Human Small-Intestinal β -Galactosidases

SEPARATION AND CHARACTERIZATION OF THREE FORMS OF AN ACID β -GALACTOSIDASE

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1. An acid β -galactosidase, optimum pH 4.0–4.5, in the human small-intestinal mucosa was separated and characterized. 2. Autolysis of mucosal homogenates at acid pH inactivated the lactase and hetero β -galactosidase; the total activity of the acid β -galactosidase was only slightly depleted, but a greater proportion of the enzyme was solubilized by this treatment. 3. Separation on a Sephadex G-200 column revealed that the acid β -galactosidase could occur in at least three different forms, probably representing monomer, dimer and octamer or polymer of the enzyme. 4. The properties of the different forms of the acid β -galactosidase were studied with regard to pH optimum, K_m , rate of hydrolysis of different substrates, and sensitivity to *p*-chloromercuribenzoate and tris as inhibitors. All these properties were the same for the different forms of the enzyme. 5. The acid β -galactosidase hydrolyses lactose as well as hetero β -galactosides and contributes to the lactase activity of intestinal biopsies also when measured at pH 6. This enzyme may therefore be responsible for a considerable part of the residual lactase activity found in lactose-intolerant patients.

The separation by gel-filtration chromatography and the characterization of two β -galactosidases in human small-intestinal mucosa was reported in a previous paper (Asp, Dahlqvist & Koldovsky, 1969). One of the enzymes, the lactase, was particle-bound and could be partially solubilized with papain. The other enzyme, the hetero β -galactosidase, was mainly soluble and had no activity against lactose, though synthetic β -galactosides were split readily. Indications of the existence of a third β -galactosidase with acid pH optimum were also reported.

In parallel work Gray & Santiago (1969) separated three β -galactosidases in the human small intestine by using gradient ultracentrifugation. Their enzyme I corresponded to the lactase in our study, and enzyme III to the hetero β -galactosidase. The characterization of enzymes I and III gave findings very similar to those presented for the corresponding enzymes in our paper. Enzyme II had an acid pH optimum and hydrolysed lactose as well as synthetic hetero β -galactosides. Gray, Santiago, Colver & Genel (1969) also studied the biochemical lesion in human lactase deficiency. Lactose-intolerant patients were found to be completely lacking enzyme I (lactase) and some of the patients lacked enzyme III (hetero β -galactosidase) as well. It was suggested that enzyme III might be a fragment or a precursor of enzyme I.

Alpers (1969) presented evidence for the existence of one lysosomal β -galactosidase with acid pH optimum corresponding to enzyme II and one other enzyme, presumably of brush-border origin, with properties corresponding to the lactase (enzyme I).

In the present study the acid β -galactosidase of the human intestinal mucosa was isolated from the other β -galactosidases by autolysis and gel-filtration chromatography on Sephadex G-200. In this way three different forms of the enzyme were separated and characterized to obtain more complete information on the properties of this enzyme, this being necessary for the development of specific assay methods for the three different β -galactosidases in small-intestinal biopsies (N.-G. Asp & A. Dahlqvist, unpublished work).

MATERIALS AND METHODS

Preparation of mucosal homogenates. Pieces of human small intestine were obtained at autopsy from patients without known history of gastrointestinal disease within 8 h after death. The intestines were examined in a dissection microscope and were found to have normal configuration of villi with intact epithelial cells. The pieces of intestine were chilled as soon as possible after removal, rinsed with cold 0.9% NaCl and gently dried with a piece of soft filter paper. The mucosa was then scraped off with a metal spatula and homogenized in

4 vol. (v/w) of water. The homogenate was either used immediately or stored at -20°C until use. The β -galactosidases remained practically unchanged for at least 6 months.

Preparation of 'soluble fractions'. Three kinds of preparative procedures were used to obtain 'soluble fractions': (i) ultracentrifugation at 100000g for 60 min at 4°C ; (ii) autolysis at 37°C for 90 min after addition of 0.12M- KH_2PO_4 and adjustment to various pH values with M-HCl or M-NaOH, followed by ultracentrifugation at 100000g for 60 min at 4°C ; (iii) autolysis at 37°C for 90 min after addition of 0.12M- KH_2PO_4 and adjustment to pH4 with M-HCl, followed by freezing and thawing ten times, followed by ultracentrifugation at 100000g for 60 min at 4°C .

