Rat Small-Intestinal β -Galactosidases

INFLUENCE OF pH ON THE HYDROLYSIS OF DIFFERENT SUBSTRATES

By ARNE DAHLQVIST AND NILS GEORG ASP

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

(Received 8 August 1966)

1. The influence of pH and the kind of buffer on the hydrolysis of lactose and four hetero- β -galactosides (phenyl β -galactoside, *o*-nitrophenyl β -galactoside, *p*-nitrophenyl β -galactoside and 6-bromo-2-naphthyl β -galactoside) by homogenates of rat small-intestinal mucosa has been studied. 2. There are at least two β -galactosidases present in the homogenates, one with optimum pH 3–4 and another with optimum pH 5–6. 3. The enzyme with the lower pH optimum is mainly a heterogalactosidase. It hydrolyses lactose slowly. The other enzyme is mainly a disaccharidase, since it hydrolyses lactose much more rapidly than the heterogalactosides. 4. Under the conditions used, citrate had an inhibitory effect on the 6-bromo-2-naphthyl β -galactosidase activity at pH 3–4, but did not influence the 6-bromo-2-naphthyl β -galactosidase activity at pH 5–6 or the hydrolysis of the other substrates at any pH.

During the last few years observations have been made that indicate the existence of more than one β -galactosidase in the rat small-intestine. Dahlqvist, Bull & Gustafsson (1965*a*) and Dahlqvist, Bull & Thomson (1965*b*) separated the 6-bromo-2-naphthyl β -galactosidase activity from the lactase activity in small-intestinal homogenates from adult rats, thus showing that these two β -galactosides are hydrolysed by different enzymes. Doell & Kretschmer (1962) and Koldovský *et al.* (1965) found two pH optima for the lactase and the *o*-nitrophenyl β -galactosidase activities of small-intestinal homogenates from rats a few days old, indicating that both substrates are hydrolysed by more than one enzyme. Furth & Robinson (1965) separated rat small-intestinal β -galactosidase (measured with four different heterogalactoside substrates) into several fractions by ion-exchange chromatography.

Heterogeneity of intestinal β -galactosidase has been described also in man. Hsia, Makler, Semenza & Prader (1966) separated two β -galactosidases in human mucosal extracts by gel-filtration chromatography. One of the enzymes hydrolysed 6-bromo-2-naphthyl β -galactoside more rapidly than lactose, and was assumed to be mainly a heterogalactosidase, whereas the other one only hydrolysed the sugar. The pH optima for the two human β -galactosidases were not reported.

In attempts to elucidate the specificity of the small-intestinal β -galactosidases, we have now measured the activity of crude rat mucosal homo-

genates with five different β -galactoside substrates (lactose, phenyl β -galactoside, *o*-nitrophenyl β -galactoside, *p*-nitrophenyl β -galactoside and 6-bromo-2-naphthyl β -galactoside). The influence of pH and the kind of buffer on the hydrolysis of these substrates have been studied.

MATERIALS AND METHODS

Mucosal homogenates. Adult albino rats weighing 150–200 g. were starved overnight but were given a free allowance of water. The animals were then anaesthetized with ether, the small intestine was removed and cut open and the mucosa was scrapped off with a glass slide. If faeces were present in the distal part of the small intestine this part was discarded. The mucosa was homogenized with 4 vol. of cold 0.9% NaCl in an Ultra-Turrax homogenizer. The tube was chilled with crushed ice during the homogenization. The homogenate, which contained about 20 mg. of protein/ml., was either used immediately or stored at -20° .

Assay of protein concentration. The protein concentration of the homogenates was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) with human serum albumin as a standard. The modified reagent B of Eggstein & Kreutz (1955) was used.

Enzyme activity assay. The reaction mixture contained 29 mM-lactose, 20 mM-phenyl β -galactoside, *o*-nitrophenyl β -galactoside or *p*-nitrophenyl β -galactoside, or 0.39 mM-6-bromo-2-naphthyl β -galactoside. [This last substrate has a very low solubility in water, and when it was used as substrate the reaction mixture also contained 10% (v/v) of methanol.]

The amount of protein used in the reaction mixture was about 5 mg./ml. when phenyl β -galactoside was the sub-

strate, 1–2.5 mg./ml. with lactose, and 0.2–0.5 mg./ml. with the other substrates. The degree of hydrolysis of the substrate after 1 hr. of incubation was below 14% for phenyl β -galactoside or 6-bromo-2-naphthyl β -galactoside and below 6% for the other substrates. Control experiments showed that zero-order kinetics were followed under the conditions used.

