbo cornutus*, one of two forms of

β-D-galactosidase purified by gel filtration and ion exchange chromatography was also found to have β-D-fucosidase, β-D-glucosidase, and α-L-arabinosidase activities [2].

In the rat, an epididymal β-D-galactosidase exhibited β-D-fucosidase and α-L-arabinosidase activities and was inhibited by β-D-fucono- and α-L-arabinolactones [1]. A rat kidney β-D-galactosidase purified by gel filtration [3] or starch gel electrophoresis [4] contained β-D-glucosidase activity and was inhibited by β-D-gluconolactone, and a β-D-glucosidase purified by ion exchange chromatography was shown by substrate inhibition to have β-D-xylosidase activity [5]. Similar results were obtained with rat liver [6]. Furth and Robinson [7] separated at least four forms of β-D-galactosidase from rat liver, but no β-D-glucosidase activity was detected. In mouse liver [8], each of three forms of β-D-galactosidase exhibited β-D-fucosidase activity, but only one of four forms of β-D-galactosidase isolated from pig kidney had this extra activity [9].

Several studies have been performed on human tissues. In liver, β-D-glucosidase activity could not be separated from one form of β-D-galactosidase activity by starch gel electrophoresis or gel filtration, nor could the influence of heating or chloride ion concentration show that the activities were due to different enzymes [10]. This enzyme was recently suggested to be different from a form of β-D-galactosidase which has β-D-fucosidase and α-L-arabinosidase activity [11]. Öckerman [12] separated the β-D-galactosidase activity of liver into three forms, one of which was associated with β-D-glucosidase and β-D-xylosidase activities. In liver tissue from a patient with Gaucher's disease, in which β-D-glucosidase is the primary enzyme deficiency, this β-D-glucosidase and the associated β-D-galactosidase and β-D-xylosidase were absent. It was therefore proposed that these three activities were due to the same enzyme. Human kidney has been demonstrated by starch gel electrophoresis to have two forms of β-D-galactosidase, one of which had β-D-glucosidase activity and was thought to be the same enzyme on the basis of inhibition by lactones and its absence from urine [13].

Human leucocytes have a β-D-fucosidase associated with a β-D-galactosidase [14], and cultured fibroblasts from a patient with Gaucher's disease had very low levels of β-D-glucosidase, β-D-xylosidase, and α-L-arabinosidase activity [15].

It is evident that either a great diversity of glycosidase specificity exists between different organisms and in some cases between different tissues from the same animal or else some of the methods frequently used give results which are difficult to interpret. For example the inability to differentiate between two or more enzymic activities on the basis of lack of separation by physical methods, or on reaction to certain external influences, such as heat, pH change, or ionic strength, provides only circumstantial evidence that the activities are due to a single entity. The most commonly used method, that of inhibition by aldonolactones, can give useful information but the results are often difficult to interpret correctly [16]. The best single method available is to study the competition for mixed substrates using as pure an enzyme preparation as can be obtained.

Normal human liver and liver from patients with Gaucher's disease were used in this study to demonstrate whether one or more enzymes are responsible

for β-D-galactosidase, β-D-glucosidase, β-D-fucosidase, β-D-xylosidase, and α-L-arabinosidase activity.

Materials

4-Methylumbelliferyl and *p*-nitrophenyl substrates were obtained from Sigma Chemical Co., St. Louis, U.S.A., and Koch-Light Laboratories, Colnbrook, England.

Normal human liver was obtained at autopsy 6—10 h after death, and kept at −20°C until required. Liver tissue was also obtained at autopsy from 3 patients with Gaucher's disease (two males, aged 3 and 6 years, and one female, aged 3 years).

Methods

Enzyme preparation

1 g of liver tissue was thawed and homogenized in 10 ml of glass-distilled water in an all glass Potter-Elvehjem homogenizer at 0°C. This homogenate, suitably diluted, was used to compare the enzymic activities of normal and Gaucher liver. All subsequent procedures were performed at 4°C unless stated otherwise.

