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Substrate specificity of small-intestinal lactase: Study of the steric effects and hydrogen bonds involved in enzyme–substrate interaction

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Abstract

Milk lactose is hydrolysed to D-galactose and D-glucose in the small intestine of mammals by the lactase–phlorizin hydrolase complex (LPH, EC 3.2.1.23-62). Lactase activity has broad substrate selectivity and several glycosides are substrates. Recently, using the monodeoxy derivatives of methyl β-lactoside (**1**), we have shown the importance of each hydroxyl group in the substrate molecule concerning the interaction with the enzyme. Now we have studied the corresponding *O*-methyl derivatives, as well as some of the halo derivatives of **1**. We have found that the enzyme presents steric restrictions to the recognition of substrates modified in the galactose moiety. In contrast, the binding site for the aglycon part of the substrate is looser. On the other hand, we have previously shown that HO-3′ and HO-6 were important for the recognition of the substrate by the enzyme. Now we have found that the corresponding fluorine derivatives are not, or very poorly, recognized. This suggests that the HO-3′ and HO-6 participate, as donors, in hydrogen bonds in the interaction with the enzyme.

Keywords: Intestinal lactase; Methyl β-lactoside; Galactosidase; Substrate specificity; Molecular recognition

1. Introduction

Lactase–phlorizin hydrolase (LPH) is a membrane protein located in the brush border membranes of the small intestine [1,2]. It hydrolyses lactose to give D-galactose and

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D-glucose, allowing its absorption through the intestine. In mammals, this enzymatic activity is of vital necessity. Lactose is the major β-glycoside in milk, the only food during the nursing period. The absence or malfunction of this enzymatic activity is the cause of certain cases of lactose intolerance. In humans, the most frequent genetically based syndrome, known as adult-type alactasia, consists of a substantial decrease of this enzymatic activity in adulthood [3,4]. These reasons have made LPH the subject of considerable investigation.

LPH is a β-glycosidase with broad selectivity [2,5]. It displays two enzymatic activities which are located on different active sites: lactase activity, which is thought to be selective towards glycosides (with either *galacto* or *gluco* configuration) with a hydrophilic aglycon [6–8], and phlorizin hydrolase activity, which seems to be more selective to glycosides with a hydrophobic aglycon [9]. The LPH gene has recently been cloned and the sequence indicates that both active sites are present on the same polipeptide chain [10]. In addition both active sites have been affinity-labelled [11].

We are involved in a systematic study of the lactase activity, aimed at the understanding of the factors that govern the interaction between the protein and the carbohydrate. For this purpose we have used substrates modified at every hydroxyl group of the molecule. Recently, we have described the behaviour of all the monodeoxy derivatives of methyl β-lactoside and of other lactose analogues with respect to lactase activity [12]. We found that the HO-3′ and HO-2′ groups on the galactopyranose moiety of the substrate were essential for recognition and hydrolysis of the substrate, respectively. In contrast, the glucopyranose moiety was less demanding than the galactose part in the requirements for being a substrate of intestinal lactase. In fact, although the HO-6 group seemed to be required for an efficient interaction with the enzyme, no hydroxyl group in the glucose part was absolutely essential. We have now extended our previous work with deoxy derivatives to the study of the *O*-methyl derivatives and some halo derivatives of methyl β-lactoside as potential substrates of lactase.

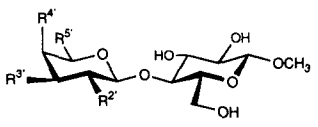
Hydroxyl group substitutions by hydrogen, combined with substitutions by fluorine or *O*-methyl groups, have been used in some cases to gain information about the role of particular hydroxyl groups in carbohydrate–protein interactions [13–17]. On the basis of its electronegativity and size, fluorine has been proposed as a surrogate of the hydroxyl group in order to examine the participation of the latter in hydrogen bonds, either as acceptor or as donor, in carbohydrate–protein interactions [13,18]. Fluorine cannot act as a hydrogen donor, but it retains the capacity to act as acceptor. However, it should be noted that fluorine substitution can introduce new interactions, as has been shown in the binding of 6-deoxy-6-fluoro-D-galactose to the L-arabinoside-binding protein [19], and therefore conclusions should be drawn cautiously. *O*-Methyl substitution is also able to sustain hydrogen bonds in which the oxygen acts as acceptor. In addition, the bigger size of the *O*-methyl group compared to the hydroxyl group can give valuable information with regard to the steric restrictions of the binding site for a carbohydrate. This property has been elegantly applied by Lemieux in his studies of the lectin IV of *Griffonia simplicifolia* and its carbohydrate ligand [14,15].

