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ENZYMATIC PROPERTIES OF RAT LACTASE–PHLORIZIN HYDROLASE

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SUMMARY

1. A lactase–phlorizin hydrolase complex has been purified from the intestinal mucosa of neonatal rats. Polyacrylamide gel electrophoresis of "pure" lactase–phlorizin hydrolase reveals four isoenzymes, all having similar enzymatic properties.

2. Lactase–phlorizin hydrolase is a glycoprotein containing 17% carbohydrate, consisting of galactose, mannose, fucose, and *N*-acetylglucosamine.

3. At neutral pH and 37 °C, purified lactase–phlorizin hydrolase rapidly loses its ability to hydrolyze lactose. Loss of lactase activity is prevented by *p*-chloromercuribenzoate. Lactase–phlorizin hydrolase still attached to its brush border membrane is relatively stable at 37 °C.

4. The lactase–phlorizin hydrolase complex contains two distinct enzymatic active sites, one of which (lactase) hydrolyzes β-glucosides excepting phlorizin, whereas the other (phlorizin hydrolase) is specific for phlorizin alone.

5. The developmental patterns of lactase and phlorizin hydrolase specific activities differ. Lactase declines after birth, whereas phlorizin hydrolase activity is maximum at 15 days after birth.

6. These data suggest that lactase is truly a β-glycosidase, and that phlorizin hydrolase may represent an active site which is more specific for the phloretin portion of the substrate than for the glucose.

INTRODUCTION

Rat lactase exists in an enzyme complex containing two enzymatic sites, lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23) and phlorizin hydrolase (phlorizin glucohydrolase, EC 3.2.1.62). This enzyme complex has been purified to a high degree [1], and kinetic and mixed inhibition experiments have suggested that the two active sites are separate, but closely related [2]. Lactase and phlorizin hydrolase are equally affected in human lactase deficiency [3], but developmental data from rats suggest separate biological control of these two activities in one study [4], but not in another [5]. Activity of highly purified rat lactase is relatively unstable at 37 °C in neutral buffer [1]. We have utilized the relative stability of the two enzymatic

Abbreviations: PCMB, *p*-chloromercuribenzoic acid; NPh-Glc, *p*-nitrophenyl-β-D-glucoside; MeUM-Gal, 4-methylumbelliferyl-β-galactopyranoside.

activities of lactase–phlorizin hydrolase purified from the neonatal rat to determine substrate specificities of the two catalytic sites. In addition, we have found that the thermal instability of lactase can be prevented by *p*-chloromercuribenzoic acid (PCMB). Since phlorizin hydrolase activity was stable at 37 °C, we were able to measure enzyme activity at each of the two active sites in the lactase–phlorizin hydrolase complex. We found that one site (lactase) hydrolyzes all substrates, β-glucosides and β-galactosides, except phlorizin, whereas the other site (phlorizin hydrolase) splits only phlorizin. The different developmental patterns of lactase and phlorizin hydrolase activities in neonatal rats confirm that these two enzymatic activities represent distinct and separate active sites. We have further characterized the purified rat enzyme by demonstrating the presence of isoenzymes and by determining the carbohydrate and amino acid composition.