Ion exchange chromatography

A homogenate was prepared as described above except that homogenisation was performed in 4 ml of 0.01 M sodium phosphate buffer, pH 6.0, instead of water. The homogenate was then centrifuged at 105 000 × *g* for 60 min and 2 ml of the resulting supernatant were applied to a column (0.9 cm × 15 cm) of DEAE-cellulose (DE-52, W and R Balston, Maidstone, England) pre-equilibrated with the same buffer. 40 ml of buffer were passed through the column at a flow rate of 60 ml/h followed by a linear gradient of NaCl (0.5 mM/ml). 4-ml fractions were collected.

Large scale preparation of glycosidases

400 g of human liver were homogenized in 2 l of distilled water using a Waring Blendor. The homogenate was centrifuged at 16 000 × *g* for 60 min, the supernatant was concentrated to 300 ml using polyethylene glycol (PEG 23/100 Kebo-Grave, Sweden), then dialysed against 0.05 M Tris · HCl, pH 7.4. 150 ml of the dialysed preparation were applied to a column (2.5 cm × 45 cm) of DEAE-cellulose (DE-52). The column was eluted with 500 ml of 0.05 M Tris·HCl, pH 7.4, followed by 500 ml of the same buffer, containing 0.7 M NaCl. 5 ml fractions were collected and assayed for β-D-galactosidase and β-D-glucosidase activity. The fractions containing both these activities were pooled and concentrated to 30 ml using PEG and then applied to a column (3 cm × 95 cm) of Sephadex G-150. 5-ml fractions were collected.

The fraction containing most β-D-glucosidase activity was used in the competition studies described.

Enzyme assays

β-D-*Galactosidase.* 4-Methylumbelliferyl-β-D-galactoside, 1 mM, 100 μl; sodium acetate buffer, pH 4.5 or pH 5.5, 1 M, 25 μl; and liver homogenate or column fraction, 25 μl, were incubated at 37°C for various times up to 120 min. The reaction was stopped by adding 3 ml of glycine/NaOH buffer, 0.2 M, pH 10.7, and the fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 448 nm is an Aminco-Bowman fluorimeter. 4-Methylumbelliferone in glycine buffer was used as the standard. The enzymic activity was directly proportional to the amount of enzyme preparation used. Non-enzymic hydrolysis of the substrate was very low but corrected for.

α-L-*Arabinosidase.* 4-Methylumbelliferyl-α-L-arabinoside, 0.5 mM, 100 μl; sodium citrate buffer, pH 5.0, 1 M, 25 μl; and column fraction, 25 μl, were incubated at 37°C for various times up to 240 min. Subsequent procedures were as described above.

β-D-*Xylosidase.* As α-L-arabinosidase, except that 4-methylumbelliferyl-β-D-xyloside was used as substrate.

β-D-*Glucosidase.* 4-Methylumbelliferyl-β-D-glucoside, 1 mM in 10 mM histidine buffer, pH 6.5, 50 μl; sodium citrate buffer, pH 5.0, 1 M, 25 μl; and liver homogenate or column fraction, 25 μl, were incubated at 37°C for various times up to 40 min. Subsequent procedure was as above.

α-D-*Mannosidase.* 4-Methylumbelliferyl-α-D-mannoside, 12.5 mM, 50 μl; sodium citrate buffer, pH 4.5, 1 M, 10 μl; and liver homogenate, 10 μl, were incubated at 37°C for various times up to 10 min. Subsequent procedure was as above.

α-D-*Glucosidase.* 4-Methylumbelliferyl-α-D-glucoside, 1 mM, 100 μl; sodium citrate buffer, pH 4.5, 0.2 M, 25 μl; and liver homogenate, 25 μl, were incubated at 37°C for various times up to 120 min. Subsequent procedure was as above.

β-D-*Fucosidase.* *p*-Nitrophenyl-β-D-fucoside, 8 mM, 150 μl; sodium citrate/phosphate buffer, pH 7.0, 1 M, 150 μl; and liver homogenate or column fraction, 50 μl, were incubated at 37°C for various times up to 24 h. The reaction was stopped by addition of 150 μl of 5% trichloroacetic acid. The precipitate was removed by centrifuging and 200 μl of the resulting supernatant were mixed with 3 ml of 0.2 M glycine buffer, pH 10.7. Liberated *p*-nitrophenol was measured at 400 nm in a Zeiss spectrophotometer. *p*-Nitrophenol in the glycine buffer was used as the standard.

