Noninvasive Evaluation of Intestinal Lactase with 4-Galactosylxylose: Comparison with 3- and 2-Galactosylxylose and Optimization of the Method in Rats

CARMEN HERMIDA,[1] GUILLERMO CORRALES,[2] OSCAR H. MARTÍNEZ-COSTA,[1] ALFONSO FERNÁNDEZ-MAYORALAS,[2] and JUAN J. ARAGÓN[1]*

Background: Urinary excretion of D-xylose by suckling rats after ingestion of a mixture of 4-, 3-, and 2-galactosylxyloses reflects lactase activity in vivo. We aimed to select the most convenient of these disaccharides for detecting changes of the enzyme activity in vivo and to optimize the method.

Methods: 4-, 3-, and 2-Galactosylxyloses were synthesized and purified, then orally administered to suckling rats of different ages. D-Xylose was measured colorimetrically by the phloroglucinol reaction in urine and plasma. Lactase activity was determined in extracts of small intestine mucosa with lactose, galactosylxyloses, and phlorizin as substrates.

Results: D-Xylose appeared in the urine in a dose-dependent manner after ingestion of any of the 3 galactosylxylose disaccharides. Correlation between D-xylose elimination and intestinal lactase activity was highest with 4-galactosylxylose ($r = 0.97$; $n = 24$), lower with 2-galactosylxylose ($r = 0.89$; $n = 24$), and lowest with 3-galactosylxylose ($r = 0.34$; $n = 23$). The kinetic properties of intestinal lactase accounted for these differences. D-Xylose concentration in plasma after administration of 4-galactosylxylose also correlated with lactase activity ($r = 0.93$; $n = 33$).

Conclusions: 4-Galactosylxylose is the most suitable compound for the evaluation of lactase activity in vivo. Measurement of the derived D-xylose in either urine or blood gives an estimate of the total lactose digestive capacity of the small intestine. The optimized method holds promise for development of a simple, low-cost, and reliable new test for the noninvasive diagnosis of hypolactasia.
© 2006 American Association for Clinical Chemistry

Lactase phlorizin hydrolase (EC 3.2.1.23) is an intestinal brush border enzyme responsible for the hydrolysis of lactose to galactose and glucose, which are absorbed by the intestinal mucosa. Very low lactase activity, or hypolactasia, leads to malabsorption of lactose, which leads to symptoms of lactose intolerance, such as flatus, pain, and diarrhea, after ingestion of milk or dairy products *(1, 2)*. This disorder can be caused by the genetically determined adult-type hypolactasia, which affects over one half of the world population and is predominantly found in non-Caucasian people *(3, 4)*. It can also be the result of the rare congenital lactase deficiency and secondary hypolactasia caused by atrophy or damage of the small intestine mucosa *(1, 2)*.

Evaluation of lactase activity can be performed directly by measuring the enzyme activity in a small-intestine biopsy or indirectly by noninvasive, diagnostic tests. These methods rely on a load of lactose followed by determination of plasma glucose and galactose *(5, 6)*, urinary galactose *(7)*, or the breath concentrations of H_2 *(5, 6, 8)*, $^{14}CO_2$ *(9)*, or $^{13}CO_2$ (derived from ^{14}C-lactose or ^{13}C-lactose, respectively) *(10)*. Measurement of plasma ^{13}C-glucose after consumption of ^{13}C-lactose *(11)*, or of $^{13}C/^2H$-glucose by simultaneously administering ^2H-glucose *(12)*, has also been used. However, these methods have important drawbacks such as *(a)* low reliability,

---

[1] Departamento de Bioquímica and Instituto de Investigaciones Biomédicas Alberto Sols Universidad Autónoma de Madrid-Consejo Superior de Investigaciones Científicas, Facultad de Medicina de la Universidad Autónoma de Madrid, Madrid, Spain.

[2] Instituto de Química Orgánica General, Consejo Superior de Investigaciones Científicas, Madrid, Spain.

* Address correspondence to this author at: Departamento de Bioquímica, Facultad de Medicina de la Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain. Fax 34-91-4975353; e-mail juanjose.aragon@uam.es.

because they can be influenced by many factors *(3, 5, 6, 8)*, as is well documented for the breath hydrogen test *(1, 6, 10–13)*; and (*b*) the abdominal discomfort produced in intolerant patients by the unphysiologically high doses of lactose [usually 50 *(5–10)* or 25 g *(11, 12)*]. In some cases, highly sophisticated equipment is required *(10–12)*. Therefore, a new method for evaluation is necessary.

We developed a new, noninvasive method based on oral administration of 4-galactosylxylose, a close structural analog of lactose, which is hydrolyzed by intestinal lactase yielding 2 physiologic products, galactose and xylose *(14, 15)*. D-Xylose is passively absorbed from the small intestine *(16, 17)* and is accumulated in the urine *(14, 15)*, where it could be measured by a simple colorimetric method *(18)*. To overcome the complexity and inefficiency of the chemical synthesis of 4-galactosylxylose, we carried out an enzymatic synthesis involving only 1 reaction step *(19)*, which produced a final preparation with high yield, but consisting of a mixture of 2-, 3-, and 4-galactosylxyloses (Fig. 1), although urinary excretion of D-xylose followed administration of this mixture *(19)*. Distinct kinetic properties of the 3 regioisomers, observed with lamb intestinal lactase in vitro *(19)*, suggested that they could behave differently in vivo.

In view of the potential advantages of this method, we examined the efficiency of each of the 3 galactosylxylose disaccharides in detecting the changes of rat lactase activity in vivo, so that we might select the one most suitable for noninvasive evaluation of the enzyme.

