Rat Small-Intestinal β -Galactosidases

KINETIC STUDIES WITH THREE SEPARATED FRACTIONS

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1. Three fractions of β -galactosidase activity from the rat small-intestinal mucosa were separated chromatographically. Two of these fractions had an acid pH optimum at 3–4, and the third one had a more neutral pH optimum at 5·7. 2. The two 'acid' β -galactosidase fractions had considerably lower K_m values for hetero β -galactosides than for lactose. The V_{max} values were similar for all the substrates used (lactose, phenyl β -galactoside, *o*-nitrophenyl β -galactoside, *p*-nitrophenyl β -galactoside and 6-bromo-2-naphthyl β -galactoside). No difference could be detected between the two 'acid' fractions with respect to their enzymic properties (pH optimum, K_m for the different substrates, K_i for lactose as an inhibitor of the hydrolysis of hetero β -galactosides, K_i for phenyl β -galactoside as an inhibitor of the hydrolysis of lactose, and relative V_{max} for the hydrolysis of different substrates). These two fractions probably represent different forms of the same enzyme. 3. The 'neutral' fraction had similar K_m values for all the substrates hydrolysed, but with lactose as substrate the V_{max} was much higher than with the hetero β -galactosides. This fraction did not split phenyl β -galactoside or 6-bromo-2-naphthyl β -galactoside at a measurable rate. 4. Lactose was a competitive inhibitor of the hetero β -galactosidase activities of all the three fractions, and K_i for lactose as an inhibitor in each case was the same as K_m for the lactase activity. Phenyl β -galactoside was a competitive inhibitor of the lactase activity of all the three fractions. These facts strongly indicate that in all the three fractions lactose is hydrolysed by the same active sites as the hetero β -galactosides. 5. Human serum albumin stabilized the separated enzymes against inactivation by freezing and thawing.

In a previous paper (Asp & Dahlqvist, 1968) the separation of three fractions of β -galactosidase activity from the small-intestinal mucosa of adult rats was reported. Five different β -galactosides were used as substrates (lactose, phenyl β -galactoside, *o*-nitrophenyl β -galactoside, *p*-nitrophenyl β -galactoside and 6-bromo-2-naphthyl β -galactoside). Two of the fractions had higher activity at pH 3–4 than at pH 5·0–5·5, and are called 'acid' fractions. The third fraction was assayed at pH 5·0–5·5 but had no activity at pH 3–3·5. This fraction is called the 'neutral' fraction.

To characterize these three fractions of β -galactosidase activity further we have now studied (1) the pH-activity curves, (2) the influence of the substrate concentration on the hydrolysis of the different substrates and (3) the inhibition of the hydrolysis of hetero β -galactosides by lactose and of the hydrolysis of lactose by phenyl β -galactoside.

The effect of human serum albumin on the stability of the separated fractions was also investigated.

MATERIALS AND METHODS

Preparation of the 'acid' fractions. Albino rats 8–12 days old were decapitated. The distal third of the small intestine was removed, rinsed with about 5 ml. of cooled 0·9% NaCl and placed on an ice-cold glass dish. The intestine was cut open and the mucosa was scraped off and homogenized with 10–20 parts (v/w) of 0·9% NaCl in an Ultra-Turrax homogenizer. The tube was chilled with crushed ice during the homogenization.

The homogenate was centrifuged at 100 000*g* for 60 min. at 4° in a Spinco preparative ultracentrifuge. The supernatant, which contained 50–68% (mean 60%) of the 'acid' β -galactosidase activity of the crude homogenate measured with *o*-nitrophenyl β -galactoside or *p*-nitrophenyl β -galactoside as substrate at pH 3·5, was then dialysed overnight at 4° against 10 mM-sodium phosphate buffer, pH 6·0.

During the dialysis 15–37% of the activity of the supernatant was lost.

A portion (2–5 ml.) of the dialysed supernatant was chromatographed on a DEAE-cellulose column (10 cm. long $\times$ 1 cm. diam.) with a buffer concentration gradient at constant pH, as described by Asp & Dahlqvist (1968).

