Human Small-Intestinal β -Galactosidases

SEPARATION AND CHARACTERIZATION OF ONE LACTASE AND ONE HETERO β -GALACTOSIDASE

BY NILS-GEORG ASP, ARNE DAHLQVIST AND OTAKAR KOLDOVSKÝ

*Research Department of the Hospital and Department of Physiological Chemistry,
University of Lund, Lund, Sweden*

(Received 28 March 1969)

1. Two β -galactosidases from human small-intestinal mucosa were separated by gel-filtration chromatography and the properties of the two enzymes were studied. Lactose and four hetero β -galactosides were used as substrates. 2. One of the enzymes was particle-bound and could be partially solubilized with papain. Of the substrates hydrolysed by this enzyme, lactose was hydrolysed most rapidly. This enzyme is thus essentially a disaccharidase and is named lactase. It is presumably identical with the 'lactase 1' described earlier. 3. The other enzyme was mainly soluble and hydrolysed all artificial substrates used, whereas no lactase activity could be detected. This enzyme has therefore been designated hetero β -galactosidase. 4. *p*-Chloromercuribenzoate (0.1 mM) inhibited the hetero β -galactosidase completely but did not influence the activity of the lactase. Tris was a competitive inhibitor of both enzymes. 5. The residual lactase activity in the mucosa of lactose-intolerant patients may be exerted by a small amount of remaining lactase as such, or possibly by a third enzyme with a more acid pH optimum.

Heilskov (1956) reported evidence for the hydrolysis of lactose and 6-bromo-2-naphthyl β -galactoside by two different enzymes in the small-intestinal mucosa of the calf. Subsequently the small intestine of several other mammals has been shown to contain more than one β -galactosidase (Doell & Kretchmer, 1962, 1963; Dahlqvist, Bull & Thomson, 1965; Koldovský *et al.* 1965; Koldovský, Heringová & Jirsová, 1966a; Koldovský, Heringová, Jirsová, Chytil & Hošková, 1966b; Asp & Dahlqvist, 1968a; Swaminathan & Radhakrishnan, 1967; Hore & Messer, 1968). One of these enzymes is mainly soluble and has high affinity for hetero β -galactosides as substrates. In the mouse and rat this enzyme has optimum pH 3–4 (Maio & Rickenberg, 1960; Koldovský *et al.* 1965, 1966a,b; Dahlqvist & Asp, 1967; Asp & Dahlqvist, 1968b). The other enzyme is localized in the brush border of the mucosal epithelium (Doell, Rosen & Kretchmer, 1965; Koldovský *et al.* 1965) and hydrolyses lactose more rapidly than hetero β -galactosides. It has optimum pH 5.5–6.0.

The human small intestine has been shown to contain one soluble β -galactosidase, hydrolysing 6-bromo-2-naphthyl β -galactoside, and one insoluble β -galactosidase, responsible for most of the lactase activity (Hsia, Makler, Semenza & Prader,

1966). The 6-bromo-2-naphthyl β -galactosidase activity has been reported not to be lowered, or to be only moderately lowered, in patients with lactose malabsorption (Zoppi, Hadorn, Gitzelmann, Kistler & Prader, 1966; Cook & Dahlqvist, 1968).

Semenza, Auricchio & Rubino (1965) separated two lactases ('lactase 1' and 'lactase 2') by gel-filtration chromatography. The 6-bromo-2-naphthyl β -galactosidase studied by Hsia *et al.* (1966) was assumed to be identical with 'lactase 2'.

The purpose of the present study was to characterize the different human intestinal β -galactosidases with respect to pH optimum, substrate specificity and the effect of some inhibitors. Knowledge about such properties of the enzymes would enable us to evaluate the contribution of each of the different β -galactosidases to the total lactase activity found in small-intestinal biopsy specimens, which is of special interest in connexion with lactose intolerance.

MATERIALS AND METHODS

Preparation of homogenates. Macroscopically normal pieces of human small intestine were obtained during surgical section (Table 1).

The pieces of intestine were chilled with crushed ice as soon as possible after removal (usually within 1 hr.). The

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

Sample no.	Age (years) and sex of patient	Diagnosis	Level of small intestine	β -Galactosidase activities (units/g. wet wt. of mucosa)			Protein (mg./g wet wt. of mucosa)
				Lactase	<i>p</i> -Nitrophenyl β -galactosidase	6-Bromo-2-naphthyl β -galactosidase	
1	55; ♂	Pancreatic tumour	Duodenum	0.11	1.02	0.090	157
2	52; ♀	Uretero- ileostomy	Distal ileum	0.30	0.77	Not measured	Not measured
3*	58; ♂	Hepatic cirrhosis	Middle part of small intestine	3.38	0.86	0.057	107
4	55; ♀	Pancreatic tumour	Proximal jejunum	5.27	2.68	0.229	185
5	55; ♀	Large-intestinal tumour	Distal ileum	20.76	4.54	0.180	162
6†	51; ♂	Jejunal tumour	Proximal jejunum	5.45	2.51	0.122	167

* Obtained at autopsy about 8 hr. after death.