Gel-filtration chromatography. Sephadex G-200 was left to swell for 3 days at room temperature in excess of buffer. Two dimensions of columns were used, either 95 cm $\times$ 1 cm or 95 cm $\times$ 1.5 cm. Buffers used for elution are specified in the text and legends. The flow rate was 3 ml/h for the smaller column and 4.5 ml/h for the larger one. Fractions were collected every 40 min.

Enzyme-activity assay. The measurement of enzyme activity was performed as described by Asp *et al.* (1969) with the following modifications.

(1) For the determination of free phenol liberated from phenyl β -galactoside the following modified reagents were added to the incubation mixture (0.2 ml): first 0.15 ml of a reagent containing M-tris-HCl buffer, pH 8.5, 1.33% (v/v) of ethanol and 0.27 mg of 4-aminoantipyrine/ml, and then 0.05 ml of $\text{K}_3\text{Fe}(\text{CN})_6$ solution (4 mg/ml).

(2) Incubations were performed for 10–30 min at 37°C , since the reactions of separated enzyme did not always obey zero-order kinetics for longer time of incubation.

(3) A new substrate, 2-naphthyl β -galactoside (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was introduced. The incubation mixture (0.2 ml) contained 0.001 M substrate, 10% (v/v) methanol, 0.05 M-sodium citrate buffer, pH 4 or 4.5. Incubation was performed at 37°C for 30 min and stopped by addition of 2 ml of 0.1 M-glycine-NaOH buffer, pH 12. The fluorescence was measured in a filter fluorimeter with primary filter giving exciting light with maximum at 360 nm and with secondary filter of 'cut-off'-type passing light above 415 nm.

Specific determination of the three different intestinal β -galactosidases in homogenates and 'soluble fractions' (Tables 1 and 2 and Fig. 1). (1) The lactase was assayed with *p*-nitrophenyl β -galactoside or lactose as substrate at pH 5.5 in the presence of 0.1 mM-*p*-chloromercuribenzoate; (2) the acid β -galactosidase was assayed with 2-naphthyl β -galactoside as substrate at pH 4.5; (3) the hetero β -galactosidase was assayed with *p*-nitrophenyl β -galactoside as substrate. The activity inhibited by *p*-chloromercuribenzoate was measured at pH 5.5, representing the sum of the hetero β -galactosidase and the acid β -galactosidase. The contribution of the latter enzyme was then calculated from the assay with 2-naphthyl β -galactoside as substrate and subtracted.

Measurement of protein concentration. The protein concentration of homogenates was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955). In the effluent of chromatograms the transmittance at 280 nm was measured

Table 1. Data on the samples of human small intestine used in the investigation

Sample no.	Age (years) and sex of patient	Diagnosis	Part of small intestine	β -Galactosidase activities (units/g of protein)			Protein (mg/g wet wt.)
				Lactase (substrate lactose)	Acid β -galactosidase (substrate 2-naphthyl β -galactoside)	Hetero β -galactosidase (substrate <i>p</i> -nitrophenyl β -galactoside)	
1	50; ♂	Bronchial tumour	Middle part of small intestine	47.4	4.3	4.6	94
2	83; ♂	Senile demensis	20 cm distal to ligament of Treitz	20.0	3.3	1.3	95

continuously with a Uvicord II Ultraviolet Absorptiometer. Human serum albumin (KABI AB, Stockholm, Sweden) was used as standard.

Calculations. Enzyme activities were calculated as μmol of substrate hydrolysed/min at 37°C . Michaelis constants, K_m , and the maximal rates of hydrolysis, V_{max} , were calculated by the method of Lineweaver & Burk (1934) as modified by Dixon (1953). K_{av} is defined from the formula: $K_{\text{av}} = (V_e - V_0)/(V_t - V_0)$ where V_e is the elution volume of the substance under investigation, V_0 is the void volume and V_t is the total volume of the column.

RESULTS

Solubility of acid β -galactosidase and effect of autolysis on different β -galactosidases. Although reported to be lysosomal (Alpers, 1969) only 30–40% of the acid β -galactosidase remained in the supernatant after ultracentrifugation of a water homogenate of intestinal mucosa. Different methods were tried to discover a solubilization procedure that gave a better yield of acid β -galactosidase.

