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SMALL INTESTINAL PHLORIZIN HYDROLASE: THE "β-GLYCOSIDASE COMPLEX"

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SUMMARY

1. In baby rats small intestinal phlorizin hydrolase activity (phlorizin glucohydrolase, EC 3.2.1.62) is the highest at birth and decreases to adult levels at the time of weaning.

2. In rat and hamster small intestine phlorizin hydrolase follows neutral lactase during purification. It is associated with it, but the two β-glycosides are split by at least partially independent catalytic sites.

3. Phlorizin inhibits intestinal sugar uptake more in baby rats than in adult rats.

INTRODUCTION

Malathi and Crane[1,2] have reported the existence in the membrane of small intestinal brush borders of a phlorizin hydrolase (phlorizin glucohydrolase, EC 3.2.1.62) activity (see also ref. 3). This activity has been the object of various papers which have provided information on its substrate specificity, inhibitors and other kinetic properties[2,4,5] on its absence in human lactose intolerance[6] and on its possible role in the mechanism of phlorizin inhibition of sugar uptake in the small intestine[3]. The present paper reports other properties this enzyme activity including its age dependence and its association with "neutral" lactase. Some observations were published in a preliminary form[7–9].

MATERIAL AND METHODS

Phlorizin hydrolase activity was measured according to Malathi and Crane[2]; the incubation medium was 0.83 mM in phlorizin, 33.3 mM in McIlvaine buffer, pH 6.0.

The present paper is dedicated to Professor F. Leuthardt on the occasion of his 70th birthday.

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Deproteinization was carried out either with $Ba(OH)_2/ZnSO_4$ (refs 10 and 11) or, when only small amounts of protein were present, by heating the samples for 2 min in a boiling water bath.

Liberated glucose was determined according to the method of Nelson–Somogyi[10,11] or with the hexokinase–glucose-6-*P*-dehydrogenase method[12]. The latter method is superior in sensitivity and thus in accuracy at low enzyme activities.

When nitro-phenyl-β-glycosides were used as substrates, their final concentration was in general 3.3 mM; 33.3 mM sodium maleate buffer, pH 6.0 was used. The reaction was stopped with 0.1 M Na_2CO_3 buffer, pH 10.5, and the phenolate determined from its absorbance at appropriate wavelength (*o*-nitro-phenolate at 420 nm, *m*-nitro-phenolate at 392 nm and *p*-nitro-phenolate at 405 nm).

Lactase was determined according to Dahlqvist[13] (substrate lactose 114 mM in 0.1 M sodium maleate buffer, pH 6.0). Liberated glucose was then measured by the Tris–glucose oxidase method[13] and galactose with galactose dehydrogenase[14]. Protein was determined according to Lowry *et al.*[15].

The samples of essentially pure intestinal lactase used in the experiments were prepared in our laboratory[5]. Simultaneously and independently a very similar method for preparing rat lactase was reported by others[4,16].

Partial purification of oligosaccharides from cows colostrum was obtained as follows: the colostrum was dialyzed for 24 h against a 4-fold volume of water. The dialyzate was desalted by ion exchange. The mixture was concentrated and separated by paper chromatography (Whatman W1 paper; *n*-butanol–pyridine–water (30:10:15, by vol.). The sugars were detected on a strip with $AgNO_3$ in NH_4OH and NaOH. The oligosaccharides were eluted with water; a second paper chromatography showed that they were free of monosaccharides.

Intestinal sugar uptake was measured *in vitro* in Krebs–Henseleit buffer, using 6-deoxy-D-glucose (2 mM) as a substrate, with either 2-deoxy-D-glucose (1.5 mM) or [^{3}H]mannitol (0.25 μM) as a marker for the extracellular space. Everted small-intestinal segments were mounted in appropriate frames, so that the uptake was an accurate approximation of the unidirectional flux[17]. The extracellular space amounted to less than 10%. The small intestine of rats younger than approx. 10 days proved, however, too fragile to be mounted in these frames. Randomized rings from baby rats were, therefore, incubated as such[18]. Under these conditions, the extracellular space was approx. 30%. Incubation at 37 °C lasted 15 min. After deproteinization with $Ba(OH)_2/ZnSO_4$, 6-deoxy-D-glucose was measured according to Dische and Shettles[19], 2-deoxy-D-glucose according to Waravdekar and Saslaw[20]; ^{3}H-labelled mannitol was determined by scintillation counting. The data are expressed as μmoles substrate taken up/min per ml tissue water, assuming that the latter amounted to 80% of the fresh weight[18].

