

STUDIES ON THE ULTRASTRUCTURAL LOCALIZATION OF INTESTINAL DISACCHARIDASES

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It is now firmly established that disaccharidases are an intrinsic part of the mammalian intestinal (1) and renal (2) brush border. Isolated brush borders of hamster and rabbit intestine (3-6) are studded with knobs (65 Å in diameter) or doughnut-shaped particles (45-110 Å in diameter) respectively, when viewed as negatively stained preparations. Isolated brush borders of rabbit kidney do not have such particles (2). Treatment of the intestinal brush borders with papain removes the particles and solubilizes the disaccharidases, sucrase and maltase (3-6). It was concluded that these enzymic activities are localized on the knobs, in the glycocalyx, external to the plasma membrane of the microvillus. Crane (7, 8) has incorporated this view in his schematic models for digestive-absorptive function.

Recent investigations on sugar transport systems in kidney and intestine (2, 9) prompted us to question the conclusion that the different disaccharidases of the brush border are localized exclusively on particles external to the microvillus membrane. Moreover, it is possible that the results of the earlier studies (3-6) may have been overinterpreted, since the precise conditions required to remove (a) the enzymic activities and (b) the 60 Å particles were not described. For example, the number of units of papain used to treat a particular quantity of brush borders was not specified. Thus, it is not clear whether or not the disaccharidases and the knobs were removed at the same time. The present paper is a correlated kinetic study of the papain treatment of hamster intestinal brush borders. The removal of disac-

charidase activities and of the 60 Å particles (as determined by electron microscopy) have been monitored simultaneously with time. The results show that the disaccharidases cannot be correlated quantitatively with the number of knobs on the brush border membrane.

METHODS AND MATERIALS

Disaccharidase activities were measured at 37°C with an incubation mixture containing 30 mM disaccharide, 1.13 mM adenosine triphosphate (ATP), 1.13 mM MgCl₂, 1.61 mM NADP⁺, 20 mM sodium phosphate, pH 6.0, and excesses of hexokinase and glucose-6-phosphate dehydrogenase. In the case of sucrase, the concentration of Na⁺ was increased to a minimum of 50 mM by the addition of NaCl in order to obtain maximal velocities (10). Isomaltase, also activated by Na⁺ (11), was assayed at 33 mM Na⁺. Reduction of NADP⁺ was followed spectrophotometrically. Control experiments showed that the initial rates observed in the presence or absence of the mixture of ATP, MgCl₂, and NADP⁺ were the same. Measurements were done in triplicate, and the reproducibility of replicates was within 1-2%.

The intestinal brush borders of adult male golden hamsters were isolated by the method of Miller and Crane (12), as modified by Forstner et al. (13). Minor changes of this procedure were made. After homogenization in the Waring Blendor, the homogenate was passed first through a thin layer of Pyrex wool to remove large particles and then through 200-mesh bolting silk. Two centrifugations at 100 g for 10 min at 0°C were made to sediment the brush borders from contaminating small fragments. Phase-contrast microscopy (×400) was used to estimate the effectiveness of the procedure.

Protein was measured by the method of Lowry et al. (14).

Papain was covalently complexed to cellulose by the method of Eichholz (15) and is referred to as papain-cellulose in this paper. The specific activity of the complex was 0.0423 μ moles of α -benzoylarginine ethyl ester hydrolyzed per minute per milligram of complex, at 23°C. Isolated brush borders were incubated with papain-cellulose, as follows: Papain-cellulose (51 mg) was activated in 2.5 ml of freshly prepared 100 mM cysteine containing 20 mM sodium EDTA, final pH 6.5, at 0°C for 30 min (16). To this was added 50 ml of 12 mM potassium phosphate, pH 6.5, containing 5 mM sodium EDTA and 2.5 mM cysteine. This mixture was brought to 37°C and 4.0 mg of brush border protein in 2.5 ml of 5 mM sodium EDTA, pH 7.4, was added to start the hydrolysis. The final concentration of K^+ was 14.4 mM (15). At timed intervals, 10 ml samples were removed from the incubation. The papain-cellulose was rapidly removed by centrifugation at 100 g for 1 min at 0°C. The supernatant was recentrifuged at 43,000 g for 40 min at 0°C. The supernatant was decanted and the small pellet was suspended in 1.0 ml of sodium EDTA, pH 7.4. Samples were taken for electron microscopy and each fraction was assayed for the different disaccharidases. In the experiments in which soluble papain (2.15 units at 23°C/4.0 mg brush border protein) was used, the procedure was the same except that the initial centrifugation at 100 g for 1 min was omitted.