In the present study, we have used this strategy of sequential modification of each hydroxyl group on the substrate molecule to obtain insights on the role of the hydroxyl groups of lactose in its interaction with intestinal lactase.

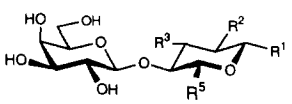
2. Results and discussion

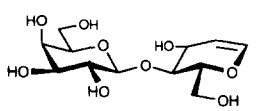
Intestinal lactase was obtained from lamb small intestine. As in our previous study [12], the enzyme was purified following the procedure described by Semenza and co-workers [6] with minor modifications. The SDS-PAGE (sodium dodecylsulphate-polyacrylamide gel electrophoresis) of the purified preparation under denaturing and reducing conditions showed a major band of molecular mass 120 kDa as expected for LPH. Although the final specific activity for lactose hydrolysis was lower than in the previous work, the K_m values corresponding to lactose, methyl β-lactoside, and phlorizin were practically identical to those reported in the former study. Furthermore, following the Dahlqvist criteria [20], no other intestinal β-galactosidase activity was detected: first, 6-bromo-2-naphthyl β-D-galactoside, a substrate of lysosomal acid β-D-galactosidase, was not hydrolysed by the purified LPH preparation, and second, *p*-chloromercuribenzoate (PCMB), a known inhibitor of the lysosomal galactosidase and the cytoplasmic hetero-β-galactosidase, did not inhibit the glycosidase activities of the LPH preparation.

Table 1 shows the results obtained from the kinetic experiments carried out on all the methyl β-lactoside analogues (Scheme 1):



Compound	$R^{2'}$	$R^{3'}$	$R^{4'}$	$R^{5'}$
1	OH	OH	OH	CH_2OH
2[a]	H	OH	OH	CH_2OH
3	OCH_3	OH	OH	CH_2OH
4[a]	OH	H	OH	CH_2OH
5	OH	OCH_3	OH	CH_2OH
6	OH	F	OH	CH_2OH
7[a]	OH	OH	H	CH_2OH
8[a]	OH	OH	*epi*-OH	CH_2OH
9	OH	OH	OCH_3	CH_2OH
10	OH	OH	F	CH_2OH
11[a]	OH	OH	OH	CH_3
12	OH	OH	OH	CH_2OCH_3
13	OH	OH	OH	CH_2F
14	OH	OH	OH	CH_2Br



Compound	R^1	R^2	R^3	R^5
1	OCH_3	OH	OH	CH_2OH
16[a]	α-OCH_3	OH	OH	CH_2OH
17[a]	OCH_3	H	OH	CH_2OH
18	OCH_3	OCH_3	OH	CH_2OH
19[a]	OCH_3	OH	H	CH_2OH
20[a]	OCH_3	OH	OCH_3	CH_2OH
21[a]	OCH_3	OH	OH	CH_3
22	OCH_3	OH	OH	CH_2OCH_3
23	OCH_3	OH	OH	CH_2F
24	OCH_3	OH	OH	CH_2I
15[a]				

Scheme 1. Structures of methyl β-lactoside derivatives. [a]These compounds were studied previously [12].

with modifications either on the galactose or on the glucose moiety. The kinetic parameters of the monodeoxy derivatives used in the preceding work [12] are also included for reference. As LPH has been shown to be a retaining glycosidase [21], these results will be discussed on the basis of the reaction sequence shown in Fig. 1 expected for retaining glycosidases. Modifications on the galactoside moiety can affect all the