MATERIALS AND METHODS

Cellobiose, *p*-nitrophenyl-β-glucoside (NPh-Glc) iodoacetate, cysteine, papain, glucose oxidase, phenazine methasulfate, tetranitroblue tetrazolium, human albumin, and Coomassie blue were obtained from Sigma Chemical Corp. (St. Louis, Mo.). PCMB and 4-methylumbelliferyl-β-D-galactopyranoside (MeUm-Gal) were products of Schwarz-Mann (Orangeburg, N.Y.); lactose was purchased from Fisher Scientific Corp. (Chicago, Ill.); 4-methylumbelliferone and β-mercaptoethanol were obtained from Eastman-Kodak Co. (Rochester, N.Y.); dithiothreitol was purchased from Calbiochem Co. (Chicago, Ill.); and phlorizin from K and K Laboratories (Plainview, N.Y.). Sephadex G-200 was purchased from Pharmacia Co. (Piscataway, N.J.), Bio-Gel A-5m 100–200 mesh from Bio-Rad Co. (Richmond, Calif.), and DEAE-cellulose microgranular from Whatman Co. (Kent, England). Peroxidase was purchased from Worthington Co. (Freehold, N.J.). All chemicals were reagent grade. Biochemicals 2-fucosyl lactose and lacto-*N*-fucopentaose were generously provided by Dr R. Kornfeld. 3-fucosyl lactose and lacto-*N*-tetraose were prepared by Dr V. Ginsburg. Lactase was obtained from homogenates of whole intestines of albino rats, age 4 or 15 days by the method of Schlegel-Haueter et al. [1]. This preparation, shown in Fig. 1, was used for all experiments in which isolated or "purified" lactase–phlorizin hydrolase was designated. No differences were found in the electrophoretic pattern of enzyme from four day old rats, when compared with that from 15-day-old rats. Brush borders were prepared by the method of Forstner et al. [6].

Whole intestines of neonatal rats (5–25 days) used for developmental studies were homogenized in a 2 fold volume of 50 mM potassium phosphate buffer, pH 6.0. The homogenates were centrifuged at 30 000 × *g* for 20 min, the supernatants discarded, and the pellets rehomogenized in buffer and centrifuged a total of three times. The final pellet which contained over 85% of the total lactase and phlorizin hydrolase activity was used for enzymatic studies. Lactase and cellobiase activity were measured according to Dahlqvist [7]. Hydrolyses of MeUm-Gal were measured flurometrically [8]. Phlorizin hydrolase and β-nitrophenyl glucosidase activity were determined according to the method of Malathi and Crane [9]. Hydrolysis of milk sugars was carried out using a concentration of substrate equivalent to 0.04 μmole of terminal galactose per assay tube in 50 mM potassium phosphate buffer, pH 6.0. Released galactose was determined using galactose dehydrogenase [10]. Protein determinations were by

the method of Lowry et al. [11]. Disc electrophoresis was performed using 5% acrylamide gels with a pH 6.5 discontinuous buffer system described by Rodbard and Chrambach [12]. All lower gels were pre-run with lower gel buffer for 1 h at 4 mA per tube to remove the ammonium persulfate. Gels were stained for lactase activity using lactose as substrate and a histochemical method [13] to detect glucose release. All protein stains were with Coomassie blue dye 0.05% in 12% trichloroacetic acid. Gels were extruded using a Savant model AGD-30A extruder and fractions equivalent to 1.0 mm of gel length were collected with cold 10 mM potassium phosphate buffer, pH 6.0, as the extruding fluids. After shaking for 12 h at 4 °C, the acrylamide was separated from the buffer by centrifugation.

Acrylamide gel electrophoresis in sodium dodecylsulfate was performed by a modification of the method of Dunker and Rueckert [14] as previously described [15]. Molecular weight standards were the same as reported earlier [15].

Amino acid analysis was performed on a Technicon NC-1 amino acid analyzer after hydrolysis of the glycoprotein in 6 M HCl for 22 h at 105 °C in an evacuated sealed tube. Carbohydrate analysis was performed using gas–liquid chromatography as described by Reinhold [16]. Sulfhydryl group titrations were performed in 6 M guanidine using Ellman's reagent [17].

Fig. 1. Disc-gel electrophoresis of rat lactase. 30 μg of purified lactase were applied to a 5% polyacrylamide gel and electrophoresed as described in Materials and Methods. The anode is at the bottom of the gel.

