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Intestinal Glycosidase Activities in One Adult and Two Suckling Echidnas: Absence of a Neutral Lactase (β-D-Galactosidase)

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Abstract

The activities of various glycosidases in homogenates of the small-intestinal mucosa of one adult and two suckling echidnas, *Tachyglossus aculeatus*, were investigated. The activities of lactase (*β*-D-galactosidase), *β*-*N*-acetylglucosaminidase, neuraminidase and α-L-fucosidase were higher in the sucklings than in the adult animal. Maltase and isomaltase activities were lower. Sucrase and cellobiase activities were absent or present in trace amounts only. The lactase activity had a pH optimum of 4·0–4·5, was predominantly in the soluble fraction following ultracentrifugation and was inhibited by *p*-chloromercuribenzene sulfonate, suggesting that it was due to a lysosomal acid *β*-galactosidase and not a brush-border neutral lactase. The maltase activity of the sucklings also had the characteristics predominantly of a lysomal acid hydrolase. It is proposed that in suckling echidnas, the oligosaccharides (mainly neuraminyllactose and fucosyllactose) of the mother's milk are digested intracellularly by lysosomal enzymes, rather than at the brush border, of the epithelial cells of the small-intestinal mucosa.

Extra keywords: disaccharides, monotremes.

Introduction

Numerous studies with eutherian mammals have shown that the mucosa of the small intestine contains a variety of glycosidases. These include neutral hydrolases, located on the microvillous membrane of the brush border, which act on oligosaccharides such as lactose, trehalose, maltose and sucrose (Semenza 1968), as well as intracellular lysosomal acid hydrolases including *β*-D-galactosidase, EC 3.2.1.23, *β*-*N*-acetyl-D-glucosaminidase, EC 3.2.1.30 (Koldovsky *et al.* 1972) and neuraminidase, EC 3.2.1.18 (Dickson and Messer 1978).

Kerry (1969) and Walcott and Messer (1980) showed that most of these enzyme activities are found also in adult and pouch-young marsupials, but quantitative data on monotremes are limited to two adult echidnas, *Tachyglossus aculeatus*, studied by Kerry (1969). Since the milk of monotremes contains significant amounts of unusual oligosaccharides (Messer and Kerry 1973), studies on the intestinal glycosidases of suckling monotremes are of special interest. In addition, recent findings suggesting that a neutral intestinal lactase (*β*-D-galactosidase) is absent in pouch-young and adult tammar wallabies (Walcott and Messer 1980; Walcott *et al.* 1980) raise the question of whether this enzyme is found in monotremes.

In this paper, we report on the levels and pH optima of the intestinal glycosidase activities of three echnidas, two of which were sucklings. We also present data pertaining to the cellular localization of the lactase activity.

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Materials and Methods

Suckling No. 1, which originated from Boxall State Forest, near West Wyalong, N.S.W. was killed in late October 1978 when its age was estimated to be 67 days. The intestine was stored in a sealed plastic bag at −60C° for 6 months, and then assayed for glycosidase activities. Suckling No. 2, from Kangaroo Island, S.A., was killed in November 1980 when its age was estimated to be 81 days. Its intestine was similarly stored at −60C° for 20 months, before assay. The adult echidna, from Kangaroo Island, was killed in November 1981; its intestine was stored at −60°C for 7 months. Owing to the long periods of storage, it is possible that there was some loss of activity of intestinal glycosidases, though previous work with rat small intestine has shown that there was no loss during storage at −20°C for 3 months (Walcott and Messer 1980). The enzyme likely to be the least stable is neuraminidase (Dickson and Messer 1978), but control experiments with rat intestine stored at −20°C for 28 and 36 months showed no loss of neuraminidase activity.

After thawing, the small intestine of each animal was severed from the hindgut at the ileocaecal junction and from the pseudoduodenum (Griffiths 1968) at the entry point of the common bile duct. It was then divided into three sections of equal length (proximal, middle and distal to the stomach). The contents of each section were removed by rinsing with ice-cold 0·15 M NaCl, and the mucosa then squeezed out by placing the section on a chilled glass plate and drawing a glass rod firmly along along its length. The mucosa was homogenized in 5 vol. (w/v) of ice-cold water, with a Potter–Elvehjem homogenizer. Homogenates were either assayed immediately (for neuraminidase and the experiments shown in Table 2) or stored at −20°C in the presence of toluene (10 μl ml^{-1}) for up to 4 days, before enzyme assay. Control experiments showed that, except for neuraminidase, there was no loss of glycosidase activity during this time.

