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THE PREPARATION OF LACTASE AND GLUCOAMYLASE OF RAT SMALL INTESTINE

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SUMMARY

1. Both lactase and glucoamylase were obtained in a highly purified form from the small intestine of baby rats, by papain solubilisation followed by chromatography on Sepharose, Sephadex and DEAE-cellulose. Some properties of these enzymes were investigated.

2. Attempts at affecting the levels of small intestinal lactase activity to the diet of baby rats failed.

3. The multiplicity of human intestinal lactases was also briefly investigated (see APPENDIX).

INTRODUCTION

Small intestinal lactase ("neutral" β-galactosidase) (β-D-galactoside galactohydrolase, EC 3.2.1.23) was purified to a very high degree by WALLENFELS AND FISCHER[1], by ALPERS[2] and by SWAMINATHAN AND RADHAKRISHNAN[3] from calf, rat and monkey, respectively. Partial purification was also obtained by other authors (for a review, see ref. 4). In the following we report a rather simple procedure to prepare rat intestinal lactase in a homogeneous, or almost homogeneous, form. A part of our work was presented in a preliminary form[5].

As a byproduct of lactase, intestinal glycoamylase (γ-amylase, α-1,4-glucan glucohydrolase, EC 3.2.1.3) can also be isolated by a procedure described below. To the best of our knowledge, ours is the first report of a homogeneous intestinal glucoamylase. (Partial purification from rat[6,7,8] and monkey[40] was reported by previous authors.)

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METHODS

Unless otherwise stated, lactase and cellobiase activities were measured at pH 5.5 in 0.1 M sodium maleate buffer at a substrate concentration of 114 mM at 37°. Glucoamylase activity was measured at 37° at pH 6.0 in 0.05 M sodium maleate buffer, which was 2.5 mM in EDTA and contained 10 mg of soluble amylose per ml. For the first two steps of the purification procedure, the α-amylase(s) had first to be denaturated by heating the samples in 5 mM EDTA, 0.1 M maleate buffer (pH 6.0), 2.5 mM NaCl for 25 min at 50° (ref. 7). The glucose liberated was determined with the Tris–glucose oxidase–peroxidase reagent[9]. Phlorizin hydrolase activity was measured in McIlvane buffer at pH 6, as suggested by MALATHI AND CRANE[10]. The released glucose was determined either according to SOMOGYI[11], or with the hexokinase method as described by SCHMIDT[12]. Protein was determined by the method of LOWRY *et al.*[13] or from absorbance at 280 nm (in the chromatographic fractions).

Disc electrophoresis[14,15] was performed in 0.1 M sodium phosphate buffer (pH 7.2) in 7.5 and 5% gels. More alkaline systems, particularly those containing Tris, were avoided with lactase, because they were found to dissociate the sucrase–isomaltase complex (A. EBERLE, unpublished results); perhaps for similar reasons they consistently gave two bands of similar intensities with "pure" lactase. Disc electrophoresis of glucoamylase was carried out in the Tris–glycine system (pH 8.9) (refs. 14, 15).

The sedimentation analysis was performed in a Spinco Model E analytical ultracentrifuge with Schlieren optics. The isoelectric point was estimated in a LKB electrofocusing system 8100-10 in a 110-ml column with ampholine in a pH gradient from 3.0 to 6.0 stabilised by a sucrose gradient (0 to 40%, w/w).

MATERIALS

All chromatographies were developed with the help of a peristaltic pump (W. Meyer, Lucerne), Sepharose 2B and Sephadex G-200 were obtained from Pharmacia, Uppsala, Sweden; DEAE-cellulose from Serva, Heidelberg, W. Germany; papain (2 times cryst.) from Mann Res. Laboratories, New York; the hexokinase and the glucose-6-phosphate dehydrogenase for glucose determination were from Boehringer, Mannheim, W. Germany; all other reagents were commercial preparations of reagent grade.

The standard diet on which the rats were kept after weaning was obtained from Latz, Euskirchen, W. Germany.

RESULTS

The purification procedure for lactase

The procedure eventually adopted will be described first. A few comments on the individual steps will follow.

For each preparation approximately 50 baby rats, of either sex, 10 to 15 days old, were used. They were killed by a blow on the neck and the first half of the small intestine was excised. If the intestines were not processed immediately, they were frozen, kept for a few days at approx. −20°, and thawed shortly before use. Unless otherwise stated, all steps were carried out at approx. 4°.