RESULTS

Lactase heterogeneity

The most purified lactase preparation from 4- or 15-day-old rats contained several molecular components which were separated only by electrophoresis, and each component was capable of hydrolyzing lactose. Disc electrophoresis at pH 6.5 and 8.5 produced four electrophoretically distinct protein bands (Fig. 1). The fourth band migrated behind the three shown in Fig. 1, but was faint and reproduced poorly. Histochemical staining of the gels demonstrated that lactase activity was present in areas corresponding to location of the protein bands. When gels were fractionated by extrusion and enzyme activity measured, two main peaks (B and C) and two minor peaks (A and D) were found (Fig. 2). Peaks B, C, and D corresponded to the three

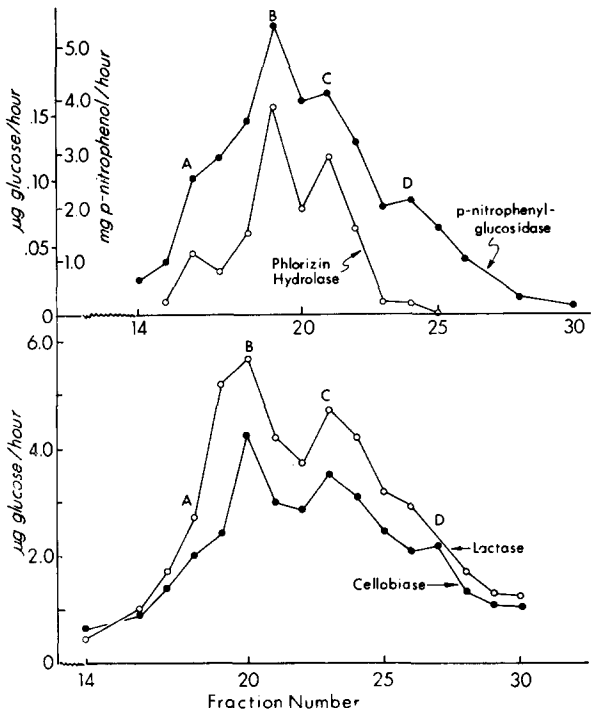

Fig. 2. Enzymatic activity of lactase–phlorizin hydrolase isoenzymes. 37 μg of purified lactase–phlorozin hydrolase were electrophoresed as described in Fig. 1. One gel was stained with Coomassie blue, another gel was extruded and the enzyme eluted as described in Materials and Methods. The two parts of the figure are derived from separate electrophoretic runs, and the letters refer to the protein bands seen in the stained gels. Bands B–D correspond to the three major bands seen in Fig. 1. Band A was faint and reproduced poorly in gel pictures.

protein bands seen in Fig. 1. Hydrolysis of MeUm-Gal, cellobiose, NPh-Glc, and phlorizin occurred in exactly the same fractions, and in nearly the same proportion. Phlorizin hydrolase activity of peak D was proportionally lower than in other peaks, but was definitely present in at least two other experiments. Since it is possible that peak D represents a partial separation of lactase and phlorizin hydrolase activity,

the electrophoretic pattern seen in Fig. 1 might represent a composite of two different proteins migrating together. This seems unlikely since the two enzyme activities also migrate together on column chromatography (Sephadex and DEAE). Thus, it was impossible to distinguish each enzyme peak by substrate specificity. Furthermore, the major peaks (B and C) demonstrated identical heat inactivation properties (see below). Since all of the proteins (A–D) obtained from the final step of lactase–phlorizin hydrolase purification had lactase activity, the enzyme preparation used for all subsequent studies was not subjected to electrophoretic separation first.

Acrylamide gel electrophoresis in sodium dodecylsulfate of rat lactase–phlorizin hydrolase separated five protein components (Fig. 3) with a dominant component

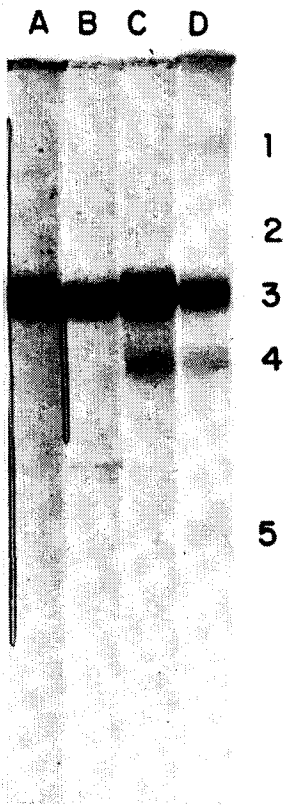

Fig. 3. Acrylamide gel electrophoresis in sodium dodecylsulfate of lactase–phlorizin hydrolase. Isoenzymes of lactase–phlorizin hydrolase were eluted from polyacrylamide gels as outlined in Fig. 2. Peaks A, B, C, and D were pooled and treated with 2% sodium dodecylsulfate and 1% β-mercaptoethanol at 37 °C for 1 h. The samples were then electrophoresed in sodium dodecylsulfate on a 5% acrylamide gel as described in Materials and Methods.