† Homogenate stored at -20° for 2 years.

mucosa was scraped off with a glass slide or a metal spatula and then homogenized with 4 vol. (v/w) of 0.05 M-potassium-sodium phosphate buffer, pH 7.0 (equal concentrations of K^{+} and Na^{+}), in an Ultra-Turrax homogenizer. The tube was chilled with crushed ice during the homogenization. The homogenate was either used immediately or stored at -20° until use (up to a few months).

Ultracentrifugation and solubilization. The homogenate was centrifuged at 100000g for 60 min. at 4° in a Spinco preparative ultracentrifuge. The supernatant contained the spontaneously soluble enzyme fraction of the homogenate. The pellet was resuspended in 0.05 M-potassium-sodium phosphate buffer, pH 7.0, to the original volume of the homogenate and then treated with papain in the following way. To each 1 ml. of resuspended pellet was added 0.125–5.0 mg. of crystalline papain (Mann Research Laboratories, New York, N.Y., U.S.A.) and 0.2 mg. of cysteine hydrochloride. The tube was incubated for 60 min. at 37° and then chilled with crushed ice. The preparation was again centrifuged at 100000g for 60 min. at 4° . The supernatant contained the originally insoluble, but papain-solubilized, enzyme fraction.

Gel-filtration chromatography. A 3.5 g. portion of Sephadex G-200 was mixed with about 500 ml. of 0.05 M-potassium-sodium phosphate buffer, pH 7.0, and left to swell for 3 days at room temperature. The gel was then packed into a column (95 cm. $\times$ 1 cm.) and washed with at least 150 ml. of buffer. A portion (1–2.5 ml.) of enzyme preparation was applied to the column. The flow rate was 4–5 ml./hr. and half-hour fractions were collected. In some experiments a column 95 cm. $\times$ 2.5 cm. was used, in which case 4–6 ml. of enzyme preparation was applied and the flow rate was about 10 ml./hr.

Enzyme-activity assay. The buffer concentration of the reaction mixture was 50 mM. For the pH-activity curve measurements, the following buffers were used: glycine-HCl, pH 1.5–2.0; sodium citrate, pH 2.6–6.0; sodium phosphate, pH 6.5–8.0. The final pH of the reaction mixture was measured. For the other experiments sodium citrate buffer, pH 5.5, was used.

The following substrate concentrations were used for the routine determinations: lactose, 28 mM; phenyl β -galactoside, 20 mM; *o*-nitrophenyl β -galactoside, 12 mM; *p*-nitrophenyl β -galactoside, 12 mM; 6-bromo-2-naphthyl β -galactoside, 0.39 mM. We also used two β -glucosides: cellobiose, 29 mM; *p*-nitrophenyl β -glucoside, 12 mM. For the K_m determinations the substrate concentrations were varied within the following limits: lactose, 3–64 mM; phenyl β -galactoside, 0.5–16 mM; *o*-nitrophenyl β -galactoside, 0.25–16 mM; *p*-nitrophenyl β -galactoside, 0.25–32 mM; 6-bromo-2-naphthyl β -galactoside, 0.1–1 mM.

When PCMB* (KEBO AB, Stockholm, Sweden) was tested as an inhibitor 36 mg. was dissolved in 5.0 ml. of 0.1 M-NaOH, giving a stock solution of 0.02 M-PCMB. This was then diluted with 0.1 M-sodium citrate buffer, pH 5.5, to appropriate concentrations.

When tris was tested as an inhibitor, 0.025–1 M-tris-HCl was dissolved in the stock buffer and the pH was adjusted to 5.5. The reaction mixture contained 0.0125–0.5 M-tris.

The incubations were performed at 37° for 30–60 min., over which period rates were constant. Blanks were prepared in the same way as the samples, but not incubated. The following methods were used to measure the degree of hydrolysis of the different substrates.

(a) Lactose and cellobiose. The incubations were stopped by placing the reaction mixture (0.2 ml.) in a boiling-water bath for 2 min. Then 0.4 ml. of tris-glucose oxidase reagent (Dahlqvist, 1964) was added and the tube incubated at 37° for 60 min. The extinction was measured at 420 nm. with a Beckman spectrophotometer or with a Vitatron filter-photometer at 425 nm.

During the attempts to find out whether the hetero β -galactosidase had any lactase activity, experiments were also performed in which liberated glucose was assayed with a hexokinase-ATP-glucose 6-phosphate dehydrogenase-NADP⁺ system as described by Asp & Dahlqvist (1968b).

Standard curves were prepared with 1–20 μ g. of glucose.

