Intestinal β-Galactosidases

I. SEPARATION AND CHARACTERIZATION OF THREE ENZYMES IN NORMAL HUMAN INTESTINE

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ABSTRACT Previous studies based on work in the rat and preliminary experiments with human intestine have suggested that two β-galactosidases are present in small intestine, and it is believed that only one of these enzymes is a lactase important for the digestion of dietary lactose. The high prevalence of intestinal lactase deficiency in man prompted more complete study of these enzymes.

Human intestinal β-galactosidases were studied by gel filtration on Sephadex G-200 and Biogel P-300 as well as by density gradient ultracentrifugation. Gel filtration produced partial separation into three peaks of enzyme activity, but much activity against synthetic substrates was lost. Only the trailing peak with specificity for synthetic β-galactosides was completely separated from the other enzymes. Thus gel filtration was not a suitable preparative procedure for biochemical characterization.

Density gradients separated the enzymes more completely, and they were designated according to their sedimentation rates and further characterized. Enzyme I has a molecular weight of 280,000, pH optimum of 6.0, and specificity for lactose of at least five times that for cellobiose or synthetic substrates. A second lactase, enzyme II, possesses slightly greater activity against lactose than for some synthetic substrates and is incapable of splitting cellobiose. Further, it has a lower pH optimum (4.5) and is present in two molecular species (molecular weights 156,000 and 660,000). Enzyme III shows specificity only for synthetic β-galactosides but has a pH activity curve identical with enzyme I and a molecular weight of 80,000. Whereas human liver and kidney contain a β-galactosidase with the same biochemical characteristics as intestinal enzyme II, enzymes I and III appear to be peculiar to intestine, and enzyme I most probably represents the lactase of importance in the mucosal digestion of dietary lactose. The following paper considers this further in terms of the biochemical change in intestinal lactase deficiency.

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INTRODUCTION

The lactose in milk is a principal source of calories for children, and this disaccharide is important food for adult nutrition in many areas of the world. However, man's ability to digest lactose is limited, presumably because intestinal mucosal lactase is normally less active than the other disaccharidases (1, 2). In fact lactose is split so slowly that the hydrolysis step is rate limiting for the over-all process of hydrolysis-transport in both normal adults (3) and children.[1] This is in contrast to the hydrolysis of other oligosaccharides by intestinal mucosal enzymes which occurs rapidly enough to provide for saturation of the intestinal monosaccharide transport mechanism (3, 4). Perhaps partly because of the relatively low lactase levels in normal man, frank deficiency of intestinal lactase, and consequent intolerance to lactose-containing foods has been found to be frequent in individuals from certain population groups (5, 6) as well as in healthy people who appear to retain this defect as a permanent residual of previous intestinal disease (7, 8).

Information about the intestinal β-galactosidases is derived mainly from animal studies which have suggested that there may be two enzymes confined to the intestinal digestive-absorptive cell that are responsible for the hydrolysis of lactose. Doell and Kretchmer (9), using homogenates of rat intestine, found a discrepancy in the

[1] Genel, M., and G. M. Gray. To be published.

pH optimum of different fractions after differential centrifugation, and later Koldovský, Heringová and Jirová (10) demonstrated a higher pH optimum for enzyme localized to brush border than for that found in the remainder of the cell. But man differs from other animals studied as regards lactase. Whereas the rat shows a postsuckling decline of activity (9), human intestinal lactase remains at high levels into adulthood (1). Previous studies of human intestinal mucosa by Semenza, Auricchio, and Rubino (11) and Hsia, Makler, Semenza, and Prader (12) have suggested that there may be two intestinal β-galactosidases. Unfortunately these enzymes were not differentiated by study of their biochemical characteristics such as pH optimum, kinetics, or heat inactivation; furthermore, separation techniques other than gel filtration have not been applied to human intestine.

Our purpose was to determine the number and kinds of human β-galactosidases by study of their biochemical characteristics in order to ascertain their role in the digestion of dietary lactose with a view toward defining the enzymatic defect in lactase deficiency.

METHODS

Lactase assay was performed as previously described (8) except that two modifications were made to increase sensitivity: (*a*) lactose [2] substrate was 56 mmoles/liter in the reaction mixture, and (*b*) after incubation of the 0.2 ml reaction mixture for 10–60 min at 37°C followed by boiling for 2 min to stop the reaction, 0.5 ml of glucose oxidase reagent was added directly to the tube and the light absorption then determined 60 min later in a semimicro curvette (0.9 ml capacity, 10 mm light path). This provided a 5-fold increase in sensitivity with linearity for 1–10 μg of glucose in the reaction mixture.

Cellobiase activity was assayed as described for lactase except that substrate was 7 mmoles/liter in the reaction mixture. The cellobiose [3] had a significant glucose impurity that was removed by filtration on a 25 × 45 cm column of Sephadex G-15 [4] before its use as substrate.

6-bromo-2-naphthyl-β-galactosidase [5] *(BNGase) activity* was determined according to a modification (13) of Cohen's method (14) except that (*a*) 0.15 M citrate-phosphate buffer was used for the assay step, (*b*) substrate was 0.30 mg/ml of reaction mixture, and (*c*) sample and all reagents were decreased in volume by a factor of 4. After extraction of the coupled 6-bromo-2-naphthol [6] in chloroform and centrifugation for 10 min at 3000 rpm in an International CS centrifuge with a No. 831 head (International Equipment Co., Needham Heights, Mass.), samples were stoppered to prevent evaporation, heated in a 56°C water bath for 2–3 min, and transferred to a curvette holder that had been kept on the spectrophotometer power supply (60°C air temperature) between determinations. Heating prevented coalescence of water droplets on the walls of the curvettes. The reaction was linear when 1–10 μg of 6-bromo-2-naphthol was in the curvette.

Nitrophenyl-β-galactosidase activities were assayed as described by Lederberg (15) with *o*-nitrophenyl-β-galactoside [6] (ONPG) and *p*-nitrophenyl-β-galactoside [6] (PNPG) as substrates at 5 mmoles/liter in the reaction mixture.

Protein was measured by the Folin phenol method (16).