Preparation of the 'neutral' fraction. Albino rats about 16 days old were decapitated. The proximal third of the small intestine was removed, rinsed with about 5 ml. of cooled 0.9% NaCl and placed on an ice-cold glass dish. The intestine was cut open and the mucosa was scraped off and homogenized with 4 parts (v/w) of 0.9% NaCl in an Ultra-Turrax homogenizer. The tube was chilled during the homogenization.

The homogenate was then solubilized with papain as described earlier (Dahlqvist, Bull & Thomson, 1965; Asp & Dahlqvist, 1968) and ultracentrifuged at 100 000g for 60 min. at 4°. The supernatant contained 49–75% (mean 62%) of the 'neutral' β -galactosidase activity of the crude homogenate, measured with *o*-nitrophenyl β -galactoside as substrate at pH 5.5, the 'acid' activity being inhibited with *p*-chloromercuribenzoate (Koldovský, Asp & Dahlqvist, 1968). The supernatant was dialysed against 50 mM-sodium phosphate buffer, pH 7.0, which gave a loss of 6–36% (mean 17%) of its 'neutral' activity.

A portion (1.5–2.5 ml.) of the dialysed supernatant was chromatographed on a Sephadex G-200 column (95 cm. long $\times$ 1 cm. diam.), as described by Asp & Dahlqvist (1968).

Enzyme activity assay. The buffer concentration of the reaction mixtures was 50 mM. The following buffers were used for the pH-activity-curve measurements: glycine-HCl, pH 1.5–2.0; sodium citrate, pH 2.3–6.0; sodium phosphate, pH 6.5–7.0. The pH of the reaction mixtures was measured with a glass micro electrode (Radiometer, Copenhagen, Denmark) and differed from the pH of the stock buffer by 0.0–0.3 pH unit. For the kinetic studies sodium citrate buffer, pH 3.5 or 5.5, was used. Control experiments with sodium acetate buffer gave practically the same results.

The reaction mixtures contained 0.5 mg. of human serum albumin (KABI AB, Stockholm 30, Sweden)/ml. to stabilize the enzymes (see the Results section). When 6-bromo-2-naphthyl β -galactosidase activity was assayed, however, the albumin concentration was only 4–10 μ g./ml., since 0.05 mg. or more of albumin/ml. interfered with the determination of liberated 6-bromo-2-naphthol.

The substrate concentrations were varied within the following limits: lactose, 3–64 mM; phenyl β -galactoside, 0.33–8 mM; *o*-nitrophenyl β -galactoside, 0.2–16 mM; *p*-nitrophenyl β -galactoside, 0.1–32 mM; 6-bromo-2-naphthyl β -galactoside, 0.1–1 mM. When lactose was tested as an inhibitor of the hydrolysis of hetero β -galactosides the lactose concentration was 5–50 mM. When phenyl β -galactoside was tested as an inhibitor of the hydrolysis of lactose the concentration of phenyl β -galactoside was 2–100 mM.

The incubations were performed at 37°. In each experiment one series of tubes was incubated for 30 min. and another series for 60 min. to check that zero-order kinetics were obeyed under the conditions used. Blanks were prepared in the same way as the samples but not incubated.

The degree of hydrolysis was never allowed to exceed 20%. In most of the samples it was far below 20%. The

following methods were used to measure the degree of hydrolysis of the different substrates.

(a) Lactose. The incubation was stopped by boiling the reaction mixture in a water bath for 2 min. The amount of glucose liberated was usually measured with tris-glucose oxidase reagent (Dahlqvist, 1964). The reaction mixture (0.2 ml.) and 0.4 ml. of tris-glucose oxidase reagent were incubated at 37° for 60 min. The colour was then measured at 420 m μ on a Beckman Spinco 151 spectrophotometer with an ultramicro cuvette (volume 100 μ l., light-path 0.6 cm.). A standard curve was prepared with 1–20 μ g. of glucose.