Table 1
Substrate specificity of intestinal lactase

Compound	K_m (mM)	% V_{max} [b]	V_{max}/K_m [c]
1	16.0 ± 2.0	100	1
Galactose moiety			
2 [a]	I [d]	—	—
3	I	—	—
4 [a]	N.O. [e]	—	—
5	N.O.	—	—
6	N.O.	—	—
7 [a]	14.4 ± 0.6	23.2	0.26
8 [a]	5.3 ± 2.0	10.2	0.31
9	N.O.	—	—
10	I	—	—
11 [a]	0.4 ± 0.05	34.4	13.8
12	123 [f]	1.4	0.0018
13	1.27 ± 0.08	14.6	1.84
14	N.A. [g]	—	—
Glucose moiety			
15 [a]	23.2 ± 1.6	73.8	0.50
16 [a]	4.5 ± 0.5	74.8	2.7
17 [a]	50.8 ± 5.4	88	0.27
18	8.07 ± 1.3	52	1.03
19 [a]	51.3 ± 4.5	122	0.38
20 [a]	153 ± 25	7.2	0.007
21 [a]	246 ± 48	42.6	0.027
22	210 [f]	90	0.069
23	292 [f]	170	0.089
24	N.A.	—	—

[a] Data are from Ref. [12]. [b] V_{max} values are expressed as % of the V_{max} obtained for methyl β-lactoside (V_{max} 5 μM min^{-1} mg^{-1}). [c] Relative values normalized to methyl β-lactoside $V_{max}/K_m = 3.1 \times 10^{-4}$ min^{-1} mg^{-1}. [d] I, Inhibitor. [e] N.O., No hydrolysis was observed. [f] Estimated values are given since saturation conditions could not be reached. [g] N.A., The compounds were very poor substrates and were not further analysed.

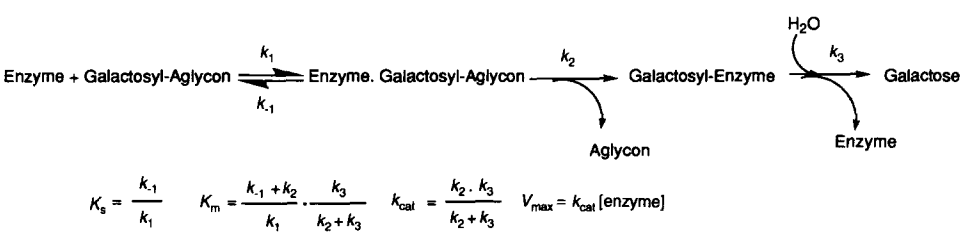


Fig. 1. Reaction sequence and kinetic equations for retaining glycosidases.

steps in the reaction sequence: the initial binding (K_s), the enzyme glycosidation step (k_2), and the deglycosidation step (k_3), altering in consequence the values of both K_m and V_{max}. However, the last step (k_3) should be essentially independent of the aglycon. For this reason, modifications on the glucose moiety should only affect the initial

binding and the enzyme glycosidation step. In these cases the change in the V_{max} value with respect to the reference compound should reflect only variations in k_2, while the change in the K_m value can be due to alteration of the initial binding (K_s) or of the enzyme galactosidation step (k_2).

On the other hand, as the two active sites of LPH are on the same polypeptide chain [11], it is not possible to study them separately. According to the currently accepted hypothesis [11], the lactase site is more selective towards glycosides (either galactoside or glucoside [6,7]) with a hydrophilic aglycon, although *o*-nitrophenyl β-D-galactopyranoside is also a good and characteristic substrate for this site [11], while the phlorizin hydrolase site is more selective towards glycosides with a large hydrophobic aglycon [9]. This hypothesis is based on the differential thermal inactivation of both activities [6,22,23] and on competition studies [7,9,24]. Those experiments are not fully conclusive, due in part to the large difference betwen the K_m of phlorizin (μmolar range) and lactose (mmolar range), and some discrepancies have been described [25]. We have carried out a series of experiments to address this problem. In our previous work [12] we showed that methyl β-lactoside (**1**), the reference compound used in the present study, was a competitive inhibitor of lactose hydrolysis. On the other hand, it was also shown that lactal, probably the analogue with the most hydrophobic aglycon studied there, was hydrolysed at the same site as lactose. In the present work we have studied a series of compounds with at least two hydroxyl groups free on the glucose moiety which we consider are essentially directed to the lactase site.