Step 1. Homogenisation at high ionic strength: The small intestines were cut into small pieces and homogenised in 1 M NaCl (3–4 ml/g fresh weight) in an apparatus of the Waring blendor type (Ultraturrax Bühler). This homogenate will be referred to as "starting material". It was spun at 105 000 × *g* for 60 min. The sediment was suspended in the same volume of 10 mM potassium phosphate buffer (pH 6.8) and spun as before. Both supernatants were discarded.

Step 2. Papain solubilisation: The sediment was resuspended in 10 mM potas-

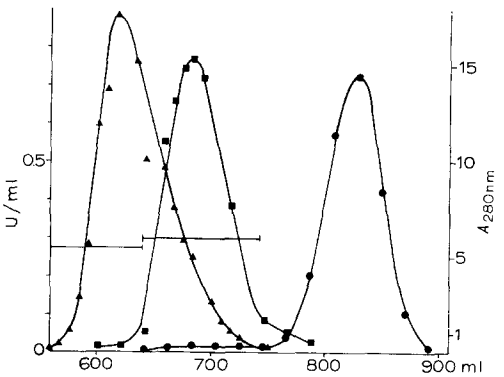

Fig. 1. Sepharose-2B chromatography of the solubilised material. The total volume of the column was approx. 900 ml. For details see text. ▲—▲, glucoamylase; ■—■, lactase; ●—●, protein ($A_{280\ nm}$).

sium phosphate buffer, pH 6.8 (1 ml per original g fresh weight), to which 4 mg papain and 0.4 mg cysteine·HCl per original g fresh weight were added. After incubation at 37° for 40 min with shaking, the mixture was spun at 105 000 × *g* for 90 min. The sediment was discarded.

Step 3. Sepharose chromatography: The supernatant (15–25 ml) was applied to a Sepharose 2B column (3.5 cm × 94 cm) which had been equilibrated with 10 mM potassium phosphate buffer, pH 6.0. The column was developed with the same buffer (flow rate 9 ml/h). Fractions were collected every 30 min. The fractions containing most of the lactase activity (as indicated in Fig. 1) were pooled and concentrated by

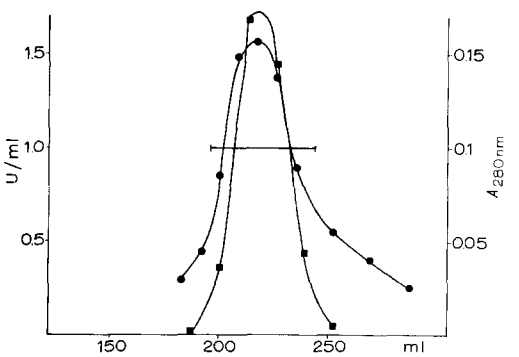

Fig. 2. Sephadex G-200 chromatography of the lactase pool from the sepharose column. The total volume of the column was approx. 500 ml. For conditions see text. ■—■, lactase; ●—●, protein.

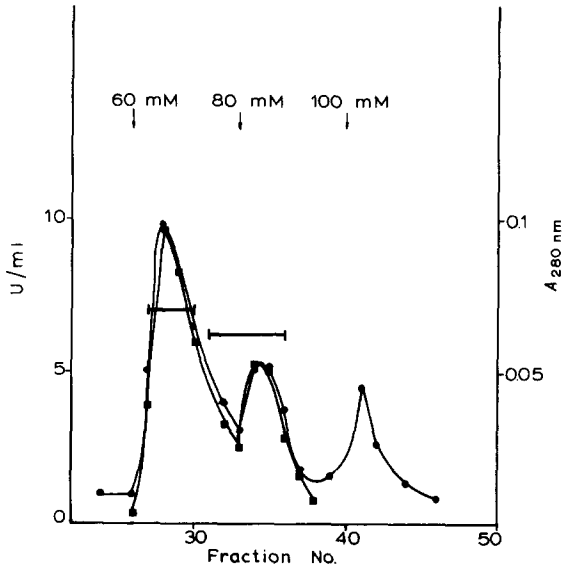

Fig. 3. DEAE-cellulose chromatography of the lactase pool from the Sephadex column. For conditions see text. ■—■, lactase; ●—●, protein.

ultracentrifugation at 140 000 × *g* overnight. The centrifuge was allowed to come to a stop with the brakes turned off. Some nine tenths of the supernatant were carefully sucked off and discarded.