Since phenol is known to inhibit the tris-glucose oxidase reagent (Dahlqvist, 1960) another method for glucose determination had to be used when phenyl β -galactoside was tested as an inhibitor of the hydrolysis of lactose. We then used the hexokinase-ATP-glucose 6-phosphate dehydrogenase-NADP⁺ system to yield NADPH, which was measured spectrophotometrically (Slein, Cori & Cori, 1950; Cori & Lerner, 1951; Schmidt, 1961). After incubation, the reaction mixture (0.2 ml.) was boiled for 2 min. in a water bath and brought to neutral pH with *m*-NaOH by adding 10 μ l. to an incubation mixture at pH 5.5 or 25 μ l. to an incubation mixture at pH 3.5. A 50 μ l. sample of the incubation mixture was then mixed with 300 μ l. of an enzyme reagent prepared by mixing 6.5 ml. of 50 mM-triethanolamine buffer, pH 7.6, 0.2 ml. of 0.1 M-MgCl₂, 10.3 mg. of ATP, 20 μ l. of hexokinase suspension (10 mg./ml.) and 40 μ l. of glucose 6-phosphate dehydrogenase (5 mg./ml.), and the mixture was added to a micro cuvette of an Eppendorf photometer with a filter giving maximum transmission at 336 m μ . The apparatus was adjusted to show zero extinction. Then 5 μ l. of a solution of NADP⁺ (10 mg./ml. in water) was added to start the reaction. After the contents had been mixed with a small plastic rod, the increase in extinction was followed until the reaction was completed, which took 3–8 min., depending on the amount of glucose present. Standard curves were prepared with 2.5–10 μ g. of glucose.

(b) Phenyl β -galactoside. The reaction was stopped and the colour was developed by adding to 0.2 ml. of incubation mixture: 0.1 ml. of a reagent containing *m*-NH₃, 2% (v/v) ethanol and 0.4 mg. of 4-aminoantipyrine/ml.; then 0.1 ml. of K₃Fe(CN)₆ solution (2 mg./ml.) (Martin, 1949; Baruah & Swain, 1957). The colour was measured on a Beckman Spinco 151 spectrophotometer at 505 m μ . Standard curves were prepared with 1–5 μ g. of phenol.

(c) *o*-Nitrophenyl β -galactoside and *p*-nitrophenyl β -galactoside. The reaction was stopped and the colour was developed by making the sample (0.2 ml.) alkaline in the following way. When the incubation mixture contained sodium citrate buffer, pH 3.5 or 5.5, 0.1 ml. of *m*-tris buffer, pH 9.0 or pH 8.5 respectively, was added. When the incubation mixtures contained other buffers, 0.1 ml. of *m*-K₂CO₃ was added. This gave some spontaneous hydrolysis, especially of *p*-nitrophenyl β -galactoside, which increased the blank values if the extinction was not measured immediately. The colour was measured on a Beckman Spinco 151 spectrophotometer at 420 m μ . Standard curves were prepared with 0.5–10 μ g. of *o*-nitrophenol and 0.5–5 μ g. of *p*-nitrophenol.

(d) 6-Bromo-2-naphthyl β -galactoside. The incubation mixture (1 ml.) contained 10% (v/v) of methanol to increase the solubility of the substrate. After the reaction had been stopped by boiling the mixture for 2 min. in a water bath, 0.2 ml. of 2 M-tris and then 0.2 ml. of freshly prepared

Naphthanil Diazo Blue B solution (1 mg./ml.) were added. After about 3 min., 0.4 ml. of 80% (w/v) trichloroacetic acid was added and the colour was then extracted into 2.0 ml. of chloroform. The chloroform phase was then removed and centrifuged in a bench centrifuge for 10 min. to remove water droplets. The extinction was measured in a Beckman model B spectrophotometer at 560 m μ in a cuvette with 1 cm. light-path. Standard curves were prepared with 2.5–10 μ g. of 6-bromo-2-naphthol (Cohen, Tsou, Rutenburg & Seligman, 1952; Dahlqvist, Bull & Gustafsson, 1965).

In a previous paper on the effect of pH on the hydrolysis of different β -galactoside substrates by rat small-intestinal mucosal homogenates (Dahlqvist & Asp, 1967), the inhibition of the 6-bromo-2-naphthyl β -galactosidase activity by citrate at low pH was reported. It has now been found that this was in fact an inhibition of the diazotization reaction used to determine the liberated 6-bromo-2-naphthol. The inhibition was due to too weak a buffering

capacity of the M-tris-HCl buffer, pH 7.8, added to adjust the pH of the reaction mixture before the diazotization.