having an apparent molecular weight of 132 000. This dominant component was the only protein found in peaks A and B obtained from disc electrophoresis (Fig. 2) and was the major constituent for peaks C and D. However, peaks C and D contained other minor components when electrophoresed in gels containing sodium dodecylsulfate (Fig. 3). It is unlikely that these minor components corresponded to phlorizin

hydrolase since this enzyme activity was present in peaks A and B. However, the presence of minor components again points out the possible heterogeneity of the final lactase–phlorizin hydrolase preparation. Thus, the electrophoretic differences in Figs 1 and 2 are not entirely related to differences in molecular weight. Moreover, the lactase–phlorizin hydrolase complex (at least peaks A and B) resists breakdown by sulfhydryl reagents and sodium dodecylsulfate.

Lactase inactivation and PCMB protection

Purified lactase, although stable for months at −20 °C, was readily inactivated at 37 °C and at neutral pH. Much of this inactivation could be prevented by certain sulfhydryl reagents (Table I). The interaction of lactase with PCMB and cysteine was

TABLE I

THE EFFECT OF SULFHYDRYL REAGENTS ON THE INACTIVATION OF RAT LACTASE

Lactase activity was assayed as described in Materials and Methods using lactose as substrate and purified lactase–phlorizin hydrolase as enzyme.

Sulfhydryl reagent	Concentration (mM)	$T^1/_2$ (min)
Control	—	41
p-Chloromercuribenzoate	0.25	250
Cysteine	4	108
β-Mercaptoethanol	2.3	49
Iodoacetate	100	51
N-Ethylmaleimide	10	48
Dithiothreitol	10	47
Mercuric chloride	0.01	49

quite specific since the other sulfhydryl agents tested had no effect (Table I). PCMB protected activity better than cysteine but did not completely prevent loss of activity. The concentrations recorded were maximal for PCMB and cysteine, and corresponded to effective concentrations for the other sulfhydryl reagents. Lactase activity was lost rapidly at 37 °C only when removed from its position in the brush border (Fig. 4). The rate of inactivation of lactase bound to brush border membranes was almost identical to the activity of solubilized enzyme in the presence of PCMB. Lactase activity was relatively stable at 25 °C. PCMB protection was noted as low as 0.013 mM and was maximally effective at 0.120 mM.

The protection afforded by PCMB appeared rapidly after addition of the sulfhydryl reagent (Fig. 5). However, PCMB could not restore enzyme activity once lost; that is, lactase was irreversibly heat-inactivated. The PCMB-mediated protection of lactase inactivation was pH dependent (Fig. 6). Although lactase activity diminished at pH below 6.0 when incubated at 37 °C, PCMB had no effect on this inactivation. However, at pH 7 and 8, PCMB had a marked effect on the stability of enzyme activity.

When lactase was heat inactivated, the loss of activity was due entirely to a change in V, falling from 5.00 to 3.13 μg/h in 30 min. PCMB prevented this change in V and had no effect on the K_m, which remained 41.7 mM. When the major electro-