(b) Phenyl β -galactoside. The reaction was interrupted,

* Abbreviation: PCMB, *p*-chloromercuribenzoate.

and the colour developed, by adding to the reaction mixture (0.2 ml.): 0.1 ml. of a reagent containing 1 M-NH₃, 2% (v/v) ethanol and 0.4 mg. of 4-aminoantipyrine/ml., and then 0.1 ml. of K₃Fe(CN)₆ solution (2 mg./ml.) (Martin, 1949; Baruah & Swain, 1957). The extinction was measured on a Beckman spectrophotometer at 505 nm. or on a Vitatron filter-photometer at 508 nm. Standard curves were prepared with 1–5 μ g. of phenol.

(c) *o*-Nitrophenyl β -galactoside and *p*-nitrophenyl β -galactoside. When the incubation mixture contained 50 mM-sodium citrate buffer, pH 5.5, the reaction was stopped, and the colour developed, by addition of 0.1 ml. of *m*-tris-HCl buffer, pH 8.5, to the incubation mixture (0.2 ml.). When the incubation mixture contained other buffers, or tris, 0.1 ml. of *m*-K₂CO₃ was added instead. The extinction was measured on a Beckman spectrophotometer at 420 nm. or on a Vitatron filter-photometer at 425 nm. Standard curves were prepared with 0.5–10 μ g. of *o*-nitrophenol or 0.5–5 μ g. of *p*-nitrophenol.

(d) 6-Bromo-2-naphthyl β -galactoside. The hydrolysis of this substrate was measured by the method of Cohen, Tsou, Rutenburg & Seligman (1952) performed as described by Asp & Dahlqvist (1968b).

Measurement of protein concentration. The protein content of homogenates was measured with the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955). The protein concentration in the eluate of the chromatograms was estimated by measuring the E_{280} . In both cases human serum albumin (KABI AB, Stockholm, Sweden) was used as standard.

Calculations. Enzyme activities were calculated as μ moles of substrate hydrolysed/min. Michaelis constants, K_m , inhibitor constants, K_i , and the maximal rates of hydrolysis, V_{max} , were calculated by the method of Lineweaver & Burk (1934) as modified by Dixon (1953).

RESULTS

Solubility of the β -galactosidase activity. When a mucosal homogenate had been ultracentrifuged at 100 000 *g* for 60 min. only 2–5% of the lactase activity but 60–80% of the 6-bromo-2-naphthyl β -galactosidase activity were found in the supernatant. The rest of the activities could be recovered in the resuspended sediment. With *p*-nitrophenyl β -galactoside as the substrate 17–61% (mean 39%) of the activity was soluble.

The lactase activity of a homogenate was not inhibited when 0.1 mM-PCMB was added to the reaction mixture. The 6-bromo-2-naphthyl β -galactosidase activity, however, was completely inhibited. The *p*-nitrophenyl β -galactosidase activity was partially inhibited by PCMB and 81–97% of the remaining (i.e. PCMB-stable) activity sedimented during ultracentrifugation. The PCMB-inhibited activity was calculated as the difference between the total and the PCMB-stable *p*-nitrophenyl β -galactosidase activity. About 80% of this activity remained in the supernatant during ultracentrifugation.

Solubilization of the particle-bound β -galactosidase

Fig. 1. Effect of digestion with papain on the particle-bound β -galactosidase activity (lactase). The resuspended pellet after ultracentrifugation of a mucosal homogenate from sample 4 was incubated for 60 min. at 37° without papain and with different concentrations of papain. After the incubation the remaining activity (O) and the activity soluble after ultracentrifugation (●) were measured. 12 mM-*p*-Nitrophenyl β -galactoside was used as the substrate and the assay was performed in the presence of 0.1 mM-PCMB. The activities are given as percentages of the original activity of the resuspended pellet. In the sample where no papain was present, some solubilization and inactivation occurred owing to 'autolysis'.

activity. Incubation with papain partially solubilized the originally particle-bound β -galactosidase activity (Fig. 1). Optimum solubilization occurred when the papain concentration was 70–140 mg./g. of pellet protein. Therefore the following procedure was used as a standard method for solubilization in the subsequent experiments: 2.5 mg. of crystalline papain, about 15–20 mg. of pellet protein and 0.2 mg. of cysteine hydrochloride were incubated for 60 min. at 37° in 1 ml. of 0.05 M-potassium-sodium phosphate buffer, pH 7.0. The preparation was then ultracentrifuged at 100 000 *g* for 60 min. The clear supernatant was used as solubilized enzyme preparation.

After this procedure 30% of the originally particle-bound β -galactosidase (measured with *p*-nitrophenyl β -galactoside as the substrate in the presence of 0.1 mM-PCMB) appeared in the supernatant, and 20–30% of the activity still sedimented with the pellet. About 40–50% of the original activity was inactivated during the incubation with papain.

The PCMB-inhibited activity was sensitive to incubation with papain. Thus when a homogenate had been incubated with 69 mg. of papain/g. of protein for 60 min. at 37° all the remaining β -galactosidase activity was resistant to PCMB.