Materials and Methods

CHEMICALS

Phlorizin, phloretin, sugars, and phloroglucinol (1, 3, 5-trihydroxybenzene) were from Sigma. All other chemicals were from commercial sources and were of analytical grade.

SYNTHESIS OF GALACTOSYLXYLOSES

The synthesis of 4-, 3-, and 2-*O*-β-D-galactopyranosyl-D-xylose disaccharides (4-, 3-, and 2-galactosylxylose, respectively) was performed in 1 reaction step by enzymatic β-D-galactosylation of D-xylose, following our previous work on this topic *(19–21)*. Nevertheless, further modifications were introduced to obtain pure disaccharides in gram scale that might facilitate a simple and inexpensive scaling up of their production (G. Corrales, C. Hermida, J.J. Aragón, F.J. Cañada, and A. Fernández-Mayoralas, unpublished manuscript). Purity was above 98% for 4-galactosylxylose and ~90% for 3- and 2-galactosylxylose, as assessed by gas chromatography.

ANIMALS

Sprague–Dawley rats were purchased from Harlan and housed at the animal facilities of the Medical School of Universidad Autónoma de Madrid. We followed the institution's guide with register no. EX-021-U for the care and use of laboratory animals, which conforms to guide-
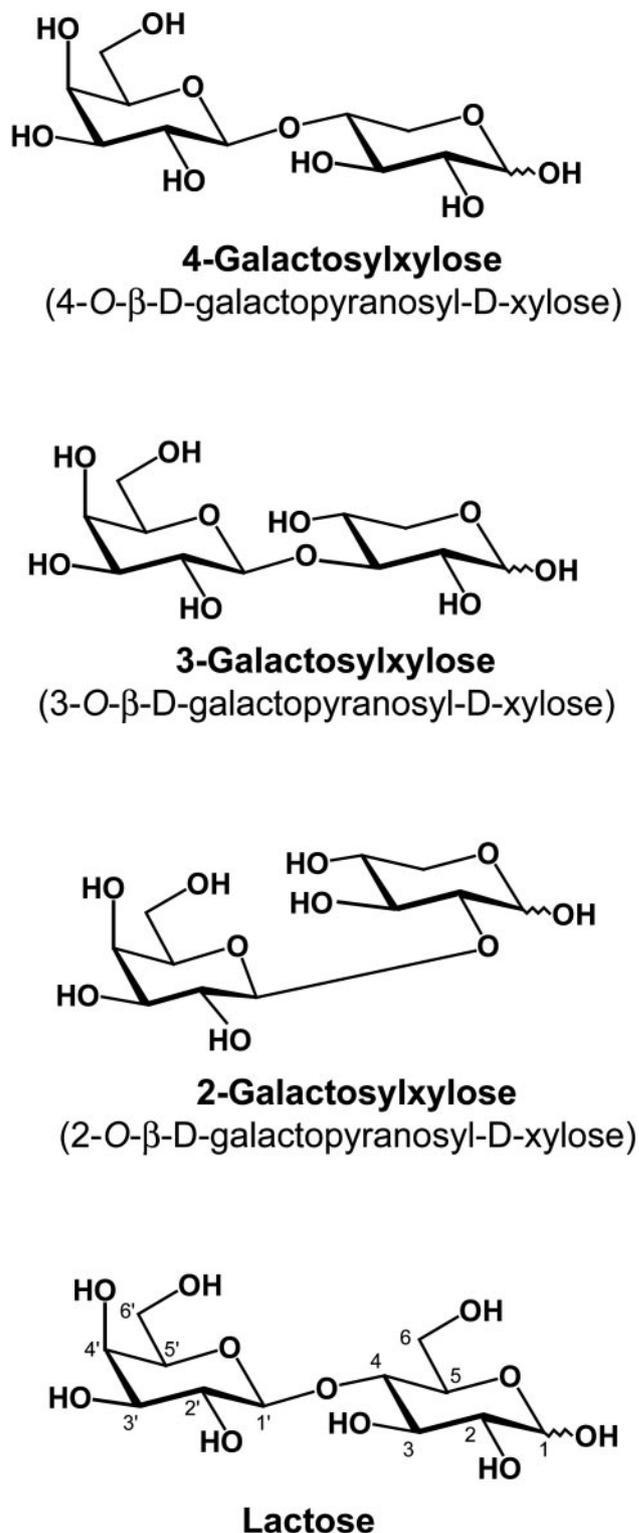

Fig. 1. Chemical structures of the galactosylxyloses used in this work.
The structure of lactose is included for comparison.

lines issued by the European Economic Community [Journal Officiel des Communautés Européennes (Déc 18) 1986; L358/26].

ADMINISTRATION OF GALACTOSYLXYLOSES TO
ANIMALS AND SAMPLE COLLECTION

Suckling rats 12–35 days of age were fasted for 6 h in metabolic cages at 30 °C and received an oral gavage of 0.3 mL of an aqueous solution containing the disaccharides or D-xylose. Urine was collected by intermittent transabdominal bladder pressure before and at the time indicated after administration of the compound. Blood samples of ~0.4 mL were obtained via cardiac puncture, and plasma was immediately separated by centrifugation at 1000g for 5 min. Urine and plasma samples were stored at −20 °C until analyzed.