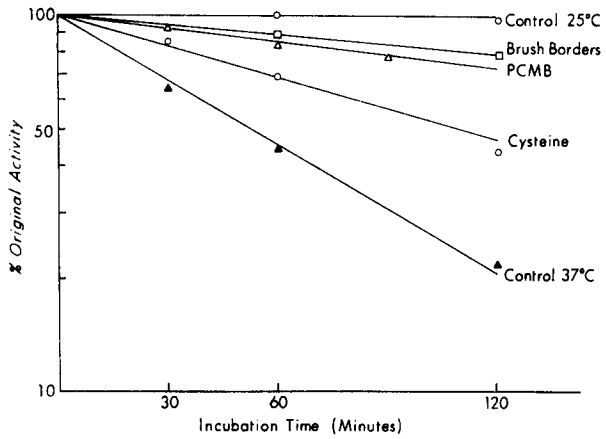

Fig. 4. Effect of sulfhydryl reagents on heat inactivation of lactase activity. Purified lactase–phlorizin hydrolase was incubated at 25 °C and 37 °C in 10.0 mM potassium phosphate buffer, pH 7.0. For all assays, except when PCMB was present, the substrate was lactose and 100% enzyme activity corresponded to between 4.0 and 5.0 μg glucose liberated per h. Because PCMB affects the glucose oxidase reagent, when PCMB was present the substrate was MeUm-Gal and 100% enzyme activity corresponded to 12.0 ng 4-methyl-umbelliferone liberated per h. PCMB was added to 0.25 mM final concentration; cysteine to 4.0 mM.

phoretic isoenzymes of lactase corresponding to peaks B and C (Fig. 2) were tested separately, each one showed the same PCMB protection of heat inactivation.

Chemical properties of lactase–phlorizin hydrolase

Purified lactase–phlorizin hydrolase (corresponding to the preparation applied to gels in Fig. 1) was analyzed for carbohydrate composition, and contained per mg of protein: 6 μg fucose, 37 μg mannose, 123 μg galactose, 36 μg *N*-acetyl glucosamine, and 4 μg *N*-acetyl galactosamine. No glucose or sialic acid was found. Thus, 17% of

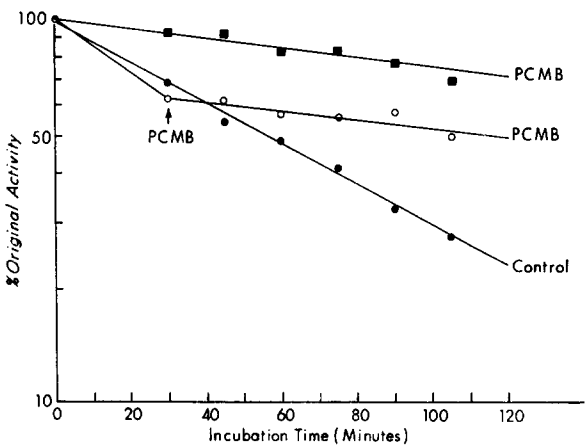

Fig. 5. PCMB protection of lactase activity. Lactase–phlorizin hydrolase was incubated at 37 °C in the presence and absence of 0.25 mM PCMB. Conditions of assay were the same as in Fig. 4. PCMB was added either at 0 (■—■) or 30 min (○—○).

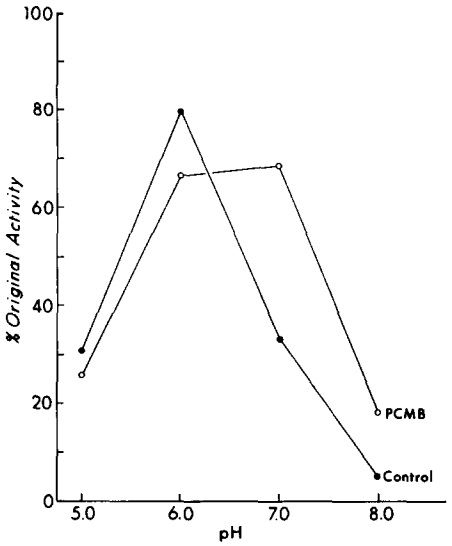

Fig. 6. Effect of pH on PCMB protection of lactase activity. Purified lactase–phlorizin hydrolase was incubated at pH 5, 6, 7, and 8 for 30 min at 37 °C in the presence or absence of 0.25 mM PCMB. Buffer used was potassium phosphate 60 mM. Enzymatic assays were performed as described in Fig. 4.

the molecule by weight was attributed to carbohydrate. This compared with 29.4% and 16.7% as carbohydrate found in rat sucrase and maltase [18]. The amino acid composition of purified lactase–phlorizin hydrolase is shown in Table II. The percentage of hydrophobic amino acids (Ala, Val, Met, Ile, Leu, Phe) was 32%, even lower than that found in surface membrane proteins in erythrocytes [19]. No free SH groups were detected in the undenatured enzyme, but 4 μmoles of cysteine/μmole protein were found after incubation in 6 M guanidine.