6-Deoxy-D-glucose was a generous gift of Professor E. Hardegger, ETH Zurich; 2-deoxy-D-glucose was obtained from Sigma, St. Louis, Mo.; phlorizin from Fluka, Buchs, Switzerland, and was recrystallized twice from water; [^{3}H]mannitol was obtained from Amersham, England. All other chemicals were reagent grade, and were obtained from commercial sources.

RESULTS AND DISCUSSION

Age dependence

Fig. 1 shows the age dependence of phlorizin hydrolase activity in rat small intestine. Clearly, it is highest at birth and decreases at the time of weaning, reaching the low adult level at about 20 days of age. This age dependence is very similar to that of small intestinal lactase[21,22] and is quite different from that of the small intestinal α-glucosidases (maltase, sucrase, isomaltase and trehalase) which are essentially absent at birth and begin to develop between the 15th and 16th day of life[22].

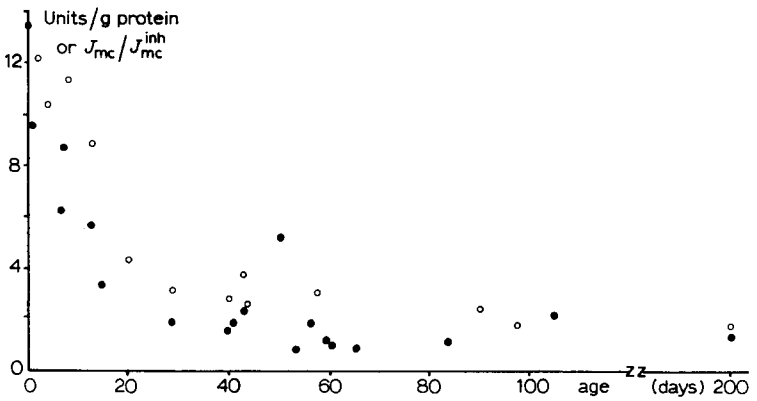

Fig. 1. Age dependence of phlorizin hydrolase activity (●) and of phlorizin inhibition of sugar uptake (○) in the small intestine of baby rats. Age, in days after birth. Phlorizin hydrolase activity as units per g protein. Sugar (6-deoxy-D-glucose, 2 mM) uptake as the ratio between the uptake in the absence of phlorizin (J_{mc}) and that in the presence of 10^{-5} M phlorizin (J_{mc}^{inh}).

The similar age dependence of lactase and phlorizin hydrolase, together with a strong correlation between these two activities in a random sample of human peroral biopsies[6] and the absence of phlorizin hydrolase activity in the small intestine of patients affected by lactase deficiency[6] indicate that phlorizin hydrolase and lactase activities are either due to the same catalytic site, or if due to more than one enzyme that they are subjected to similar or related biological control mechanisms.

After our present work was completed Ramaswamy and Radhakrishnan[23] reported a different age dependence of phlorizin hydrolase activity in baby rats. However, these authors determined the enzyme in the absence of phosphate (which activates brush-border phlorizin hydrolase[2,*]) and at a different pH value than we did. They may, therefore, have measured a statistical average of various phlorizin hydrolases, since it is known that rat small intestine possesses more than one phlorizin splitting enzymatic activities[4].

* Ramaswamy and Radhakrishnan[43] have shown recently that phlorizin hydrolase activity is stimulated at pH 3.3 by a number of organic acids.

The relationship between phlorizin hydrolase and lactase activities: the "β-glycosidases complex"

"Neutral" (brush-border bound) lactase was isolated from the small intestine of baby rats according to a procedure already described[5] and involving: (i) papain solubilization of the membrane-bound ensyme, (ii) Sepharose, (iii) Sephadex and (iv) DEAE-cellulose chromatography. The procedure leads to an almost homogeneous product. Phlorizin hydrolase activity was measured during the isolation along with lactase activity. Table I shows that the lactase/phlorizin hydrolase ratio remains essentially constant during those fractionation steps which bring about an almost 70-fold purification. Under the conditions of our determinations the lactase/phlorizin hydrolase ratio of the final rat preparation is approx. 50:1* (Kraml *et al.*[4], have shown that in addition to this lactase-associated phlorizin hydrolase, brush borders possess another, minor, phlorizin hydrolase which is also released by papain).