Activities of lactase, cellobiase, maltase (α-D-glucosidase, EC 3.2.1.20), isomaltase (oligo-1,6-glucosidase, EC 3.2.1.10), sucrase (sucrose α-D-glucohydrolase, EC 3.2.1.48) and trehalase (α,α-trehalase, EC 3.2.1.28) were determined using the appropriate disaccharide substrates, as described by Dahlqvist (1964). Sodium acetate buffer, 0·1 M, was used for the pH range 3·5–5·5, and sodium maleate for pH 5·5–6·5. In the assays of the lactase activities of suckling No. 2 and the adult animal, NaCl (0·1 M) was included to stabilize the activity during incubation (Heyworth *et al.* 1981). Glucose was initially determined using *o*-dianisidine as chromogen; in later experiments, ABTS [2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulfonate] was used (Bergmeyer and Bernt 1974). Phenyl β-galactosidase activity was assayed as described by Asp (1971*a*) except that 0·1 M sodium acetate buffer, pH 4·5 was used instead of citrate. β-*N*-Acetyl-D-glucosaminidase activity was assayed as described by Koldovsky and Herbst (1971), except that the final concentration of substrate (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) was 2·5 mM, the pH was 5·0 and the reaction was stopped with 1 M Tris-HCl, pH 8·3 (Asp 1971*b*). α-L-Fucosidase, EC 3.2.1.51, was assayed with *p*-nitrophenyl-α-L-fucoside as substrate (Levvy and McAllan 1961). The assay mixture, 0·8 ml, contained 2 vol. of sodium citrate, 0·1 M, pH 5·5, 1 vol. of substrate solution, 4 mM, and 1 vol. of diluted homogenate. The reaction was stopped with 2 vol. of 1 M Tris-HCl, pH 8·3. Neuraminidase was assayed with *N*-acetylneuraminyl-D-lactose as substrate as described by Dickson and Messer (1978) except that dimethylsulfoxide was used instead of acid butanol in the thiobarbituric acid determination of sialic acid (Skoza and Mohos 1976).

All enzyme assays were done on duplicate samples of homogenate; duplicate determinations did not differ from each other by more than 5%. One unit of enzyme activity is defined as that which hydrolyses 1 μmole of substrate per minute at 37°C.

pH optima were determined using the middle section of the intestine only, except that in suckling No. 1 all three sections were studied and no differences were found between the pH optima of the three sections.

Protein was estimated by the method of Lowry *et al.* (1951); bovine serum albumin was used as the standard.

For *in vitro* hydrolysis experiments, neuraminyllactose and fucosyllactose isolated from the water-soluble fraction of echidna milk by gel-permeation chromatography on Sephadex G-15 (Messer and Kerry 1973) were used. The neuraminyllactose was further purified by preparative paper chromatography using Whatman No. 3 paper and pyridine–ethyl acetate–acetic acid–water (5 : 5 : 1 : 3, v/v) as solvent to separate the major component, 4-*O*-acetyl-*N*-acetylneuraminyl-lactose, from the minor component, *N*-acetylneuraminyllactose (Messer 1974). 4-*O*-Acetyl-*N*-acetylneuraminyllactose, 400 μg in 20 μl of 50 mM sodium acetate, pH 4·5, or fucosyllactose, 400 μg in 20 μl of 50 mM sodium citrate, pH 5·5, was mixed with 20 μl of a 20% (w/w) homogenate of

intestinal mucosa from a suckling echidna, plus 1 μl of toluene as preservative. Incubation was done at 37°C and 1-μl samples taken for thin-layer chromatography (t.l.c.) at various times up to 24 h. T.l.c. was done on silica-gel plates (Merck, Darmstadt, Art. 5553). For the neuraminyl-lactose experiments, isopropanol–acetone–0·1 M lactic acid, 4 : 4 : 2 (v/v), was used as a solvent (Hansen 1975). For fucosyllactose experiments, n-butanol–acetic acid–water, 2 : 1 : 1 (v/v), was used in a 16-h run; the solvent, when it reached the top of the plate, was absorbed in an attached pad of Whatman No. 3 paper. The latter solvent, unlike the former, separated fucosyllactose from lactose.

Results

pH of Optimum Activity

In eutherian mammals, the intestinal lactase and other disaccharidases of both suckling and adult animals have pH optima between 5·5 and 6·0 (Rubino *et al.* 1964; Semenza 1968). Fig. 1 shows that the pH of optimum activity of lactase of all three echidnas was considerably lower, *viz.* 4·0–4·5; similarly the pH optimum for the maltase activity of the two sucklings was low at 5·0–5·5. The maltase activity of the adult echidna (Fig. 1) and the other enzyme activities studied (Table 1) had pH optima that were similar to those of eutherian mammals.