TABLE II

AMINO ACID COMPOSITION OF RAT LACTASE–PHLORIZIN HYDROLASE

100 μg purified rat lactase were analyzed for amino acid composition as described in Materials and Methods.

Amino acid	μg/mg Protein	Amino acid	μg/mg Protein
Lysine	34	Glycine	29
Histidine	22	Alanine	28
Arginine	49	Half Cysteine*	5
Aspartic Acid	64	Valine	25
Threonine	37	Methionine	18
Serine	39	Isoleucine	22
Glutamic Acid	61	Leucine	55
Proline	17	Tyrosine	26
		Phenylalanine	37

* Determined by sulfhydryl analysis in 6 M guanidine, as described in Materials and Methods.

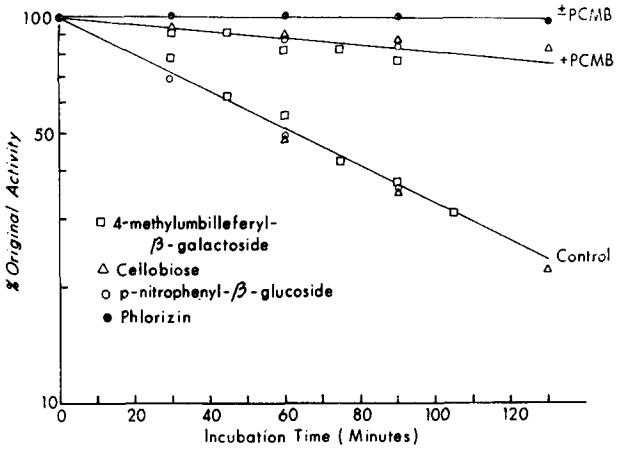

Fig. 7. Effect of PCMB on heat inactivation of β-galactosidase and β-glucosidase activity of rat lactase. Purified lactase–phlorizin hydrolase was incubated at 37 °C in the presence or absence of 0.5 mM PCMB. Hydrolysis of the various substrates was performed as described in Materials and Methods.

Substrate specificity of lactase–phlorizin hydrolase

Other workers have shown that lactase is really a hetero-β-glycosidase, hydrolyzing β-glucosides and β-galactosides [2]. Yet, phlorizin hydrolase activity, most likely due to a separate active site or protein [1, 2], has been equated with β-glucosidase using a non-specific β-glucoside, NPh-Glc, as substrate [9]. The ability to distinguish the two activities, lactase and phlorizin hydrolase, by heat inactivation provided a means for examining substrate specificity. Fig. 7 shows that after heat inactivation at 37 °C and pH 7, the only stable enzyme activity was directed against phlorizin. Ability to hydrolyze cellobiose and NPh-Glc, both β-glucosides, was lost identically with lactase activity. Furthermore, all of these activities were protected by incubation in the presence of PCMB.

TABLE III

HYDROLYSIS OF MILK OLIGOSACCHARIDES BY ISOLATED RAT LACTASE–PHLORIZIN HYDROLASE

Purified rat lactase was incubated at 37 °C for 2 h in the absence of PCMB to destroy most of the lactase activity (cf. Fig. 7). Untreated enzyme was used for control. Hydrolysis of sugars was assayed as described in Materials and Methods.