In any case, we should point out that the analogues studied here are of similar or lower potency as substrates than the reference compound **1**, with the exception of the compounds modified in the 6′ position (Table 1). It was interesting to check if the phlorizin hydrolase site had any participation in the hydrolysis of the analogues with modifications at the 6′ position. By means of competitive assays (see Experimental section) it was confirmed that **1** and the 6′F (**13**) derivative, when used at concentrations around their respective K_m, did not inhibit significantly the phlorizin hydrolase activity, but inhibited the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (ONPG) with inhibition constants in the range of their respective K_m (Table 2). This result excludes a major participation of the phlorizin site in their hydrolysis.

Table 2
Inhibition constants for lactose analogues

Compound	K_i (mM) [a]
1	12.6 ± 1.3
2 [b]	10.0 ± 2.6
3	125 ± 13
10	47 ± 4
12	105 [c]
13	0.6 ± 0.07

[a] Calculated as described in the Experimental section using ONPG as substrate; under these conditions the analogues behave as competitive inhibitors. [b] Data from Ref. [12] with lactose as substrate. [c] Value estimated from a triplicate measurement of the % inhibition of 5 mM ONPG hydrolysis in the presence of 100 mM **12** considered as a competitive model.

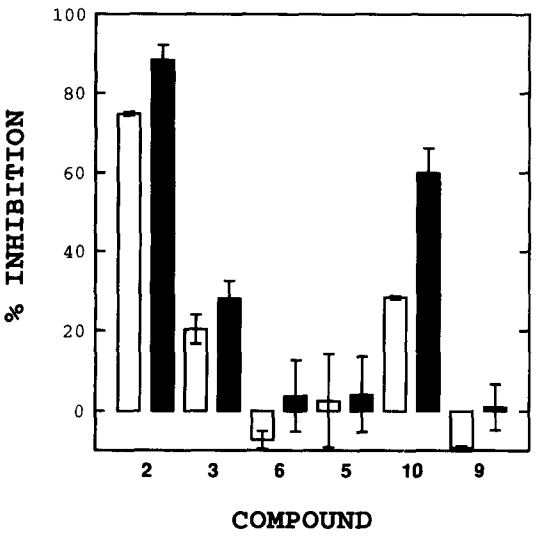


Fig. 2. Inhibition of lactase activity by methyl β-lactoside analogues which are not lactase substrates. Values are expressed as % inhibition of methyl β-lactoside (10 mM) hydrolysis in the presence of compounds **2**, **3**, **5**, **6**, **9**, and **10** at 40 mM (□) or 80 mM (■).

However, the above-formulated hypothesis will not be definitively confirmed until the substrate selectivity of each active site can be studied independently.

Galactopyranose moiety.—We have previously observed that the absence of either HO-2′ or HO-3′ in the substrate molecule abolishes its hydrolysis by lactase while the HO-4′ or the HO-6′ are nonessential. Interestingly, the 2′-deoxy derivative (**2**) behaved as a competitive inhibitor (K_i 10 mM) of lactose hydrolysis but the 3′-deoxy derivative (**4**) did not show significant inhibition even at concentrations higher than 100 mM [12]. From those results it was concluded: first, the HO-3′ was essential for the initial recognition by the enzyme and second, the HO-2′, although not necessary for initial recognition, was important for the subsequent steps of the enzymatic reaction. Whether the HO-2′ participates in the stabilization of the transition state at the active site of the enzyme or is necessary for the proper alignment of the substrate with the catalytic groups of the enzyme, as has been proposed in the case of other glycosidases [26,27], is not yet clear.

Now we have studied the corresponding *O*-methyl derivatives at positions 2′ and 3′. Neither the 2′-OMe (**3**) nor 3′-OMe (**5**) derivatives were hydrolysed by the enzyme within the sensitivity of our analytical method by GC (0.1 nmol of monosaccharide) that covers a range around four orders of magnitude in the V_{max}/K_m parameter (from 4.3×10^{-1} min^{-1} mg^{-1} for **11** to 5.7×10^{-5} min^{-1} mg^{-1} for **12**). In addition, the 3′-deoxy-3′-fluoro derivative (**6**) was also not hydrolysed. When these compounds were assayed as potential inhibitors of methyl β-lactoside hydrolysis, only **3** showed a weak inhibition of the enzymatic activity (Fig. 2). This inhibition followed a competitive pattern with K_i 120 mM, calculated using ONPG as substrate (Table 2). These results confirm the importance of both hydroxyl groups on positions 2′ and 3′ of the substrate.