TABLE I

Lactase–phlorizin hydrolase ratios during the major steps of the preparation of small intestinal "neutral" lactase, by papain solubilisation, Sepharose, Sephadex and DEAE-cellulose chromatography.

	From small intestine of baby rats (ref. 5)		*From small intestine of adult hamsters*	
	Lactase/ phlorizin hydrolase ratio	*Phlorizin-hydrolase units/mg protein*	*Lactase/ phlorizin-hydrolase ratio*	*Phlorizin-hydrolase units/mg protein*
Total homogenate (approx.)	(20)	(0.006)		
After Sepharose 2B	52	0.022	3.8	0.1
After Sephadex G-200	53	0.077	(This step omitted)	
After DEAE-cellulose	49	0.13	4.5	0.1

Most of the studies on the phlorizin hydrolase activity of the gut have involved the used of adult hamster small intestine and a partial purification of the enzyme from this source was, therefore, attempted. This could not be pursued to homogeneity because of the low activity of the starting material. However, essentially similar results were obtained as in the rat (Table I). The lactase/phlorizin hydrolase ratio in the hamster is approx. 4–5:1, a figure, however, which should not be given great significance due to the heterogeneity of the final preparation.

These observations could be interpreted in three ways: (i) lactose and phlorizin are split by the same catalytic site(s); (ii) phlorizin and lactose are split by two independent catalytic sites, both occurring in the same protein or protein complex; (iii) a combination of (i) and (ii). Evidence is given below which suggests that possibilities (ii) and (iii) are the most likely.

* Kraml *et al.*[24] have reported very recently an improved procedure for the isolation of rat intestinal lactase. Their product is homogeneous in both polyacrylamide electrophoresis and immuno electrophoresis and has a slightly higher specific activity than our preparation. Their new preparation also splits phlorizin, with the same lactase/phlorizin hydrolase ratio as our lactase does. (Kraml, J., personal communication, 1973). Thus, the minor impurity(ies) still present in our preparation do not invalidate the conclusions of the present paper.

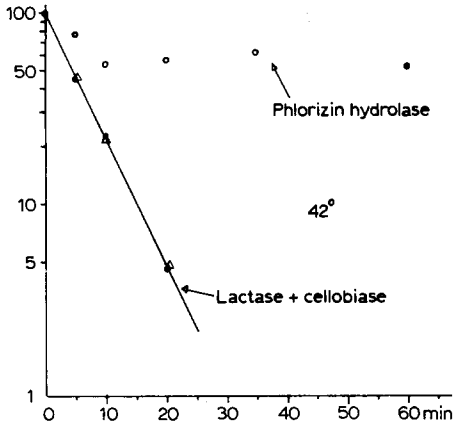

Fig. 2. Time course of inactivation of partially purified β-glycosidase complex from adult hamster at 42 °C. ○, phlorizin hydrolase activity; ●, lactase activity; △, cellobiase activity.

The different time-course of heat inactivation of the two glycosidase activities in essentially pure rat intestinal lactase[5] in partially purified hamster intestinal lactase (Fig. 2) as well as in total homogenate of human small intestine[6] shows that in these species the two glycosides (at the concentrations used in the essay) are not split by a single common catalytic site, ruling out possibility (i) above*.

However, some phlorizin hydrolase is lost along with the inactivation of lactase activity. At least in the case of the human enzymes, the kinetics of inactivation of phlorizin hydrolase and lactase activities at two temperatures have suggested that lactase itself has phlorizin hydrolase activity, albeit small compared to lactase (some 2.8% of the lactase activity, but accounting for some 28% of the total phlorizin hydrolase of human intestinal homogenate[6]).

Experiments of mixed substrate inhibition (Table II) are not compatible with

TABLE II

Mixed substrate incubations with partially purified β-glycosidase complex from adult hamster. Incubation at 37 °C in 0.1 M sodium maleate buffer, pH 6.0. The results are expressed as n moles liberated/ml per min^{-1}; liberated phloretin was calculated from glucose and galactose, assuming that transglycosidation was negligibly small compared with hydrolysis.