Source and preparation of intestine. Fresh specimens of intestinal mucosa were obtained either from surgery or autopsy and either processed immediately or wrapped in Parafilm (American Can Co., Neenah, Wis.) and stored at −20°C. Condition of intestinal villi and cells could be readily monitored by viewing preparations by light microscopy with minimal substage lighting. 10 different tissues with intact villi and cells were studied. Though activity was maximal in jejunum, the same enzymes were also found in duodenum and ileum. Behavior of the enzymes was the same whether obtained at operation or within 1–8 hr after death, and storage in the frozen state did not affect the enzymes. The tissue was scraped gently with a glass microscopic slide, homogenized in 0.02 M sodium–potassium phosphate (NaH_2PO_4, K_2HPO_4) buffer pH 5.5, and centrifuged at 100,000 *g* for 60 min. The supernatant material was then studied either without further preparation or after precipitation in 60% saturated $(NH_4)_2SO_4$ and resuspension in the same buffer. The particulate was solubilized by incubating 1 g of material with 20 mg of papain [7] and 10 mg of cysteine-HCL [8] per 6 ml of buffer for 30 min (11). Some loss (20–30%) of β-galactosidase activity occurred during this procedure. Both supernatant and solubilized particulate fractions contained appreciable enzyme activity, but the distribution varied greatly from tissue to tissue, with 10–70% of lactase and 40–80% of BNGase being in soluble form before papain treatment.

Gel filtration chromatography (17) was carried out at 4°C on a 2.5 × 45 cm column of cross-linked dextran (Sephadex G-200) [4] or polyacrylamide (Biogel P-300) [8] with 0.02 M sodium–potassium phosphate buffer, pH 6.0, or 0.02 M citrate phosphate buffer, pH 4.5. Longer columns did not improve separation of the enzymes. Blue Dextran [4] was used to determine void volume for Sephadex and, since the Blue Dextran adhered to the polyacrylamide, purified thyroglobulin [9] was used for Biogel columns. Flow rates were maintained at 2–5 ml/hr by a pressure of 10 cm of water, and the effluent was collected in 1.0- to 3.0-ml fractions.

Density gradient ultracentrifugation. 0.1 ml of supernatant or solubilized particulate material of the intestinal homogenate was layered on a 5.0 ml linear gradient of 5–20% sodium chloride or mannitol in 0.01 M sodium–potassium phosphate buffer, pH 5.5, and centrifuged at 44,000 rpm for 9 hr in a SW 50-L rotor in the Beckman model L preparative instrument. A small amount of mannitol precipitated during the centrifugation, but this did not affect the sedimentation of the enzymes. Fractions (0.1 ml) were collected at 4°C by gravity from the bottom of the tube after puncture by a hollow needle.

Determination of approximate molecular weights. Density gradient centrifugation was carried out as described above and crystalline alcohol dehydrogenase [10] and catalase [11]

[2] 4-*O*-β-D-galactopyranosyl-β-D-glucose, Merck & Co., Inc., Rahway, N. J.

[3] 4-*O*-β-D-glucopyranosyl-β-D-glucose, Sigma Chemical Co., St. Louis, Mo.

[4] Pharmacia Fine Chemicals Inc., Piscataway, N. J.

[5] Substrate used was 6-bromo-2-naphthyl-β-D-galactoside, Sigma Chemical Co., St. Louis, Mo.

[6] Sigma Chemical Co., St. Louis, Mo.

[7] Purified powder, Mann Research Labs Inc., New York.

[8] Calbiochem, Los Angeles, Calif.

[9] Courtesy of Dr. Gordon C. Sharp.

[10] From yeast, stock No. 340-26, Sigma Chemical Co., St. Louis, Mo.

[11] Stock No. C-100, Sigma Chemical Co., St. Louis, Mo.

were included as proteins of known molecular weight. Assay of these standard enzymes and calculation of approximate molecular weights of the intestinal β-galactosidases were carried out as described by Martin and Ames (18) for gradient centrifugation on 5–20% sucrose. In our experiments sucrose could not be used because it is attacked by intestinal sucrase. Preliminary gradient experiments for 3, 6, and 9 hr with 5–20% NaCl or mannitol revealed that the standard enzymes and intestinal β-galactosidases migrated linearly from the meniscus with respect to time, and hence the ratio of the distances travelled for any two of these proteins was always constant. If it is assumed that the molecules are roughly spherical proteins containing little lipid, an approximate molecular weight (MW) can be obtained from the sedimentation constants (S) (18):

$$R = \frac{\text{Distance Migrated}_{\text{unknown}}}{\text{Distance Migrated}_{\text{standard}}} = \frac{S_{\text{unknown}}}{S_{\text{standard}}} = \left(\frac{MW_{\text{unknown}}}{MW_{\text{standard}}}\right)^{\frac{1}{3}}$$

$$MW_{\text{unknown}} = MW_{\text{standard}} \times R^{\frac{1}{3}}$$

pH Optimum studies. The relationship of enzyme activity to pH was determined for the enzymes isolated from gel filtration and density gradient techniques. The pH of the substrate-enzyme incubation mixture was determined by a combined calomel-glass electrode, the following buffers being used at 0.07 mole/liter: acetate and citrate-phosphate (pH 3.0–5.0), sodium–potassium phosphate and sodium maleate (pH 5.5–6.8), sodium–potassium phosphate (pH 6.8–7.5), and glycine-NaOH (pH 7.5–8.5). All buffers contained 154 meq/liter of NaCl. At least two buffers were used at the upper and lower points of each pH range. No inhibitory or stimulatory effect on activity was found for any of these buffers.

RESULTS

Gel filtration chromatography. Sephadex G-200 filtration with 0.02 M sodium–potassium phosphate buffer pH 6.0 demonstrated three peaks of β-galactosidase activity for intestinal supernatant (Fig. 1). Recovery in the effluent was 70–95% of the lactase added, but only 30–60% of the BNGase irrespective of column conditions even though protein recovery was complete. Similar results were found for solubilized particulate material. The first peak was eluted immediately after the void volume and contained activity against both lactose and the artificial substrate BNG. The second peak of activity (peak A) followed shortly thereafter and was active only against lactose. There was also a trailing peak that showed activity for the artificial substrate BNG but not for lactose. Unfortunately, glucose was also eluted in a broad peak from the column close to, and in some experiments coincident with, the third peak of activity (Fig. 1). It was found in concentrations comparable to those produced in the lactase assay for the first two peaks, thereby producing high blanks for that assay and making determination of small amounts of lactase activity very difficult to detect. This glucose probably results from attack on the dextran gel column by α-dextrinase (isomaltase) in the intestinal tissue (19).

Polyacrylamide gel (Biogel P-300) was then used in a similar manner as Sephadex and was found to be more satisfactory. Recovery of enzymes and total protein was the same as for Sephadex. Fig. 2 demonstrates that the three peaks of β-galactosidase activity were in approximately the same position as that found with Sephadex gel filtration. However, the leading peak was in the void volume itself rather than appearing after it, and more important, no glucose was released from the column. The specificity of the enzymes was the same as that found on Sephadex and activity per unit of protein was increased about 5-fold by gel filtration.