Samples were negatively stained for electron microscopy with 1% potassium phosphotungstate, pH 6.5. Preparations were examined with a Siemens-Halske Elmiskop I electron microscope.

Disaccharides were obtained from commercial sources. When necessary, they were purified from glucose by the glucose oxidase method (17). ATP, NADP⁺, and crystalline hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer-Mannheim. The hexokinase preparation contained some sucrase activity and the appropriate control was always conducted. Phosphoglucose isomerase, assayed at pH 6.0, was not found as a contaminant of the hexokinase or glucose-6-phosphate dehydrogenase. Papain was bought from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

The solubilization of the different disaccharidases of the brush border with time of treatment with papain-cellulose is described in Fig. 1. It is evident that the total activity of each enzyme (soluble plus insoluble) remains constant throughout the period of digestion, and also that the disaccharidases, sucrase, isomaltase, and maltase, are largely released from the brush border by papain-

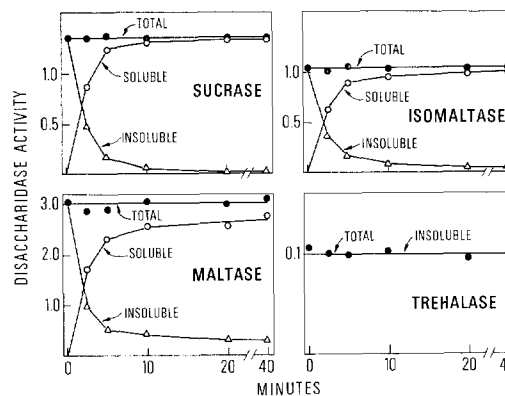
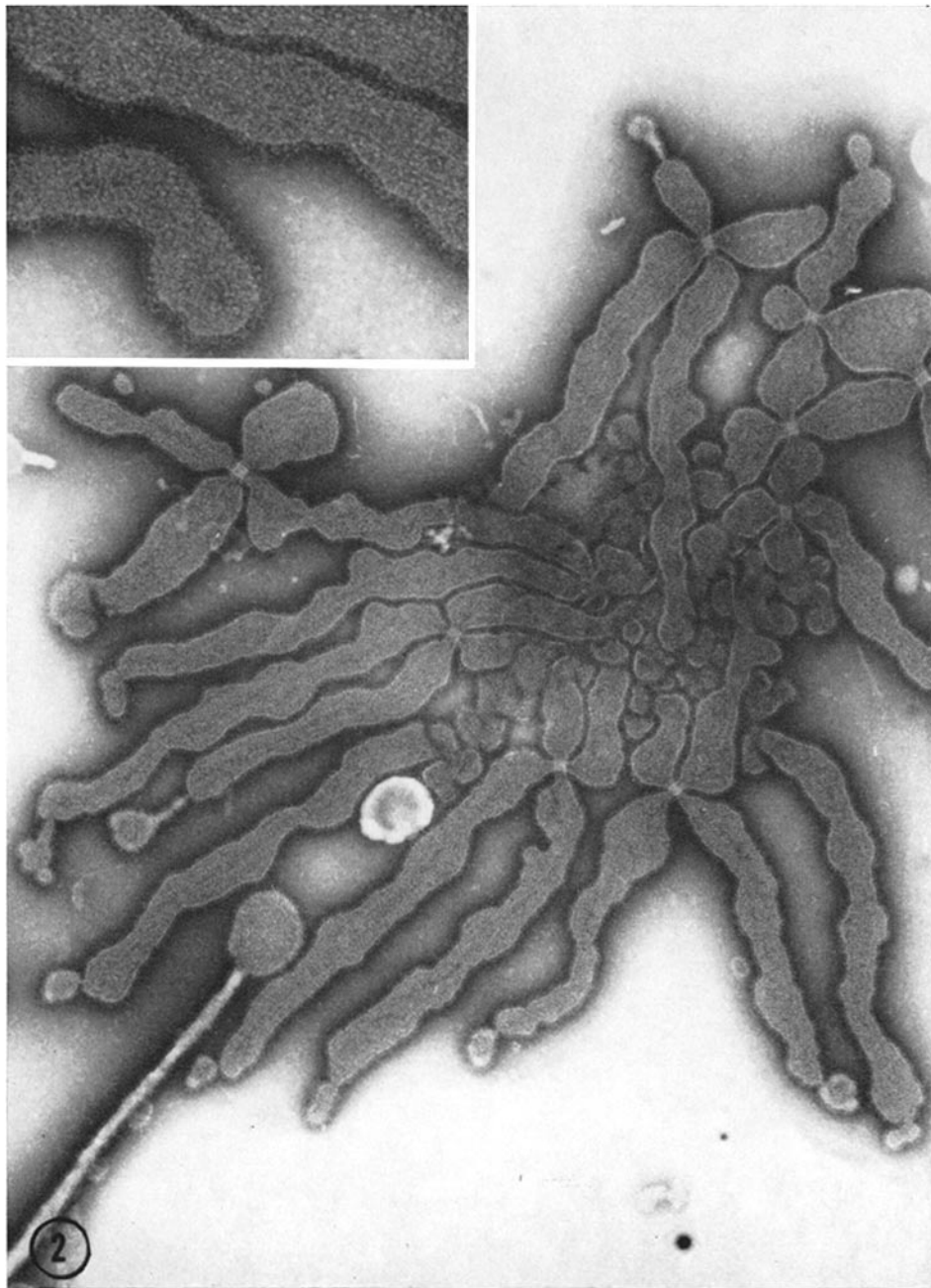


