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THE DISACCHARIDASE ACTIVITY OF A MEMBRANE FRACTION OBTAINED FROM THE RABBIT RENAL CORTEX

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SUMMARY

An investigation has been made into the disaccharidase activities found in the rabbit renal cortex with particular reference to their selective solubilization by the use of proteolytic enzymes.

1. A membrane fraction which carries high specific activities of the enzymes maltase and trehalase (0.14 and 0.52 μmole substrate hydrolyzed/min per mg protein) has been isolated from the rabbit renal cortex. It appears to represent brush border membrane together with its associated external coat.

2. Brief treatment of this particulate material with papain releases a soluble fraction which is excluded from Sephadex G-100, and which contains 8 % of the total protein and 90 % of the total maltase activity. The specific activity of this soluble enzyme is increased by 10–20 times. Almost all (99 %) of the trehalase activity remains on the membrane.

3. Treatment of the membranes with trypsin also releases a soluble fraction excluded from G-100 but in smaller yield (approx. 3 % of total protein). However, no maltase and only a trace of trehalase activity could be detected in this fraction, almost all the activities being retained in the particulate residue.

INTRODUCTION

Attempts to study the renal brush border have been hampered by difficulties in isolation and characterization, although several worker have obtained morphologically distinct preparations, *e. g.* Kinne and Kinne-Saffran[1] working with rat tissue. Recently, however, it has been shown that purified, morphologically identifiable brush borders from the rabbit carry with them high activities of the disaccharidases, maltase and trehalase, and that these enzymes are not found elsewhere in the kidney[2]. This finding has provided marker enzymes for the brush border as well as posing the problem of the physiological function, if any, of these disaccharidases in a situation where they might not contact their substrates. The suggestion that trehalase is involved in glucose transport has been made[3], although the intestinal trehalase has been definitely located external to the transport barrier[4].

This paper describes a simple method of preparing good yields of membranes with associated disaccharidase activities very close to those of Berger and Sacktor[2].

Proteolytic enzymes have then been used in an attempt to remove these disaccharidases from the membrane.

MATERIALS AND METHODS

Preparation of membrane

The method used was based on that of Hillman and Rosenberg[5]. Kidneys were obtained under Nembutal anaesthesia from random-bred male rabbits weighing 2–3 kg. Chopped cortices, approx. 7 g wet wt. from a single rabbit, were then incubated with collagenase (Type A, Sigma), final concn. 0.4 mg/ml, in 10 ml of the gassed and supplemented Krebs–Ringer bicarbonate–saline buffer (pH 7.4) described by these authors. Incubation was for 3 h at 37° with gentle shaking. At the end of this time very little solid tissue remained. The digest was centrifuged at 10300 × *g* in a Sorvall SS-1 centrifuge at 4° for 20 min. The supernatant was discarded, the sediment washed with 10 ml cold Krebs–Ringer buffer and centrifuged again. The sediment was then taken up in 30 ml cold Krebs–Ringer buffer and filtered through 4 layers of surgical gauze (Johnson and Johnson). The filtrate was homogenized (10 strokes) by hand in a stainless steel in glass homogenizer with a minimum clearance of 0.01 cm. Aliquots of this homogenate were frozen for subsequent enzyme assays. The remainder was centrifuged at 45000 × *g* in an MSE Superspeed 50 centrifuge at 4°. The lowest layer of sediment consisted of red blood cells, therefore the upper layers were taken off and homogenized (5 strokes) in 30 ml cold 5 mM EDTA (disodium salt) at pH 7.5. This suspension was left to stand overnight at 4° in order to lyse intracellular organelles. It was then centrifuged again, washed with the cold EDTA, followed by cold Krebs–Ringer buffer and centrifuged. The sediment was resuspended in 6 ml cold Krebs–Ringer buffer and stored in this at 4°. The yield from 7 g wet wt. of cortex was approx. 83 mg dry wt. of this membrane suspension.

Density gradient centrifugation

A discontinuous density gradient of glycerol in 0.5 M $MgCl_2$ (3.8 ml each of 20, 30, 40, 50 and 60 %) was prepared in a 23-ml polycarbonate centrifuge tube by using a Pasteur pipette. A 1-ml aliquot of membrane suspension was then layered onto this gradient. Centrifugation was carried out at 4° in an MSE Superspeed 50 centrifuge for 10 min at 12 000 × *g*, using the 3 × 23 ml swing-out head. Fractions were collected from the base of the centrifuge tube after puncturing with a needle.