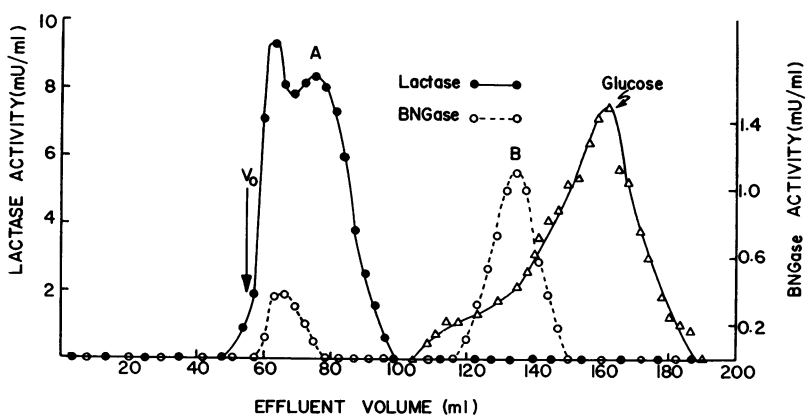

FIGURE 1 Sephadex G-200 chromatography of intestinal supernatant material. 1 ml containing 250 mU of lactase, 80 mU of BNGase, and 50 mg of protein was layered on the 45 × 2.5 cm column, and the effluent rate was 2.0 ml/hr. V_o = void volume as determined by Blue Dextran. mU = millimicromoles per minute. Glucose is plotted with units equivalent to those for the lactase assay (millimicromoles per milliliter).

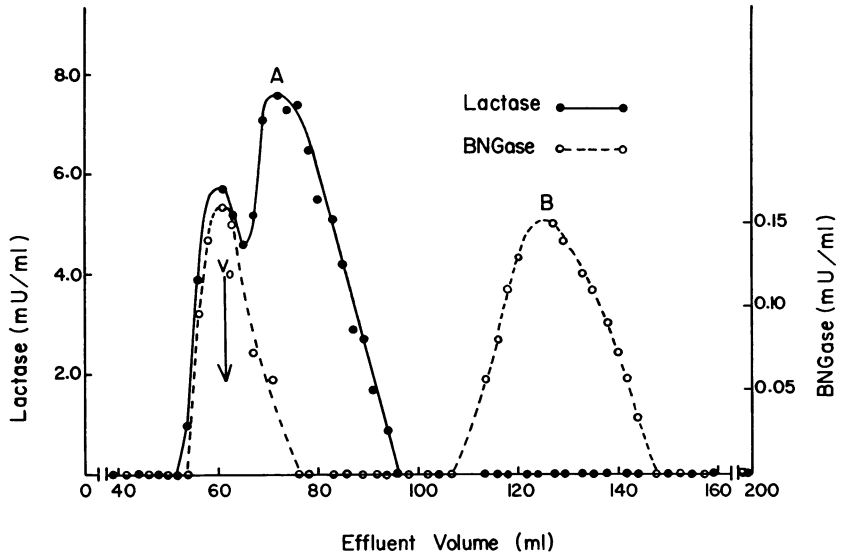

FIGURE 2 Biogel P-300 chromatography of intestinal supernatant material. 1 ml containing 190 mU of lactase, 20 mU of BNGase, and 40 mg of protein was used. Conditions are otherwise the same as for Fig. 1. Void volume was measured with purified thyroglobulin.

The pH activity of gel filtration peaks. In order to determine whether the two lactases found on gel filtration represented different enzymes, activities were studied as a function of pH of the reaction mixture of peaks V_0 and A isolated from polyacrylamide gel filtration. Fig. 3 shows that though both peaks showed the same pH optimum of about 5.5, the void volume activity had a broader pH activity curve in the acid range, and subtraction of the two curves resulted in a difference curve with optimal pH at 4.5. This may indicate that both peaks of lactase activity have an enzyme in common,

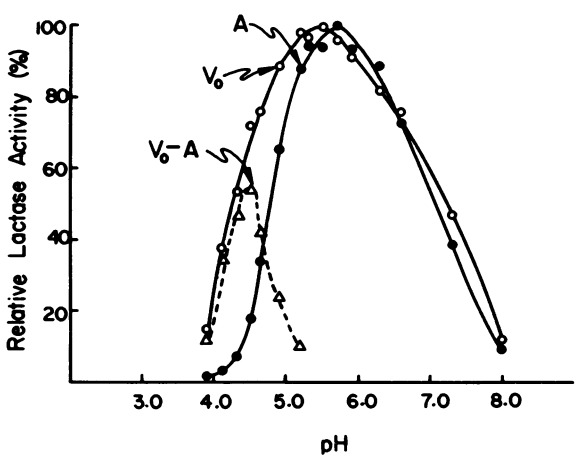

FIGURE 3 pH activity relationships for lactase isolated from void volume (V_0) and peak *A* of Biogel P-300 gel filtration experiment. $V_0 - A$ designates difference curve. See Method section for details of buffers.

a finding not unexpected since they were eluted very close together. Furthermore, these pH activity differences also suggest that the void volume contains a second enzyme with a lower pH optimum. When this is taken together with the fact that BNGase activity in void volume was found to have a pH optimum identical with that of lactase for the V_0-A difference curve, it appears that the second enzyme in void volume might be a β-galactosidase with specificity for both lactose and BNG at low optimal pH.

Density gradient ultracentrifugation of intestinal preparations. The incomplete separation of the intestinal β-galactosidases by gel filtration chromatography prompted the use of other techniques. Fig. 4 compares density gradient centrifugation for supernatant and solubilized particulate fractions of human intestine. Supernatant material separated on 5–20% sodium chloride or mannitol gradients into three distinct peaks of β-galactosidase activity (Fig. 4, top). Mannitol showed essentially identical results as sodium chloride. The initial peak (*I*) showed activity against lactose and cellobiose, and the second peak (*II*) was able to hydrolyze both lactose and BNG. The trailing enzyme (*III*) was incapable of splitting lactose but did hydrolyze BNG. Separation of the enzymes was more complete than that found by chromatography on Sephadex or Biogel and, notably, the first two peaks of β-galactosidase activity were in the reverse order from that found on gel filtration. Particulate preparations contained two β-galactosidases that appeared identical with enzymes I and II of the intestinal supernatant, but enzyme III was either absent or pres-