Step 4. Sephadex G-200 chromatography: The concentrated lactase, as obtained in the previous step, was applied to a Sephadex G-200 column (2.5 cm × 90 cm), which had been equilibrated with 10 mM potassium phosphate buffer. The column was developed upwards (9 ml/h) with the same buffer. Fractions were collected every

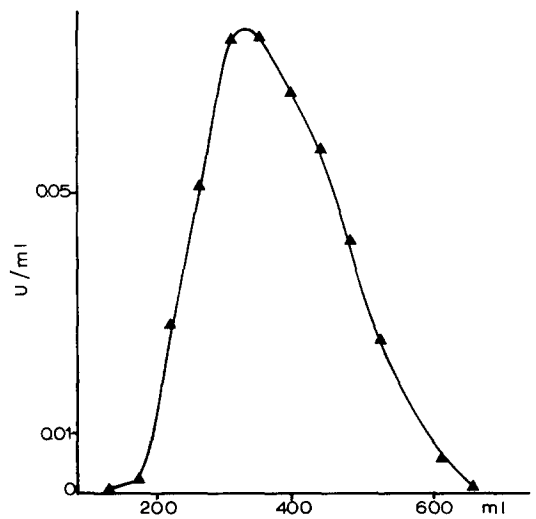

Fig. 4. Sephadex G-200 chromatography of the glucoamylase pool from the Sepharose column. The total volume was approx. 500 ml. For conditions see text. The $A_{280\ nm}$ of the fractions was almost zero. ▲—▲, glucoamylase.

30 min. The lactase-rich fractions (see Fig. 2) were pooled and concentrated by ultracentrifugation as in the previous step.

Step 5. DEAE-cellulose chromatography: The preparation was applied to a DEAE-cellulose column (0.9 cm × 7.5 cm), which had been equilibrated with 10 mM potassium phosphate buffer, pH 6.0. The column was developed stepwise with 10, 40, 60, 100 and 300 mM potassium phosphate buffers (approx. 30 ml each; flow rate: 13.7 ml/h). Fractions were collected every 8.5 min. (Fig. 3). The lactase-containing fractions were pooled and concentrated by ultrafiltration or by ultracentrifugation. The lactase preparation was divided into small aliquots and kept frozen until required.

The purification procedure for glucoamylase

Steps 1 to 4 were identical with the steps for lactase preparation, and actually Steps 1 to 3 were used for the preparation of both lactase and glucoamylase. The only difference in Sepharose chromatography (Step 3) was that for glucoamylase other fractions were pooled (see Fig. 1), concentrated and applied to another Sephadex G-200 column as in Step 4 of the lactase preparation. The glucoamylase was abnormally retained by Sephadex and was therefore purified very effectively, but it was also very much diluted (Fig. 4). The pooled fractions (approx. 400 ml) were concentrated by absorption on a DEAE-cellulose column in 10 mM potassium phosphate buffer, pH 6.8 and sharp elution with 0.3 M buffer. Additional concentration was carried out by ultracentrifugation as in Step 3.

Comments on the procedure

Choice of the animals. Intestinal disaccharidase activities in mammals are related to age, stage of development and diet (for reviews, see refs. 4, 16). Baby rats 10 to 15 days old have high lactase activity and detectable maltase (glucoamylase) activity, whereas they are essentially devoid of sucrase and isomaltase activities[17–20]. Older rats have, of course, a more developed small intestine; the specific activity of lactase is, however, much lower. In addition, they also have other α-glucosidases.

On the other hand, rats younger than 10 days, besides being poorer sources of glucoamylase, provide lesser quantities of small intestine. The age chosen (10 to 15 days) is, therefore, a convenient optimum for the purpose of preparing either lactase or glucoamylase, or both. It is possible that other mammalian species could provide equally convenient small intestine at a corresponding stage of development.

Step 1. Homogenisation at high ionic strength: As we have reported previously[21], papain treatment of a sediment rich in nucleohistone brings about solubilisation of a DNA-containing material which may render the solution too viscous for the subsequent chromatography. The purpose of the homogenisation at high ionic strength with subsequent centrifugation is to dissociate the thymonucleohistone and thereby to discard most of the DNA with the supernatant.

In the supernatant much hetero-galactosidase activity (presumably of lysosomal origin), some lactase activity and much α-amylase activity were eliminated. The determinations of membrane-bound lactase and glucoamylase were not accurate in the presence of high "soluble" galactosidase and α-amylase, respectively. Therefore the yields given in Table I were calculated on the basis of activity measured in the sediment from this ultracentrifugation, rather than in the total homogenate.

TABLE I

SUMMARY OF A PREPARATION OF LACTASE AND GLUCOAMYLASE FROM THE SMALL INTESTINE OF BABY RATS

The specific activity is expressed as international units per mg protein. One I.U. of either lactase or glucoamylase activity liberates 1 μmole of glucose per min from either lactose or amylase, respectively, under the conditions of the assay.