Separation by gel-filtration chromatography. When the supernatant after ultracentrifugation of a crude

Fig. 2. Separation of human small-intestinal β -galactosidases by Sephadex G-200 column chromatography. Protein concentration ($\circ$) was measured by the E_{280} . (a)–(c) A mucosal homogenate from sample 4 was ultra-centrifuged and 2 ml. of the supernatant was applied to the column (1 cm. diam.). For clarity the results are shown in three parts. β -Galactosidase activity was measured with 28 mM-lactose ($\bullet$), 12 mM-*p*-nitrophenyl β -galactoside (Δ) and 0.39 mM-6-bromo-2-naphthyl β -galactoside ($\blacktriangle$) as substrate (a and b). For comparison β -glucosidase activity was also measured with 29 mM-cellobiose ($\blacksquare$) and 12 mM-*p*-nitrophenyl β -glucoside ($\square$) as substrate (c). One small peak of lactase (fraction 1a) and one peak of hetero β -galactosidase (fraction 2) were obtained. (d) and (e) The pellet after ultracentrifugation was resuspended, solubilized with papain and ultra-centrifuged again. Of the supernatant thus obtained 2.5 ml. was applied on the column. For clarity the results are shown in two parts. β -Galactosidase activity was measured with 28 mM-lactose ($\bullet$) and 12 mM-*p*-nitrophenyl β -galactoside (Δ) as substrate (d). For comparison the β -glucosidase activity was also measured with 29 mM-cellobiose ($\blacksquare$) and 12 mM-*p*-nitrophenyl β -glucoside ($\square$) as substrate (e). One peak of lactase (fraction 1b) was obtained in the same position as fraction 1a above. No hetero β -galactosidase was recovered. (f) Blue Dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) ($\blacktriangle$) and glucose (Δ) were run as reference substances to determine the void volume and the total volume of the column.

Table 2. *Relative rates of hydrolysis of different substrates*

The activity, V_0 , was measured with the substrate concentrations given in the Materials and Methods section. Theoretical $V_{\max}$ values were calculated from Lineweaver–Burk plots. The values are given as percentages of the lactase activity (fraction 1b) or *p*-nitrophenyl β -galactosidase activity (fraction 2).

Substrate	Measured V_0 values		Theoretical $V_{\max}$ values	
	Fraction 1b	Fraction 2	Fraction 1b	Fraction 2
Lactose	100	< 0.3	100	—
Phenyl β -galactoside	< 0.2	21	—	26
<i>o</i> -Nitrophenyl β -galactoside	24	46	21	48
<i>p</i> -Nitrophenyl β -galactoside	16	100	23	100
6-Bromo-2-naphthyl β -galactoside	< 0.2	11	—	23

mucosal homogenate was chromatographed on Sephadex G-200 two peaks of β -galactosidase activity were separated (Fig. 2). The first peak

(referred to as fraction 1a) was eluted close to the void volume ($K_{av} = 0.17$). The enzyme in this peak hydrolysed lactose most rapidly. *o*-Nitrophenyl

β -galactoside and *p*-nitrophenyl β -galactoside were also hydrolysed. Phenyl β -galactoside or 6-bromo-2-naphthyl β -galactoside were not hydrolysed at a measurable rate. The substrate specificity at pH 5.5 was the same as for fraction 1b (see below).

Fig. 3. (a) pH-activity curve of fraction 1a. 12mM-*p*-Nitrophenyl β -galactoside was used as substrate and the assays were performed without inhibitor (○) and in the presence of 0.1mM-PCMB (●). (b) pH-activity curve of fraction 1b. 12mM-*p*-Nitrophenyl β -galactoside (●) and 28mM-lactose (○) were used as substrates. (c) pH-activity curve of fraction 2. 12mM-*p*-Nitrophenyl β -galactoside (●) and 0.39mM-6-bromo-2-naphthyl β -galactoside (○) were used as substrates.

The second peak ($K_{av} = 0.53$) had very high *p*-nitrophenyl β -galactosidase activity. The enzyme in this peak also hydrolysed phenyl β -galactoside, *o*-nitrophenyl β -galactoside and 6-bromo-2-naphthyl β -galactoside. Lactase activity, however, could not be demonstrated with any of the methods used for assay of liberated glucose (see the Materials and Methods section), even after 20-fold concentration of the peak material (Fig. 2 and Table 2). The β -galactosidase activity of this peak is referred to as fraction 2.

The protein was eluted in three main peaks, one at the void volume, one between the void volume and the total volume and one at the total volume of the column (Figs. 2 and 5).

Chromatography of the solubilized pellet gave one single peak of β -galactosidase activity in practically the same position as fraction 1a ($K_{av} = 0.14$) (Figs. 2 and 5). The enzyme in this peak, which is referred to as fraction 1b, hydrolysed lactose, *o*-nitrophenyl β -galactoside and *p*-nitrophenyl β -galactoside, but not phenyl β -galactoside or 6-bromo-2-naphthyl β -galactoside (Table 2). The relative rates of hydrolysis of the different substrates at pH 5.5 were the same as in fraction 1a.

One major peak of protein was eluted close to the total volume.