FIGURE 1 The solubilization of disaccharidases of the hamster intestinal brush border with time of treatment with papain-cellulose. Activity is reported as μ moles of disaccharide hydrolyzed per minute at 37°C in the 10 ml sample removed for centrifugation at the end of each time period. The 10 ml sample (Total) contained the equivalent of 0.727 mg of original brush border protein. The specific activities of the isolated brush borders varied slightly among different preparations. The specific activities of the disaccharidases (international units) were similar to published values: maltase, 4.14; isomaltase, 1.43; sucrase, 1.86; and trehalase, 0.157.

cellulose. However, each of these disaccharidases shows unique kinetics with respect to its solubilization. Essentially all (99%) of the sucrase activity is found in the 43,000 g supernatant after 20 min of incubation with papain-cellulose. At 5 and 10 min, 90 and 96%, respectively, of the enzyme is solubilized. The pattern of solubilization of isomaltase resembles that of sucrase. Over 90% of the activity is released in 10 min. A small portion (6%) of the isomaltase is not solubilized, remaining bound to the brush border after 20 min of incubation with papain-cellulose. A significantly larger percentage of the maltase activity of the brush border resists solubilization. Only 89% of the total activity is solubilized at 20 min; 86% is found in the supernatant after 10 min of incubation. In marked contradistinction to the other disaccharidases, none of the trehalase activity of the brush border is solubilized by papain-cellulose, even after an incubation of 40 min. The failure of papain-cellulose to solubilize trehalase agrees with the finding of Eichholz (15).

The action of papain-cellulose on the morphology of isolated brush borders was examined concurrently with the kinetic studies. Some of these observations are illustrated in Figs. 2-4.



FIGURES 2-4 Electron micrographs of negatively stained brush border preparations. Effect of papain-cellulose digestion with time. Stained with potassium phosphotungstate, pH 6.5.

FIGURE 2 Control brush border, freshly isolated, undigested. Low magnification shows intact brush border and uniform distribution of 60 Å particles over the surface of the preparation. *Inset*: Particles are more clearly seen at higher magnification. $\times 39,000$. *Inset*, $\times 97,500$.