Fig. 1. Effect of autolysis at different pH values on human intestinal β -galactosidases. The homogenate was incubated at 37°C for 90 min in the presence of $0.12\text{M-KH}_2\text{PO}_4$ at various pH values. The activity of each enzyme before the incubation was arbitrarily set at 100. ●, Lactase; ○, acid β -galactosidase; ▲, hetero β -galactosidase.

Methods such as incubation with papain or trypsin and treatment with chloroform or detergent (Triton X-100) were not successful.

Autolysis at acid pH offered several advantages as a first step for the isolation of acid β -galactosidase. Fig. 1 shows the remaining activity of the different β -galactosidases after autolysis at various pH values. At pH 6 all the three enzymes were relatively stable against autolysis, not more than 10% being inactivated after incubation for 90 min at 37°C . At pH 5 the lactase began to be inactivated and at pH 4 as much as 80% of the lactase and all the hetero β -galactosidase were inactivated, whereas about 90% of the acid β -galactosidase still remained active. At pH lower than 4 the acid enzyme was inactivated also. Ultracentrifugation after autolysis at pH 4 revealed that about 50% of the remaining acid β -galactosidase was now soluble. Freezing in solid CO_2 -ethanol mixture and thawing ten times after the autolysis increased the solubility of the acid β -galactosidase to about 60%. The proportions of the activities of the other β -galactosidases rendered soluble by this procedure were very low (Table 2). Incubation for 45 or 60 min rather than 90 min gave a little higher yield of acid β -galactosidase but also increased the remaining soluble lactase. If potassium dihydrogen phosphate was excluded or replaced with equimolar sodium chloride the lactase was partially protected from inactivation during the autolysis. Thus procedure (iii) described above (see the Materials and Methods section), gave optimum yield of acid β -galactosidase in the 'soluble fraction' with minimal contamination by the other β -galactosidases and was therefore used for the further experiments.

Separation by gel-filtration chromatography. Column chromatography on Sephadex G-200 of the spontaneously soluble fraction (i) of preparation 1 is shown in Fig. 2. In Fig. 2(a) elution was performed with 0.05M -potassium-sodium phosphate buffer, pH 7.0, i.e. as used by Asp *et al.* (1969). Fractions 1a and 2 were eluted as described by

Table 2. Effect of autolysis at pH 4 with 0.12M -potassium dihydrogen phosphate on different β -galactosidases

The total and soluble activity of each enzyme after autolysis is expressed as a percentage of the activity in untreated homogenate. The autolysis was performed according to procedure (iii).

Preparation	Acid β -galactosidase activity after autolysis		Lactase activity after autolysis		Hetero β -galactosidase activity after autolysis	
	Total	Soluble	Total	Soluble	Total	Soluble
1	88 (82–96)	62 (59–66)	21 (13–36)	<1	Not measurable*	
2	88 (84–95)	60 (55–68)	19 (13–24)	<1	Not measurable*	

* Less than 0.001 unit/ml expressed as *p*-nitrophenyl β -galactosidase activity.

Fig. 2. Separation of the spontaneously soluble material (i) of preparation 1 (Table 1) on a Sephadex G-200 column (95 cm $\times$ 1 cm) with (a) 0.05 M-sodium-potassium phosphate buffer, pH 7.0, and (b) the same buffer containing 0.34 M-NaCl as eluent. $\circ$, *p*-Nitrophenyl β -galactosidase at pH 4.0; $\bullet$, *p*-nitrophenyl β -galactosidase at pH 5.5; $\blacktriangle$, *p*-nitrophenyl β -glucosidase at pH 5.5. ----, Protein concentration.

Asp *et al.* (1969). At the void volume there was also a small peak of β -galactosidase with acid pH optimum. In Fig. 2(b) 0.34 M-sodium chloride had been added to the elution buffer, but otherwise the experiment was performed as in Fig. 2(a). The activity of the acid peak at the void volume (fraction 3a) was now fivefold higher. Fraction 1a was unchanged, but in front of fraction 2 another acid β -galactosidase peak appeared (fraction 3c). Fraction 2 was unchanged when measured at pH 5.5, but at pH 4 its activity was considerably decreased. The elution pattern of the protein was essentially the same in the two chromatograms.