Assay of protein concentration. The extinction of the samples at 280 m μ was measured. Human serum albumin (KABI AB) was used as standard.

Calculations. The enzyme activities were calculated as μ moles of substrate hydrolysed/min./ml. of enzyme solution.

The substrate constant, K_m , the inhibitor constant, K_i , and the maximal rate of hydrolysis, V_{max} , were calculated graphically by the method of Lineweaver & Burk (1934) as modified by Dixon (1953).

RESULTS

Isolation of the β -galactosidase fractions and determination of the pH optima. A typical DEAE-cellulose chromatogram of an ultracentrifuged homogenate from the ileum of suckling rats is shown in Fig. 1. Two peaks of β -galactosidase activity were separated. The activity in the first peak was not retained on the column whereas that in the second one reached a maximum when the buffer concentration had increased to approx. 50 mM. The broken lines indicate how fractions I and II were pooled. Fig. 2 shows that these two fractions had identical pH-activity curves with optima at pH 3–4. Thus fractions I and II are 'acid' enzymes. The protein content of fractions I and II was in each case 0.1–0.5 mg./ml.

Fig. 3 shows a typical Sephadex G-200 chromatogram of a papain-solubilized and ultracentrifuged preparation from the jejunum of suckling rats. Two peaks of β -galactosidase activity were separated. The broken lines show how fraction III was pooled from the first peak. This fraction had its pH optimum at pH 5.6–5.8 (Fig. 4) and is therefore a 'neutral' enzyme. The second peak had an acid pH optimum. The protein content of fraction III was 0.5–1 mg./ml.

Stabilization of the enzymes. When they were isolated as described above, the β -galactosidase

Fig. 1. DEAE-cellulose chromatography of an ultracentrifuged homogenate from the ileum of suckling rats. $\circ$, β -Galactosidase activity assayed with 12 mM-*o*-nitrophenyl β -galactoside as the substrate at pH 3.5. The broken lines indicate how fraction I and fraction II were pooled. $\blacktriangle$, Eluting buffer concentration.

Fig. 2. pH-activity curves for fraction I ($\bullet$) and fraction II ($\circ$) with 28 mM-lactose (a), 20 mM-phenyl β -galactoside (b) and 12 mM-*o*-nitrophenyl β -galactoside (c) as substrates. The activity at optimum pH was arbitrarily set to 100.

Fig. 3. Sephadex G-200 chromatography of a papain-solubilized and ultracentrifuged preparation from the jejunum of suckling rats. The β -galactosidase activity was assayed with 12mM-*o*-nitrophenyl β -galactoside as the substrate at pH 3.5 ($\circ$) and pH 5.5 ($\bullet$). The broken lines indicate how fraction III was pooled. At the substrate concentration used the 'acid' enzyme was saturated but the 'neutral' enzyme only half-saturated. Therefore the contamination by 'acid' enzyme in fraction III was smaller than it seems from the Figure.

Fig. 4. pH-activity curve for fraction III with 28mM-lactose ($\bullet$), 12mM-*o*-nitrophenyl β -galactoside ($\odot$) and 12mM-*p*-nitrophenyl β -galactoside ($\circ$) as substrates. The activity at optimum pH was arbitrarily set at 100.

activity of the 'acid' fractions was not stable. Only 2–54% (mean 30%) of the activity of fraction I and 6–40% (mean 22%) of the activity of fraction II, but 75–95% of the activity of fraction III, remained after the fractions had been frozen in an ethanol–solid carbon dioxide mixture and thawed in water (10–15°) ten times. To stabilize the enzymes 2mg. of human serum albumin/ml. was added to the enzyme solutions. The addition of albumin increased the β -galactosidase activities by 10–40%, although the albumin used had no β -galactosidase activity, and stabilized the enzymes so that 80–103% (mean 93%) of the activity of fraction I, 96–101% (mean 98%) of the activity of

fraction II and 95–99% of the activity of fraction III remained after the fractions had been frozen and thawed ten times.

Substrate constants. The K_m was determined for each fraction with five different substrates (Table 1). Fractions I and II had in all cases the same K_m values. The hetero β -galactosidase activities of these fractions had 8–50 times lower K_m values than the lactase activity. Fraction III, on the other hand, had similar K_m values for lactase and for the hetero β -galactosidase activities.