	Protein (total mg)	*Lactase*			*Glucoamylase*		
		Total units	*Specific activity*	*Recovery (%)*	*Total units*	*Specific activity*	*Recovery (%)*
Lactase and glucoamylase:							
Starting material	3580	375	0.10		245	0.068	
Membrane-bound	480	130	0.27	100	50.4	0.10	100
Supernatant after papain solubilisation	192	62	0.32	48	42	0.22	83
Lactase alone:							
After Sepharose	7.1	34	4.7	26			
After Sephadex G-200	4.0	29	7.2	22			
After DEAE-Cellulose	0.95	21	22	16			
Glucoamylase alone:							
After Sepharose	2.6				19	7.4	38
After Sephadex-G-200 and DEAE-Cellulose	0.5				12	36*	24

* Uncertain, protein concentration and amount too low for exact assay.

Step 2. Papain solubilisation: This procedure, originally introduced by us for the solubilisation of human intestinal disaccharidases[22,23], has already been used for rat lactase[24,25] and has proved of value in the solubilisation of most disaccharidases in many species (for a review, see ref. 4). The yield of this step is only apparently low (see Table I). It can be increased easily by washing the sediment. This, however, increases the volume which in turn requires either a concentration step or an increase in the size of the Sepharose column.

Step 3. Sepharose chromatography: Since agarose, the matrix of Sepharose gels, is a linear polysaccharide composed of 3,6-anhydro-L-galactose and D-galactose units[26] it was first used in the hope that lactase might be retarded on such columns by enzyme–substrate like interactions to such an extent that it would be eluted last. However, it was found that although lactase was retarded slightly it was eluted ahead of the main protein band and perhaps in its correct position in relation to its molecular weight. The interactions between lactase and the gel were not investigated further but this step was retained as it still resulted in a considerable purification of glucoamylase and lactase.

Step 4. Sephadex chromatography: A number of carbohydrases are retained abnormally by this gel, presumably through an enzyme–substrate like interaction[27]. The sequential use of two gels having similar hydrodynamic properties but displaying different additional interactions with one given component affords a very efficient means of purifying that component. We have already made use of this principle in the purification of the small intestinal sucrase–isomaltase complex, which is retained by

Sephadex but not by Biogel[21,28]. In the present paper, we have obtained an efficient purification of glucoamylase by the sequential use of Sepharose (which excludes this enzyme) and of Sephadex (which retards it very much). The high dilution was found acceptable in view of the efficiency of the step (Table I). Some free glucose—presumably arising from the dextran matrix—emerges from the column along with glucoamylase (see also refs. 21 and 28).

Step 5. DEAE-chromatography: With substances having strongly curved isotherms (such as proteins), stepwise development is usually not the procedure of choice. Nor is the use of anionic buffers on anion exchangers[29]. The impurities present in the lactase preparation at this stage, however, seem to have such different chromatographic behaviours on DEAE-cellulose to warrant the use of a simple and quick procedure, such as stepwise development. Gradient elution did not give better results.

The concentration steps. Whenever the volume of the sample(s) permitted, concentration of lactase and glucoamylase by ultracentrifugation overnight was found

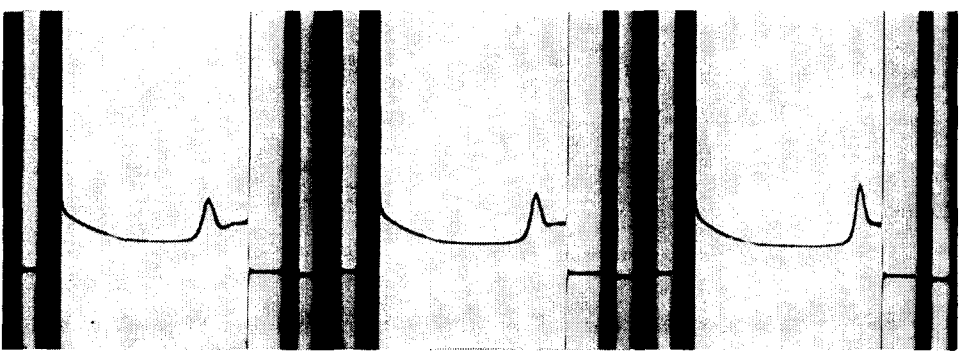

Fig. 5. Sedimentation of purified lactase. Conditions: 3 mg protein/ml; 0.1 M NaCl; 59 780 rev./min; rotor AnD. Pictures shown here: after 16, 24, 32 min.

to be a convenient and mild procedure and was performed routinely. Naturally, other concentration procedures could be used as well, if compatible with the stability of lactase (see below).