MEASUREMENT OF D-XYLOSE

Unless otherwise indicated, D-xylose was determined colorimetrically with phloroglucinol (18), except that the final reaction volume was 1 mL. Absorbance at 554 nm was measured in a Perkin-Elmer Lambda 5 spectrophotometer. In some experiments, a micromethod was also used for D-xylose determination, using nonsterile, 96-well plates resistant to acid and heat (polypropylene plates; Costar). Each well contained 190 μL of the color reagent (0.5 g of phloroglucinol, 100 mL of glacial acetic acid, and 10 mL of concentrated hydrochloric acid) and up to 10 μL of the sample in a final volume of 200 μL. The plate was sealed with Thermowell™ (Costar), and the reaction was allowed to proceed for 20 min at 100 °C in an oven (Heraeus Model T5042E) and then stopped by a 10-min incubation at 4 °C. Absorbance at 554 nm was read in a microtiter plate reader (Molecular Devices; Model EL340). D-Xylose determination could be carried out with confidence within a linear interval of 0.25 to 20.0 μg of this compound ($r = 0.99$). In some experiments, D-xylose was also determined by gas chromatography as described below.

ASSAY OF INTESTINAL LACTASE ACTIVITY

To measure rat intestinal lactase, the mucosa from the medium region of the small intestine was obtained as described (14) and homogenized with 10 volumes of 100 mmol/L sodium maleate buffer, pH 6.0. Lactase activity was determined in the homogenate, designated the extract, as described (14) by measuring the released galactose with galactose dehydrogenase (22). One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 μmol of substrate/min at 25 °C.

To study the kinetic properties of rat intestinal lactase with 4-, 3-, and 2-galactosylxylose and lactose, substrate hydrolysis was evaluated as the xylose (from galactosylxyloses) or galactose (from lactose) released, measured by gas chromatography as follows. Samples (50 μL) of the assay mixture were removed at 0, 10, and 20 min, then boiled for 2 min, frozen, and lyophilized. The dry residue was dissolved in pyridine (20 μL) containing 1 mmol/L benzyl β-xyloside as internal standard, and 20 μL of the derivatizing agent trymethylsilylimidazol was added. Af-

ter sonication for 60 s and incubation for 30 min at 60 °C, 1–2 μL of sample was injected on a glass capillary column ultra-2 [12 m $\times$ 0.2 mm (i.d.)] attached to a flame ionization detector (Hewlett-Packard Model 5890).

The phlorizin hydrolase activity of intestinal lactase was measured in a reaction mixture containing 50 mmol/L sodium phosphate, pH 6.0; 10 μmol/L phlorizin; and 10 μL of extract in a final volume of 200 μL. The phloretin released was determined as described (23). Protein concentration was determined as described (24).

STATISTICS

Results are presented as the mean (SE). Correlations were determined by linear regression analysis (least-squares method). Statistical significance was set at $P < 0.05$.

Results

TIME COURSE AND DOSE DEPENDENCE OF THE
URINARY ELIMINATION OF D-XYLOSE DERIVED FROM
4-GALACTOSYLXYLOSE

As shown in Fig. 2, oral administration of 16 mg of 4-galactosylxylose to suckling rats led to progressive elimination of D-xylose in the urine, which reached a peak 3 h after ingestion of the disaccharide and was completed after 7 h, so that 98% of the pentose accumulated in the urine after 6 h. The latter time was selected as the optimal period for urine collection.

Elimination of urinary D-xylose increased linearly with increasing doses (from 2 to 32 mg) of the disaccharide (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem/content/vol52/issue2). However, the percentage of D-xylose eliminated was similar for all doses, averaging
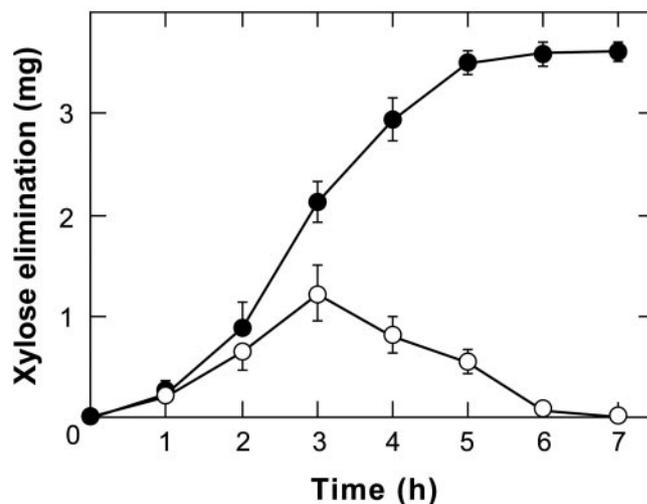

Fig. 2. Elimination of D-xylose in the urine of suckling rats after oral administration of 4-galactosylxylose.

Four suckling rats of the same litter and age (15 days) were orally administered 16 mg of 4-galactosylxylose. Urine was collected at the time indicated after administration of the disaccharide, and D-xylose was determined in the urine samples colorimetrically. *Plotted points* represent means (SE; *error bars*) of D-xylose eliminated in each sample (○) and in cumulative urine (●). In some cases, the SE of the means does not exceed the height of the symbols.

22.8 (0.4)% of the administered amount of 4-galactosylxylose. Similar results were obtained with 3- and 2-galactosylxylose (data not shown), indicating that they are also substrates of intestinal lactase in vivo. Therefore, the disaccharide dose could be reduced to at least 4 mg for highly confident measurements of urinary D-xylose.

When 1, 2, 4, 8, and 16 mg of D-xylose were given to suckling rats 15, 18, and 30 days of age (4 animals for each dose and age), the proportion of the pentose recovered in the urine averaged 52.1 (0.1)% of the amount given, being independent of dose and age. This percentage was above that of the urinary D-xylose resulting from 4-galactosylxylose [22.8 (0.4)%], indicating that D-xylose uptake was not saturated under the conditions in Table 1 in the online Data Supplement. Endogenous metabolization may account for most of the remaining 48% of administered D-xylose. In humans, D-threitol is the main end product of D-xylose catabolism *(25)*, but the exact metabolic pathway is unknown in mammals.