	Galactose	*Glucose*	*Phloretin*
Phlorizin (2.1 mM)		36.4	36.4
Lactose (3 mM)	72.4	76.8	
Lactose (3 mM) + phlorizin (2.1 mM)	61.4	101.2	ca. 40

* Kraml *et al.*[25] have made the interesting observation that the velocities of disappearance of β-glucosidase and β-galactosidase activities at 43 °C are superposable if the subsequent determinations are carried out at substrate concentrations well above their respective K_m values. They are not superposable, instead, if the determinations are carried out at low substrate concentrations, the β-galactosidase activity disappearing faster. This observation, which is a warning against the indiscriminate use of heat inactivation in studies of this sort, can be explained, as suggested, by a partial reactivation by the substrate. It can also indicate, however, that one of the substrates, *e.g.* *p*-nitrophenyl-β-galactoside, is split by both lactase and phlorizin hydrolase, one enzyme having very low K_m and V and the other large K_m and large V for this substrate. Fig. 3 shows that this latter possibility is real.

one common catalytic site but rather with two catalytic sites *plus* an inhibition of lactase by phlorizin. (Kraml *et al.*[4] have already reported that phlorizin is a competitive inhibitor of rat intestinal lactase).

We failed to detect any inhibition of phlorizin hydrolase activity by lactose (Table II) (in the hamster) as would have expected if some phlorizin hydrolase activity were due to lactase (as indicated by the heat inactivation of human small intestine quoted above). However, since in these preparations much more glucose is liberated from lactose than from phlorizin, a minor inhibitory effect of lactose on (total) phlorizin hydrolase activity might have been masked by a high rate of lactose hydrolysis.

The Lineweaver–Burk plot with *p*-nitro-phenyl-β-galactoside and essentially homogeneous lactase (Fig. 3) indicates the possible existence of two kinds of active sites splitting this substrate. We want to make clear that these data are still preliminary.

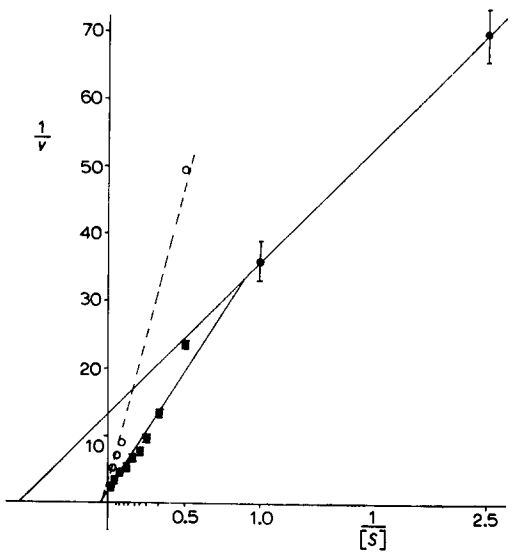

Fig. 3. Lineweaver–Burk plot of *p*-nitro-phenyl-β-galactoside hydrolysis by rat intestinal lactase–phlorizin hydrolase. ●, by the native enzyme (average of four determinations, S.E. indicated by bars); ○, by the same enzyme preparation after partial heat inactivation (at 46 °C for 33 min in 0.01 M potassium phosphate buffer, pH 6.8; these data are single determinations and should thus be considered as preliminary). The results with the native enzyme are best described by assuming the action of two kinds of active sites on this substrate, the two K_m values being approximately 30 and 1.6 mM. The low K_m site(s) would also have a V equal to approx. 1/20 of the V of the large K_m site(s). The large K_m is in fair agreement with that reported by Kraml *et al.*[2], *i.e.* 22.3 mM (range 15.5–30 mM). After nearly total inactivation of lactase with partial inactivation of phlorizin hydrolase (○, preliminary data) the large-K_m site(s) are still partially active with no indication of the small K_m site(s): the points at low s fall on the same line as the others (data not shown). s, substrate concentration (mM); v, reaction velocity (μmoles substrate hydrolyzed/min per ml).