Criteria of purity. The limited amount of enzymes available prevened an extensive investigation. However, Fig. 5 reports a sedimentation analysis of lactase and Fig. 6 a gel electrophoresis of both lactase and glucoamylase. It can be seen that glucoamylase is essentially homogeneous. Lactase, although homogeneous in the ultracentrifuge, often yielded one major and one minor band in the gel electrophoresis. It could not be established whether the minor band indicated impurity, denaturation or aggregation.

Summing up. Lactase and glucoamylase were obtained in a highly purified or homogeneous form. The specific activity of our lactase compares favourably with that reported by other workers for preparations with similar degrees of homogeneity[1,2]. In the present paper, this result was obtained by a 170–200-fold increase in specific activity, because of the favourable starting material. An efficient and quick step introduced was Sepharose chromatography, particularly in combination with Sephadex. As far as intestinal glucoamylase is concerned, ours is, to the best of our

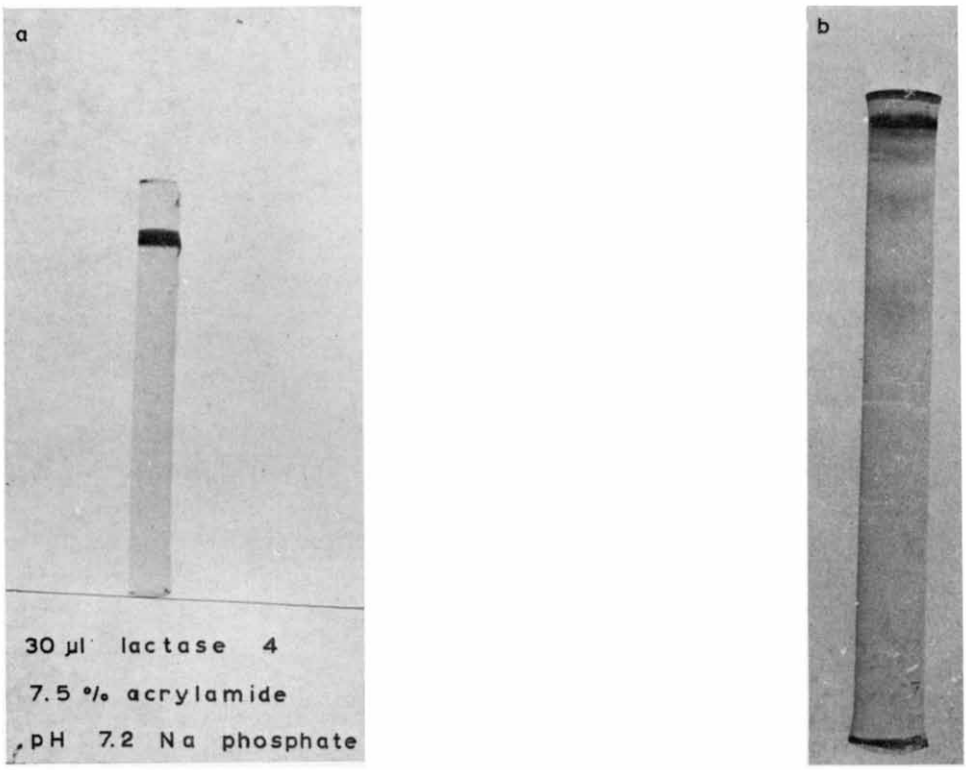

Fig. 6. Gel electrophoresis of purified lactase and glucoamylase. a, approx. 130 µg lactase in a 7.5% gel, pH 7.2, 0.1 M sodium phosphate buffer; b, approx. 140 µg glucoamylase on a 7.5% gel, pH 8.9, Tris–glycine buffer.

knowledge, the first procedure reported for the preparation of an essentially homogeneous enzyme.

Properties of lactase

The following data were collected using lactase preparations which were carried through all the purification steps.

Isoelectric point. From ion focusing experiments in ampholine gradients from pH 6 to 3 in sucrose (not reported in detail), the isoelectric point of rat intestinal lactase was found to be in the range 4.3–4.5.

Heat stability and pH stability. When kept frozen at −20°, lactase does not show detectable loss of activity for several months. At 46°, and also at 37°, it is inactivated even at neutral pH (Fig. 7). At pH values below 5 at 37°, it is even less stable (Fig. 8).

pH-activity. With 10 min incubations, the curve given in Fig. 9 was obtained. With longer incubations (*e.g.* 60 min), very little enzyme activity was detected below pH 4.8 (Fig. 9), probably due to the poor stability of the enzyme below pH 5 (see above, Fig. 8). Thus, the exact pH–activity curve cannot be determined accurately.