Substrate	Enzymatic activity (μmoles galactose/h)		Activity lost
	Control	Incubated 37 °C	
Lactose	2.7	0.6	78
3-Fucosyllactose	0.6	0.12	80
Lacto-*N*-tetraose	1.2	0.2	83
2′-Fucosyllactose	—	—	—
Lacto-*N*-fucopentaose	—	—	—

Since phlorizin is itself a β-glucoside, it seemed possible that phlorizin was not the natural substrate for this heat stable enzymatic activity. There are oligosaccharides other than lactose in the milk which might serve as substrates. Using sugars present in milk, we found that only oligosaccharides containing galactose as the glycone in β-linkage (lactose, 3-fucosyllactose, and lacto-*N*-tetraose) were hydrolyzed by isolated lactase–phlorizin hydrolase, and that most of this activity was lost after incubation at 37 °C (Table III). 2′-fucosyl lactose and lacto-*N*-fucopentaose were not hydrolyzed to any detectable extent. Thus, there was no evidence that phlorizin hydrolase activity (i.e. enzymatic activity stable at 37 °C) was active against any of these naturally occurring milk sugars. Phlorizin was, therefore, the only substrate hydrolyzed by the phlorizin hydrolase active site.

Phlorizin hydrolase also differed from the other β-glucosidases by having a unique developmental pattern. Fig. 8 shows the rapid decline in lactase specific

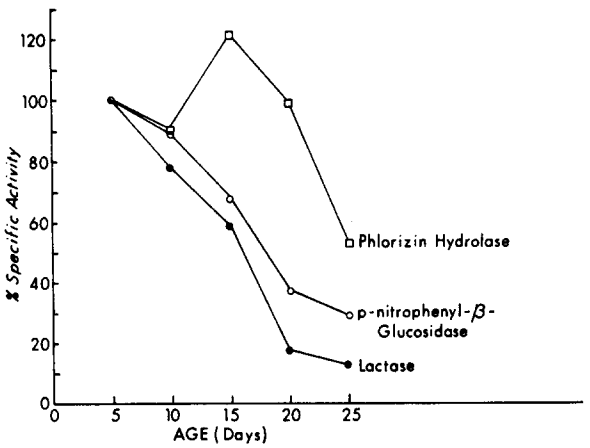

Fig. 8. Developmental patterns of lactase and β-glucosidases. Rats of varying ages were sacrificed and the small intestine removed and treated as described in Materials and Methods. Activity at 5 days of life was used as a reference point and corresponded to 14.6 mg glucose liberated/h per ml homogenate from lactose, 14.3 mg glucose/h per ml homogenate from NPh-Glc, and 291 μg glucose/h per ml from phlorizin. Each point corresponds to material obtained from a homogenate containing intestines from five separate animals, and is the mean of duplicate determinations.

activity reported previously [4]. *P*-Nitrophenyl-β-glucosidase activity had a similar developmental pattern. However, phlorizin hydrolase differed markedly, having a maximum specific activity at 15 days after birth, in agreement with the results of Ramaswamy and Radhakrishnan [4] but not of Colombo and Semenza [5].

DISCUSSION

Isoenzymes of β-galactosidases have been reported in bacteria [20] and in animals [21] but not previously regarding the intestinal brush border lactase. Schlegel-Haueter et al. [1] reported finding a major and a minor protein band in their purified lactase from infant rats after disc electrophoresis in a phosphate buffer, pH 7.2, but did not pursue the finding further.

Alpers reported a single protein band on a polyacrylamide electrophoresis when lactase was isolated from adult rats [22]. We report here at least three and probably four separate isoenzymes of lactase from neonatal rats. The reasons for these differences are not apparent. They might be related to the age of the animals, the length of time used for papain solubilization, or the electrophoretic system used. The latter seems unlikely since we obtained the same findings at pH 9.5 (Tris–glycine) and pH 6.5 (Pyridine–cacodylic acid). It is possible that with varying degrees of papain solubilization, different pieces of membrane or glycocalyx are solubilized along with the enzyme itself. The lack of any sialic acid on the enzymes makes it unlikely that pieces of membrane might be attached, but additional small pieces of glycocalyx might account for the isoenzymes. Age difference is a possibility, especially since young animals have the highest lactase levels, and might benefit from multiple enzyme forms. However, between days 4 and 15 after birth, the isoenzyme pattern does not change, although even at day 15 the lactase level is still three to four times the adult level. Other chemical differences in brush border composition of newborn and adult rats have been reported [23]. However, preliminary work in our laboratory suggests that infant and adult human lactase occurs in only one form (Alpers, D. H. and Goodwin, C. L., unpublished observations). Thus, the multiplicity of lactases may be peculiar to one species or group of species.