Finally, the K_i values of various competitive inhibitors (Tris, galactonolactone, gluconolactone) are different for rat phlorizin hydrolase and lactase (Kraml *et al.*[4]). This observation also indicates that lactase and the major phlorizin hydrolase activity are due to different catalytic sites.

We conclude, therefore, that lactase and phlorizin hydrolase activities are due to the same protein or protein complex (which will be referred to as the "β-glycosidases complex"); however, they are due to at least two independent or partially independent catalytic sites. To this basic pattern one must add the minor phlorizin hydrolase activity of the lactase site itself (at least in man, ref. 6) which may be reflected in the inhibition of lactase by phlorizin (competitive in nature, as Kraml *et al.*[4] have shown for the rat enzyme), as well as the fact that some hetero-β-glycosides are split by both lactase and phlorizin hydrolase (see later).

These conclusions may be subjected to partial revision in the future, when more will be known on the β-glycosidase complex. However, as it stands now, the biological and biochemical relationships between small intestinal lactase and phlorizin hydrolase are reminiscent of those between two other small-intestinal carbohydrases (sucrase and isomaltase)[26], which also occur as an oligoenzyme complex. Sucrase and isomaltase also are subjected to the same or to related biological control mechanisms as indicated by (i) the constancy of the sucrase–isomaltase ratio in a number of samples of human intestines[27], (ii) the simultaneous appearance of sucrase and isomaltase activities in the small intestine of baby rats[22] and of human fetuses[28] and (iii) the lack of both sucrase and isomaltase in human sucrose–isomaltose malabsorption[29,30] a monofactorial recessive genetic disease[31]. In the case of the sucrase–isomaltase complex, the two subunits have been separated, partially characterized and shown to be rather similar proteins[32,33].

K_m values, Tris inhibition

Table III reports the K_m values of the β-glycosidase complex from various species with lactose, phlorizin and *m*-nitro-phenyl-β-glucoside as substrates. The species differences are of the same order as those found in the K_m values of other intestinal carbohydrases (for a review, see ref. 34).

TABLE III

K_m values of the β-glycosidase complex from various species (mM at 37 °C at the pH values indicated).

	Lactose (pH 5.5)	*Phlorizin*		*m-Nitrophenyl-β-glucoside*
		(pH 6.0)	*(pH 5.5)*	*(pH 6.0)*
Rat (homogeneous or purified complex)	21 (ref. 5), 25 (ref. 42) 40 (ref. 4)	0.68 (this paper)	0.4 (ref. 4)	
Hamster (brush borders)		0.1 (ref. 2)		
Hamster (solubilized and purified complex)	3 (this paper)	0.33 (this paper)		2.3 (this paper)
Man (total homogenate)	18 (ref. 27)	0.5 (ref. 6)		

Tris inhibits phlorizin hydrolase competitively[4]. In addition, we have observed that Tris inhibition is larger the higher the pH, being negligible below pH 6.5 (Table IV). Similar observations have been made for small intestinal isomaltase[35] (oligo-1,6-α-glucosidase), sucrase[36] and lactase[37]. Therefore, if the pH–activity curve is determined for phlorizin hydrolase in the absence of Tris, the pH optimum is found to be at a more

TABLE IV

pH DEPENDENCE OF TRIS INHIBITION OF PHLORIZIN HYDROLASE

A partially purified preparation from adult hamster was used; 0.83 mM phlorizin ±50 mM Tris.

pH	*Buffer*	*% Inhibition*
6.0	0.1 M dimethylglutarate–KOH	0
6.5	0.1 M dimethylglutarate–KOH	0
7.0	0.1 M triethanolamine–HCl	10
7.5	0.1 M triethanolamine–HCl	23
8.1	0.1 M triethanolamine–HCl	50
8.6	0.1 M triethanolamine–HCl	82

alkaline pH value then that found by previous authors, *i.e.* around neutrality, see footnote on p. 414.