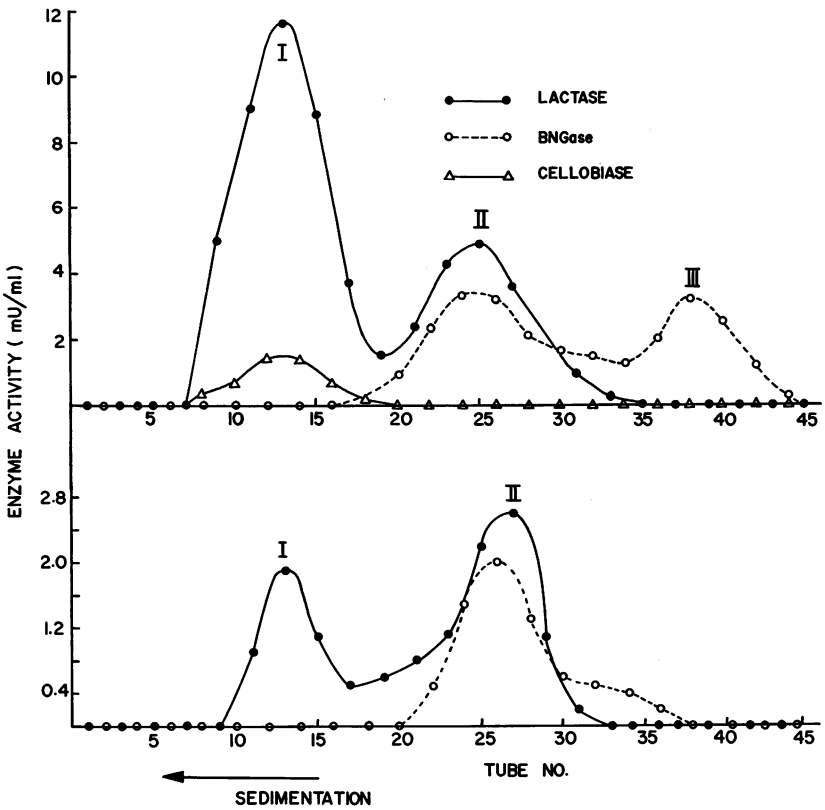

FIGURE 4 Density gradient ultracentrifugation on 5–20% NaCl at 44,000 rpm (158,000 *g*) for 9 hr. 0.1 ml of supernatant containing 10 mU of lactase, 1.5 mU of cellobiase, 6.5 mU of BNGase, and 1.0 mg of protein was placed on the 5 ml gradient (top of figure), and an adjacent tube with the same volume of solubilized particulate from the same tissue containing 3.0 mU of lactase, 1.8 mU of BNGase, and 0.5 mg of protein was run concomitantly (bottom). Note that both preparations show the two enzymes with lactase activity. Although not shown, enzyme I in particulate also had specificity for cellobiose.

ent in relatively low amounts (Fig. 4, bottom). Intestinal preparations from a 2 month old child showed the same pattern for the β-galactosidases as found for adult tissue (Fig. 5). As was found for gel filtration, the specific activity of all enzymes was increased 5-fold by density gradient centrifugation.

Density gradient centrifugation of liver and kidney preparations. Lactase from human liver or kidney showed the same density gradient pattern as intestinal enzyme II (Fig. 6). No other β-galactosidase was found.

Transfer of enzymes from gel filtration to density gradients. The discrepancy between gel filtration and density gradient techniques for enzymes I and II despite use of the same starting material suggested that aggregation or fragmentation of the β-galactosidases may have occurred during one of these separation procedures. Therefore, the material eluted in the Biogel P-300 void volume (fractions 52–62 of Fig. 2) was concentrated in a dialysis bag under reduced pressure, dialyzed against 0.02 M sodium–potassium phosphate buffer, pH 5.5, and the final 0.1 ml placed on a density gradient. As shown in Fig. 7, enzyme I was found in the expected position, but the lactase-BNGase was of much greater molecular size than enzyme II that is present in supernatant or solubilized particulate starting material. Transfer of the other gel filtration peaks of activity (A and B) to density gradients revealed peak A to be enzyme I and peak B to be enzyme III, but a β-galactosidase with migration characteristics of enzyme II was not found.

Identification of a labile macromolecular form of enzyme II by short-term density gradient centrifugation. Since a large enzyme in intestinal starting material might migrate to the bottom of the gradient tube during a 9 hr centrifugation and become inactivated by the high concentration of NaCl, several fractions from the bottom of the tube were pooled and dialyzed against 0.02 M sodium–potassium phosphate buffer, pH 5.0, but no β-galactosidase activity was found after removal of the salt.

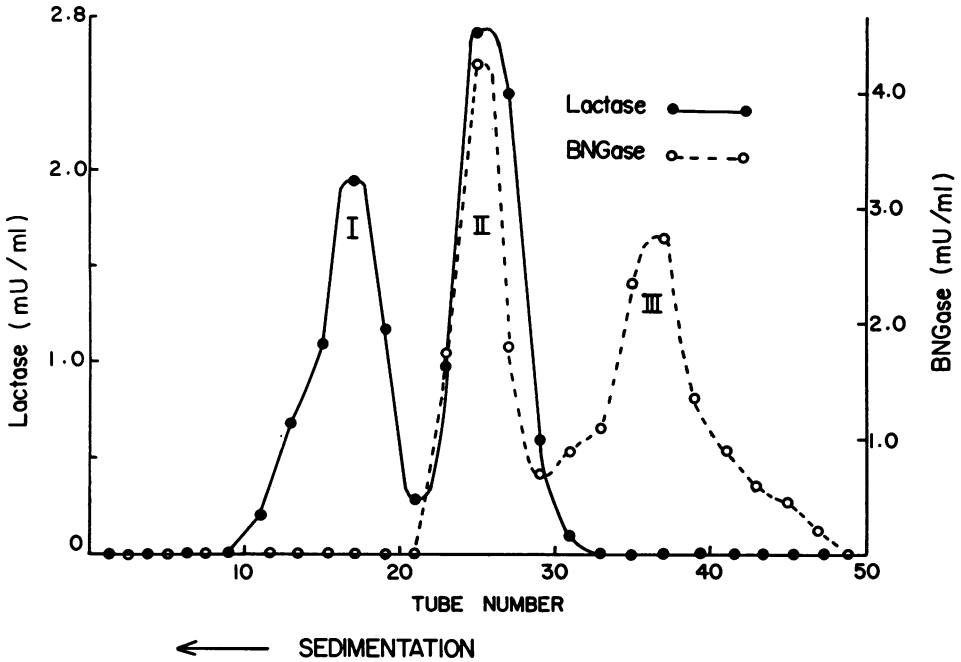

FIGURE 5 Density gradient ultracentrifugation of intestinal supernatant from a 2 month old child. Conditions were the same as described in Fig. 4. 0.1 ml containing 2.5 mU of lactase, 2.3 mU of BNGase, and 0.4 mg of protein was added.