Enzyme assays

Maltase and trehalase were assayed by the method of Dahlqvist[6] using maltose (Sigma) and trehalose (B.D.H.) as substrates, and glucose oxidase (Type V, Sigma) to measure glucose released. Alkaline phosphatase was assayed by hydrolysis of *p*-nitrophenyl phosphate (B.D.H.) at pH 10.0 (ref. 7). β-glucuronidase assay was by hydrolysis of phenolphthalein mono-β-glucuronic acid (Sigma)[8].

Protein determination was by the method of Lowry *et al.*[9] using crystalline bovine serum albumin (B.D.H.) as a standard. Dry weights were determined on samples after drying for 15 h at 102°. If buffer was present, samples of buffer only were also dried down, and then subtracted.

Incubation with papain

Purified membranes (13 mg dry wt.) were suspended in 0.5 ml of 50 mM Tris–maleate buffer, pH 6.2, containing 2 mM EDTA and 10 mM cysteine, and warmed to 37°. Papain (2 times crystallized, Sigma) 0.45 mg was activated by incubation at 37° in 0.18 ml of the above buffer for 15 min, and was then added to the membrane suspension. After incubating for 5 min the mixture was put into ice to stop enzyme action.

Incubation with trypsin

Purified membranes (9 mg dry wt. were suspended in 0.5 ml of the Krebs–Ringer buffer (pH 7.4). Trypsin (B.D.H., crystalline), 0.25 mg dissolved in 0.05 ml 0.001 M HCl, was added to the pre-warmed suspension and incubation carried out for 10 min at 37°. The substrate control was incubated with 0.05 ml 0.001 M HCl only. At the end of incubation, the mixture was put into ice.

Before use both papain and trypsin were tested for activity using benzoyl arginine ethyl ester (B.D.H.) as substrate and following hydrolysis by the $\Delta A_{253\ nm}$[10].

Electron microscopy

Pellets of membrane preparations were fixed in osmic acid, dehydrated with graded alcohols and embedded in Epikote resin. For staining with ruthenium red, the pellet was first fixed in 1 % buffered glutaraldehyde containing 1 mg/ml ruthenium red (Gurr), then washed 2 times with cacodylate buffer, pH 7.4, containing ruthenium red (0.6 mg/ml). It was then placed in 1 % osmic acid containing ruthenium red (0.6 mg/ml). Dehydration and embedding was then as usual.

RESULTS

Table I shows the results of enzyme assays on the various fractions obtained during the purification procedure. The homogenate of the collagenase digest has quite high specific activities of maltase and trehalase when compared to those Berger and Sacktor[2], who used homogenized whole cortex as their initial material. Their values were 0.01 and 0.02 μmole substrate hydrolyzed/min per mg protein for maltase and trehalase, respectively. In order to compare values for the collagenase digest

TABLE I

ENZYMIC ACTIVITIES OF FRACTIONS OBTAINED DURING THE PURIFICATION PROCEDURE

Specific activities are reported as μmoles substrate hydrolyzed/min per mg protein. The ranges given are standard errors of the mean, with the number of readings in parentheses.

Enzyme	*Specific activity*			*Recovery from the homogenate in Band 1–2* (%)
	Homogenate	*Membrane suspension*	*Band 1–2*	
Maltase	0.05 ± 0.01 (3)	0.08 ± 0.02 (5)	0.14 ± 0.02 (7)	25.4 ± 0.2 (2)
Trehalase	0.22 ± 0.04 (3)	0.35 ± 0.06 (5)	0.52 ± 0.07 (7)	22.4 ± 0.7 (2)
Alkaline phosphatase		0.15 ± 0.01 (4)	0.27 ± 0.02 (4)	
Percentage protein		62.1 ± 2.6 (4)	46.3 ± 1.5 (3)	8.2 ± 0.1 (2)

directly with those of whole cortex, fresh cortical material from one rabbit kidney was chopped finely and homogenized (50 strokes) in the same homogenizer as used for the collagenase digest. Aliquots were then assayed for protein and disaccharidase activity, and were found to be 0.02 and 0.12 μmole substrate hydrolyzed/min per mg protein for maltase and trehalase, respectively. Clearly a concentration of activity has been brought about by the collagenase digestion and filtration. However, 50 % of the disaccharidase activity of the whole cortex is lost during preparation of the homogenate of the collagenase digest, 22 % of this in the supernatant and washings of the digest, and the remainder presumably in material retained on the gauze.