When corresponding amounts of supernatant and solubilized pellet were chromatographed, fraction 1b contained 5–6 times as much lactase activity as fraction 1a.

pH optima. The pH optimum of fraction 1a and 1b was at pH 5.6 (Figs. 3a and b). The shape of the pH-activity curves of the two fractions was similar at the alkaline side of the pH optimum. At acid pH, however, fraction 1a had higher activity than fraction 1b. The pH-activity curve of fraction 1a was studied also in the presence of 0.1mM-PCMB. This substance inhibited the activity at pH values lower than 5 but did not influence the activity at higher pH values (Fig. 3a).

Table 3. K_m values of fractions 1b and 2 with different substrates

The values given represent the means of four to six experiments with the ranges in parentheses.

Substrate	K_m (mM)	
	Fraction 1b	Fraction 2
Lactose	19 (17–23)	—
Phenyl β -galactoside	—	10.0 (8.9–11.0)
<i>o</i> -Nitrophenyl β -galactoside	6.0 (4.5–7.2)	3.5 (2.9–5.3)
<i>p</i> -Nitrophenyl β -galactoside	17 (11–24)	2.7 (2.0–3.1)
6-Bromo-2-naphthyl β -galactoside	—	0.54 (0.49–0.60)

Fraction 2 had a pH optimum between pH 5.5 and 6. The pH-activity curve was broader with *p*-nitrophenyl β -galactoside as substrate than with 6-bromo-2-naphthyl β -galactoside (Fig. 3c).

K_m values. From the solubility studies, the relative rates of hydrolysis of different substrates, the behaviour on Sephadex chromatography, the pH-activity curves, the β -glucosidase activities (see below) and the stability to PCMB (see below) it was thought reasonable to assume that the β -galactosidase activity of fraction 1a was exerted mainly by the same enzyme as that eluted in fraction 1b. Therefore the estimation of apparent *K_m* values with different substrates was made only with fractions 1b and 2 (Table 3).

Fraction 1b had a lower *K_m* with *o*-nitrophenyl β -galactoside as the substrate than with lactose and *p*-nitrophenyl β -galactoside.

Fraction 2 had the lowest *K_m* with 6-bromo-2-naphthyl β -galactoside as the substrate. The *K_m* value given for *p*-nitrophenyl β -galactoside as substrate was obtained with substrate concentrations above 2mM. With 0.25–1mM substrate the apparent *K_m* was lower (0.15–0.56mM).

The relative *V_{max}* values for the hydrolysis of different substrates are given in Table 2. Fraction 1b hydrolysed lactose much more rapidly than hetero β -galactosides. Fraction 2, on the other hand, hydrolysed *p*-nitrophenyl β -galactoside much more rapidly than other hetero β -galactosides, and lactose was not hydrolysed at a measurable rate.

β -Glucosidase activity of the different fractions. β -Glucosidase activity was measured with 29mM-cellobiose or 12mM-*p*-nitrophenyl β -glucoside as substrate. Fractions 1a and 1b hydrolysed both substrates. The cellobiose/lactase activity ratio was in each case about 0.2. The *p*-nitrophenyl β -glucosidase/lactase activity ratio was also about 0.2. Fraction 2 hydrolysed *p*-nitrophenyl β -glucoside but not

cellobiose. The *p*-nitrophenyl β -glucosidase/*p*-nitrophenyl β -galactosidase activity ratio varied in different experiments between 0.5 and 1.3.

Effect of PCMB and tris. Fraction 1b was not influenced by PCMB up to 0.1mM concentration. Fraction 1a was not influenced when measured at pH 5.5, but at low pH values the activity of this fraction was partially inhibited (Fig. 3a). The β -galactosidase activity of fraction 2 was partially inhibited by 0.1–10 μ M-PCMB and completely inhibited by 0.1mM-PCMB (Fig. 4).

Tris competitively inhibited the β -galactosidase activity both in fraction 1b and in fraction 2. The *K_i* was about 25mM in fraction 1b with 8–64mM-lactose as substrate and 12.5–50mM-tris as inhibitor, and about 60mM in fraction 2 with 2–16mM-*p*-nitrophenyl β -galactoside as substrate and 31–500mM-tris as inhibitor.

DISCUSSION

From the values of β -galactosidase activity of the different tissue specimens used (Table 1), it is obvious that samples 3–6 had normal lactase activity (Auricchio *et al.* 1963; Dahlqvist, Hammond, Crane, Dunphy & Littman, 1963; Dunphy *et al.* 1965; Haemmerli *et al.* 1965; Zoppi *et al.* 1966; McMichael, Webb & Dawson, 1966). Samples 1 and 2, however, had low lactase activity. For sample 1 this was probably due to the fact that the sample was taken too high up in the duodenum (Auricchio *et al.* 1963). In the Sephadex G-200 chromatogram of sample 1 no fraction 1a could be detected, whereas in sample 2 fractions 1a and 1b were detectable but very small.