But irreversible denaturation of the enzyme could still have occurred, and short-term (2- and 4-hr) density gradient centrifugation experiments did reveal a β-galactosidase at 2 hr that sedimented much more rapidly than enzymes I, II, and III (Fig. 8 top). Its activity was 10–90% (average 25%) relative to enzyme I. This macromolecular enzyme was apparently inactivated as it moved into more concentrated NaCl since it was not found in 4-hr gradients (Fig. 8 bottom) even after removal of the NaCl. The enzyme could be completely separated from the other β-galactosidases by centrifugation of intestinal preparations on a 12 ml linear gradient of 5–20% NaCl in a SW41-Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 40,000 rpm for 6 hr. After pooling of appropriate fractions from six gradient tubes and dialysis at 4°C for 4 hr against 2 M urea and 0.001 M mercaptoethanol to effect denaturation, it was precipitated in 60% saturated $(NH_4)_2 SO_4$ and

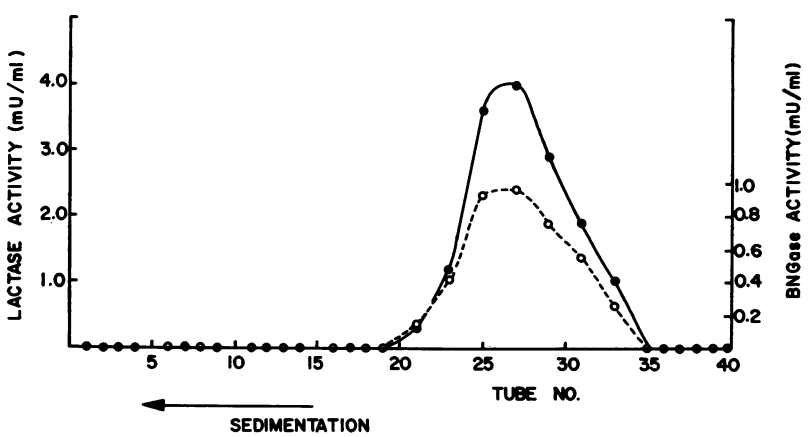

FIGURE 6 Density gradient ultracentrifugation of human liver supernatant. Conditions were the same as in Fig. 4. 3.5 mU of lactase, 1.0 mU of BNGase, and 0.7 mg of protein were placed on the gradient.

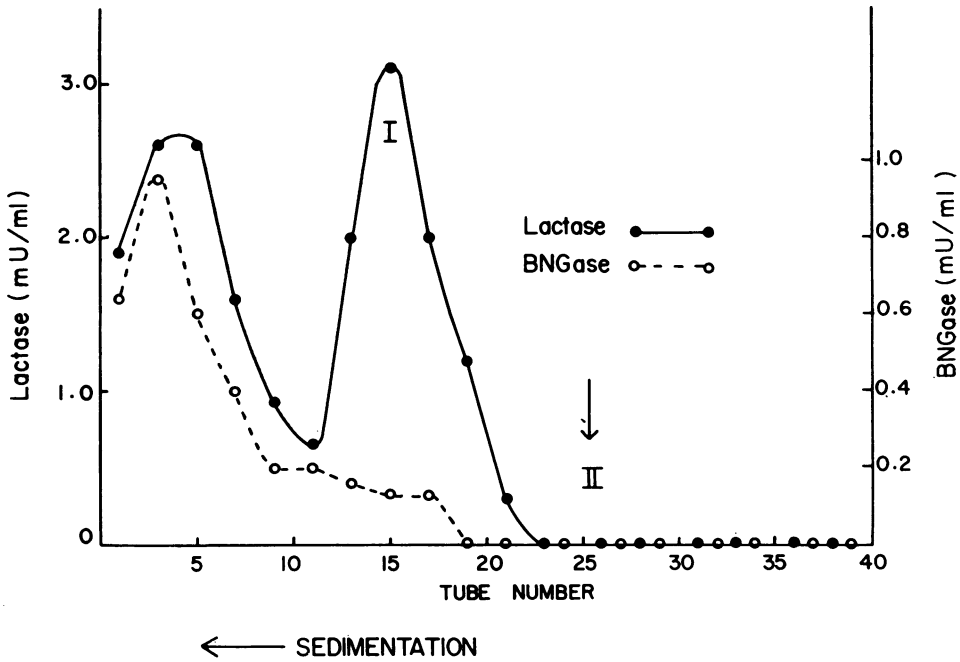

FIGURE 7 Density gradient ultracentrifugation of void volume peak from a Biogel P-300 gel filtration experiment. Conditions are as in Fig. 4. See text for details.

washed twice with 5 ml of additional 60% saturated $(NH_4)_2 SO_4$ to remove any residual urea and mercaptoethanol. The precipitate was then dissolved in 0.5 ml of 0.02 M $NaKPO_4$ buffer, pH 5.0. Approximately 70% of the original enzyme activity was recovered after exposure to and removal of urea and mercaptoethanol. When the dissolved material was studied by the conventional density gradient technique (0.1 ml layered on 5 ml of 5–20% NaCl and centrifuged at 44,000 rpm for 9 hr), a small peak of β-galactosidase activity was found in the bottom of the tube, as might have been expected. Notably however, a second peak of lactase-BNGase activity was located at fraction number 25, the usual position of enzyme II. Thus it appeared that enzyme II might be the active monomeric unit of the macromolecular β-galactosidase. This was supported by comparative studies on pH activity, substrate specificity, and molecular weight of the enzymes, as outlined below.

Characteristics of the intestinal β-galactosidases isolated from density gradients

Each peak of β-galactosidase activity was collected from appropriate density gradient centrifugation experiments such as shown in Figs. 4, 5, and 8. The enzymes were then dialyzed at 4°C against 0.01 M sodium–potassium phosphate buffer with 154 mEq of NaCl at pH 5.5 for 24 hr with several buffer changes. Addition of saline to the buffer prevented significant loss of activity, and the isolated enzymes were then studied for a variety of biochemical characteristics as outlined below.

Substrate specificity. Table I outlines the relative activity of the three enzymes against five different β-galactosides. Activity against lactose was arbitrarily assigned unity for enzymes I and II, and BNG was the reference substrate for enzyme III. The enzymes showed a great deal of difference in their substrate specificity. Notably, enzyme I was five times more active against lactose than against cellobiose or artificial substrates and had no detectable ability to hydrolyze BNG. Although enzyme II also showed much activity against lactose, it had appreciable activity against the three synthetic substrates (BNG, ONPG, PNPG) with the most activity found for one of the artificial substrates, PNPG. The macromolecular β-galactosidase showed specificity for the same substrates with very similar relative activities as those found for enzyme II. This is further evidence that it is a macromolecular form of enzyme II. Enzyme III showed no specificity for either of the natural sugars, but was quite active against BNG and the other artificial substrates, especially PNPG. As can be readily seen from Table I, the use of lactose, cellobiose, and BNG as substrates was of help in comparing and differentiating the three β-galactosidases.

pH activity relationships. Enzyme activity is shown as a function of pH in Fig. 9. The optimal pH for enzyme II of 4.5 was appreciably lower than that for enzyme I (pH 6.0) and it was found to have an essentially

identical pH activity curve whether lactose or BNG was used as substrate. Although not shown, the macromolecular β-galactosidase had a pH activity curve identical with enzyme II, again suggesting that it is a different large molecular unit of the same enzyme. It is also interesting that enzyme III with BNG as substrate showed a pH activity curve that is essentially equal to that for enzyme I with lactose as substrate. Lactase from human liver, which migrated identically to enzyme II on density gradients (Fig. 6), had the same pH activity curve as enzyme II from intestine (Fig. 9, top).