CORRELATION BETWEEN URINARY ELIMINATION OF D-XYLOSE DERIVED FROM 4-, 3-, AND 2-GALACTOSYLXYLOSE AND LACTASE ACTIVITY

Fig. 3 shows how D-xylose elimination in 6-h urine after administration of 4 mg of each galactosylxylose to suckling rats correlates with the physiologic decrease of lactase activity measured directly in intestinal mucosa extracts along animal growth. As seen in Fig. 3A, D-xylose elimination reached a maximum of ~22% at the 15th day with either disaccharide, followed by a gradual decrease, which was highly different with the 3 compounds. The pattern of 4-galactosylxylose reflected the normal decrease of lactase activity in the postweaning period,

whereas the decrease of D-xylose elimination was lower than the enzyme decrease with 2-galactosylxylose and much lower with 3-galactosylxylose, so that the urinary pentose excretion at day 35 diminished by 65.6 (2.1)% and 42.2 (1.8)%, respectively, compared with the values at day 15. In contrast, 4-galactosylxylose elicited a decrease of 93.1 (0.3)% during the same period, which practically coincided with that of lactase activity [93.7 (0.5)%]. Therefore, correlation between D-xylose elimination and lactase activity was the highest with 4-galactosylxylose, lower with 2-galactosylxylose, and very low with 3-galactosylxylose (Fig. 3B).

KINETIC CHARACTERISTICS OF RAT INTESTINAL LACTASE WITH GALACTOSYLXYLOSE DISACCHARIDES

The different behaviors of the 3 galactosylxyloses in vivo suggested that intestinal lactase hydrolyzed them with distinct catalytic efficiencies. This was tested by determining the kinetic parameters of the enzyme for these substrates and for lactose. As demonstrated in Table 1, rat lactase exhibited the highest affinity for 3-galactosylxylose ($K_m = 8.5$ mmol/L) and the lowest for 4-galactosylxylose ($K_m = 58.8$ mmol/L), which also yielded the lowest V_{max} value (11% of that with lactose). Thus, the V_{max}/K_m ratio indicated a much lower specificity of the enzyme for 4-galactosylxylose (3.5% with respect to that for lactose) than for 3-galactosylxylose, which gave a ratio (187%) even higher than lactose. The kinetic properties with 2-galactosylxylose were somewhere in between those found with the 2 other regioisomers.

The marked differences observed in the kinetic behaviors of 4- and 3-galactosylxylose led us to analyze the contribution of the 2 catalytic activities of the enzyme,
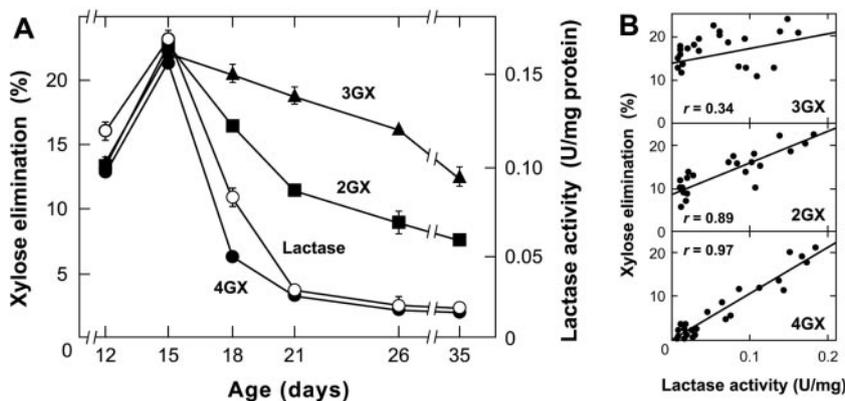

Fig. 3. Elimination of D-xylose in the urine of suckling rats after oral administration of 4-galactosylxylose (*4GX*), 3-galactosylxylose (*3GX*), and 2-galactosylxylose (*2GX*), and intestinal lactase activity as a function of age.

(*A*), a distinct group of suckling rats from 2 different litters and of the same age (12 days) was used for each disaccharide. These groups were composed of 24 animals each for 4GX and 2GX, and of 23 animals for 3GX. Rats were orally administered 4 mg of the corresponding disaccharide. D-Xylose derived from 4GX (●), 3GX (▲), or 2GX (■) and eliminated in the first 6-h urine collected after disaccharide administration was determined. The intestinal lactase activity (○) was determined post-mortem in 4 rats from each group of animals, except for the group that received 3GX, in which 3 animals were killed on the day 12. The remaining animals were returned to maternal nursing, and the experiment was repeated at the indicated age until all the rats were killed. Urinary D-xylose in each group of animals was determined in 20, 20, 16, 12, 8, and 4 rats at the age points of day 12, 15, 18, 21, 26, and 35, respectively. D-Xylose elimination is represented as a percentage of the amount of the administered disaccharide. Data at each age point are given as means (SE) of 4 to 20 animals for D-xylose elimination and of 11–12 animals for lactase activity. (*B*), linear regression analysis of urinary D-xylose after oral administration of each disaccharide and intestinal lactase activity. For 3GX (*top*), regression equation is $y = 26.14x + 15.71$; $r = 0.34$ ($r^2 = 12\%$); n = 23; P >0.1. For 2GX (*middle*), regression equation is $y = 74.39x + 9.18$; $r = 0.89$ ($r^2 = 79\%$); n = 24; P <0.0001. For 4GX (*bottom*), regression equation is $y = 104.93x + 0.12$; $r = 0.97$ ($r^2 = 93\%$); n = 24; P <0.0001.