Inhibition by one substrate of the hydrolysis of another substrate. Lactose was a competitive inhibitor of all the hetero β -galactosidase activities of the three different fractions. As an example the inhibition of the *o*-nitrophenyl β -galactosidase activities by lactose is shown in Fig. 5. The K_i values for lactose as an inhibitor are shown in Table 2. In all the fractions the K_i was the same as the K_m for the lactase activity. Phenyl β -galactoside was a competitive inhibitor of the lactase activity of each fraction (Fig. 6). The K_i with fractions I and II was the same as the K_m of the phenyl β -galactosidase activities of these fractions. Fraction III had practically no phenyl β -galactosidase activity (Table 3). Therefore the K_m could not be determined. The K_i for phenyl β -galactoside as an inhibitor of the lactase activity of fraction III was 34–50mM (mean 41mM).

Rates of hydrolysis of different substrates. In Table 3 the maximal rate of hydrolysis, $V_{max.}$, calculated graphically, of the different substrates is given. Fractions I and II had, for each substrate, practically the same $V_{max.}$ The values for lactose and the hetero β -galactosides were comparable. Fraction III, on the other hand, had a considerably higher $V_{max.}$ for lactose than for the hetero β -galactosides. This fraction did not split phenyl β -galactoside or 6-bromo-2-naphthyl β -galactoside at a measurable rate.

DISCUSSION

The purpose of this study was to characterize the three fractions of β -galactosidase activity obtained by gel filtration and ion-exchange chromatography of extracts from rat small-intestinal mucosa (Asp & Dahlqvist, 1968). Since we knew from other studies (e.g. Doell & Kretschmer, 1962; Koldovský & Chytil, 1965; Koldovský, Heringová & Jirsová, 1966; Asp, Koldovský & Hošková, 1968) that the small intestine of suckling rats contains considerably higher β -galactosidase activities than that of adult rats, suckling rats were used in this investigation to obtain enzyme preparations that were as active as possible. The ileum of suckling rats contained the highest amount of 'acid' β -galactosidase activity per g. wet wt. Therefore

Table 1. K_m values for the three fractions of β -galactosidase activity measured with different substrates

The incubations were performed in 50 mM-sodium citrate buffer, pH 3.5 (for fractions I and II) or pH 5.5 (for fraction III). The K_m values given represent the means of four to ten experiments, with the ranges in parentheses.

Substrate	K_m (mM)		
	Fraction I	Fraction II	Fraction III
Lactose	10.2 (9.5–10.7)	9.8 (8.6–10.7)	25 (22–30)
Phenyl β -galactoside	1.28 (1.04–1.50)	1.24 (1.02–1.43)	—
<i>o</i> -Nitrophenyl β -galactoside	0.40 (0.32–0.51)	0.40 (0.35–0.48)	11.1 (8.5–13.0)
<i>p</i> -Nitrophenyl β -galactoside	0.21 (0.17–0.24)	0.21 (0.20–0.25)	21 (17–25)
6-Bromo-2-naphthyl β -galactoside	0.31 (0.25–0.43)	0.30 (0.28–0.30)	—

Fig. 5. Lactose as an inhibitor of the hetero β -galactosidase activities of the three enzyme fractions I (a), II (b) and III (c). *o*-Nitrophenyl β -galactoside was used as the substrate. The activity was measured without inhibitor (●) and with 20 mM-lactose as an inhibitor (○). v , Reaction velocity (units/ml. of enzyme solution); s , substrate concentration (mM).

this part of the intestine was used to prepare fractions I and II. As source of fraction III, we used only the jejunum, to avoid the high 'acid' activity of the ileum. The elution patterns of the chromatograms (Figs. 1 and 3) were very similar to those obtained for the adult rat small intestine (Asp & Dahlqvist, 1968).