Kinetic studies. The relationship of substrate concentration to rate of the hydrolytic reaction was determined at optimal pH for the enzymes isolated from density gradients. Data were analyzed by the double reciprocal method of Lineweaver and Burk (20) to determine maximal rate of hydrolysis (V_{max}) and Michaelis constant (K_m). Table II shows the K_m values for the three intestinal enzymes. The K_ms for ONPG and PNPG were not determined since all three enzymes showed some specificity for these substrates, and therefore further study of the characteristics using the nitrophenyl galactosides did not serve to differentiate the enzymes. Interestingly enough, the K_m for both enzyme I and II with lactose as substrate was essentially identical (18–19 mmoles/liter). However, these enzymes differed in that enzyme I hydrolyzed cellobiose with a very low K_m (approximately one-tenth of that for lactose), whereas enzyme II showed specificity for BNG. The K_m for enzyme III with BNG as substrate was consistently

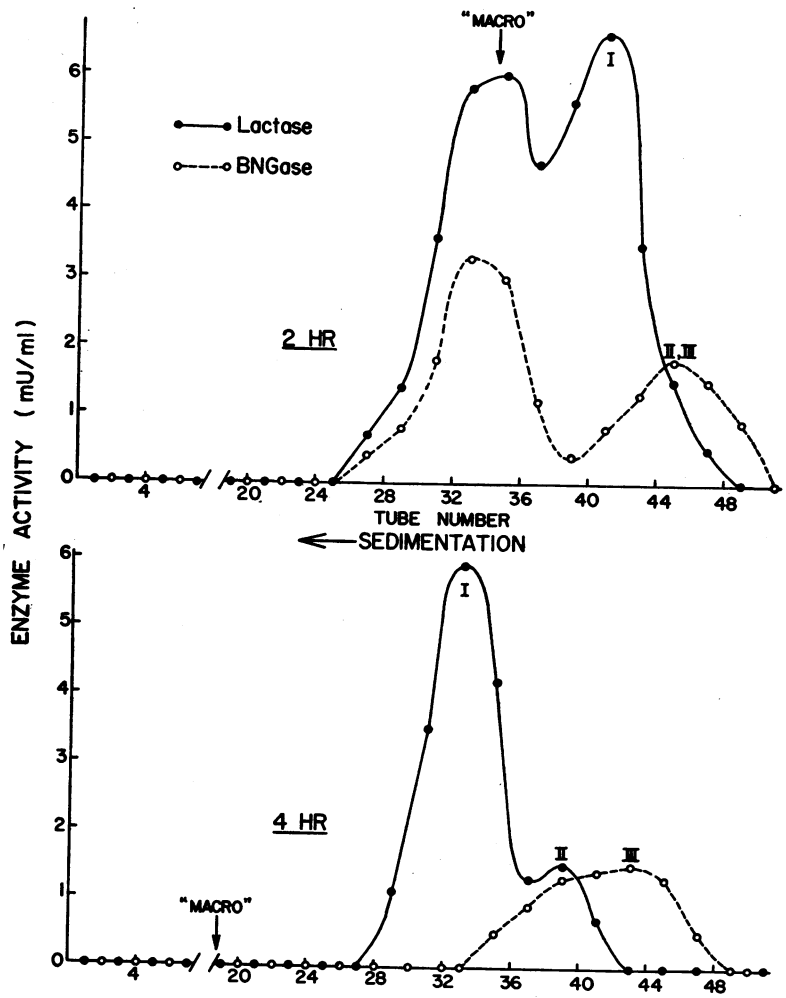

FIGURE 8 Short-term density gradient ultracentrifugation demonstrating a macromolecular β-galactosidase ("macro") at 2 hr that is not detectable by 4 hr. Enzymes I, II, and III can be seen to be migrating at rates predictible from 9-hr gradient experiments. Except for time, all other conditions were the same as in Fig. 4.

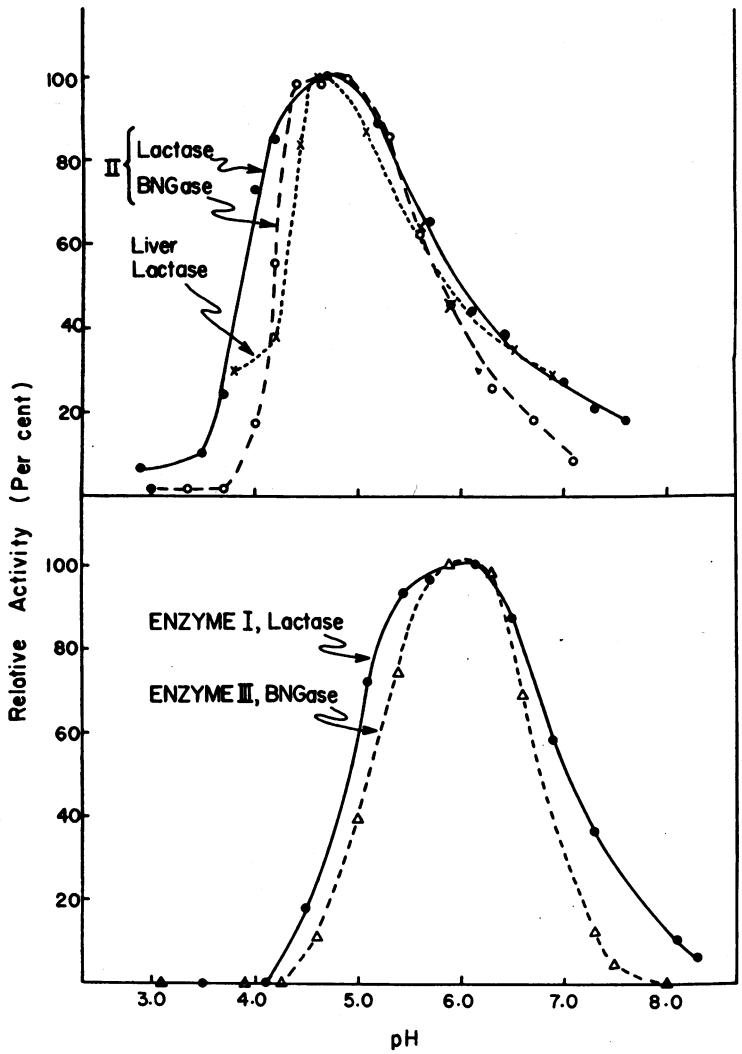

FIGURE 9 pH activity relationships for intestinal β-galactosidases and liver lactase isolated from density gradient experiments. See Methods for preparative details and buffers used.

twice that for enzyme II. Competitive inhibition occurred between BNG and lactose for enzyme II (Fig. 10), a finding verifying that this enzyme is capable of splitting the two substrates at a single active site.