Other substrates of phlorizin hydrolase

Malathi and Crane[2] as well as Kraml *et al.*[4] have tested a number of β-glycosides as possible substrates of what we now know to be a β-glycosidase complex. We have made some preliminary investigations on whether some of these β-glycosides are split by the phlorizin hydrolase moiety. To this end we examined the hydrolysis of a number of hetero-β-glycosides by a partially purified preparation of hamster lactase–

TABLE V

HYDROLYSIS OF β-GLYCOSIDES BY NATIVE OR PARTIALLY HEAT-INACTIVATED HAMSTER β-GLYCOSIDASE COMPLEX

Incubation at 37 °C in 0.125 M potassium maleate buffer, pH 6.0; 2.5 mM substrates. The results are expressed as μmols of substrate hydrolysed/min per g protein and are the average of four determinations. The enzyme preparation was used either native or after partial heat inactivation (20 min at 42 °C, see Fig. 2). Values in parentheses represent percent activity remaining.

	Native	*After partial heat inactivation (%)*	
Expt a			
Phlorizin	150	108	(72)
Arbutin	4.1	0	(0)
Salicin	12.9	6.0	(46)
Cellobiose	54	0	(0)
Methyl-β-D-glucoside	1.5	0.7	
Phenyl-β-D-glucoside	1.9	1.3	
o-Nitrophenyl-β-D-glucoside	51	19	(37)
m-Nitrophenyl-β-D-glucoside	173	139	(80)
p-Nitrophenyl-β-D-glucoside	120	88	(73)
Expt b			
Phlorizin	107	81	(75)
o-Nitrophenyl-β-D-galactoside	215	62	(35)
p-Nitrophenyl-β-D-galactoside	7.6	1.6	(20)
p-Nitrophenyl-β-D-mannoside	2.2	0	(0)
o-Nitrophenyl-β-D-fucoside	235	82	(35)
p-Nitrophenyl-β-D-fucoside	226	76	(34)
p-Nitrophenyl-β-L-fucoside	0	0	
p-Nitrophenyl-β-D-xyloside	0.85	0.45	
p-Nitrophenyl-β-D-*N*-acetyl-glucosamide	1.7	0	

phlorizin hydrolase, before and after partial heat denaturation. Treatment at 42 °C for 20 min was convenient because this destroys lactase (and cellobiase) activity almost completely (see Fig. 2), leaving behind some 70% of the phlorizin hydrolase activity. Clearly, *m*- and *p*-nitro-phenyl-β-glucosidase activities survive the treatment essentially to the same extent as phlorizin hydrolase activity (Table V), making them suitable substrates for a further study. It is likely, but not proved, that those glycosides which are not split any further after the partial heat inactivation were originally split by the lactase moiety, and that those that are still split, but to a lesser extent, after the heat treatment, were originally split by both moieties.

Experiments of mixed substrate inhibition between phlorizin and *m*-nitrophenyl-β-glucoside are reported in Table VI. Clearly, these β-glucosides compete mutually for the same catalytic site and the contribution of the lactase moiety to *m*-nitro-phenyl-β-glucosidase activity must be minimal or zero. At pH 6.0 the K_m of *m*-nitro-phenyl-β-glucoside is 2.3 mM; its V is three times as large as that of phlorizin. The pH optimum is 6–6.5.

TABLE VI

MUTUAL INHIBITION BETWEEN PHLORIZIN AND *m*-NITRO-β-D-GLUCOSIDE PARTIALLY PURIFIED β-GLYCOSIDASE COMPLEX FROM ADULT HAMSTERS

Incubation at 37 °C in 0.1 M potassium phosphate buffer, pH 6.0. The results are expressed as nmols liberated/min per ml.

	m-Nitrophenol	*Glucose*	*Phloretin*
Part a			
Phlorizin (5 mM)		15.5	15.5
m-Nitrophenyl-β-D-glucoside (3 mM)		26.4	
Phlorizin (5 mM) *plus* *m*-nitrophenyl-β-D-glucoside (3 mM)		26.4	
Phlorizin (5 mM) *plus* *m*-nitrophenyl-β-D-glucoside (3 mM) (calculated for common catalytic site)		18.1	
Part b			
Phlorizin (5 mM)		15.5	15.5
m-Nitrophenyl-β-D-glucoside (30 mM)	45.9	42.4	
Phlorizin (5 mM) *plus* *m*-nitrophenyl-β-D-glucoside (30 mM)	25.5	29.6	4.1
Phlorizin (5 mM) *plus* *m*-nitrophenyl-β-D-glucoside (30 mM) (calculated for common catalytic site(s))	22.2	29.1	8.5