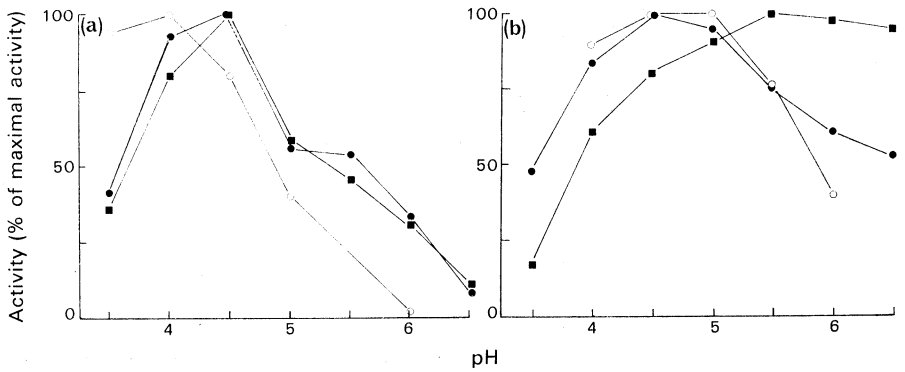

Fig. 1. Activity as a function of pH, of lactase (*a*) and maltase (*b*) in the middle third of the small intestine. ■ Adult echidna. ○ Suckling No. 1. ● Suckling No. 2.

Intestinal Glycosidase Activities

Table 1 lists the specific glycosidase activities of mucosal homogenates of the proximal, middle and distal thirds of the small intestine, all determined at their optimum pH. The results of Kerry (1969) previously obtained with two adult echidnas are included for comparison. In the two sucklings, the activities were considerably higher than in the adult animals, except for maltase and isomaltase, which were lower, and sucrase and cellobiase, which were absent or found in only trace amounts. In both sucklings, the isomaltase and trehalase activities were highest in the proximal part of the intestine, whereas the lactase and maltase activities were evenly distributed. The results for the two animals differed from each other in that in suckling No. 1 the neuraminidase, fucosidase and *N*-acetylglucosaminidase activities were highest in the distal intestine, whereas in suckling No. 2 they were more or less evenly distributed. In the adult animal, the maltase, isomaltase and trehalase activities were much higher in the proximal section than in the distal section.

Solubility of the Intestinal Disaccharidases of Echidna

In eutherian mammals, the intestinal disaccharidases are firmly bound to the limiting membrane of the microvillous brush border (Semenza 1968), and their activities are therefore found predominantly in the particulate (insoluble) fraction of an homogenate following ultracentrifugation. Table 2 shows that over 80% of the intestinal trehalase and isomaltase activities of both the adult and a suckling echidna were in the particulate fraction. In the adult echidna, the maltase activity was similarly associated mainly with the particular fraction, but in the suckling it was predominantly found in the supernatant (soluble) fraction. The lactase activity, like that of the α-L-fucosidase (a lysosomal enzyme), was predominantly soluble in both animals.

Table 1. Intestinal glycosidase activities of suckling echidnas aged 67 days (No. 1) and 81 days (No. 2) and an adult echidna

Activities, given as units per gram of tissue protein, are arithmetic means of duplicate determinations (see Materials and Methods). The pH of optimum activity, at which assay was done (see Materials and Methods), is also given

Glycosidase	Animal No.	Suckling pH	Suckling Glycosidase activity in: Proximal intestine	Middle intestine	Distal intestine	Adult pH	Adult Glycosidase activity in: Proximal intestine	Middle intestine	Distal intestine	Data of Kerry (1969)[A]
Lactase	1	4·0	6·0	9·3	11	4·5	2·7	1·5	1·1	0·07
	2	4·5	23	23	13					
Cellobiase	1	6·0	<0·1	<0·1	<0·1	6·0	0·23	0·15	<0·1	0·19
	2	6·0	0·17	0·19	<0·1					
Maltase	1	5·0	21	22	28	5·5	93	27	4·4	55
	2	4·5	15	16	18					
Isomaltase	1	5·5	7·8	2·4	1·3	6·0	80	23	5·5	44
	2	6·0	3·4	1·0	0·90					
Trehalase	1	6·0	102	46	18	6·0	22	6·7	1·0	27
	2	6·0	103	42	6·3					
Sucrase	1	6·0	<0·1	<0·1	<0·1	6·0	<0·1	<0·1	<0·1	0
	2	6·0	<0·1	<0·1	<0·1					
Neuraminidase	1	4·5	4·8	8·3	16	4·5	<0·5	<·05	<0·5	n.d.
	2	4·5	6·7	8·1	4·5					
α-L-Fucosidase	1	5·5	36	56	108	5·5	3·6	1·8	1·6	n.d.
	2	5·5	153	140	103					
β-*N*-Acetyl-D-glucosaminidase	1	5·0	95	182	352	5·0	64	42	36	n.d.
	2	5·0	317	359	337					