In the competition experiments the conditions of assay were as follows: sodium citrate/phosphate buffer, 1 M, 100 μl at the pH given in Table II; 50 μl of most active column fraction (diluted 1 : 4) obtained from the large scale glycosidase preparation; and 5—200 μl of the appropriate glycosidase substrate in water (*p*-nitrophenyl-β-D-fucoside, 8 mM; *p*-nitrophenyl-β-D-glucoside, 7.5 mM; 4-methylumbelliferyl-α-D-mannoside, 12.5 mM; 4-methylumbelliferyl-β-D-glucoside, -α-L-fucoside, -α-L-arabinoside, -β-D-galactoside, -β-D-xyloside, 1 mM) in a final volume of 350 μl. Incubation was carried out at 37°C for various times up to 60 min. The liberated *p*-nitrophenol or 4-methylumbelliferone was determined as described above. When mixed substrate experiments were performed, one substrate was the *p*-nitrophenyl derivative and the other the 4-methylumbelliferyl derivative. The liberated aglycones were then measured

spectrophotometrically or fluorimetrically. No interference between the two aglycones was found.

The enzyme activities are expressed in nmol of aglycone liberated per min.

Results

Chromatography on DEAE-cellulose

The distribution of enzymic activity after ion exchange chromatography is shown in Fig. 1. The β-D-galactosidase activity was eluted in two peaks, the first (peak A) being more active at pH 5.5 than pH 4.5. The reverse was true for the second peak of activity (peak B). Virtually all of the β-D-glucosidase, β-D-fucosidase, and β-D-xylosidase activities coincided in peak A. α-L-Arabinosidase activity was also associated with peak A but most of the total activity appeared to be associated with the β-D-galactosidase in peak B. However, re-analysis of the fractions after storage at 4°C for 4 weeks resulted in the activities associated with peak A remaining virtually unaltered, whereas the β-D-galactosidase in peak B was completely inactivated and the associated α-L-arabinosidase in peak B retained about half of its activity, indicating that in peak B the β-D-galactosidase and α-L-arabinosidase are different enzymes (Fig. 2).

Studies on Gaucher's disease

The enzyme deficient in Gaucher's disease has been demonstrated as being a β-D-glucosidase, glucocerebrosidase. The activity of several glycosides in normal and Gaucher liver is shown in Table I. The Gaucher livers had very low β-D-glu-