In DEAE-cellulose chromatograms of solubilized homogenates from the adult rat intestine, the

'neutral' enzyme appeared mixed with the second peak of 'acid' β -galactosidase activity. Thus fraction II used in this investigation would be expected to contain also some 'neutral' activity. This contamination by 'neutral' enzyme was very small and therefore without practical significance for two reasons. First, the homogenates used as the source of the 'acid' fractions (e.g. fractions I and II) were not solubilized. Therefore 80–90% of the

Fig. 6. Phenyl β -galactoside as an inhibitor of the lactase activities of the three enzymes fractions I (a), II (b) and III (c). The activity was measured without inhibitor (●) and with the following concentrations of phenyl β -galactoside: ○, 2 mM; ⊙, 5 mM; △, 50 mM; ▲, 100 mM. v , Reaction velocity (units/ml. of enzyme solution); s , substrate concentration (mM).

Table 2. K_i values for lactose as an inhibitor of the hetero β -galactosidase activities of the three fractions

The inhibition was always competitive. The K_i values given represent the means of two to five experiments with the ranges in parentheses. The conditions were the same as in Table 1 except that the incubation mixtures also contained 5–50 mM-lactose.

Substrate	K_i for lactose as inhibitor of hetero β -galactosidase (mM)		
	Fraction I	Fraction II	Fraction III
Phenyl β -galactoside	9.3 (8.4–10.2)	8.8 (8.2–9.3)	—
<i>o</i> -Nitrophenyl β -galactoside	9.8 (8.9–11.6)	9.2 (8.9–9.5)	25 (22–29)
<i>p</i> -Nitrophenyl β -galactoside	9.3 (8.4–9.9)	9.8 (8.6–11.6)	29 (27–32)
6-Bromo-2-naphthyl β -galactoside	12.1 (11.1–13.1)	10.0 (9.8–10.2)	—

'neutral' enzyme, which is associated with the microvilli fraction, sedimented during the ultracentrifugation (Doell & Kretchmer, 1962, 1963;

Koldovský *et al.* 1965, 1966; Hsia, 1966). Secondly, the kinetic studies of the 'acid' fractions were performed at pH 3.5. At this pH the 'neutral'

Table 3. $V_{\max}$ values for the hydrolysis of the different substrates

$V_{\max}$ for the hydrolysis of lactose in each fraction was arbitrarily set at 100. The activities were measured at pH 3.5 for fractions I and II and at pH 5.5 for fraction III. The ranges are shown in parentheses.

Substrate	$V_{\max}$ (% of that for lactase activity)		
	Fraction I	Fraction II	Fraction III
Lactose	100	100	100
Phenyl β -galactoside	64 (52-71)	68 (52-80)	<0.4*
<i>o</i> -Nitrophenyl β -galactoside	91 (85-97)	85 (80-88)	32 (26-42)
<i>p</i> -Nitrophenyl β -galactoside	119 (107-130)	113 (103-124)	22 (17-29)
6-Bromo-2-naphthyl β -galactoside	86 (69-115)	91 (86-97)	<0.4†

* V_0 with 20 mM-substrate.

† V_0 with 0.39 mM-substrate.

enzyme had practically no activity (Asp & Dahlqvist, 1968; Koldovský *et al.* 1968).

The contamination of the 'neutral' fraction (III) by 'acid' activity also was small, since the last part of the 'neutral' peak, which occurred close to the 'acid' peak in the Sephadex G-200 chromatograms, was discarded.

The pH optima of the different fractions agree with previous reports on the pH optima measured in crude homogenates from rat small intestine (e.g. Koldovský & Chytil, 1965; Koldovský *et al.* 1965, 1966; Dahlqvist & Asp, 1967). The K_m values of the 'acid' and 'neutral' β -galactosidases also are similar when measured in crude homogenates (Koldovský *et al.* 1966, 1968) and with the separated peaks. This gives good evidence that the characteristics of the β -galactosidases do not change during the solubilization and isolation.

The instability of the isolated 'acid' fractions could be due to the low protein concentration of these preparations (0.1-0.5 mg./ml.). Walker & Whelan (1960) found that purified β -amylase, stable in concentrated crystalline suspension, lost its stability when diluted with water; addition of human serum albumin (0.05%) also rendered this enzyme stable. Whether other proteins have the same effect has not been studied.