[A] Means of results for whole small intestine of two animals, recalculated from data of Kerry (1969) on the assumption that protein content of mucosal homogenates was 10% (w/v).

Further Studies on the Lactase Activity

The low pH optimum and the solubility of the lactase activity suggested that it was due to a lysosomal acid β-galactosidase (Alpers 1969) rather than a brush-border neutral lactase. We therefore tested the effect of *p*-chloromercuribenzene sulfonate (*p*-CMBS), which inhibits rat and human intestinal acid β-galactosidase but has no effect on the neutral lactase (Koldovsky *et al.* 1969). We also examined the activity of the intestinal homogenate towards phenyl β-galactoside, which is hydrolysed by rat and human acid β-galactosidase but not by the lactase (Asp and Dahlqvist 1968*a*). We found that 0·1 mM *p*-CMBS produced 100% inhibition of the lactase activity of all three sections of the intestine of suckling No. 2 and that phenyl β-galactoside was a good substrate. The V_{max} for phenyl β-galactoside was 50% of that for lactose, which is comparable with values of 66 and 65% for the rat

and human acid β-galactosidases, respectively (Asp and Dahlqvist 1968*b*; Asp 1971*b*).

Since 30% of the intestinal lactase activity of the suckling was insoluble (Table 2), it was considered that this activity might be due to a neutral lactase. We therefore tested the effects of pH and *p*-CMBS on the activity of this particulate fraction, but found that the activity had a pH optimum of 4·0–4·5 and was completely inhibited by *p*-CMBS.

Table 2. Distribution of glycosidase activities between the particulate and supernatant fractions after centrifugation at 105 000 *g* for 60 min

Fractions from the proximal section of the small intestine were assayed. The particulate fractions were assayed after resuspension in 0·15 M NaCl. Activities are given as percentages of total activity

Glycosidase	Adult		Suckling No. 2	
	Glycosidase activity in:		Glycosidase activity in:	
	Particulate fraction	Supernatant fraction	Particulate fraction	Supernatant fraction
Lactase	8·0	92	30	70
Maltase	86	14	29	71
Isomaltase	85	15	83	17
Trehalase	84	16	96	4·0
α-L-Fucosidase	—	—	15	85

These results thus confirmed that the intestinal lactase activity in the echidna was due to an acid β-galactosidase and showed that the neutral lactase of eutherian mammals was absent.

Degradation of Oligosaccharides of Echidna Milk by Intestinal Mucosa in vitro

When fucosyllactose (isolated from echidna milk) was incubated for up to 24 h with an homogenate of intestinal mucosa from a suckling echidna, it was partially degraded to lactose, fucose, glucose and galactose (detected by t.l.c.). Similar incubation of 4-*O*-acetyl-*N*-acetylneuraminyllactose resulted in complete hydrolysis to lactose, *N*-acetylneuraminic acid, glucose and galactose with the intermediate formation of *N*-acetylneuraminyllactose. No 4-*O*-acetyl-*N*-acetylneuraminic acid could be detected. There was no hydrolysis of 4-*O*-acetyl-*N*-acetylneuraminyllactose when this was incubated (under identical conditions) with an homogenate of intestinal mucosa from a suckling rat.

Discussion

Our results for the adult echidna were similar to those of Kerry (1969), except for the lactase activity, which we found to be an order of magnitude higher. However, Kerry (1969) assayed lactase at pH 5·8 instead of the pH optimum of 4·0–4·5, and used incubation times of up to 16 h, during which the enzyme may have lost activity. The absence of sucrase activity, previously observed by Kerry (1969), is of interest in view of the presence of isomaltase; in eutherian mammals, the intestinal sucrase and isomaltase activities are usually associated, being due to two subunits, respectively, of a single protein (Eggermont 1981).