Interestingly, the methylation of either of these hydroxyl groups reduced drastically the interaction of the corresponding analogues with the enzyme; **5** was not recognized at all and **3** was poorly recognized.

The fact that the 3′-H (**4**), 3′-F (**6**), and 3′-OMe (**5**) derivatives were neither substrates nor inhibitors of lactase would suggest the involvement of the HO-3′ group of the substrate in a hydrogen bond, probably as donor, in the active site of the enzyme. With regard to position 2′, methylation reduced the initial recognition by the enzyme as denoted by the significantly high K_i observed (120 mM), when compared to the K_i of the reference compound **1** (12 mM) or the 2′-deoxy analogue **2** (10 mM) (Table 2). This suggests that the enzyme must present steric hindrance to substituents on the oxygen at position 2′. Yet the exact role of the HO-2′ group, once the enzyme–substrate complex is formed, remains an open question.

Regarding the 4′ position, it was already known that it could be modified without a significant loss of the capacity of the resulting analogues to act as substrates [5,8]. Thus, the *epi* analogue, methyl β-cellobioside (**8**) [12], and cellobiose itself [22] were substrates for the enzyme. Equally, the 4′-deoxy derivative (**7**) was a substrate comparable to **1** [12]. Now we have found that, when the HO-4′ group is replaced by OMe or fluorine, the corresponding derivatives are not substrates of lactase. It is possible to infer from the behaviour of the 4′-OMe derivative (**9**) (neither substrate nor inhibitor) together with that of **7** and **1** (both substrates) that the HO-4′ group of lactose is not involved in hydrogen bonds important for the stabilization of the enzyme–substrate complex. However, these results would suggest that the enzyme has a restricted access for large substituents on position 4′ of the substrate. The results obtained with the 4′-deoxy-4′-fluoro derivative (**10**) are more difficult to interpret. Unexpectedly, when **10** was assayed with lactase it did not behave as substrate but as a moderate inhibitor of the enzymatic activity (Fig. 2 and Table 2). This inhibition followed a competitive pattern (K_i 45 mM) when ONPG was used as substrate. These results indicate that **10** retains some capacity to bind to the enzyme. This is compatible with the observation that the HO-4′ is not necessary for the hydrolysis (compound **7** is substrate) and with the fact that fluorine substitution should not introduce strong steric hindrance since it is of similar or even smaller size than the hydroxyl group [28]. A reason for the lack of detectable hydrolysis of **10** could be the high electronegativity of fluorine, which would destabilize the reaction pathway through a glycosyl carbonium cation (a step proposed in the mechanism of retaining glycosidases [29]). In this regard, it has been shown that fluorine substitution reduces the hydrolysis rate in the case of the deoxyfluoro-D-glucopyranosyl phosphates [30]. Also as a consequence of the higher electronegativity, the fluorine at the 4′ position could introduce new and nonstabilizing interactions with the enzyme, which are absent in methyl β-lactoside and its 4′-deoxy derivative. These interactions would prevent the correct alignment of the substrate with the catalytic groups of the enzyme and consequently halt the hydrolysis. As an example in relation with this, it has been shown in the case of the L-arabinose-binding protein of *E. coli* that fluorine substitution in galactose introduces instability in the interaction with the lectin due to the repulsion with a negative charge of the binding site [19].

Finally, with respect to the HO-6′ group we have previously shown not only that it is dispensable, but that its removal from the substrate molecule [6′-deoxy derivative (**11**)]