The studies on the influence of the substrate concentration on the rate of hydrolysis of different substrates (Table 1) show that the 'acid' fractions have considerably higher affinity (i.e. lower K_m) for the hetero β -galactosides than for lactose, and thus justify the view expressed earlier that the 'acid' fractions should be characterized as hetero β -galactosidases. The 'neutral' fraction had a much higher maximal rate of hydrolysis of lactose than of *o*-nitrophenyl β -galactoside and *p*-nitrophenyl β -galactoside. Phenyl β -galactoside and 6-bromo-2-naphthyl β -galactoside were not split at a measurable rate. Thus the 'neutral' enzyme is essentially a disaccharidase (lactase). Its localiza-

tion in the brush border (Koldovský *et al.* 1965) indicates its physiological function in the digestion of dietary lactose.

The fact that lactose and phenyl β -galactoside were competitive inhibitors of the other β -galactosidase activities, with the same K_i as their K_m when used as substrate, strongly indicates that in each fraction lactose is hydrolysed by the same active site as the hetero β -galactosides.

The $V_{\max}$ values for the different activities of the fractions correlate well with the specificity Tables given in a previous paper (Asp & Dahlqvist, 1968). With the substrate concentrations used in that study, 74% of the $V_{\max}$ of the lactase, 97% of the *o*-nitrophenyl β -galactosidase, 98% of the *p*-nitrophenyl β -galactosidase, 94% of the phenyl β -galactosidase and 56% of the 6-bromo-2-naphthyl β -galactosidase of the 'acid' enzyme were measured. For the 'neutral' enzyme, 54% of the $V_{\max}$ of the lactase, 52% of the *o*-nitrophenyl β -galactosidase and 36% of the *p*-nitrophenyl β -galactosidase activities were measured.

No difference between the two acid fractions could be found in any of the properties studied (e.g. pH optimum, K_m , K_i and $V_{\max}$ for the different substrates). Therefore these fractions seem to contain different forms of the same enzyme. They do not seem to differ in molecular size either, since the 'acid' peak obtained on Sephadex G-200 chromatography was homogeneous. Furth & Robinson (1965) and Robinson, Price & Dance (1967) studied the β -galactosidase activity of different organs in the rat. The elution patterns on DEAE-cellulose chromatograms from different organs including the small intestine were very similar to that of the 'acid' activity studied by us. The pH optimum and K_m were reported for liver β -galactosidases with *p*-nitrophenyl β -galactoside and phenyl β -galactoside as substrates. The results were very similar to those reported in the present paper for the 'acid' fractions from the small

intestine. It seems therefore, as suggested by Asp & Dahlqvist (1968), that the 'acid' enzyme (or enzymes) is a β -galactosidase that is not a specifically intestinal enzyme but is present in many different organs. The metabolic function of this enzyme is not known.

The small-intestinal β -galactosidases of different species seem to be similar. Maio & Rickenberg (1960) studied the β -galactosidase activity of different infant mouse organs and strain-L cells with 1mm-*o*-nitrophenyl β -galactoside as the substrate (Lederberg, 1950). The pH optimum for the intestinal β -galactosidase, the K_m determined in different organs and the K_i for lactose as an inhibitor of the strain-L-cell β -galactosidase were very similar to our results on the 'acid' enzyme. Owing to the low substrate concentration used, any 'neutral' activity of the small intestine would not be expected to have been found in the work of Maio & Rickenberg (1960).

In the calf (Heilskov, 1956), rabbit (Doell & Kretchmer, 1962), monkey (Swaminathan & Radhakrishnan, 1967) and domestic cat (Hore & Messer, 1968), the existence of at least two intestinal β -galactosidases with different specificities has been reported on the basis of heat-inactivation, pH and solubility studies.

In the human small intestine there are also at least two β -galactosidases. One of them is soluble and has much higher hetero β -galactosidase than lactase activity. This enzyme seems to correspond to the 'acid' enzyme in the rat, but its pH optimum is at pH 6 and the splitting of lactose by this enzyme is much slower than that by the 'acid' enzyme of the rat. The other human β -galactosidase is particle-bound and its enzymic properties such as pH optimum and substrate specificity are very similar to those of the 'neutral' enzyme in the rat (Hsia, Makler, Semenza & Prader, 1966; N.-G. Asp, O. Koldovský & A. Dahlqvist, unpublished work).

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