The results show that in echidnas, as in other mammals (Koldovsky 1972), the activities of intestinal lactase and of the acid hydrolases are higher in suckling than in adult animals. In suckling echidnas, the high activities of intestinal lactase, neuraminidase and fucosidase presumably reflect the need to digest the oligosaccharides, i.e. mainly 4-*O*-acetyl-*N*-acetylneuraminyllactose and fucosyllactose (Messer and Kerry 1973; Messer 1974; Kamerling *et al.* 1982) of the mother's milk. The fucosidase activity in the suckling echidnas was an order of magnitude higher than in suckling tammar wallabies (Walcott and Messer 1980), which is consistent with the fact that fucosyl oligosaccharides are not found in the milk of marsupials (Messer and Mossop 1977; Messer and Green 1979).

In eutherian mammals, the digestion of lactose and other oligosaccharides takes place at the brush-border surface membrane of the enterocytes (Gray 1975), but the present results suggest that in suckling echidnas, as in pouch-young tammar wallabies (Walcott and Messer 1980), the milk oligosaccharides are digested by intracellular lysosomal acid hydrolases. Intestinal neuraminidase (Gossrau *et al.* 1977; Dickson and Messer 1978) and α-L-fucosidase (Barrett and Heath 1977) are probably lysosomal enzymes, and therefore the initial digestion of neuraminyllactose or fucosyllactose to *N*-acetylneuraminic acid or fucose, respectively, plus lactose, is likely to be lysosomal. Our results demonstrate that the further digestion of lactose to galactose and glucose is catalysed by an enzyme with the characteristics of a lysosomal acid β-galactosidase rather than a brush-border neutral lactase, as shown by its low pH optimum, lack of cellobiase activity, inhibition by *p*-CMBS, solubility and action on phenyl β-galactoside. Therefore, we suggest that, *in vivo*, the oligosaccharides of the milk of the echidna are digested within the enterocytes of the suckling young by the combined actions of neuraminidase, α-L-fucosidase and the acid β-galactosidase.

Since we were unable to detect neutral lactase activity in any part of the small intestine of either suckling or adult echidnas, and since this activity is also absent in tammar wallabies (Walcott and Messer 1980), it could be that brush-border lactase is found only in eutherian mammals.

As mentioned previously (Dickson and Messer 1978; Walcott and Messer 1980), lysosomal digestion of milk oligosaccharides requires a mechanism for their entry into the absorptive cells. It may well be that the extensive network of tubules in the apical cytoplasm of the intestinal absorptive cells in suckling echidnas observed by Krause (1972) provides such a mechanism. These tubules are in communication with invaginations of the apical cell membrane located between the microvilli and lie close to small spherical vacuoles (probably lysosomes) and a large supranuclear vacuole. This endocytic complex is similar to that found in the ileal absorptive cells of suckling eutherian mammals, in which the supranuclear vacuoles are considered to be giant lysosomes (Gossrau *et al.* 1977) and in which extensive pinocytosis leading to the absorption of macromolecules is known to occur (Henning and Kretchmer 1973).

During the *in vitro* degradation of 4-*O*-acetyl-*N*-acetylneuraminyllactose by intestinal mucosa from a suckling echidna we detected the intermediate formation of *N*-acetylneuraminyllactose, showing that the *O*-acetyl group is removed from the 4-*O*-acetylneuraminyllactose before the action of neuraminidase. Since *O*-acetylation of the C4 hydroxyl group makes neuraminyllactose relatively resistant to the action of neuraminidases (Messer 1974), this observation suggests the presence of an esterase in the small-intestinal mucosa of suckling echidnas which, by removing the 4-*O*-acetyl group, permits the hydrolysis of the neuraminyllactose. 4-*O*-Acetylneur-

aminyllactose, which is not present in rat milk, was not degraded by intestinal mucosa from a suckling rat.

In contrast to lactase, the trehalase and isomaltase activities of both the suckling and adult specimens had the properties expected of brush-border enzymes, *viz.* a pH optimum of 5·5–6·0 and localization mainly in the particulate fraction. The maltase activity of the adult echidna similarly appeared to be due mainly to a brush-border enzyme. That of the sucklings, however, had a pH optimum of 4·5 and was predominantly soluble. It appears, therefore, that in the echidna, as in the tammar wallaby (Walcott and Messer 1980), the intestinal maltase activity of the sucklings is due mainly to a lysosomal acid maltase similar to that described in suckling rats (Galand and Forstner 1974).

The low activity of intestinal maltase of adult echidnas, compared with that of adult rats (cf. Rubino *et al.* 1964), may be related to their diet; this consists exclusively of ants and termites (Griffiths 1968) and is therefore low in carbohydrate.

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