The isoenzymes of lactase do not seem to differ by molecular weight in acrylamide gel electrophoresis in sodium dodecylsulfate (Fig. 3) as they do in bacteria [20]. The most likely basis for their separate identity is a difference in charge. It is also possible that each isoenzyme contains a different amount of additional glycocalyx and glycopeptides, explaining the separation of several minor components in two of the lactase isoenzymes (C and D) on sodium dodecylsulfate gels.

The carbohydrate content of lactase–phlorizin hydrolase was higher than that (15%) reported by Cogoli et al. [24] for rabbit sucrase–isomaltase and similar to that of rat maltase [18]. The carbohydrate composition of rat sucrase (29%) is the highest for all rat disaccharidases thus far examined [18]. Amino acid composition showed 23% acid residues (aspartic and glutamic) and 32% hydrophobic residues (Ala, Val, Met, Ile, Leu, Phe). The percentage of hydrophobic residues is even lower than that found (35%) on the surface of red cell membranes [19]. Proteins integral to the red cell membrane itself and more difficult to extract had a 49% hydrophobic amino acid content [19]. Thus, amino acid analysis confirms the peripheral localization of lactase–phlorizin hydrolase on the brush border membrane. Despite the fact that PCMB affects lactase–phlorizin hydrolase activity, no free thiols could be measured. This could be due to the fact that only certain –SH agents affect lactase–phlorizin hydrolase, related perhaps to variables such as size, shape and charge of the reagent. The four –SH groups/molecule were similar to six/molecule found in rabbit sucrase [24]. Since attachment to the brush border membrane also confers stability at 37 °C to the enzyme complex (Fig. 4), it is possible that the important –SH group(s) may be near the attachment site.

The relationship between the two active sites of lactase–phlorizin hydrolase is still uncertain. Kraml et al. [2] used mixed inhibition studies and found a close relationship between the β-galactosidase and β-glucosidase. However, these authors felt that there might be more than one binding site for artificial substrates. Schlegel-Haueter et al. [1] demonstrated that rat lactase (a β-galactosidase) and cellobiase

were equally heat inactivated, whereas phlorizin hydrolase was more stable. Colombo and Semenza [5] showed that, in the hamster, both phlorizin hydrolase and *p*-nitrophenyl-β-glucosidase survived heating to 42 °C for 20 min, suggesting that some of the activity against synthetic β-glucosides is related to the phlorizin hydrolase activity. These authors also concluded that the lactase and phlorizin hydrolase catalytic sites were at least partially independent. However, the developmental patterns of rat lactase and phlorizin hydrolase were identical, suggesting related biological control mechanisms.

Our data support the hypothesis that in the rat, one site is responsible for all the enzyme activity except for the hydrolysis of phlorizin. These data include (a) identical heat inactivation at 37 °C for all substrate hydrolysis except phlorizin (Fig. 7), and (b) a developmental curve which shows close correlation between β-glucosidase activity different from phlorizin hydrolase (Fig. 8). The pattern seen for phlorizin hydrolase agrees with earlier data from one group of workers [4] but not for another [5]. Colombo and Semenza [5] explain the differences as resulting from a predominance of the non-lactase–phlorizin hydrolase-associated phlorizin hydrolase activity [9] which would have been favored under the conditions used by Ramaswamy and Radhakrishnan [4]. However, we assayed phlorizin hydrolase under conditions similar to those used by Colombo and Semenza [5], yet obtained different results. The only difference was that we measured only membrane bound phlorizin hydrolase. The developmental curve of phlorizin hydrolase, a β-glucosidase, seems the more valid since it does not coincide with the curve for *p*-nitrophenyl-β-glucosidase activity. Thus, our data suggest separate sites under different biological controls, at least in the developing rat. Another possibility is, of course, that lactase and phlorizin hydrolase are completely separate proteins which have thus far not been separated during purification.

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