improves the interaction with the enzyme and produces a better substrate (relative V_{max}/K_m 13.8) than **1** itself. This observation was interpreted as the existence of an hydrophobic area in the complementary part of the enzyme. Now we have found that the 6′-deoxy-6′-fluoro derivative (**13**) is a substrate slightly better than **1** (relative V_{max}/K_m 1.8). Furthermore **13** is a strong inhibitor of the ONPG hydrolysis (K_i 0.6 mM). We interpreted these results as an indication of the increased affinity of the enzyme for substrate analogues more hydrophobic at the 6′-position than the reference compound. Interestingly, the 6′-OMe (**12**) and the 6′-Br (**14**) derivatives, when assayed with lactase, were poorly hydrolysed. In the case of **12**, the K_m and V_{max} were estimated to be ca. 10 times higher and 70 times lower than those of **1**, respectively (Table 1). When the hydrolysis of ONPG (5 mM) was assayed in the presence of **12** (100 mM) only a 31% inhibition was observed. This value can account, considering a competitive inhibition pattern, for a K_i 105 mM which is close to the estimated K_m. These results indicate that the effect of methylation on O-6′ has a significant effect on the initial binding, together with important consequences on the catalytic steps as denoted by the decrease in the V_{max}. It seems that the enzyme is not able to accommodate properly substrates with bigger substituents than OH at C-6′, as was observed with the other positions.

Glucopyranose moiety.—It is known that intestinal lactase has a broad selectivity regarding the aglycon part, although it shows preference towards hydrophilic aglycons [2]. In this regard, we have previously shown, using the monodeoxy derivatives of methyl β-lactoside (**17**, **19**, and **21**), lactal (**15**), and methyl α-lactoside (**16**) [12], that none of the hydroxyl groups on the glucose part of the substrate molecule is absolutely essential for the interaction with lactase or for hydrolysis. Only when the HO-6 was absent was the interaction of the enzyme with the corresponding derivative severely impaired [12].

It was previously reported that the 2-deoxy (**17**) and 3-deoxy (**19**) derivatives of methyl β-lactoside had somewhat lower affinity for the enzyme (the K_m for compounds **17** and **19** increased ca. 3 times with respect to the K_m of **1**, while the V_{max} remained almost unchanged, being 88 and 122% of that of **1** for **17** and **19**, respectively (Table 1) [12]. Now, when the 2-OMe derivative (**18**) was assayed, the K_m value obtained was half of that of the reference compound **1** and ca. 6 times lower than that of the deoxy derivative **17**. On the other hand, the variation on the V_{max} is less significant (Table 1). These results would indicate a recovery in the affinity when going from the deoxy to the *O*-methylated derivative. Since the differences in the kinetic parameters are moderate, the HO-2 group could be an acceptor of hydrogen bonds with a minor contribution to the stabilization of the enzyme–substrate complex.

Concerning position 3, we had previously found that 3-OMe derivatives, 3-*O*-methyl-lactose [31,32] and methyl 3-*O*-methyl-β-lactoside (**20**) [12] were substrates for intestinal lactase, although they were poorer than the natural substrate [12]. The V_{max} of the hydrolysis of compound **20** is 14 times lower than the V_{max} for **1**, but the K_m value increases around 10 times. As both parameters change in similar proportion it is not possible to discriminate whether the methylation of O-3 generates steric hindrance for the initial recognition or whether the main reason for the lower affinity is the lack of hydrogen donor capacity. In any case, these results suggest that the methylation introduces a significant restriction on the enzyme galactosidation step (k_2 in Fig. 1).

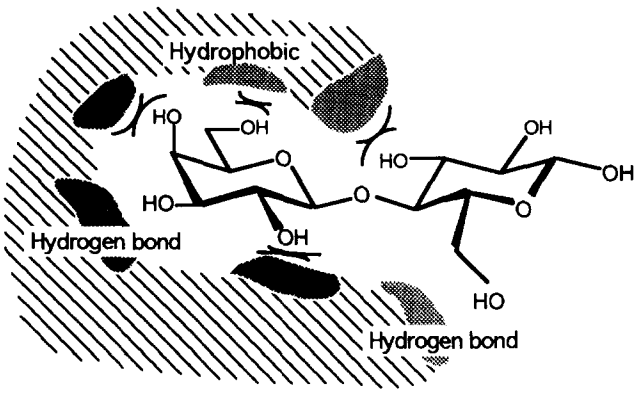


Fig. 3. Schematic representation of lactose–lactase interactions. Methylation of hydroxyl group at position 2′, 4′, 6′, or 3 limits substrate recognition. Hydroxyl groups at positions 3′ and 6 probably act as hydrogen bond donors. The hydroxyl group at position 6′ is placed in a hydrophobic environment. Modifications of hydroxyl groups at positions 1 and 2 have minor consequences in the interaction.