The 6-bromo-2-naphthyl β -galactosidase activity of the different samples is comparable with that reported by Cohen *et al.* (1952) and Cook & Dahlqvist (1968), but much lower than in the patients studied by Zoppi *et al.* (1966) and Hsia *et al.* (1966). Zoppi *et al.* (1966) also studied the distribution of lactase and 6-bromo-2-naphthyl β -galactosidase activities from jejunal mucosa of adults in the supernatant and the pellet after centrifugation at 113000g for 75min. They found about 60% of the 6-bromo-2-naphthyl β -galactosidase activity in the supernatant and about 20% in the pellet. The lactase activity, on the other hand, was found mainly in the pellet. Doell & Kretschmer (1962) reported that no lactase activity remained in the supernatant after centrifugation at 105000g for 1hr. of a small-intestinal homogenate from a 3-day-old child.

Our results thus confirm the earlier reports on the solubility of different β -galactosidase activities in mucosal homogenates from the human small intestine. The number of pieces of intestine investigated by us was too small to permit any conclusions

Fig. 4. Effect of PCMB on the β -galactosidase activity of fractions 1b (●) and 2 (○). 12mM-*p*-Nitrophenyl β -galactoside was used as substrate.

on possible differences in the distribution of the β -galactosidases at different levels of the small intestine.

The results of this investigation support the idea that at least two different β -galactosidases exist in the small-intestinal mucosa of the human. One of the enzymes, eluted in fractions 1a and 1b and at least 95% particle-bound, is resistant to 0.1 mM-PCMB, and most active with lactose as the substrate. This enzyme should be characterized as the lactase of the small-intestinal mucosa. The other enzyme, eluted in fraction 2, is mainly soluble and is inhibited by 0.1 mM-PCMB. This enzyme hydrolyses all the hetero β -galactoside substrates tested but has no detectable lactase activity. Therefore it should be characterized as the hetero β -galactosidase of the intestinal mucosa. The differences in the pH-activity curves of fractions 1a and 1b indicate the possible existence of a third β -galactosidase with low pH optimum. This enzyme might be responsible for the activity measured at pH 3.0–3.5 by Cook & Dahlqvist (1968).

For the solubilization of the human lactase a much higher concentration of papain was required than for the solubilization of the corresponding enzyme of the rat small intestine (i.e. the 'neutral' β -galactosidase; Dahlqvist *et al.* 1965; Asp & Dahlqvist, 1968*a,b*), and even under optimum conditions only 30% of the originally particle-bound activity could be recovered in soluble form.

Gel-filtration chromatography of the initially soluble material and of the material soluble after digestion with papain both gave a single peak of lactase, which was eluted early ($K_{av} = 0.14$ and 0.17 respectively). This lactase is presumably identical with the 'lactase 1' reported by Semenza *et al.* (1965). 'Lactase 1' had $K_d = 0.14$ and a lactase/cellobiose activity ratio about 5. The same ratio was found for the lactase in this study. Further, Hsia *et al.* (1966) reported the K_m of 'lactase 1' to be 18 mM, i.e. practically the same value found for the lactase in this study.

However, the 'lactase 2' reported by Semenza *et al.* (1965) could not be demonstrated in our separations. 'Lactase 2' could not be identical with the hetero β -galactosidase isolated in this study for several reasons. First, no lactase activity could be demonstrated in the hetero β -galactosidase peak. Secondly, this peak was eluted earlier ($K_{av} = 0.53$) than 'lactase 2' (K_d about 0.8). Thirdly, 'lactase 2' was demonstrated also when the preparation had been solubilized by incubation with 200 mg. of papain/g. of pellet protein for 30 min. at 37°. According to our experience the hetero β -galactosidase would have been completely inactivated by this treatment.

We could also not demonstrate any cellobiase activity in the expected position of 'lactase 2'.

A prominent glucose peak was, however, eluted close to the total volume of the Sephadex G-200 column (Fig. 5). This glucose was released from the Sephadex during chromatography. The isomaltase activity was eluted in a broad peak somewhat earlier than the glucose, and this enzyme is probably responsible for the release of glucose. Kolínská & Semenza (1967) reported the liberation of glucose from Sephadex by the sucrase-isomaltase complex from the rabbit small intestine. Because of this liberation of glucose from the Sephadex gel, a separate zero-time blank had to be prepared for every disaccharidase assay in the eluate, as otherwise a spurious disaccharidase activity would be found close to the total volume of the chromatogram.

The inhibition of the hetero β -galactosidase by PCMB indicates that this enzyme, like the β -galactosidase of *Escherichia coli* (Wallenfels, Lehmann & Malhotra, 1960) and the 'acid' enzyme of the rat (Koldovský, Asp & Dahlqvist, 1969), is dependent on intact thiol groups for hydrolytic activity. The lactase, on the other hand, was not influenced by this inhibitor. The same is true for the calf intestinal lactase (Wallenfels & Fischer, 1960) and for the 'neutral' enzyme of the rat (Koldovský *et al.* 1969).