Table 1. Kinetic parameters for intestinal lactase substrates.[a]

Substrate	K_m, mmol/L	V_{max}, U/mg protein	V_{max}/K_m,[b] %
Lactose	17.9	0.322	100.0
2-Galactosylxylose	22.2	0.111	27.8
3-Galactosylxylose	8.5	0.285	186.9
4-Galactosylxylose	58.8	0.037	3.5

[a] Kinetic parameters were determined as described in *Materials and Methods*. Lactase activity was assayed in intestinal mucosa extracts of suckling rats without further purification to approach physiologic conditions.

[b] V_{max}/K_m ratios for galactosylxyloses are indicated relative to the value obtained for lactose.

lactase and phlorizin hydrolase, to the hydrolysis of both substrates in vivo. We investigated this by determining urinary D-xylose in suckling rats after giving them increasing concentrations of glucal, a competitive inhibitor of the enzyme at the lactose site *(23)*, mixed with either 4 mg of 4-galactosylxylose or 1 mg of 3-galactosylxylose. As shown in Fig. 4A, D-xylose elimination was inhibited maximally above 50–100 mmol/L glucal, decreasing no more than 40% from 4-galactosylxylose and 75% from 3-galactosylxylose. This suggested that a major proportion of 4-galactosylxylose is split at the phlorizin hydrolase site, whereas most of 3-galactosylxylose is split at the lactose site. Accordingly, in vitro studies with rat intestinal lactase (Fig. 4B) revealed that inhibition of 4-galactosylxylose hydrolysis was not higher than 35%, with a minimal effect on phlorizin hydrolysis, whereas inhibition of 3-galactosylxylose hydrolysis reached 74%, close to the 84% decrease of lactose hydrolysis. Because the lactose site splits glycosides with hydrophilic aglycons, whereas the phlorizin site preferentially splits glycosides with hydrophobic aglycons *(1)*, these findings suggest that the lack of the HO-6 group in 4-galactosylxylose provides a more hydrophobic environment for enzyme binding, thereby restraining its interaction with the lactose site.

NONINVASIVE EVALUATION OF INTESTINAL LACTASE ACTIVITY WITH 4-GALACTOSYLXYLOSE BY MEASURING D-XYLOSE IN PLASMA

As seen in Fig. 5A, D-xylose markedly accumulated in plasma after administration of 16 mg of 4-galactosylxylose to suckling rats, reaching a maximum ∼3 h after the oral gavage and gradually diminishing to values close to the initial concentrations after 6 h. When 4, 8, and 16 mg of 4-galactosylxylose were given to suckling rats (15 days of age from 2 litters and using 4 animals for each dose), the mean (SE) concentration of D-xylose in 3-h plasma increased linearly and corresponded to 3.6 (0.5), 11.7 (0.6), and 22.0 (1.1) μg/100 μL, respectively.

As shown in Fig. 5B, as observed with the urinary pentose, the concentrations of D-xylose determined in plasma 3 h after administration of 8 mg of 4-galactosylxylose to suckling rats correlated strongly with lactase activity determined directly in intestinal mucosa samples obtained post mortem.
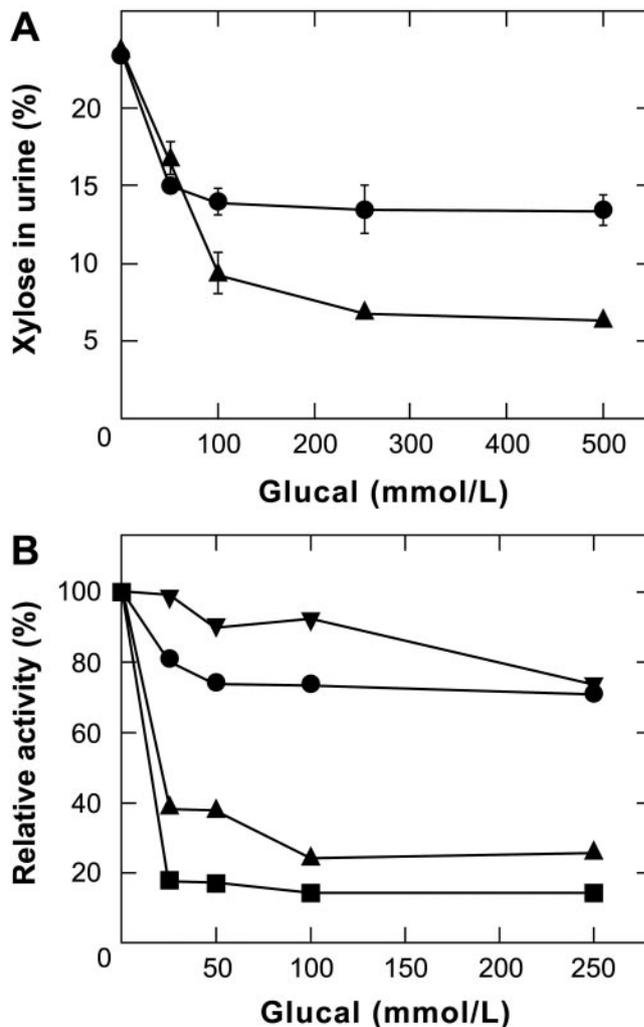

Fig. 4. Effect of glucal on the in vivo (*A*) and in vitro (*B*) intestinal lactase phlorizin hydrolase activity of suckling rats.