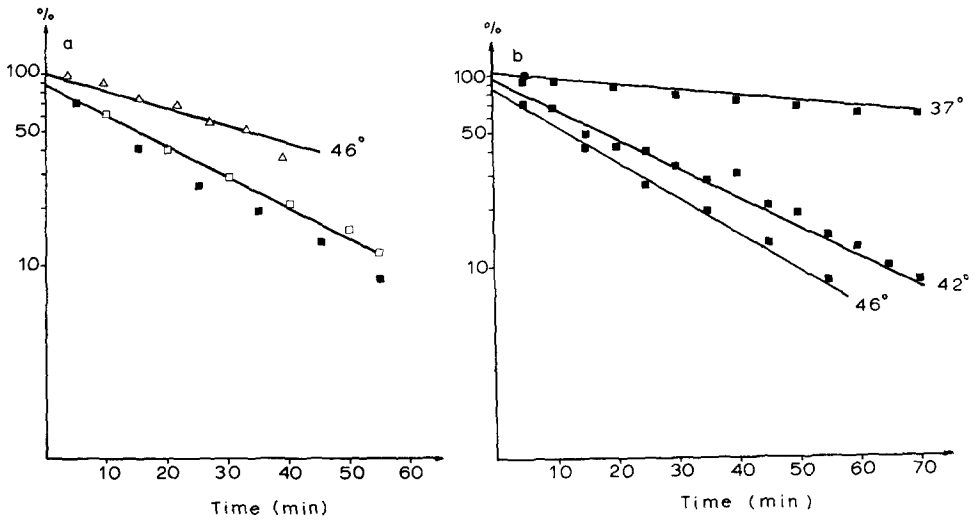

Fig. 7. Heat stability of purified lactase in 10 mM potassium phosphate buffer. The lines drawn were calculated by the least squares method. ■—■, lactase; □—□, cellobiase; △--△, phlorizin hydrolase.

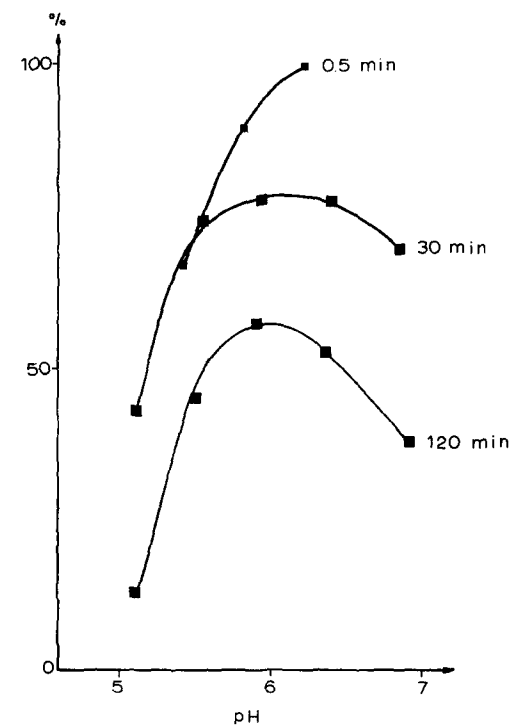

Fig. 8. pH stability of purified lactase. The enzyme (dialysed against water) was brought to the indicated pH with 0.04 M sodium acetate buffer (pH 5–5.2) or 0.04 M sodium maleate buffer (pH 5.5–7). After 0.5, 30 and 120 min the samples were incubated with the normal lactose–buffer mixture (114 mM lactose, 0.1 M sodium maleate (pH 5.5); end concentrations).