The buffer concentration in the reaction mixture was 50 mM (40 mM when 6-bromo-2-naphthyl β -galactoside was substrate). The following buffers were used: glycine-HCl at pH 1.5–3.5, sodium citrate or sodium acetate at pH 3.0–6.5, sodium phosphate at pH 6.0–8.0, and glycine-NaOH at pH 8.0–9.0; in certain experiments some of the buffers were used outside the pH range given above. In all experiments a sample (100 μ l.) of the reaction mixture was withdrawn after incubation and the pH measured with a micro glass electrode (Radiometer, Copenhagen, Denmark). The pH values given in the text are therefore those of the actual incubation mixture rather than of the stock buffer. In some control experiments the pH of the reaction mixture was measured both before and after incubation, but the difference was so small that it was regarded as sufficient to make only one measurement.

After incubation for 60 min. at 37° the degree of hydrolysis was measured as given below. Zero-time controls were used. Readings were made in a Beckman-Spinco spectrophotometer with an ultramicro cuvette (volume 100 μ l., light-path 0.6 cm.).

The following methods were used to measure the degree of hydrolysis. (a) Lactose. The amount of glucose liberated was measured with the tris-glucose oxidase reagent of Dahlqvist (1961, 1964). (b) Phenyl β -galactoside. The amount of phenol liberated was measured with 4-amino-antipyrine reagent (Martin, 1949) as described by Baruah & Swain (1957). (c) *o*-Nitrophenyl β -galactoside and *p*-nitrophenyl β -galactoside. Free *o*-nitrophenol and *p*-nitrophenol were measured spectrophotometrically at 420 $m\mu$ after making the sample alkaline with Na_2CO_3 (Lederberg, 1950). (d) 6-Bromo-2-naphthyl β -galactoside. The amount of 6-bromo-2-naphthol liberated was measured after diazotization as described by Cohen, Tsou, Rutenburg & Seligman (1952) with the modification that tris buffer, rather than phosphate, was used to adjust the pH before the coupling with the diazonium salt (Dahlqvist *et al.* 1965a).

The enzyme activity was calculated as μ moles of substrate hydrolysed/min./ml. of undiluted homogenate.

RESULTS

The pH-activity curves for the lactase, phenyl β -galactosidase, *o*-nitrophenyl β -galactosidase and *p*-nitrophenyl β -galactosidase activities are shown in Fig. 1.

The lactase pH-activity curve showed a broad peak at about pH 5.5, with an indication of an additional small peak at about pH 3.5. This small peak was seen in repeated experiments, with different kinds of buffers (see below).

The phenyl β -galactosidase pH-activity curve had a single asymmetric peak at pH 3.5–4.5. The two nitrophenyl β -galactosides were hydrolysed considerably more rapidly than the unsubstituted

Fig. 1. pH-activity curves for the lactase, phenyl β -galactosidase, *o*-nitrophenyl β -galactosidase and *p*-nitrophenyl β -galactosidase activities of a homogenate of rat small-intestinal mucosa. The buffers used are described in the text, as is the definition of activity. $\circ$, Lactase; Δ , *o*-nitrophenyl β -galactosidase; $\square$, *p*-nitrophenyl β -galactosidase; $\bullet$, phenyl β -galactosidase.

heterogalactoside at all pH values. Each of the nitrophenyl β -galactosidase activities had two more or less distinct optima, one at pH 3.0–3.5 and one at 5.0–6.0. With *o*-nitrophenyl β -galactoside as substrate the more acid peak was much the smaller, whereas with *p*-nitrophenyl β -galactoside both peaks were about equal.

In the experiments illustrated in Fig. 1 various buffers were used in different parts of the pH scale to ensure an appropriate buffering capacity (see the Materials and Methods section). To find out whether the kind of buffer used had any influence on the enzyme activity, the activities at about pH 3 and at about pH 5 were tested in separate experiments with glycine-hydrochloric acid, sodium citrate and sodium acetate buffers with all the substrates. The lactase, phenyl β -galactosidase, *o*-nitrophenyl β -galactosidase and *p*-nitrophenyl β -galactosidase activities were the same in all three kinds of buffers, provided the pH of the reaction mixture was measured and not only the pH of the stock buffer.

The 6-bromo-2-naphthyl β -galactosidase activity differed from the other activities in that the kind of buffer used strongly influenced the shape of the curve. In citrate buffer there was a single peak at about pH 5, and in glycine-hydrochloric acid buffer there was a broad peak at about pH 3.5 (Fig. 2). The activity at pH 3.5 was inhibited by citrate, rather than activated by glycine-hydrochloric acid, since it was equally low in a mixture of citrate and

Fig. 2. pH-activity curves for the 6-bromo-2-naphthyl β -galactosidase activity of a homogenate of rat small-intestinal mucosa in different buffers. ○, Glycine-HCl; ●, sodium citrate. The substrate concentration was 0.39 mM, and that of the buffers 40 mM.

glycine-hydrochloric acid buffer as in citrate alone. The activity in acetate buffer, pH 3.5, was the same as in glycine-hydrochloric acid buffer. At pH 5.0 the 6-bromo-2-naphthyl β -galactosidase activity was the same in all three kinds of buffer.