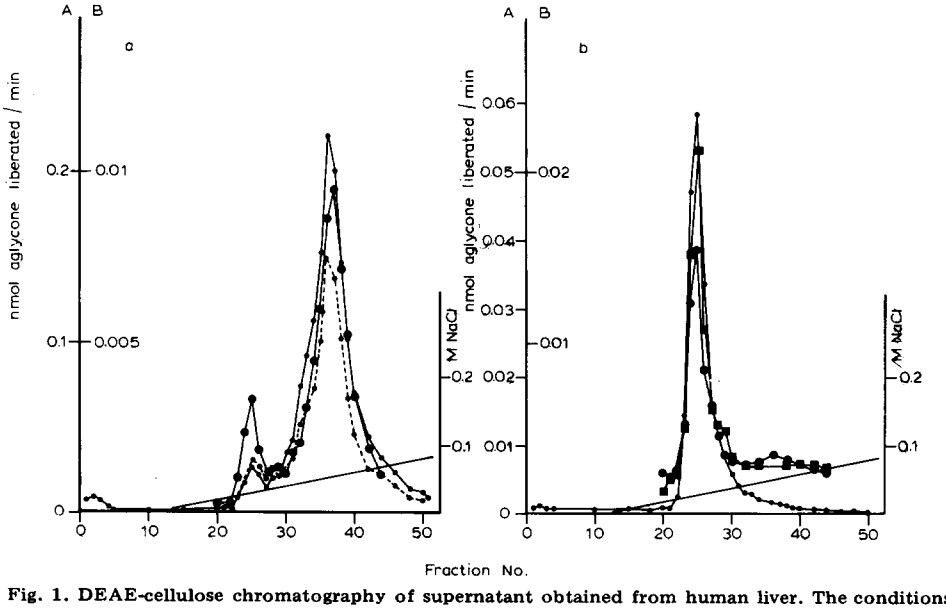


Fig. 1. DEAE-cellulose chromatography of supernatant obtained from human liver. The conditions were as given in Methods. (a) Distribution of β-D-galactosidase activity at pH 4.5 (●——●) and pH 5.5 (●- - -●), scale A; and α-L-arabinosidase activity, (●——●) scale B. (b) Distribution of β-D-glucosidase (●——●) and β-D-fucosidase (■——■) activity, scale A; and β-D-xylosidase activity (●——●), scale B. The NaCl gradient is shown by the straight line.

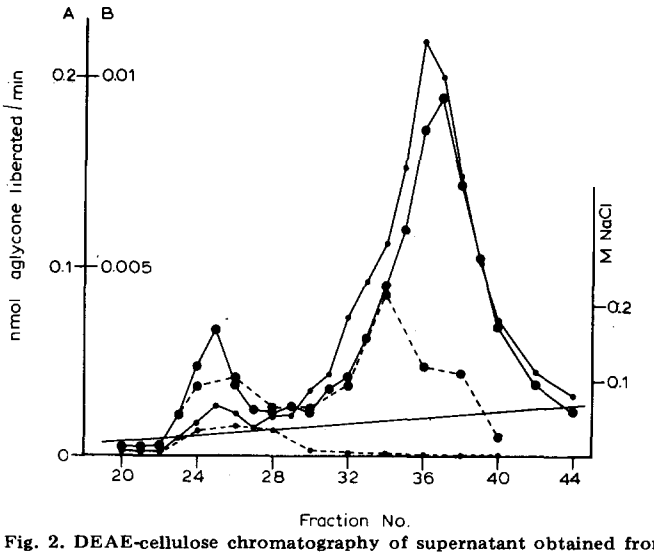


Fig. 2. DEAE-cellulose chromatography of supernatant obtained from human liver. Analysis of fractions performed before and after storage. The conditions were as given in Methods. Scale A, β-D-galactosidase activity measured at pH 4.5. (●———●), fresh fractions; (●- - - - -●), fractions kept at 4°C for 4 weeks. Scale B, α-L-arabinosidase activity. (●———●), fresh fractions; (●- - - - -●), fractions kept at 4°C for 4 weeks.

TABLE I

GLYCOSIDASE ACTIVITIES IN GAUCHER'S DISEASE

Liver homogenates were prepared and assayed as described in Methods. All values given as observed range in nmol substrate degraded/min/g protein.

	β-D-Glucosidase	β-D-Fucosidase	β-D-Galactosidase	α-D-Glucosidase	α-D-Mannosidase
Controls (n = 4)	0.19—0.59	0.07—0.56	2.5—4.3	0.8—1.2	0.4—0.7
Gaucher's disease (n = 3)	0.007—0.028	0.002—0.004	2.3—2.7	0.7—1.0	0.4—0.6

TABLE II

KINETIC PARAMETERS

Enzyme	Preparation	Substrate *	pH	K_m (mM)	V (nmol/min)
"β-D-Galactosidase"	Sephadex G-150, peak III	MU-β-D-galactoside	5.0	0.222	2.67
		MU-β-D-xyloside	5.0	0.060	0.057
		MU-α-L-arabinoside	5.0	0.012	0.209
		PNP-β-D-glucoside	5.0	0.182	2.36
		MU-β-D-glucoside	6.0	0.232	1.78
		PNP-β-D-fucoside	6.0	0.172	2.5
α-D-Mannosidase	Liver homogenate	MU-α-D-mannoside	5.0	0.615	0.142
α-L-Fucosidase		MU-α-L-fucoside	5.0	0.014	0.202
β-D-Glucosidase		PNP-β-D-glucoside	5.0	0.210	0.116

* Abbreviations: MU, 4-methylumbelliferyl; PNP, p-nitrophenyl.

cosidase and equally low β-D-fucosidase activity, but other glycosidases tested showed a normal range of activity.

Competition studies

In order to determine if the lack of separation of some of the forms of the glycosidases studied here was because the same active site was catalysing several reactions, studies were performed using a method involving the mutual competitive inhibition of two substrates [17,18].