(*A*), 40 suckling rats of 4 different litters and of the same age (15 days) were orally administered either 4 mg of 4-galactosylxylose (43 mmol/L; ●) or 1 mg of 3-galactosylxylose (11 mmol/L; ▲) and the indicated concentration of glucal in 0.3 mL of water. D-Xylose eliminated in the first 6-h urine after administration of the corresponding disaccharide and glucal was determined. Urinary D-xylose is represented as a percentage of the amount of the administered galactosylxylose. Data are given as means (SE) of 4 animals. Similar results were obtained with 4-galactosylxylose if glucal was administered 15 or 30 min before the disaccharide. (*B*), intestinal lactase-phlorizin hydrolase activity assayed in vitro at 30 mmol/L lactose (■), 15 mmol/L 3-galactosylxylose (▲), 60 mmol/L 4-galactosylxylose (●), or 0.01 mmol/L phlorizin (▼), and in the presence of increasing concentrations of glucal. The concentrations of substrates used in *A* and *B* were close to their K_m values (Table 1) *(23)*.

DETERMINATION OF D-XYLOSE BY OTHER METHODS

The concentrations of D-xylose in both 3-h plasma and 6-h urine from suckling rats 15 and 30 days of age that received 4-galactosylxylose under conditions similar to those shown in Figs. 3 and 5B, were also determined by gas chromatography and by a micromethod developed to measure the phloroglucinol reaction with a microtiter plate reader using 96-well plates, as described in the *Materials and Methods*. Both procedures gave results similar to those described above (data not shown). The phloroglucinol microassay not only facilitated the simul-
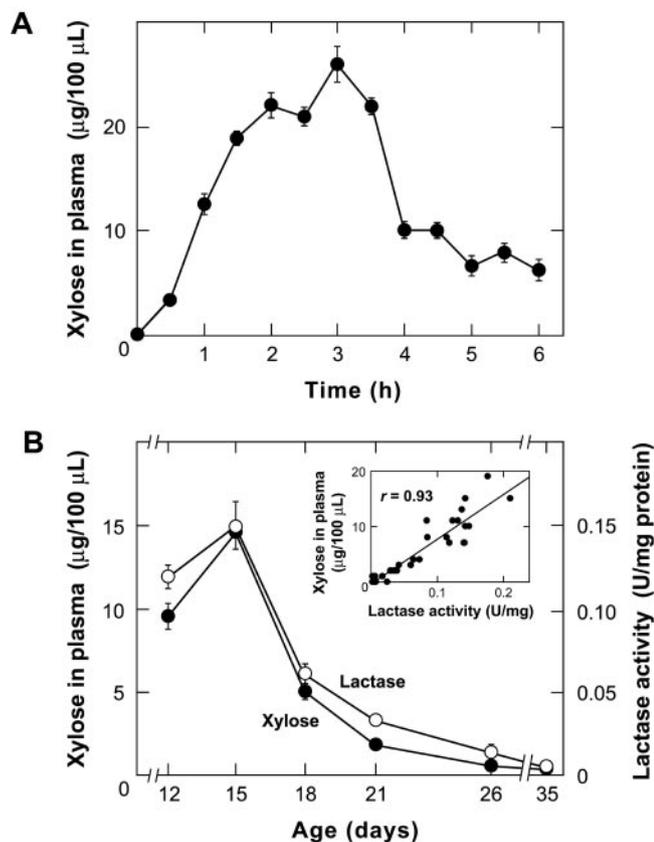
Fig. 5. Time course of plasma D-xylose in suckling rats after oral administration of 4-galactosylxylose (*A*), and correlation with intestinal lactase activity as a function of age (*B*).

(*A*), 20 rats from 2 litters and of the same age (15 days) were orally administered 16 mg of 4-galactosylxylose. Blood samples were obtained via cardiac puncture at the indicated times, and D-xylose was determined in plasma. Data are given as means (SE) of 3 to 5 animals at each time point. (*B*), 70 rats from 6 different litters and of the same age (12 days) were used. At each age point, blood samples were obtained from a group of 10 to 15 animals before and 3 h after the oral administration of 8 mg of 4-galactosylxylose. D-xylose was determined in plasma (●), and intestinal lactase activity (○) was determined post mortem in 5 to 6 animals of the same group. A disaccharide dose of 8 mg was used instead of the 4-mg dose used for urine collection, as the quantities of D-xylose detected in plasma are lower than in urine samples. Data are given as means (SE). (*Inset*), linear regression analysis of plasma D-xylose concentrations and lactase activity. Regression line: $y = 81.31x - 0.42$; $r = 0.93$ ($r^2 = 86\%$); $n = 33$; $P < 0.0001$.

taneous performance of numerous measurements, but also allowed us to use a considerably smaller volume of sample (10 µL).

Discussion

In this study, we have optimized a new method in experimental animals based in the measurement of D-xylose in either urine or blood after ingestion of the synthetic lactose analog 4-galactosylxylose. This compound was obtained with high purity after enzymatic synthesis and was compared with the regioisomers 3- and 2-galactosylxylose, which are also produced by this procedure. The 3 disaccharides behaved as substrates of intestinal lactase in vivo and yielded the same percentage of urinary D-xylose, ~22%, when administered at doses of 2–32 mg to 15-day-old rats, which had the

highest enzyme activity (Fig. 3). This value indicated that the entire oral dose of galactosylxylose was hydrolyzed by the intestinal enzyme, because it practically coincided with the percentage of D-xylose (23%) expected from 100% hydrolysis, as the urinary recovery of D-xylose was 52% of the pentose given and the D-xylose moiety accounts for 45% of the disaccharide molecular weight. However, 4-galactosylxylose was the most effective compound to evaluate lactase activity in vivo, as it elicited the highest correlation ($r = 0.97$) between urinary elimination of D-xylose and the physiologic decrease of the enzyme activity. The kinetic behavior of rat intestinal lactase with the 3 regioisomers accounted for their different behaviors in vivo. Inasmuch as the highest specificity constant (taken as the V_{max}/K_m ratio) was obtained with 3-galactosylxylose (Table 1), the catalytic efficiency in vivo was high enough to hydrolyze a relatively high proportion of this disaccharide even when the enzyme activity was lowest (day 35). Consequently, D-xylose excretion decreased after weaning to a degree lower than that of enzyme activity. The opposite was the case of 4-galactosylxylose, for which the low specificity constant allowed the change of both measurements to a similar extent. Paradoxically, it is the worst substrate that turned out to be most suitable to detect the changes in lactase activity in vivo with high reliability. These results agree with previous reports on the role of each hydroxyl group of the lactose molecule in its interaction with intestinal lactase isolated from lambs (*26, 27*).