For further characterization of the acid β -galactosidase activity the 'soluble fraction' according to

procedure (iii) was separated on Sephadex G-200 columns as shown in Fig. 3. In Fig. 3(a) 0.02 M-sodium citrate buffer, pH 4.5, containing 0.17 M-sodium chloride was used as eluent. All the β -galactosidase activity was eluted in a single peak designated fraction 3b. In Fig. 3(b) 0.02 M-sodium phosphate buffer, pH 7.0, containing 0.17 M-sodium chloride was used as eluent. The β -galactosidase activity was now separated into two peaks, 3a and 3c, as in Fig. 2(b).

Stability of separated acid β -galactosidase. Acid β -galactosidase required quite definite conditions to be stable in separated form. The experiments presented in Fig. 2 showed that a certain ionic strength was necessary for the appearance of

Fig. 3. Separation of the soluble material after autolysis (iii) of preparation 2 (Table 2) on a Sephadex G-200 column (95 cm $\times$ 1.5 cm) with (a) 0.02M-sodium citrate buffer, pH 4.5, containing 0.17M-NaCl and (b) 0.02M-sodium phosphate buffer, pH 7.0, containing 0.17M-NaCl as eluent. β -Galactosidase activity was measured at pH 4.0 with the following substrates: $\bullet$, 2-naphthyl β -galactoside; Δ , phenyl β -galactoside; $\circ$, *p*-nitrophenyl β -galactoside; $\blacktriangle$, lactose. ----, Protein concentration.

fractions 3a and 3c. But Table 3 shows that, in spite of the addition of salt, the activity of fraction 3b declined rapidly, especially after freezing. Fraction 3a was somewhat more stable, but fraction 3c less stable than fraction 3b. The effect of human serum albumin, thiols and glycerol on the stability of fraction 3b is also shown in Table 3. As much as 10mg of albumin/ml was required to make the enzyme reasonably stable when frozen and stored at -20°C . If the separated enzyme was stored at 4°C , however, 2mg of albumin/ml was enough to prevent inactivation. For further experiments therefore the addition of 2mg of albumin/ml was used

as a standard procedure. A concentrated albumin solution (40mg/ml) was added in a small volume to each tube of the chromatogram in advance. In this way the recovery of acid β -galactosidase in the chromatograms was essentially quantitative, and the activity of the different peaks remained practically unchanged for 1–2 weeks when stored at 4°C . 2-Mercaptoethanol or dithiothreitol did not stabilize the acid β -galactosidase, although the inhibition by *p*-chloromercuribenzoate (see below) indicates the importance of intact thiol groups for enzymic activity. Glycerol was a relatively good stabilizing agent.

Table 3. *Stability of separated acid β -galactosidase (fraction 3b) and influence of different substances as stabilizers*

The activity remaining is expressed as a percentage of the original activity.

Substance added for stabilization	Temperature of storage ($^{\circ}\text{C}$)	Activity remaining after storage	
		For 1 day	For 4 days
None	+4	82	82
None	-20	32	18
Human serum albumin			
(2mg/ml)	+4	100	100
(2mg/ml)	-20	70	39
(10mg/ml)	-20	99	95
2-Mercaptoethanol (0.1%)	-20	24	12
Dithiothreitol (2mg/ml)	-20	32	8
Glycerol (0.5%)	-20	94	75

Table 4. *K_{av} values and apparent molecular weights of the different human intestinal β -galactosidase fractions separable on Sephadex G-200*

Fraction no.	No. of experiments	K_{av}	Apparent mol.wt. (method of Andrews, 1965)
1a*	4	0.14 (0.11-0.16)	350 000 (310 000-420 000)
1b*	3	0.13 (0.08-0.16)	375 000 (310 000-510 000)
2*	7	0.54 (0.48-0.58)	47 000 (39 000-62 000)
3a	3	0.02 (0.00-0.04)	>700 000
3b	3	0.26 (0.21-0.30)	185 000 (150 000-235 000)
3c	3	0.38 (0.33-0.42)	100 000 (83 000-130 000)

* These fractions were studied by Asp *et al.* (1969).

Purity of separated acid β -galactosidase. Autolysis of the homogenates as shown in Table 2 resulted in hydrolysis of most of the proteins to peptides small enough to be completely excluded from the Sephadex G-200 gel. Thus very low protein concentrations were found where the acid β -galactosidase was eluted (Fig. 3), giving a considerable purification of the enzyme during the gel-filtration chromatography.