DISCUSSION

The results indicate that *o*-nitrophenyl β -galactoside, *p*-nitrophenyl β -galactoside and possibly lactose are hydrolysed by at least two enzymes in the rat small intestine, namely one with optimum pH 3–4 and another with optimum pH 5–6. If it is assumed that there is one enzyme hydrolysing all three substrates at pH 3–4 and another one hydrolysing all three substrates at pH 5–6, these enzymes must have rather different specificities, since the relative rates of hydrolysis of the three substrates at pH 3–4 and pH 5–6 are markedly different. At pH 5–6 lactose is hydrolysed more rapidly than the heteroglycosides, and at pH 3–4 the heteroglycosides are hydrolysed more rapidly than lactose. *o*-Nitrophenyl β -galactoside is hydrolysed more rapidly than *p*-nitrophenyl β -galactoside at pH 5–6, but at pH 3–4 the reverse is true.

The phenyl β -galactosidase activity does not show two separate pH-activity peaks, but on the other hand the peak of this curve is very broad. The rate of hydrolysis of phenyl β -galactoside as related to the rates of hydrolysis of the other substrates is higher in the more acid region of the curve.

Doell & Kretchmer (1962) studied the hydrolysis of lactose and *o*-nitrophenyl β -galactoside by small-intestinal homogenates from newborn rats. They also found evidence for the existence of two enzymes,

one with optimum pH about 6 that hydrolysed lactose more rapidly than *o*-nitrophenyl β -galactoside, and one with optimum pH below 2, hydrolysing the heteroglycoside more rapidly than the disaccharide. This situation is rather similar to the one observed by us, except that we found the optimum pH for the more acid peak to be 3–4, rather than below 2. Doell & Kretchmer (1962) do not state whether the pH value given by them refers to the pH of the stock buffer or of the actual pH of the incubation mixture. According to our experience these may differ considerably. If acetate buffer was used at such low pH values, as appears from the paper by Doell & Kretchmer (1962), this will have had practically no buffering capacity. These authors used rats that were only a few days old, whereas we have used rats weighing 150–200 g. Therefore the possibility exists that the enzymes that are present in the two kinds of preparations are not identical.

Koldovský *et al.* (1965) also studied the *o*-nitrophenyl β -galactosidase activity in the small intestine of newborn rats, and found evidence for the hydrolysis of this heteroglycoside by two enzymes, one that was mainly present in the insoluble fraction (especially in the brush-border membranes) and had optimum pH 5.5 and another that was chiefly soluble and had optimum pH 3.5. These values for the pH optima agree better with our data.

Interpretation of the 6-bromo-2-naphthyl β -galactosidase pH-activity curve is complicated by the inhibitory effect of citrate below pH 5. It is possible that two enzymes exist, only one of which is inhibited by citrate; however, it is perhaps more likely that citrate is a weak competitive inhibitor, and that the effect is only seen with 6-bromo-2-naphthyl β -galactoside because of the very low substrate concentration used in this case. The effect of pH on the inhibition could be due to a change in K_m for the substrate or in K_i for citrate.

This investigation has been supported by grants from the Swedish Nutrition Foundation and the Swedish Medical Research Council (project no. 13-X-157). Miss Margaretha Johannsen and Mrs Birgitta Johanneson are thanked for skilful technical assistance.

REFERENCES

- 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.
- Dahlqvist, A. (1961). *Biochem. J.* **80**, 547.
- Dahlqvist, A. (1964). *Analyt. Biochem.* **7**, 18.
- Dahlqvist, A., Bull, B. & Gustafsson, B. E. (1965a). *Arch. Biochem. Biophys.* **109**, 150.
- Dahlqvist, A., Bull, B. & Thomson, D. L. (1965b). *Arch. Biochem. Biophys.* **109**, 159.

- Doell, R. G. & Kretchmer, N. (1962). *Biochim. biophys. Acta*, **62**, 353.
- Eggstein, M. & Kreutz, F. H. (1955). *Klin. Wschr.* **33**, 879.
- Furth, A. J. & Robinson, D. (1965). *Biochem. J.* **94**, 29P.
- Hsia, D. Y. Y., Makler, M., Semenza, G. & Prader, A. (1966). *Biochim. biophys. Acta*, **113**, 390.
- Koldovský, O., Noack, R., Schenk, G., Jirsová, V., Heringová, A., Braná, H., Chytil, F. & Fridrich, M. (1965). *Biochem. J.* **96**, 492.
- Lederberg, J. (1950). *J. Bact.* **60**, 381.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Martin, R. W. (1949). *Analyt. Chem.* **21**, 1419.