If an enzyme is assayed in the presence of two competitive substrates, the activity towards each substrate will be less than that obtained if each substrate was tested separately. The calculated velocity of the reaction of one enzyme with two substrates is given by the equation:

$$V_t = \frac{\frac{V_a \cdot a}{K_a} + \frac{V_b \cdot b}{K_b}}{1 + \frac{a}{K_a} + \frac{b}{K_b}}$$

where V_t is the reaction velocity in the presence of both substrates, V_a and V_b are the maximum velocities of the two substrates, a and b are the substrate concentrations and K_a and K_b are the respective Michaelis constants.

If the two substrates are acted upon by different enzymes, then the reaction velocity, V_t, is the sum of the velocities obtained in the presence of each substrate alone.

The fraction containing most β-D-glucosidase activity obtained after DEAE-chromatography and G-150 gel filtration as described in Methods was used in all the competition studies. The enzyme in this fraction was purified 52-fold with respect to the original liver homogenate. The K_m and V values for each substrate were determined (Table II). The expected reaction rates were then calculated as described above and compared with the rates observed experimentally. The data are presented in Table III. These results clearly indicate that one enzyme is responsible for the β-D-galactosidase, β-D-glucosidase, β-D-fucosidase,

TABLE III

COMPETITION STUDIES. CALCULATED AND OBSERVED REACTION RATES

Enzyme source	Substrate a *	Substrate b *	Calculated velocity (nmol/min)		Observed velocity (nmol/min)	Number of enzymes
			1 enzyme	2 enzymes		
Sephadex G-150, peak III	MU-β-D-galactoside	PNP-β-D-glucoside	2.22	3.60	1.97	1
	MU-β-D-xyloside	PNP-β-D-glucoside	1.60	2.16	1.52	1
	MU-α-L-arabinoside	PNP-β-D-glucoside	0.90	2.30	0.99	1
	PNP-β-D-fucoside	MU-β-D-glucoside	2.28	3.13	2.26	1
Liver homogenate	MU-α-D-mannoside	PNP-β-D-glucoside	0.12	0.22	0.25	2
	MU-α-L-fucoside	PNP-β-D-glucoside	0.17	0.28	0.27	2

* Abbreviations: MU, 4-methylumbelliferyl; PNP, *p*-nitrophenyl.

β-D-xylosidase, and α-L-arabinosidase activities in this fraction. When the crude liver homogenate was tested for α-D-mannosidase, α-L-fucosidase, and β-D-glucosidase activity, the kinetic method confirmed, as expected, that these activities were due to the presence of more than one enzyme.

Discussion

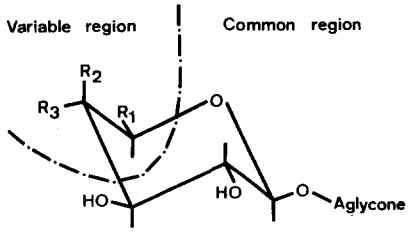
The availability of synthetic substrates has enabled extensive data to be collected on glycosidases from many sources. Frequent attempts have been made to purify these enzymes using standard biochemical techniques such as gel filtration, ion exchange chromatography, isoelectric focusing and gel electrophoresis. As separation methods have improved, increasing numbers of separate forms of many of these enzymes have been described. Whether all of these forms are present in vivo or arise during purification is not known, but the sensitive nature of some isolated enzymic forms suggests that in the natural state less heterogeneity prevails.

After partial purification of some enzyme activities it has frequently been impossible to separate the activities of one molecular form of several glycosidases, notably β-D-galactosidase, β-D-glucosidase, β-D-fucosidase, and α-L-arabinosidase, and this has led several workers to suggest that in some cases a single enzyme exists which is capable of degrading more than one type of monosaccharide glycosidically linked to a synthetic aglycone.

Further suggestions that multiple specificity may be exerted by some enzymes came from studies on inborn errors of metabolism. In G_{M1}-gangliosidosis a deficiency of β-D-fucosidase in addition to β-D-galactosidase was observed [14], and in Gaucher's disease, fibroblast cultures were deficient in β-D-xylosidase and α-L-arabinosidase as well as β-D-glucosidase [15]. The data in Table I show that the livers from Gaucher cases are also deficient in β-D-fucosidase.