Determination of molecular weight from density gradients. The approximate molecular weights of the intestinal β-galactosidases were determined from their migration in a 5 ml density gradient as compared to that for alcohol dehydrogenase and catalase, crystalline enzymes of known molecular weight. Enzymes I and II differ not only in their pH optima and substrate specificity but also, as Table III demonstrates, have an appreciable disparity in their sedimentation constants and molecular weights. Enzyme I was found to have a molecular weight of 280,000. Enzyme III, which was shown (Fig. 9) to have the same pH activity curve as enzyme I, had a molecular weight that was approximately one-fourth that of enzyme I. The molecular weight of enzyme II was intermediate at 156,000. The large form of this enzyme was determined to have a molecular weight of 660,000 as estimated from a 6 hr centrifugation in the SW41-Ti rotor at 40,000 rpm, a fact suggesting that the macromolecular form of the enzyme appears to be a tetramer of the active monomer found in the conventional density gradient experiments.

Heat inactivation. Exposure of the isolated β-galactosidases to a temperature of 46°C followed by additional heating at 56°C resulted in progressive inactivation of all three enzymes. Aliquots were removed for assay of residual activity at 10-min intervals. In con-

TABLE I

*Specificity and Relative Activity of Intestinal β-Galactosidases**

Enzyme No.	Substrate				
	Lactose	Cellobiose	BNG	ONPG	PNPG
I	1.0	0.11	0‡	0.07	0.2
II	1.0	0‡	0.5	0.7	2.7
"Macro"§	1.0	0‡	0.5	0.6	2.2
III	0‡	0‡	1.0‖	4.7	23

BNG, 6-bromo-2-naphthyl-β-galactose; ONPG, *o*-nitrophenyl-β-galactoside; PNPG, *p*-nitrophenyl-β-galactoside.

* Unity arbitrarily assigned to activity against lactose.

‡ Less than 0.01 (1%) of reference activity.

§ Macromolecular enzyme found in 2-hr density gradient experiments.

‖ BNGase activity used as reference for enzyme III.

trast to differential destruction by heat reported for intestinal maltases (21), the inactivation curves for the β-galactosidases were similar for all three enzymes (Fig. 11), and this technique did not prove to be a useful means of distinguishing them.

DISCUSSION

Previous studies (11, 12) of human intestinal lactases have emphasized that the "specific" lactase was mainly in the particulate fraction, the "nonspecific" enzyme being more soluble. Our finding that the intestinal β-galactosidases are present in variable degree (enzyme II was 20–70% as active as enzyme I) in both supernatant and papain-digested particulate (Fig. 4) points out that this crude method of separation into supernatant and particulate by 100,000 *g* centrifugation cannot be relied upon to separate the enzymes. The amount of membrane-bound enzyme probably depends on many variables including the condition of intestine before removal, time interval before freezing, and rapidity of freezing and thawing. At any rate it is encouraging to know that both the enzymes in solution and those solubilized by papain appear to have the same molecular weight, substrate specificity, and pH optimum, and it seems probable that

TABLE II

Michaelis Constants (K_m) for Intestinal β-Galactosidases*

Enzyme No.	Substrate		
	Lactose	Cellobiose	BNG
I	18	1.6	
II	19		0.2
III			0.4

BNG, 6-bromo-2-naphthyl-β-galactose.

* Units for K_m = mmoles/liter.

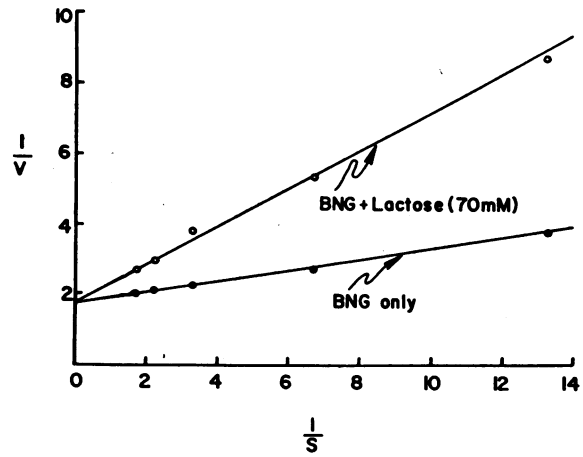

FIGURE 10 Lineweaver-Burk plot for intestinal enzyme II demonstrating competitive inhibition of BNGase activity by lactose. V = rate of hydrolysis; and S = BNG concentration. 70 mM lactose in the reaction mixture served as the inhibitor.

the native β-galactosidases can be isolated from either type of intestinal preparation.

Until now, gel filtration chromatography has been the sole method of separation of human intestinal β-galactosidases, two widely separated peaks of lactase activity having been reported by a single group of investigators (11, 12). There is a definite discrepancy between our results on gel filtration either with Sephadex or Biogel as compared to these reports. First of all, we found three peaks of β-galactosidase activity with the leading peak containing two lactases (Figs. 1–3), one of which was capable of splitting BNG at a low pH optimum; the other hydrolyzed primarily lactose and its presence in the initial peak represented spillover from its primary position (peak A) just beyond the void volume (Figs. 1, 2). Finally, rather than a lactase that was slow to elute off the column, we found a β-galactosidase that had specificity only for artificial substrates (peak B, Figs. 1, and 2). Glucose at relatively high concentrations was eluted from Sephadex columns almost coincident with

TABLE III

*Comparison of Intestinal β-Galactosidases**

Enzyme No.	Typical substrates	pH Optimum	Sedimentation constant	Molecular weight‡
I	Lactose, cellobiose	6.0	12.6	280,000 ±4,000
II	Lactose, BNG	4.5	8.2, 21	156,000 ±5,000
				660,000 ±10,000
III	BNG	6.0	5.4	80,000 ±4,000

BNG, 6-bromo-2-naphthyl-β-galactose.

* Data from density gradient ultracentrifugation.

‡ Mean ±SE for 10 different tissues.