The experiments of Table V on partial heat inactivation were carried out with a partially purified complex from hamster small intestine and the enzyme activities were measured at a single substrate concentration (3.3 mM), irrespective of their K_m values. It is quite possible that determinations carried out at other substrate concentrations lead to different conclusions[25]. Nevertheless, the intermediate inactivation observed with *p*-nitrophenyl-β-galactoside and the observations of Kraml *et al.*[25] on the peculiar behaviour of the heat inactivation of rat lactase with this substrate (footnote to page 416) prompted us to carry out a detailed K_m determination of native rat intestinal lactase–phlorizin hydrolase complex (Fig. 3). The data in a $1/v$ *vs* $1/s$

plot do not quite fall on a single straight line, but can rather be described by the existence of two active sites, the one having low K_m and low V, the other having large K_m and large V. This may be the explanation of the peculiar heat inactivation reported by Kraml *et al.*[25] (footnote to page 416). The few preliminary observations made with partially inactivated lactase (Fig. 3) suggest that the *p*-nitrophenyl-β-galactosidase having low K_m and V is lactase; that one having large K_m and large V is phlorizin hydrolase. This would again be compatible with Kraml's observations.

Thus, both kinds of active sites present in the lactase–phlorizin hydrolase complex can split glucosides and galactosides, although important quantitative—and perhaps qualitative—species differences undoubtedly exist. The heteroglycosides (either natural or synthetic) investigated are of no nutritional significance and cannot be regarded as "natural" substrates for phlorizin hydrolase.

Since phlorizin hydrolase activity is highest at birth (see below), we investigated whether sugars other than lactose occurring in the milk or in the colostrum are split by this enzyme. A crude, deionized preparation of total carbohydrates from cow colostrum was freed of monosaccharides and incubated with hamster lactase–phlorizin hydrolase (either intact or partially heat inactivated). After exhaustive incubation, the sugars were separated by paper chromatography. Only the non-heat-inactivated preparation liberated monosaccharides (glucose and galactose) in equimolar amounts. They obviously stemmed from the action of lactase on the lactose present in the colostrum: the partially heat-inactivated lactase–phlorizin hydrolase did not liberate any monosaccharide. Equally negative were tests made with individual minor oligosaccharides present in the milk and in the colostrum, *i.e.* 2′-fucosido-lactose, lacto-*N*-tetraose, lacto-*N*-fucopentaose and dilactamyl-lacto-*N*-tetraose (kindly provided by Professor Montreuil, Lille) or with two α-galactosides from soybeans (vesbascose and stachyose, kindly provided by Drs Wuhrmann and Cristofaro, Nestlé, Vevey) using the isolated lactase–phlorizin hydrolase from rat. It appears that these sugars are not split by either moiety of the complex.

The products of phlorizin hydrolase

Phlorizin hydrolase is reported to be activated by phosphate[2]. Although other β-glucosidases are also activated by phosphate[38], we investigated the possibility that phlorizin hydrolase activity might arise from the combination of a phlorizin phosphorylase *plus* small intestinal alkaline phosphatase. We can rule out this possibility, however, because: (i) the phlorizin hydrolase activity of hamster brush borders is not inhibited by 20 mM L-phenylalanine, a well-known inhibitor of intestinal phosphatase; (ii) isolated rat intestinal β-glycosidase complex liberated only phloretin and glucose from phlorizin (as identified by thin-layer chromatography); (iii) the amount of glucose liberated from phlorizin is the same, irrespective of whether it is determined as a reducing sugar or with the hexokinase–glucose-6-*P*-dehydrogenase method; (iv) partially purified hamster lactase–phlorizin hydrolase liberates equimolar amounts of *m*-nitro-phenol (measured as phenolate) and D-glucose (measured with the hexokinase–glucose-6-*P*-dehydrogenase method) (Table VI)

Correlation between phlorizin hydrolase and phlorizin sensitivity in rats of different age

As reported in the first paragraph, phlorizin hydrolase activity is the highest at birth and decreases thereafter (Fig. 1). We, therefore, investigated the effect of

10^{-5} M phlorizin on the uptake of 6-deoxy-D-glucose in the small intestine of rats of different ages. It was found (Fig. 1) that phlorizin inhibits sugar uptake in young rats more than in adult ones.