On the basis of Lemieux's studies with lectin ligands [14,15], the above results allow us to locate the interaction of the enzyme with the HO-3 substrate group more in the periphery of the binding site, where large substituents are not easily accommodated, while the HO-2 substrate group should be more exposed to the aqueous phase with no steric hindrance.

Regarding position 6, we have now assayed intestinal lactase with the corresponding methylated derivative [6-OMe (**22**) and also with some halo derivatives, 6-deoxy-6-fluoro (**23**) and 6-deoxy-6-iodo (**24**)]. Neither fluorine nor the *O*-methyl group can reverse the effects on the K_m of the absence of the hydroxyl group in the 6-deoxy (**21**) derivative (Table 1). In contrast, the values of V_{max} for the three derivatives **21**–**23** are not significantly different from the V_{max} of **1**. These results show that the HO-6 group is important for an efficient binding with the enzyme and it probably participates as a hydrogen bond donor in the periphery of the binding site.

In summary, the above results allow us to draw a formal picture of the lactase active site (Fig. 3). None of the *O*-methyl derivatives on the galactose moiety of the substrate molecule are easily recognized by the enzyme, suggesting that the active site of lactase presents a cavity where galactose lies deep. Probably, no space is available to accommodate substrate molecules with further extension from the nonreducing part. Evidently, this corroborates the disaccharidase–*exo*-glycosidase character of intestinal lactase.

The interaction of the enzyme with the 6′ position of the substrate seems to have an important hydrophobic component. The HO-3′ group probably participates, as donor, in an essential hydrogen bond with the enzyme. The roles of the HO-4′ and HO-2′ are not fully understood.

The glycosidic linkage of the substrate, logically, should be surrounded by the catalytic groups of the active site. In the case of intestinal lactase, the catalytic groups have been assigned, from sequence homology studies [33,34] and affinity labelling [11], to Glu_{1271} (lactase site) and to Glu_{1747} phlorizin hydrolase site). They would stabilize the glycosyl carbonium transition state proposed for retaining glycosidases [29]. Interest-

ingly, modifications in the substrate molecule in the vicinity of the glycosidic bond (*O*-methylation at the 3-position, or HO-2′ group abstraction) have significant effects on the chemical steps after the initial binding.

The aglycon part (glucopyranose moiety) is much less restricted towards modifications. These results suggest that hydroxyl groups at positions 3 and 6, neighbouring the glycosidic bond, interact with the periphery of the active site, and the remaining part of the molecule must be more exposed to the aqueous phase. This is in accordance with the broad selectivity of intestinal lactase towards the aglycon part of the substrate molecule.

3. Experimental

Materials.—Sepharose 2B, Sephadex G-200, and DEAE-cellulose were obtained from Pharmacia; phenylmethylsulfonyl fluoride (PMSF), *p*-chloromercuribenzoate (PCMB), Trinder reagent (glucose oxidase kit), galactose dehydrogenase, NAD, and papain were obtained from Sigma; trimethylsilylimidazole was from Aldrich. Reagents and molecular mass markers used in SDS-PAGE were products of Bio-Rad.

Substrates and other lactose analogues.—Lactose, phlorizin, *o*-nitrophenyl β-D-galactopyranoside (ONPG), 2-naphthyl β-D-galactopyranoside, D-glucose, and D-galactose were from commercial sources. Phloretin was obtained by acid hydrolysis from phlorizin. The lactose analogues methyl β-lactoside (**1**) [35], methyl 2′-*O*-methyl-β-lactoside (**3**), methyl 3′-*O*-methyl-β-lactoside (**5**), methyl 3′-deoxy-3′-fluoro-β-lactoside (**6**), methyl 4′-*O*-methyl-β-lactoside (**9**), methyl 4′-deoxy-4′-fluoro-β-lactoside (**10**), methyl 6′-*O*-methyl-β-lactoside (**12**), methyl 6′-deoxy-6′-fluoro-β-lactoside (**13**), methyl 6′-bromo-6′-deoxy-β-lactoside (**14**), methyl 2-*O*-methyl-β-lactoside (**18**), methyl 6-*O*-methyl-β-lactoside (**22**), methyl 6-deoxy-6-fluoro-β-lactoside (**23**), and methyl 6-deoxy-6-iodo-β-lactoside (**24**) were synthesized as described previously [36].