Using competitive substrate inhibition [4] it has been shown that a pig kidney β-D-glucosidase which has a neutral pH optimum also exhibits β-D-galactosidase, β-D-xylosidase, and α-L-arabinosidase activity. The experiments described here (Tables II and III) using a purified preparation from human liver demonstrate that just one active site is capable of hydrolysing β-D-galactosides, β-D-fucosides, β-D-glucosides, β-D-xylosides, and α-L-arabinosides. Superficially, these activities would appear to be very different, hydrolysing α- and β-linkages, D- and L-configurations, and both pentoses and hexoses. However, the structure of the major part of the substrate in each case is the same (Fig. 3), only the configuration at C-4 and the substituent at C-5 varying. Hence the enzyme requirement is a fixed configuration which is less than a complete monosaccharide unit (common region) and certain differences elsewhere in the molecule (variable region) do not interfere with the ability of the enzyme to effect hydrolysis. However, the K_m and V values suggest that the enzyme has a greater affinity for the smaller substrates, and this affinity slows down hydrolysis (Table II).

The biological function of this enzyme is difficult to envisage. β-D-Galactosidase activity is necessary due to the large number of cellular components containing β-linked galactose. The β-D-xylosidase activity could be utilised in hydrolysing the β-xylosyl-serine linkages of chondroitin sulphate, and a β-D-gluco-



	R_1	R_2	R_3
β-D-galactoside	$-CH_2OH$	-OH	-H
β-D-glucoside	$-CH_2OH$	-H	-OH
β-D-fucoside	$-CH_3$	-OH	-H
β-D-xyloside	-H	-H	-OH
α-L-arabinoside	-H	-OH	-H

Fig. 3. The variable and common regions of the sugars recognised by the partially specific glycosidase.

sidase is known to hydrolyse β-glucosylceramide. α-L-Arabinosides and β-D-fucosides, however, are unknown in the animal kingdom. Perhaps more unusual is the observation that the β-D-galactosidase, β-D-glucosidase, and β-D-xylosidase activities of this enzyme do not hydrolyse any of the naturally occurring compounds tested [5,6,19], the activities so far being confined to synthetic substrates. Suzuki and Suzuki [20] have shown that in GM_1-gangliosidosis type I the neutral β-D-galactosidase (multiple specificity enzyme) was not always absent. This emphasizes the complex nature of human β-D-galactosidase, of which the enzyme studied here is one form. The deficiency of the enzyme with multiple specificity in our case of Gaucher disease indicates that some forms of GM_1-gangliosidosis and Gaucher disease are biochemically closely related, the genetic mutation in each disease affecting the same enzymic form and limiting its activity. This enzyme may then be one of two or more subunits constituting some of the higher molecular weight β-D-galactosidases and β-D-glucosidases. Mutations in other subunits may cause similar enzymic deficiencies which then result in the same clinical symptoms.

The separation of the α-L-arabinosidase activity into two forms is of interest. One form is the enzyme with broad specificity and the other form elutes with the majority of the β-D-galactosidase activity after DEAE-chromatography (Fig. 1a). This latter α-L-arabinosidase activity is distinct from the major β-D-galactoside component since effectively all this β-D-galactosidase activity was lost after 1 month, whereas the α-L-arabinosidase activity was only partially destroyed (Fig. 2). This is in contrast to the results of Norden et al. [11] who also found α-L-arabinosidase activity in a highly purified β-D-galactosidase preparation free from the broad specificity enzyme (neutral β-glucosidase) and hence concluded that this arabinosidase and the highly purified β-D-galactoside were the same enzyme.

The work described here demonstrates the presence in human liver of a glycosidase which will hydrolyse the synthetic glycosides of five sugars. This en-

zyme has previously been shown to be of cytoplasmic origin, of relatively low molecular weight and to have no known action on naturally occurring glycosides. Whether this enzyme is an obsolete evolutionary remnant, or plays an as yet unknown role, for example as a precursor of the lysosomal enzymes, or is involved in the hydrolysis of naturally occurring sterol or phenolic glycosides [21] remains to be discovered.

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