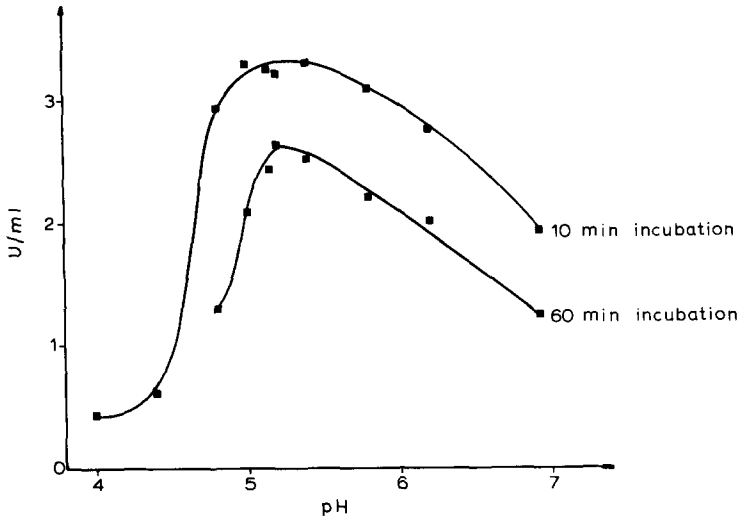

Fig. 9. pH–activity curve of purified lactase (dialysed against water). Lactase was incubated with 114 mM lactose in 0.02 M sodium acetate (pH 4–5.2) or in 0.02 M sodium maleate (pH 5–7) for 10 and 60 min.

With this limitation, our pH–activity curves agree reasonably well with those reported by others[17,30,2] for the same enzyme (rat "neutral" lactase), for the monkey enzyme[3] and for the calf enzyme[1].

The K_m at pH 5.5 is 21 mM (see also refs. 3, 25).

The anomeric form of galactose released during the hydrolysis of lactose by lactase is β-galactopyranose (ref. 31).

Relationship with phlorizin hydrolase activity. A hitherto unknown intestinal glucosidase, splitting phlorizin into phloretin and glucose was described by MALATHI AND CRANE[10], and by DIEDRICH[32]. The relationship between phlorizin hydrolase activity and lactase is examined extensively in another paper from our laboratory[33].

Fig. 7 reports the heat inactivation of lactase, cellobiase and phlorizin hydrolase activities of "pure" rat lactase. It indicates that cellobiose—contrary to what would be expected—is split by lactase rather than by phlorizin hydrolase; and that most of the phlorizin hydrolase activity in "pure" lactase is not due to the lactase catalytic site.

The lactase/phlorizin hydrolase ratio throughout the three chromatographic procedures (Sepharose, Sephadex and DEAE-chromatography) remains constant (at the pH and substrate concentrations used, 51 to 1, data not shown).

Since the specific activities of both lactase and phlorizin hydrolase increased considerably during these steps, the constant ratio indicates strongly that the two enzyme activities are bound to each other, apparently in a kind of oligo-enzyme complex. A similar situation was found previously for the sucrase–isomaltase complex from small intestine[21]. Phlorizin itself, however, does inhibit lactase activity, although less than one would expect if lactose and phlorizin were split by the same active site. KRAML *et al.*[41] have found independently that phlorizin is a competitive inhibitor of lactase (K_i 0.047 mM; pH 6.0; substrate, lactose).

TABLE II

LACK OF EFFECT OF PHENYL-β-D-GALACTOPYRANOSIDE OR METHYL-β-D-THIOGALACTOPYRANOSIDE FED TO BABY RATS ON THE LACTASE, MALTASE, SUCRASE AND TREHALASE ACTIVITIES IN THE SMALL INTESTINE

The data are expressed as international units per g protein in the total homogenate (Mean ± S.E.). I, activity in the proximal half of the small intestine. II, activity in the distal half of the small intestine. At the beginning of the treatment, the rats weaned were 4 weeks old. For details, see text.

Addition to the normal diet		*Lactase*		*Maltase*		*Sucrase*		*Trehalase*	
	n	*I*	*II*	*I*	*II*	*I*	*II*	*I*	*II*
Administration during 19 days:									
Controls (no additon)	4	13.5 ± 1.0	6.9 ± 0.16	255 ± 21	134 ± 4	44.5 ± 3.2	13.3 ± 0.2	47.1 ± 3.4	10.6 ± 0.6
Phenyl-β-D-galactopyranoside	4	11.4 ± 1.2	6.5 ± 0.35	239 ± 25	166 ± 8	46.4 ± 5.1	16.8 ± 0.12	42.5 ± 5.4	6.9 ± 0.7
Methyl-β-D-thiogalactopyranoside	2	12.3	5.8	236	176	40.9	16.4	37.9	6.1
Administration during 30 days:									
Controls (no addition)	4	13.7 ± 0.64	8.5 ± 0.3	291 ± 5	171 ± 5	40.9 ± 3	19 ± 1	51.7 ± 4.1	12.6 ± 0.9
Phenyl-β-D-galactopyranoside	4	16.1 ± 1.0	8.3 ± 0.64	332 ± 13	181 ± 14	44.6 ± 2	18.1 ± 1.3	54.0 ± 4.4	10.3 ± 1.4
Methyl-β-D-thiogalactopyranoside	4	13.7 ± 0.2	7.7 ± 0.23	309 ± 6	185 ± 8	38.2 ± 2.5	20.5 ± 2.2	49.0 ± 1.5	6.5 ± 0.5