Tris has been reported to be a competitive inhibitor of calf intestinal lactase (Wallenfels & Fischer, 1960), pig intestinal sucrase (Dahlqvist, 1958) and rabbit intestinal sucrase (Kolínská & Semenza, 1967). Other polyalcohols are also competitive inhibitors of glycosidases (Kelemen & Whelan, 1966).

In comparison of the human small-intestinal β -galactosidases with those of other species, both similarities and differences can be noted. For the human lactase and the corresponding enzyme in the rat (i.e. the 'neutral' β -galactosidase) the characteristics such as subcellular localization, behaviour during gel-filtration chromatography, pH optimum, substrate specificity, β -glucosidase activity and stability to 0.1 mM-PCMB, are very similar (Asp & Dahlqvist, 1968*a,b*; Koldovský *et al.* 1969).

The human hetero β -galactosidase, however, is different from the 'acid' β -galactosidase of the rat small intestine. The most prominent differences are found in the pH optimum, which is near neutral in the human but acid in the rat, and in the lactase activity, which is not detectable in the human hetero β -galactosidase but almost as high as the hetero β -galactosidase activities in the 'acid' enzyme of the rat. The human hetero β -galactosidase ($K_{av} = 0.53$) also seems to be a smaller molecule than the 'acid' enzyme of the rat ($K_{av} = 0.44$). In a previous paper (Asp & Dahlqvist, 1968*b*) it was shown that lactose was a competitive inhibitor of the 'acid' enzyme of the rat. The human hetero β -galactosidase, however, was not appreciably

Fig. 5. Comparison of lactase and hetero β -galactosidase with invertase, isomaltase and glucose at column chromatography on Sephadex G-200. (a) The pellet (67 mg. of protein in 4 ml.) after ultracentrifugation of a homogenate of sample 6 was solubilized with papain and ultracentrifuged again. The supernatant thus formed was applied on the column (2.5 cm. diam.). Lactase (●) and cellobiase (□) were eluted in one single peak (fraction 1b). Protein (○) was eluted mainly in a peak at the total volume of the column. The liberated glucose (Δ) occurred in a peak a little earlier than the final protein peak ($K_{av}=0.9$). (b) Isomaltase (●) and invertase (○) in the chromatogram shown in (a). To indicate the position of the lactase the *p*-nitrophenyl β -galactosidase activity is also shown (▲). The invertase-isomaltase complex was eluted a little earlier than the glucose peak shown in (a). Isomaltase and invertase were assayed with the two-step ultramicro method of Dahlqvist (1968). (c) For comparison the position of the hetero β -galactosidase after chromatography of the originally soluble fraction of a homogenate on the same column as above is shown in this part. A homogenate from sample 6 was ultracentrifuged and 4.9 ml. of the supernatant was applied to the column. β -Galactosidase activity was measured with 12 mm-*p*-nitrophenyl β -galactoside as substrate (▲). The hetero β -galactosidase (fraction 2) was eluted at 350 ml., i.e. half-way between the position of lactase (a) and the total volume of the column. Fraction 1a (at 210 ml.) was very small in this experiment. The protein (○) was eluted in three peaks. The major peak occurred at the total volume.

inhibited by lactose (N.-G. Asp & A. Dahlqvist, unpublished work). Other properties, such as solubility and sensitivity to PCMB, are similar for both enzymes.

The β -glucosidase activity of fractions 1a and 1b was probably exerted by the same enzyme as the β -galactosidase, i.e. by the lactase. The cellobiase/lactase activity ratio was the same as that reported for 'lactase 1' by Semenza *et al.* (1965) and as that reported for other species by, e.g., Dahlqvist (1961) and Swaminathan & Radhakrishnan (1967). The fact that the β -glucosidase/ β -galactosidase activity ratio varied in the hetero β -galactosidase peak may indicate the existence of a hetero β -glucosidase different from the hetero β -galactosidase.

It has been suggested that the residual lactase activity found in patients with lactose intolerance would be exerted by the soluble β -galactosidase, i.e. the hetero β -galactosidase (see, e.g., Dawson, 1968). This cannot be true since the soluble hetero β -galactosidase has no lactase activity. The residual lactase activity of these patients seems to be exerted either by a small amount of remaining lactase as such, or possibly by a third enzyme, which seems to be particle-bound and which could not be solubilized with papain. Such an enzyme might also be responsible for the insoluble fraction of the 6-bromo-2-naphthyl β -galactosidase activity and for the activity of homogenates at low pH values (Cook & Dahlqvist, 1968). To answer these questions the residual lactase activity of lactase-deficient patients should be isolated and characterized.

The skilful technical assistance of Mrs Birgitta Norén and Miss Ulla Iwarson is gratefully acknowledged. The investigation has been kindly supported by grants from the Medical Faculty of the University of Lund, the Swedish Nutrition Foundation, A. Pahlsson's Foundation, and the Swedish Medical Research Council (Project nos. B69-13X-157 and B69-13P-2262). We thank the staff of the surgical clinic of the Hospital, Lund, who have put at our disposal the human material used in the investigation. O.K. is on leave of absence from the Laboratory of Developmental Nutrition, Institute of Physiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia.