The soluble activities of lactase and hetero β -galactosidase after autolysis were very small, as shown in Table 2. In most separation experiments these activities could not be demonstrated at all in the eluate. In some experiments minor traces could be detected, but their elution volumes did not coincide with any of the fractions of acid β -galactosidase (Fig. 2 and Table 4). Therefore contamination of fractions 3a, 3b and 3c with lactase or hetero β -galactosidase could be practically excluded.

Apparent molecular weights of the different intestinal β -galactosidases. The K_{av} values of the different intestinal β -galactosidases separated and studied in the present paper (fractions 3a, 3b and 3c) and in a previous publication (fractions 1a, 1b and 2; Asp *et al.* 1969) are listed in Table 4. The apparent

molecular weights were determined by the method of Andrews (1965).

Optimum pH. The pH-activity curves of fractions 3a, 3b and 3c were identical with four different substrates (Fig. 4). The pH optimum was 4.0 with *p*-nitrophenyl β -galactoside as substrate and between 4.0 and 4.5 with phenyl β -galactoside, 2-naphthyl β -galactoside and lactose as substrates. The same pH-activity curves were found also in the 'soluble fraction' after autolysis.

K_m values. Table 5 lists the K_m values of the three fractions of acid β -galactosidase with three different substrates. For each substrate the three fractions had the same K_m value. The lowest K_m was found with *p*-nitrophenyl β -galactoside as the substrate. 2-Naphthyl β -galactoside also gave a similar value, but with phenyl β -galactoside the K_m was considerably higher. With lactose as substrate K_m values of about 20 mM were obtained for all the three fractions. The K_m with *o*-nitrophenyl β -galactoside was about 0.5 mM.

Substrate specificity. The relative rate of hydrolysis of different substrates by the different fractions was also the same. In Table 6 the substrate specificity at pH 4.5 of the 'soluble fraction' (iii) after autolysis

Fig. 4. pH-activity curves of the three fractions of acid β -galactosidase with (a) *p*-nitrophenyl β -galactoside, (b) phenyl β -galactoside, (c) 2-naphthyl β -galactoside and (d) lactose as substrates. $\circ$, Fraction 3a; $\bullet$, fraction 3b; $\blacktriangle$, fraction 3c.

Table 5. Apparent K_m values at pH 4.0 for the different fractions of acid β -galactosidase with three substrates

Substrate	$10^3 \times K_m$ (M)		
	Fraction 3a	Fraction 3b	Fraction 3c
Phenyl β -galactoside	2.5 (2.3–2.6)	2.9 (2.6–3.3)	2.5 (2.4–2.6)
<i>p</i> -Nitrophenyl β -galactoside	0.33 (0.32–0.34)	0.36 (0.33–0.40)	0.33 (0.28–0.37)
2-Naphthyl β -galactoside	0.46 (0.45–0.47)	0.48 (0.36–0.63)	0.44 (0.36–0.54)

of preparation 1 is shown. 2-Naphthyl β -galactoside was hydrolysed most rapidly. It should be noted that the β -galactosidase activities of the fractions shown in Fig. 3 were measured at pH 4.0, which explains the relatively higher activity of *p*-nitrophenyl β -galactosidase (Fig. 4).

β -Glucosidase activity. *p*-Nitrophenyl β -glucoside was not hydrolysed by any of the three fractions of acid β -galactosidase.

Effects of *p*-chloromercuribenzoate and tris. *p*-Chloromercuribenzoate was a potent inhibitor of all the three fractions of acid β -galactosidase. Tris

Table 6. *Substrate specificity at pH 4.5 of the 'soluble fraction' (iii) after autolysis of preparation 1*

The lactase activity was arbitrarily set to 100.

Substrate	Measured V_0	Theoretical V_{max}
2-Naphthyl β -galactoside (1 mM)	137	117
6-Bromo-2-naphthyl β -galactoside (1 mM)	137	96*
Lactose (28 mM)	100	100
Phenyl β -galactoside (20 mM)	98	65
<i>p</i> -Nitrophenyl β -galactoside (12 mM)	120	72
<i>o</i> -Nitrophenyl β -galactoside (12 mM)	114	69

* K_m for 6-bromo-2-naphthyl β -galactoside as substrate was estimated to 0.2 mM by Gray & Santiago (1969). This value was used for the calculation of the V_{max} for this substrate.