We obtained evidence that the rate-limiting step in the overall absorption of D-xylose from 4-galactosylxylose is not its transport but the hydrolysis of the disaccharide by lactase, and that this compound does not permeate the intestinal mucosa, as reported for lactose (*1, 28*). This was shown by the strong correlation between D-xylose from 4-galactosylxylose and intestinal lactase activity, and also by the fact that 100% of the disaccharide was hydrolyzed in vivo when administered at the age of maximal lactase content (day 15).

We found that evaluation of lactase activity in vivo with 4-galactosylxylose can also be carried out by measuring D-xylose in plasma, as indicated by the high correlation between the plasma concentrations of the pentose and enzyme activity ($r = 0.93$; Fig. 5B). This provides an alternative to urine D-xylose measurement, especially in young children and infants, from whom collection of urine can be troublesome. Additionally, determination of blood D-xylose in combination with its urinary content has been shown to improve the accuracy of the D-xylose testing of intestinal absorption (*17, 29*), as other factors such as renal dysfunction, delayed gastric emptying, or portal hypertension can give falsely low urinary D-xylose values.

The present method is a simple, low-cost, rapid colorimetric test requiring only routinely available equipment. Additionally, D-xylose determination by the phloroglucinol reaction (*18*) is highly sensitive, so that 0.25 µg of the

pentose can be confidently detected. Consequently, reliable measurements could be made even in a decrease in lactase of more than 90%, such as that observed in 35-day-old rats, by either urine or plasma determinations. This suggests that the dose of disaccharide administered to humans could be lower in comparison with other noninvasive tests and, therefore, with a possible reduction of abdominal discomfort in hypolactasia patients. Although gastrointestinal symptoms after lactose loading can be of help for further treatment, they are unreliable as a diagnostic test (2, 3, 6, 8, 9) and provide no information on the ability of the intestine to digest smaller, more physiologic quantities of lactose. In contrast, measurement of D-xylose in the urine or plasma reflects the overall lactase activity in vivo, indicating the total lactose digestive capacity of the small intestine. This can be an interesting advantage over enzyme activity data obtained from biopsy specimens, usually taken from the proximal small intestine, where lactase is low (30, 31), in addition to the possible uneven expression of the enzyme (1, 32).

Single-nucleotide polymorphisms upstream from the lactase gene have been associated with adult-type hypolactasia (33, 34) and tested to identify individuals with genetic disposition for this disorder (35, 36). A functional test of lactase activity is essential, however, for proper assessment of patient situation because both residual enzyme activity and clinical symptoms can vary within an ample range, and low lactase activity can have other causes.

The simplicity of the synthesis of 4-galactosylxylose, requiring neither toxic nor expensive chemicals (19–21), is another innovative aspect of this methodology. In fact, toxicology studies have revealed that the synthetic disaccharide is devoid of apparent side effects in animals (data not shown). The method optimized in this work is, therefore, ready for clinical trials, so that it might be applied within the primary healthcare system as a convenient, noninvasive new test for diagnosis of hypolactasia.