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Attempts at influencing the lactase levels in rat small intestine by administration of hetero-galactosides

Many authors have tried to prevent or delay the decrease in lactase activity which takes place after weaning or to stimulate its reappearance by feeding lactose. The results are to some extent conflicting[16,34,35,39]. We have made a similar attempt by feeding baby rats with either phenyl-β-D-galactopyranoside or methyl-β-D-thio-galactopyranoside (a gratuitous inducer of *Escherichia coli* β-galactosidase[36]) in addition to normal diet. 4-week-old rats of either sex were fed for 19 days or for one month with water containing either phenyl-β-galacto-pyranoside (1 g/l) or methyl-β-D-thio-galactopyranoside (0.1 g/l), or plain water (some 25 ml of liquid were put at the disposal of each animal daily). They were then sacrified, the small intestine was excised, rinsed with cold saline, and divided into proximal and distal halves. Each half was weighed and homogenised in 20 ml saline. Lactase, maltase, sucrase and trehalase activities (33 mM substrate in 33 mM sodium maleate buffer, pH 6.0) were determined. Table II reports the results, which are clearly negative: none of the disaccharidases considered was affected by the inclusion of either heterogalactoside in the diet.

A few properties of glucoamylase

Sucrose, palatinose, lactose, cellobiose and turanose (all 56 mM) are not hydrolysed to any detectable extent.

Contrary to the glucoamylase from rat liver lysosomes[37], intestinal glucoamylase is not inhibited by 30 mM turanose to a significant extent. Phlorizin (1 mM) inhibits by some 16% (substrate: 28 mM maltose). Tris (100 mM) provokes a complete inhibition.

The product released by glucoamylase from amylose is glucose, as identified by paper chromatography, gas chromatography of its persilylated derivative and by its reaction with the Tris–glucose oxidase–peroxidase reagent. The anomeric form of the glucose liberated was identified as alpha by gas chromatography (C. PINELLI, unpublished results).

The K_m for maltose in 0.025 M sodium or lithium maleate buffer (pH 6.0) was 2.5 mM $\pm$ 0.41 (Mean $\pm$ S.E., $n = 8$). The pH optimum is approximately 6.5 (see also refs. 6, 8).

APPENDIX

P. HORE, M. LANDOLT* AND G. SEMENZA

Human intestinal lactase

It was reported previously[23] that after papain solubilisation of the 105 000 × *g* sediment from homogenates of human small intestine, two enzymes splitting lactose appear in Sephadex G-200 chromatography with K_d values of approximately 0.14 ("lactase-1") and 0.8 ("lactase-2"), respectively. This observation was later questioned, however[24], a partial splitting of the lactase peak travelling near the front was reported instead[38]. Two morphologically normal pieces of human jejunum, totalling

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1.76 g, were obtained surgically and processed as described previously[23]. After homogenisation in saline, the 105 000 × *g* sediment was treated in 0.1 M potassium phosphate buffer (pH 6.8), with 1 mg papain + 1 mg cysteine·HCl per 5 mg protein, for 25 min at 37°. After centrifugation at 105 000 × *g* for 90 min, 4 ml of the supernatant were applied to a 60 cm × 2 cm Sephadex G-200 column, which was developed with 0.05 M potassium phosphate buffer (pH 6.8) at a flow rate of 3 ml/h at 4°. The sample applied contained 2.14 lactase units; 16.6 maltase units; 6.2 sucrase units and 34.4 mg protein. For the determination of disaccharidase activities in the individual chromatographic fractions, the high blank due to the glucose liberated from Sephadex[21] was taken into account (as was done previously); in addition, lactase activity was also determined after dialysis.

By neither procedure was lactase activity detected in the K_d 0.8-region of the eluate, whereas a partial splitting of the lactase-1 peak ($K_d \simeq 0.14$) was observed; this confirms, therefore, previous reports[38]. Precipitation of the solubilised extract with ethanol prior to chromatography yielded similar results.

It cannot be explained why the present results are in part at variance with those given in ref. 23, which were obtained with exactly the same technique and by taking into account the glucose blank, as here. Perhaps the artifactual lactase-2 activity arose from one of the two lactases in the lactase-1 peak ($K_d \simeq 0.14$) during storage of the biopsy specimens, or during papain solubilisation, which was carried out with another batch of this protease.

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