Fig. 5. Effects of (a) *p*-chloromercuribenzoate (PCMB) and (b) tris on the different fractions of acid β -galactosidase. $\circ$, Fraction 3a; $\bullet$, fraction 3b; $\blacktriangle$, fraction 3c. Enzyme activity was measured with *p*-nitrophenyl β -galactoside as substrate at pH 4.0 and the activity without inhibitor present was arbitrarily set at 100.

also had some inhibitory effect at high concentrations (Fig. 5). The sensitivity for the inhibitors was the same for all the three fractions of acid β -galactosidase.

DISCUSSION

The separation of three different β -galactosidases in the human small-intestinal mucosa was reported by Gray & Santiago (1969). They used gradient ultracentrifugation in a sodium chloride gradient. Gel-filtration chromatography was also tried but abandoned because of loss of activity against artificial substrates and incomplete separation. The gradient-centrifugation technique, however, has certain practical drawbacks, the most serious one being the low capacity of each separation (only 0.1 ml of tissue extract was applied in each separation). Another problem may be the long period required for ultracentrifugation.

In a previous paper on human intestinal β -galactosidases (Asp *et al.* 1969) the separation of two of the enzymes, the lactase and the hetero β -galactosidase, on Sephadex G-200 columns was reported. The acid β -galactosidase, however, was

hardly detectable in these separations. The only trace of that enzyme was a *p*-chloromercuribenzoate-inhibited acid β -galactosidase activity occurring at the void volume and not separated from the spontaneously soluble fraction of the lactase (fraction 1a; see also Fig. 2a). Essentially similar results were reported by Gray & Santiago (1969), although the acid β -galactosidase in the void volume was more obvious in their separations.

Because of the technical advantages of the gel-filtration procedure, especially for the separation of larger quantities of tissue, it was thought pertinent to try to improve the conditions for gel-filtration chromatography to separate the acid β -galactosidase also. Gray & Santiago (1969) reported that the three β -galactosidases differ considerably in molecular weight and should therefore be separable by gel-filtration chromatography.

The addition of 0.17–0.34 M-sodium chloride to the elution buffer gave a considerable increase of acid β -galactosidase activity in the void-volume peak. Further, another peak of acid β -galactosidase also appeared in front of the trailing peak of hetero β -galactosidase (fraction 3c in Fig. 2b). Thus the acid β -galactosidase required a certain ionic strength in the eluate not to be inactivated during the run. This finding explains the virtual absence of acid β -galactosidase in the separations carried out by Asp *et al.* (1969) and also the low recovery of 6-bromo-2-naphthyl β -galactosidase reported by Gray & Santiago (1969). These authors used 0.02 M-sodium phosphate buffer, pH 6.0, or 0.02 M-citrate-phosphate buffer, pH 4.5, for elution of their column, i.e. an even lower ionic strength than in the separation experiments of Asp *et al.* (1969). For storage for more than a few days it was necessary to add 2 mg of albumin/ml and to avoid freezing. The addition of sodium chloride, then, gave two fractions of acid β -galactosidase, numbered 3a and 3c. These were well separated from each other but poorly from the lactase and the hetero β -galactosidase respectively.

The influence of autolysis on the different β -galactosidases was studied in efforts to discover a solubilization procedure for the acid β -galactosidase. Since the pieces of intestine used were rinsed with saline before the mucosa was scraped off the proteolytic activity of such a homogenate would be expected to be exerted mainly by proteases in the mucosal cells. The concept of lysosomal localization of the acid β -galactosidase (Alpers, 1969) made it reasonable to believe that this enzyme would be stable against the proteolytic action of lysosomal acid proteases. Autolysis at various pH values showed, as expected, that the acid β -galactosidase was considerably more stable against autolysis at low pH than were the other two β -galactosidases. Since the yield of soluble acid β -galactosidase was considerably increased and since practically no activity of lactase or hetero β -galactosidase remained soluble after autolysis at pH 4, this procedure was very suitable as a first step for the isolation of the acid β -galactosidase.