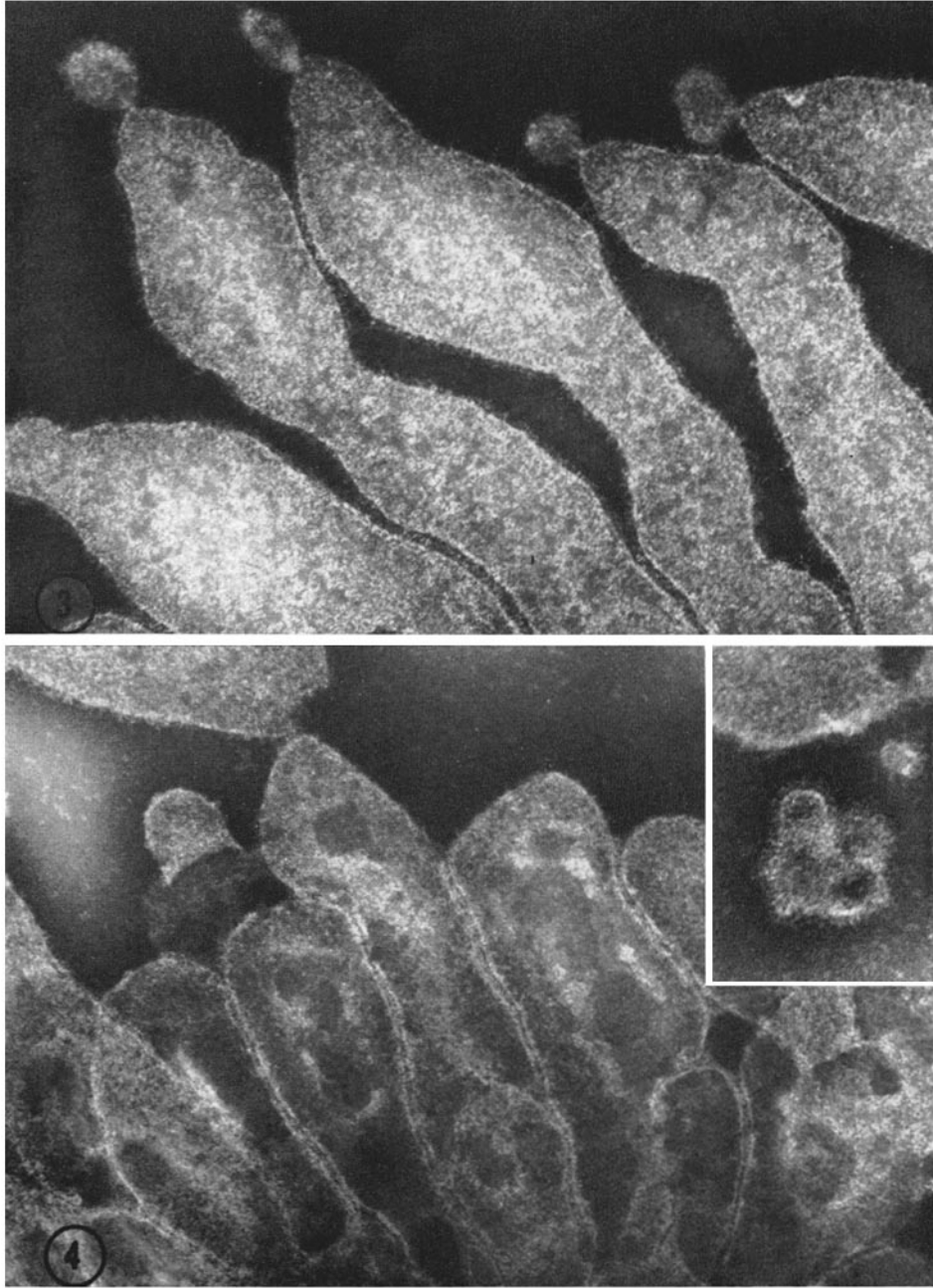


FIGURE 3 Brush border preparation after digestion for 10 min with papain-cellulose. Note the high proportion of particles remaining attached. $\times 97,500$.

FIGURE 4 Brush border after digestion with papain-cellulose for 20 min. Note the general "thinning" and fragmentation of the preparation but the retention of many attached knobs. $\times 97,500$.

Electron micrographs of negatively stained brush borders prior to their incubation with papain-cellulose (Fig. 2) show the knobs, approximately 60 Å in diameter, as they were described previously (3-6). Samples were observed in the electron microscope after digestion for 2.5, 5.0, 10.0, and 20 min. During the incubation period a progressive fragmentation of the largely intact brush borders comprising the starting preparation occurs, and a general "thinning" of the preparation is observed (Figs. 3 and 4). However, throughout the course of the experiment, fragments to which large numbers of the knobs are still attached can be found. Thus, it is not possible to correlate the release of sucrase and maltase with removal of particles, as previously suggested (3-6). This is especially apparent after 10 min of digestion when a large percentage of the knobs remain attached (Fig. 3), but approximately 90% of all of the enzyme activities measured are solubilized (Fig. 1). However, since it is difficult to quantitate with great precision the knobs remaining on the brush border, the possibility that a small specific fraction of the knobs is associated with these enzymic activities and is removed by treatment with papain-cellulose has not been excluded.