REFERENCES

- Asp, N.-G. & Dahlqvist, A. (1968a). *Biochem. J.* **106**, 841.
 Asp, N.-G. & Dahlqvist, A. (1968b). *Biochem. J.* **110**, 143.
 Auricchio, S., Rubino, A., Tosi, R., Semenza, G., Landolt, M., Kistler, H. & Prader, A. (1963). *Enzymol. Biol. clin.* **3**, 193.
 Baruah, P. & Swain, T. (1957). *Biochem. J.* **66**, 321.
 Cohen, R. B., Tsou, K.-C., Rutenburg, S. H. & Seligman, A. M. (1952). *J. biol. Chem.* **195**, 239.
 Cook, G. C. & Dahlqvist, A. (1968). *Gastroenterology*, **55**, 328.
 Dahlqvist, A. (1958). *Acta chem. scand.* **12**, 2012.
 Dahlqvist, A. (1961). *Biochim. biophys. Acta*, **50**, 55.
 Dahlqvist, A. (1964). *Analyt. Biochem.* **7**, 18.
 Dahlqvist, A. (1968). *Analyt. Biochem.* **22**, 99.
 Dahlqvist, A. & Asp, N.-G. (1967). *Biochem. J.* **103**, 86.
 Dahlqvist, A., Bull, B. & Thomson, D. L. (1965). *Arch. Biochem. Biophys.* **109**, 159.
 Dahlqvist, A., Hammond, J. B., Crane, R. K., Dunphy, J. V. & Littman, A. (1963). *Gastroenterology*, **45**, 488.
 Dawson, A. M. (1968). *Annu. Rev. sci. Basis Med.* p. 186.
 Dixon, M. (1953). *Biochem. J.* **55**, 170.
 Doell, R. G. & Kretchmer, N. (1962). *Biochim. biophys. Acta*, **62**, 353.
 Doell, R. G. & Kretchmer, N. (1963). *Biochim. biophys. Acta*, **67**, 516.
 Doell, R. G., Rosen, G. & Kretchmer, N. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 1288.
 Dunphy, J. V., Littman, A., Hammond, J. B., Forstner, G., Dahlqvist, A. & Crane, R. K. (1965). *Gastroenterology*, **49**, 12.
 Eggstein, M. & Kreutz, F. H. (1955). *Klin. Wschr.* **33**, 879.
 Haemmerli, U. P., Kistler, H., Amman, R., Marthaler, T., Semenza, G., Auricchio, S. & Prader, A. (1965). *Amer. J. Med.* **38**, 7.
 Heilskov, N. S. C. (1956). *Studier over Animalsk Lactase*, pp. 140-151. Copenhagen: Munksgaard.
 Hore, P. & Messer, M. (1968). *Comp. Biochem. Physiol.* **24**, 717.
 Hsia, D. Y.-Y., Makler, M., Semenza, G. & Prader, A. (1966). *Biochim. biophys. Acta*, **113**, 390.
 Kelemen, M. V. & Whelan, W. J. (1966). *Biochem. J.* **100**, 5r.
 Koldovský, O., Asp, N.-G. & Dahlqvist, A. (1969). *Analyt. Biochem.* **27**, 409.
 Koldovský, O., Heringová, A. & Jirsová, V. (1966a). *Biol. neonat.* **10**, 241.
 Koldovský, O., Heringová, A., Jirsová, V., Chytil, F. & Hošková, J. (1966b). *Canad. J. Biochem.* **44**, 523.
 Koldovský, O., Noack, R., Schenk, G., Jirsová, V., Heringová, A., Braná, H., Chytil, F. & Friedrich, M. (1965). *Biochem. J.* **96**, 492.
 Kolínská, J. & Semenza, G. (1967). *Biochim. biophys. Acta*, **146**, 181.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 McMichael, H. B., Webb, J. & Dawson, A. M. (1966). *Brit. med. J.* **2**, 1037.
 Maio, J. J. & Rickenberg, H. V. (1960). *Biochim. biophys. Acta*, **37**, 101.
 Martin, R. W. (1949). *Analyt. Chem.* **21**, 1419.
 Semenza, G., Auricchio, S. & Rubino, A. (1965). *Biochim. biophys. Acta*, **96**, 487.
 Swaminathan, N. & Radhakrishnan, A. N. (1967). *Indian J. Biochem.* **4**, 64.
 Wallenfels, K. & Fischer, J. (1960). *Hoppe-Seyl. Z.* **321**, 223.
 Wallenfels, K., Lehmann, J. & Malhotra, O. P. (1960). *Biochem. Z.* **333**, 209.
 Zoppi, G., Hadorn, B., Gitzelmann, R., Kistler, H. & Prader, A. (1966). *Gastroenterology*, **50**, 557.