Gray & Santiago (1969) separated two forms of acid β -galactosidase with molecular weights 660 000 and 156 000 by using gradient ultracentrifugation at pH 5.5. They concluded the different forms to be monomeric and tetrameric forms of the enzyme. The molecular weights correspond quite well to those obtained for fraction 3a, separated at neutral pH, and fraction 3b, separated at pH 4.5, in my study. These forms had apparent molecular weights 700 000 or more and 185 000 respectively. With an eluate at neutral pH it was also possible to demonstrate a third fraction, 3c, with apparent molecular weight 100 000. Thus the acid β -galactosidase can appear in at least three forms, differing in molecular weight. A probable explanation for this heterogeneity would be as follows. At pH 4.5 the enzyme occurs exclusively in dimeric form. At pH 5.5 a portion of the enzyme polymerizes further to form an octamer and at neutral pH one portion remains in the octameric form whereas the remaining enzyme depolymerizes to form the monomer. The demonstration of identical enzymic properties of the different forms with regard to pH-activity curves, K_m values, substrate specificity and sensitivity to *p*-chloromercuribenzoate and tris give further evidence that the different fractions contain the same enzyme.

The apparent molecular weight of the lactase (350 000 or 375 000) is also in reasonable agreement with the value reported by Gray & Santiago (1969) (280 000 for enzyme I). The hetero β -galactosidase (enzyme III), on the other hand, was reported by these authors to have a molecular weight of about 80 000, but the apparent molecular weight as revealed by gel filtration was only about 47 000. This finding indicates that the hetero β -galactosidase might have appeared in dimeric form in the

gradient-centrifugation experiments of Gray & Santiago (1969). Hultberg & Öckerman (1970) found that gel-filtration chromatography of the supernatant of human liver homogenate at low ionic strength gave four peaks of 4-methylumbelliferyl β -glucosidase and *p*-nitrophenyl β -glucosidase activity, which is probably exerted by an enzyme identical with the intestinal hetero β -galactosidase (N.-G. Asp, B. Hultberg, A. Dahlqvist & P. A. Öckerman, unpublished work).

2-Naphthyl β -galactoside was the substrate most readily hydrolysed by the acid β -galactosidase. This substrate was not split at any measurable rate by the other two β -galactosidases (N.-G. Asp & A. Dahlqvist, unpublished work) and therefore offers a method of determining the acid β -galactosidase specifically in the presence of the other two enzymes.

The properties of the acid β -galactosidase of the human small intestine are similar to those of the corresponding enzyme in the rat small intestine (Asp & Dahlqvist, 1968*a,b*). Both pH optimum and K_m values were slightly lower for the rat enzyme. The relative rates of hydrolysis of different substrates were similar. The molecular weight of the acid β -galactosidase in the rat was estimated by Kraml *et al.* (1969) to be 83 000–105 000, which is in good agreement with my value for the monomeric form of the human enzyme (100 000). The appearance of different molecular forms of the acid β -galactosidase in the rat has also been demonstrated (Asp, 1970). The molecular weight of neutral β -galactosidase in the rat was also virtually the same as that of the human lactase. An enzyme corresponding to the hetero β -galactosidase has not been demonstrated in the rat. In the monkey small intestine, however, Swaminathan & Radhakrishnan (1969) found, in addition to the particle-bound lactase, two β -galactosidases preferentially hydrolysing hetero β -galactosides. These enzymes seem to correspond to the acid β -galactosidase and the hetero β -galactosidase in the human.

Lactose-intolerant patients retain a low amount of lactase activity demonstrable in intestinal biopsies, and it has been suggested that this residual lactase activity might be exerted by an unspecific β -galactosidase without importance for the digestion of lactose. The properties of the acid β -galactosidase indicate that this enzyme can hydrolyse lactose also at pH 6 (Fig. 4*d*), which is usually used for assay of lactase. The enzyme will retain 20–25% of maximal activity at this pH. The hetero β -galactosidase, on the other hand, has no lactase activity and is therefore without importance in this context. Gray *et al.* (1969) demonstrated the absence of lactase (enzyme I) from lactose-intolerant patients by separating the β -galactosidases by gradient ultracentrifugation. With such an approach, however, low activities of lactase might be overlooked, since a dilution of

the enzyme activity is inevitable at the separation step.

Specific, simple and sensitive methods are desirable for the routine determination of the different β -galactosidases in intestinal biopsy specimens without separation. The differences in substrate specificity, pH optimum and sensitivity to inhibitors should offer possibilities to work out such methods. Specific assay of lactase would show the residual activity of this enzyme, if any, in different types of lactose-intolerant patients. Variation in the amount of acid β -galactosidase activity could be expected to reflect the amount of lysosomes in the intestinal epithelial cells and the determination of the hetero β -galactosidase might throw further light on the hypothesis expressed by Gray *et al.* (1969) that the hetero β -galactosidase might be a precursor of the lactase.

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