In view of the fact that the results obtained in the present studies contrast with those reported previously (3-6), attempts were made to repeat the earlier experiments with uncomplexed soluble papain rather than papain-cellulose, even though with soluble papain it is not possible to stop the reaction quickly and, thus, the times of treatment may not be recorded precisely. Nevertheless, the use of soluble papain with units of activity approximately equal to those of papain-cellulose gave essentially the same results as complexed papain.

Representative values, for 2.5- and 20-min incubations, are shown in Table I. Clearly, sucrase, isomaltase, and maltase are predominantly solubilized. However, electron microscope examinations of simultaneously sampled digestion mixtures show that many, if not all, of the 60 Å knobs are still attached to the brush border. As with incubations with papain-cellulose (Fig. 1), Table I also shows that a small amount of isomaltase activity, a somewhat greater percentage of maltase activity, and all of the trehalase activity remain firmly bound to the brush border membranes.

The finding that the different disaccharidases vary in the degree of their solubilization by papain may have additional implications. Trehalase is not released from the brush border with our experimental conditions, whereas the other disaccharidases are largely solubilized. This implies that the immediate chemical environment of trehalase, which is instrumental in integrating the enzyme into the mosaic comprising the microvillus membrane, differs substantially from the environment that binds the other hydrolytic enzymes to the membrane. Also, the data consistently show that about 10% of the total maltase activity is not susceptible to proteolytic solubilization. This suggests that the insoluble fraction may have an intramembranal localization different from that of the remaining 90% of the maltase, or that the activity, which is insoluble, may exist as a distinct isozyme or enzymic activity not measured, e.g. α -amylase. Solubilized maltase from intestines of other mammalian species has been separated into several isozymes (18). It is conceivable, therefore, that the insoluble form described in this study represents an additional isozyme. Somewhat analogous reasoning leads to the view that the

TABLE I
Removal of Disaccharidases from Isolated Hamster Intestinal Brush Borders by Soluble Papain

Activity is reported as μ moles of disaccharide hydrolyzed per minute at 37°C in the 10 ml sample removed for centrifugation at the end of each time period. The 10 ml sample (Total) contained the equivalent of 0.727 mg of original brush border protein.

Disaccharidase	2.5 min incubation			20 min incubation		
	Total	Soluble	Insoluble	Total	Soluble	Insoluble
Sucrase	1.41	1.39	0.02	1.38	1.37	0.01
Isomaltase	1.26	1.17	0.09	1.25	1.20	0.05
Maltase	3.56	3.03	0.53	3.45	3.19	0.26
Trehalase	0.11	0.00	0.11	0.13	0.00	0.13

nonsolubilized isomaltase is different from the solubilized isomaltase. Previously, a solubilized homogeneous protein complex, possessing both the isomaltase and sucrase activities of the brush border, has been described (19, 20). The present finding of a small, but significant, nonsolubilized isomaltase, not associated with sucrase activity, suggests a second isozyme of isomaltase in the intestinal brush border.

SUMMARY

The hypothesis that hamster intestinal disaccharidases are identical to the 60 A knobs seen on the brush border in the electron microscope by negative staining has been examined by a correlated kinetic study of the removal of sucrase, isomaltase, maltase, and trehalase activities and the particles from the brush border by papain. Sucrase, isomaltase, and maltase are largely solubilized; however, each of these disaccharidases shows a distinct pattern of release with time. Trehalase is not solubilized. During incubation periods when 90–100% of the sucrase, isomaltase, and maltase activities have been removed from the brush border, many of the knobs are still attached. Thus, it is not possible to correlate the release of disaccharidases with removal of particles. The finding that each disaccharidase is unique with respect to its solubilization by papain infers differences in the binding of the enzymes to the brush border membrane. A maltase and an isomaltase which differ from the known intestinal maltases and isomaltase by their inability to be solubilized by papain are reported.

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