References

1. Semenza G, Auricchio S, Mantei N. Small-intestinal disaccharidases [Review]. In: Scriber CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, et al, eds. The metabolic and molecular bases of inherited disease, Vol. 1. New York: McGraw-Hill, 2001:1623–50.
2. Villako K, Maaroos H. Clinical picture of hypolactasia and lactose intolerance [Review]. Scand J Gastroenterol 1994;202(Suppl 29):36–54.
3. Swallow DM, Hollox EJ. Genetic polymorphism of intestinal lactase activity in adult humans [Review]. In: Scriber CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, et al, eds. The metabolic and molecular bases of inherited disease, Vol. 1. New York: McGraw-Hill, 2001:1651–63.
4. Sahi T. Genetics and epidemiology of adult-type hypolactasia [Review]. Scand J Gastroenterol 1994;202(Suppl 29):7–20.
5. Newcomer AD, McGill DB, Thomas PJ, Hofmann AF. Prospective comparison of indirect methods for detecting lactase deficiency. N Engl J Med 1975;293:1232–6.
6. Arola H. Diagnosis of hypolactasia and lactose malabsorption [Review]. Scand J Gastroenterol 1994;202(Suppl 29):26–35.
7. Grant JD, Bezerra JA, Thompson SH, Lemen RJ, Koldovsky O, Udall JNJ. Assessment of lactose absorption by measurement of urinary galactose. Gastroenterology 1989;97:895–9.
8. Metz G, Jenkins DJ, Peters TJ, Newman A. Blendis LM. Breath hydrogen as a diagnostic method for hypolactasia. Lancet 1975; 1:1155–7.
9. Sasaki Y, Iio M, Kameda H, Ueda H, Aoyagi T, Christopher NL, et al. Measurement of ^{14}C-lactose absorption in the diagnosis of lactase deficiency. J Lab Clin Med 1970;76:824–35.
10. Koetse HA, Stellaard F, Bijleveld CMA, Elzinga H, Boverhof R, Van der Meer R, et al. Non-invasive detection of low intestinal lactase activity in children by use of a combined $^{13}CO_2/H_2$ breath test. Scand J Gastroenterol 1999;34:35–40.
11. Vonk RJ, Lin Y, Koetse HA, Huang CY, Zeng G, Elzinga H, et al. Lactose (mal)digestion evaluated by the ^{13}C-lactose digestion test. Eur J Clin Invest 2000;30:140–6.
12. Vonk RJ, Stellaard F, Priebe MG, Koetse HA, Hagedoorn RE, De Bruijn S, et al. The $^{13}C/^2$H-glucose test for determination of small intestinal lactase activity. Eur J Clin Invest 2001;31:226–33.
13. Koetse HA, Vonk RJ, Pasterkamp S, Pal J, de Bruijn S, Stellaard F. Variations in colonic H_2 and CO_2 production as a cause of inadequate diagnosis of carbohydrate maldigestion in breath tests. Scand J Gastroenterol 2000;35:607–11.
14. Aragón JJ, Fernández-Mayoralas A, Jiménez-Barbero J, Martín-Lomas M, Rivera-Sagredo A, Villanueva D. Evaluation of rat intestinal lactase in vivo with 4-galactosylxylose. Clin Chim Acta 1992;210:221–6.
15. Rivera-Sagredo A, Fernández-Mayoralas A, Jiménez-Barbero J, Martín-Lomas M, Villanueva D, Aragón JJ. 4-O-β-D-Galactopyranosyl-D-xylose: a new synthesis and application to the evaluation of intestinal lactase. Carbohydr Res 1992;228:129–35.
16. Crane RK. Intestinal absorption of sugars [Review]. Physiol Rev 1960;40:789–825.
17. Craig RM, Ehrenpreis ED. D-Xylose testing. [Review]. J Clin Gastroenterol 1999;29:143–50.
18. Eberts TJ, Sample RHB, Glick MR, Ehis GH. A simplified, colorimetric micromethod for xylose in serum or urine, with phloroglucinol. Clin Chem 1979;25:1440–3.
19. Aragón JJ, Cañada FJ, Fernández-Mayoralas A, López R, Martín-Lomas M, Villanueva D. A direct enzymatic synthesis of β-D-galactopyranosyl-D-xylopyranosides and their use to evaluate rat intestinal lactase activity in vivo. Carbohydr Res 1996;290:209–16.
20. López R, Fernández-Mayoralas A. Enzymatic β-galactosidation of modified monosaccharides: study of the enzyme selectivity for the acceptor and its application to the synthesis of disaccharides. J Org Chem 1994;59:737–45.
21. Montero E, Alonso J, Cañada FJ, Fernández-Mayoralas A, Martín-Lomas M. Regioselectivity of the enzymatic transgalactosidation of D- and L-xylose catalysed by β-galactosidases. Carbohydr Res 1998;305:383–91.
22. Doudoroff M. D-Galactose dehydrogenase of Pseudomonas saccharophila. Methods Enzymol 1962;5:339–41.
23. Díaz Arribas JC, Herrero AG, Martín-Lomas M, Cañada FJ, He S, Withers SG. Differential mechanism-based labeling and unequivocal

activity assignment of the two active sites of intestinal lactase/phlorizin hydrolase. Eur J Biochem 2000;267:6996–7005.

24. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

25. Pitkanen E. The conversion of D-xylose into D-threitol in patients without liver disease and in patients with portal liver cirrhosis. Clin Chim Acta 1977;80:49–54.

26. Rivera-Sagredo A, Cañada FJ, Nieto O, Jiménez-Barbero J, Martín-Lomas M. Substrate specificity of small-intestinal lactase: assessment of the role of the substrate hydroxyl groups. Eur J Biochem 1992;209:415–22.

27. Fernández P, Canada FJ, Jiménez-Barbero J, Martín-Lomas M. Substrate specificity of small-intestinal lactase: study of the steric effects and hydrogen bonds involved in enzyme-substrate interaction. Carbohydr Res 1995;271:31–42.

28. Dawson DJ, Lobley RW, Burrows PC, Miller V, Holmes R. Lactose digestion by human jejunal biopsies: the relationship between hydrolysis and absorption. Gut 1986;27:521–7.

29. Ehrenpreis ED, Salvino M, Craig RM. Improving the serum D-xylose test for the identification of patients with small intestinal malabsorption. J Clin Gastroenterol 2001;33:36–40.

30. Newcomer AD, McGill DB. Distribution of disaccharidase activity in the small bowel of normal and lactase-deficient subjects. Gastroenterology 1966;51:481–8.

31. Triadou N, Bataille J, Schmitz J. Longitudinal study of the human intestinal brush border membrane proteins: distribution of the main disaccharidases and peptidases. Gastroenterology 1983; 85:1326–32.

32. Auricchio S, Maiuri L. Cellular basis of adult-type hypolactasia. Acta Paediatr 1994;83(Suppl 395):14–7.

33. Enattah NS, Sahi T, Savilahti E, Terwilliger JD, Peltonen L, Järvelä I. Identification of a variant associated with adult-type hypolactasia. Nat Genet 2002;30:233–7.

34. Swallow DM. Genetics of lactase persistence and lactose intolerance [Review]. Annu Rev Genet 2003;37:197–219.

35. Rasinperä H, Savilahti E, Enattah NS, Kuokkanen M, Tötterman N, Lindahl H, et al. A genetic test which can be used to diagnose adult-type hypolactasia in children. Gut 2004;53:1571–6.

36. Högenauer C, Hammer HF, Mellitzer K, Renner W, Krejs GJ, Toplak H. Evaluation of a new DNA test compared with the lactose hydrogen breath test for the diagnosis of lactase non-persistence. Eur J Gastroenterol Hepatol 2005;17:371–6.