In many kinds of inhibition the J_{mc}/J_{mc}^{inh} ratio* is a linear function of the concentration of the inhibitor, the intercept on the J_{mc}/J_{mc}^{inh} axis being equal to 1. If the inhibitor arises from the action of phlorizin hydrolase activity, the concentration of the inhibitor should parallel phlorizin hydrolase activity. In this case, therefore, a plot of J_{mc}/J_{mc}^{inh} *vs* phlorizin hydrolase activity should be linear with an intercept equal to 1. This is the case (Fig. 4), the correlation coefficient being 0.98 and the intercept being barely statistically different from 1 ($P \simeq 0.02$).

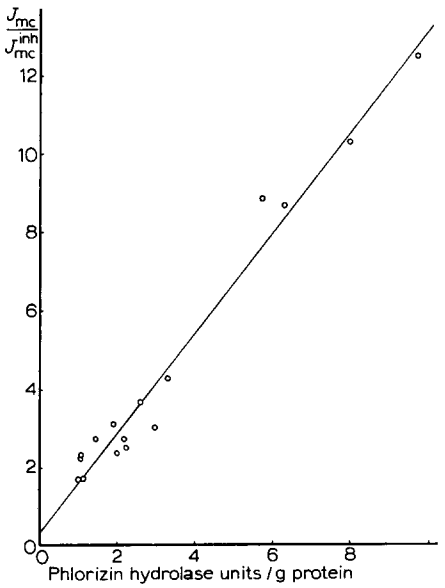

Fig. 4. Correlation between phlorizin inhibition of sugar uptake and phlorizin hydrolase activity in the same tissue (units per g protein). Data from Fig. 1; for details, see Fig. 1.

As mentioned in Material and Methods, the small intestinal rings of rats younger than approx. 7 days were too fragile to be mounted in frames[17] and had to be incubated as such[18]. This minor difference in the method used could not be responsible for the observed effect, since exposing the serosal side to the medium should have produced, if anything, an artificially low, rather than high, inhibition by phlorizin. In addition, the correlation plot between phlorizin hydrolase activity and phlorizin inhibition of sugar uptake (Fig. 4) does not show any break around the age of 7 days.

The close correlation shown in Fig. 4 can be explained in at least three ways: (i) the actual inhibitor of intestinal sugar uptake might be not phlorizin but a product arising from it through the action of phlorizin hydrolase. As far as membrane-bound

* J_{mc} is the unidirectional flux of the substrate from the mucosal medium into the small intestine in the absence of inhibitor; J_{mc}^{inh} is J_{mc} in the presence of inhibitor.

phlorizin hydrolase can be compared with the isolated enzyme, the products from this hydrolase are only glucose and phloretin (see above). It was suggested by Diedrich[3] that not phlorizin itself, but the liberated aglycone, phloretin, should be the actual inhibitor of intestinal sugar uptake. This suggestion was recently questioned by the originator himself, because of his failure to detect any phlorizin hydrolase activity in kidney tubuli, which possess phlorizin-sensitive monosaccharide transport system(s) similar, if not identical, to the system(s) of small intestine[39]; (ii) the affinity of the transport system(s) for the monosaccharides may have changed during the development of the intestine. This does not seem to be the case[9]; (iii) during the age span considered, extensive changes in the biochemical equipment of rat small intestine take place[34]. It is possible that along with the decrease of phlorizin hydrolase activity, (but independent of it), changes in the affinity of the monosaccharide carrier(s) for phlorizin also take place. This possibility, which would have appeared rather unlikely some years ago, appears now to be the most probable. In fact, Honegger[40,41] has recently provided evidence that free glucalogues are transported by at least two systems in hamster small intestine, and that the quantitative ratios between the two systems in baby hamster and in adult hamsters are different. Interestingly enough, in baby hamsters the prevailing system is more phlorizin sensitive. If these conclusions hold true for the rat also, they provide a straightforward explanation for the age-dependent correlation of Figs 1 and 4, between phlorizin hydrolase and phlorizin inhibition of sugar uptake.

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Very recently (Leese and Semenza[44]) we have obtained evidence for the identity between phlorizin hydrolase and glycosylceramidase.

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