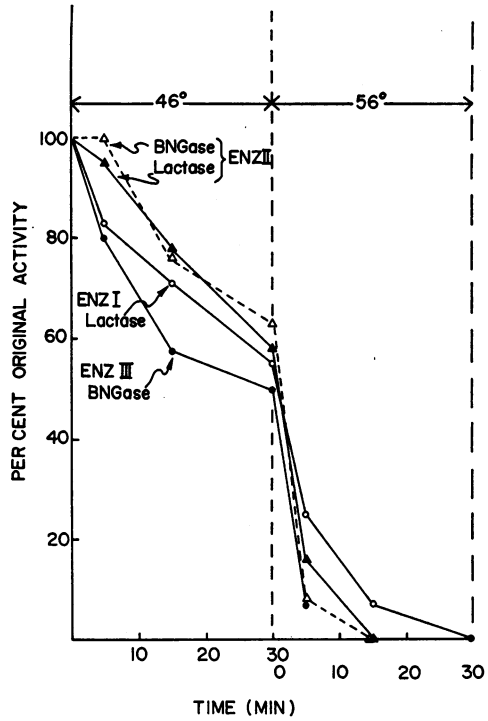

FIGURE 11 Heat inactivation of intestinal β-galactosidases. Preincubation was at temperatures (±0.1°C) and time shown before assay for residual activity.

this trailing β-galactosidase (Fig. 1). This glucose is probably the result of attack on the dextran gel column by an α-dextrinase (isomaltase) that is in the intestinal preparations studied (19). Since there was no detectable glucose in the starting material placed on the Sephadex column, there seemed to be no ostensible reason for determining the glucose content on the fractions used for lactase assays. But failure to determine glucose blanks on all fractions could lead to the inadvertent conclusion when glucose was found after disaccharidase assay that a lactase and cellobiase are present with an activity ratio of 2:1.[12] Semenza, Auricchio, and Rubino (11) reported two lactase-cellobiases by gel filtration, but the trailing enzyme had a lactase-cellobiase activity ratio of 2:1 and was at a position where we found glucose in the eluate. Furthermore, our density gradient experiments demonstrated that only a single lactase had cellobiase activity (Fig. 4). Considering the problem of incomplete separation of the enzymes as well as the appreciable loss of BNGase activity on gel filtration, other techniques had to be utilized in order to characterize these enzymes.

[12] Since two moles of glucose are released from hydrolysis of cellobiose and only one mole from lactose, the molar glucose value must be divided by 2 to determine cellobiase activity.

Density gradient centrifugation resulted in excellent separation of the intestinal β-galactosidases, all having different molecular size. Determination of molecular weight by the density gradient method (18) assumes that the enzymes are approximately spherical in shape and that they contain principally protein and little lipid. Since intestinal membrane proteins may contain lipid, the molecular weight values (Table III) may have to be altered when the exact chemical structure of each intestinal β-galactosidase becomes known. However, density gradient estimations have been found to be remarkably accurate for many enzymes in a mixture of proteins (18) such as that used in our experiments.

Of the three β-galactosidases in human intestine only two are capable of splitting lactose and presumably both could take part in the digestion of dietary lactose. However, these two enzymes have very different biochemical characteristics. Enzyme I is capable of hydrolyzing lactose at rates five times more rapid than it can hydrolyze the artificial substrates ONPG and PNPG, and it has no detectable activity against BNG (Table I). Further, it is capable of splitting cellobiose, the β-glucoside of corresponding configuration to lactose. Enzyme II, on the other hand, hydrolyzes BNG and other artificial substrates at rates that approach or exceed those for lactose, and it is not able to split cellobiose. The pH activity curves of these two enzymes are also quite different, with enzyme I having an optimal pH at 6.0 and enzyme II at pH 4.5.

Since enzyme I is peculiar to intestine, has maximum specificity for lactose rather than other β-galactosides, and has a pH optimum close to that of intraluminal intestinal fluids, it is quite likely that it is the enzyme responsible for digestion of dietary lactose. This concept is compatible with the recent demonstration by Koldovský et al. (10) that brush border lactase in the rat has a pH optimum of 6.0.

Short-term density gradient experiments demonstrated a macromolecular β-galactosidase (Fig. 8) with characteristics of pH dependence and substrate specificity (Table I) the same as for enzyme II. Furthermore, denaturation with urea and mercaptoethanol followed by renaturation produced formation of an enzyme with not only the same biochemical characteristics as enzyme II but also with the same molecular weight. This strongly suggests that there are two molecular forms of enzyme II. Comparison of molecular weights of the large (660,000) and small (156,000) species indicates that enzyme II probably occurs as both a monomer and tetramer.

The fact that both human liver and kidney contain an enzyme that appears identical with intestinal enzyme II (Fig. 6) suggests that this is a ubiquitous enzyme perhaps localized to lysosomes. The physiological role of enzyme II is unknown, but human liver β-galactosidase

has recently been shown to be deficient in patients with generalized gangliosidosis (22), and liver homogenates from such patients are unable to release galactose from a monosialoganglioside (22). Thus enzyme II may be important in ganglioside metabolism. Whether it plays a role in the digestion of dietary lactose is dubious, since intact disaccharide is considered incapable of quantitatively entering the absorptive cell (4, 23, 24), a requirement before it could come in contact with lysosomal enzymes. There is no doubt, however, that isolated enzyme II is capable of splitting lactose and could be expected to do so in vivo if intact lactose passed beyond the brush border into the depths of the intestinal digestive-absorptive cell.

Our finding that infants (Fig. 5) have the same constituent β-galactosidases as adults (Fig. 4) is not consistent with the suggestion by Huang and Bayless (25) that there is an "infantile" enzyme that wanes during childhood and an "adult" enzyme that develops later in life. Rather it is probable that man is born with a full complement of intestinal β-galactosidases two of which are lactases.

Johnson (26) has demonstrataed that 60A knobs attached to hamster intestinal brush border contain maltase and sucrase, and that both the structural characteristics and enzyme activity can be eluted shortly after Blue Dextran on a Biogel P-200 column. Our gel filtration experiments cannot be directly compared with his, however, because we used different pore size of gel (P-300) and found it necessary to use thyroglobulin rather than Blue Dextran to determine the void volume. It is of course possible that some recognizable structure is associated with the β-galactosidases. Eichholz (27) recently found that rat brush border membrane preparations release different sized disaccharidases depending on the papain treatment time. Since the molecular size of the enzymes in our study was the same for the untreated soluble supernatant as for the papain-solubilized particulate (Fig. 4), it seems likely we have studied the native β-galactosidases.

Enzyme III is a most interesting β-galactosidase with specificity only for synthetic substrates. It is present in intestine (Figs. 4 and 5) but not in liver or kidney (Fig. 6) and has the same pH activity curve as enzyme I, the lactase peculiar to intestine. This enzyme may be comparable to that isolated from rat intestine (28) by ion-exchange chromatography, but no such β-galactosidase has been found previously in man. This lightweight β-galactosidase may be related to enzyme I and is further considered in the accompanying paper.

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