Purification of the enzyme.—The lactase–phlorizin hydrolase activity was purified from lamb small intestines according to the method described by Schlegel-Haueter et al. [6] with minor variations [12]. Briefly, the enzymatic activity from the membranes of intestinal brush border cells was solubilized by proteolytic treatment with papain. The insoluble fraction was separated by centrifugation and the supernatant solution was concentrated by ultrafiltration (amicon YM-30). To avoid further proteolysis and inhibit other intestinal galactosidases, PMSF (0.5 mM) and PCMB (1 mM) were added. Lactase was then purified by subsequent chromatography on Sepharose 2B, Sephadex G-200, and DEAE-cellulose. The fractions showing lactase activity were collected. Protein concentration was determined by the Lowry method [37] using bovine serum albumin as standard. Molecular mass was estimated by SDS-PAGE, in the presence of 2-mercaptoethanol, using 7.5% acrylamide gels.

Enzymatic assays.—Lactose hydrolysis was measured using coupled enzymatic assays with galactose dehydrogenase [38] or glucose oxidase [20] as described earlier [12]. Phlorizin hydrolysis was determined by measuring the phloretin release, using a chromatographic method developed in our laboratory [12]. ONPG hydrolysis was followed by the absorbance at 417 nm of 20-μL incubation samples quenched with aq Na_2CO_3 (290 μL).

The hydrolysis of lactose analogues was measured by determining the released monosaccharides as their trimethylsilyl derivatives by gas chromatography, essentially as reported earlier [12]. The enzymatic reactions were stopped by boiling the incubation mixtures up to complete dryness (approximately 10 min). The carbohydrates were derivatized with trimethylsilylimidazole in pyridine (30 min at 60° C). The release of D-galactose or methyl β-D-glucopyranoside was measured, depending on the analogue being analysed. The monosaccharides were referred to benzyl inositol used as internal standard in the derivatization procedure. Previously, calibration curves for D-galactose and methyl β-D-glucopyranoside were obtained under the same assay conditions. The stability of the different analogues, specially the halo derivatives, during the derivatization procedure was checked by comparison with the derivatization of the crystalline compounds with trimethylsilylimidazole at room temperature.

Preliminary enzymatic assays were performed at 37° C with each substrate (at 5 and 100 mM) to determine the amount of enzyme (which ranged from 0.005 to 0.2 mg mL^{-1}) and incubation time (between 10 and 120 min) needed to obtain a measurable amount of product under linear conditions.

The kinetic parameters were obtained for compounds **1**, **12**, **13**, **18**, **22**, and **23**. Each analogue was incubated at several concentrations (from 0.5 to 20 mM or 3 to 180 mM) at a constant enzyme concentration (0.01 mg mL^{-1} for **1**, **2**, and **12**; 0.02 mg mL^{-1} for **3** and **4**; and 0.04 mg mL^{-1} for **11**) for a fixed period of time (30 min for **1** and **2**; 40 min for **12**; and 60 min for **3**, **4**, and **11**). All the assays were done in duplicate. The K_m and V_{max} values for each compound were calculated with the Enzfitter program for non-linear regression fitting [39].

Inhibition experiments.—Compounds **2**, **3**, **5**, **6**, **9**, and **10** were tested as inhibitors. Methyl β-lactoside (5 mM) was incubated with lactase (0.01 mg mL^{-1}) for 30 min in the presence of increasing concentrations (20, 40, 60, 80, 120 mM) of the corresponding disaccharide.

For the calculation of the inhibition constant of compounds **1**, **3**, **10**, **12**, and **13**, the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (at 3, 6, 10, 18, and 30 mM) was assayed in the presence of compound **1** (15, 30, and 45 mM), compound **3** (40, 80, and 120 mM), compound **10** (30, 60, and 90 mM), or compound **13** (0.75, 1.5, 3, and 6 mM). The inhibition constants were estimated with the statistical method described by Cleland [40].

The hydrolysis of phlorizin (at 2, 4, 7, 12, 20, and 40 mM) was assayed in the presence of **1** (10, 20, and 40 mM) or **13** (1, 2, and 3 mM)

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