A further advantage of the collagenase method is that it would be possible to carry out *in vitro* experiments on the tubule segments before homogenization.

The subsequent purification steps bring about a steady increase in specific activities as shown in Table I. The membrane suspension is that obtained after exposure of the homogenate to EDTA. This treatment inevitably causes some loss of alkaline phosphatase activity. Assay for β-glucuronidase, a lysosomal marker, revealed very low activities in this suspension, < 0.01 mole substrate hydrolyzed/h per mg protein. Examination of a pellet in the electron microscope showed the presence of mitochondrial and nuclear membranes as well as large numbers of small vesicles, the latter possibly formed from the brush border during exposure to hypotonic EDTA[5].

Further purification was obviously necessary, and this was brought about on a glycerol density gradient. After centrifugation, 5 bands were seen (Fig. 1), the most prominent being a double band (Band 1-2) near the top of the gradient. Band 5 had a rather granular appearance and varied in amount with each preparation. Fractions (2 ml) were collected from the base of the tube and immediately thoroughly dialyzed into cold distilled water to remove glycerol. Maltase, trehalase and alkaline phosphatase were then assayed on aliquots of each fraction. The distribution of enzymic activities through the gradient is shown in Fig. 2. The major peaks coincide with the double band seen near the top of the gradient. Attempts were made to follow the distribution of the microsomal marker enzyme, glucose-6-phosphatase, using the method of Hubscher and West[11] but it was found that in both the Krebs–Ringer buffer and in water there was a slow fall in specific activity of this enzyme

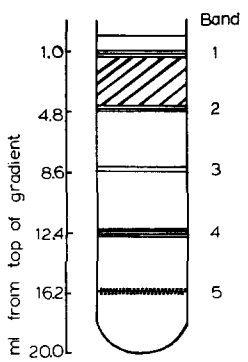

Fig. 1. Fractionation of membrane suspension on a discontinuous glycerol density gradient; visual appearance after centrifugation.

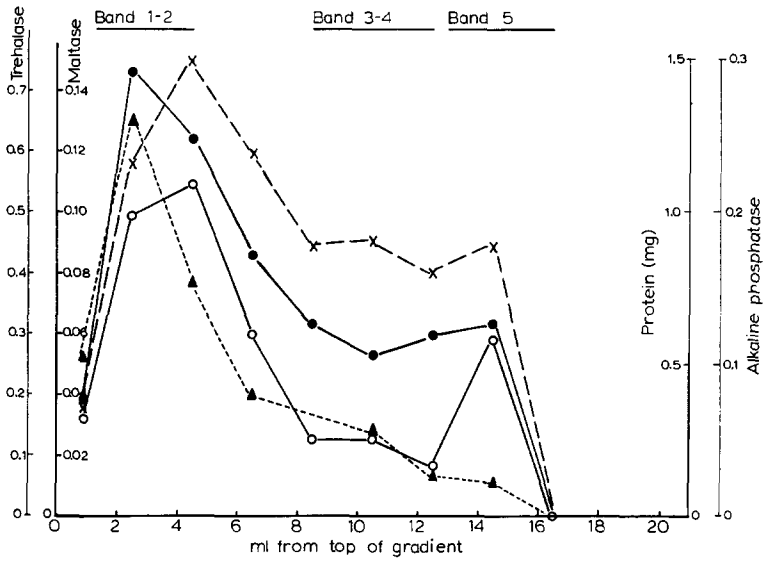

Fig. 2. Fractionation of membrane suspension on a discontinuous glycerol density gradient; distribution of enzymic activities. ○—○, maltase; ●—●, trehalase; ×---×, alkaline phosphatase; units are μmoles substrate hydrolyzed/min per mg protein; ▲---▲, protein in mg.

with time and this made comparisons of fractions very difficult. However there is undoubtedly some microsomal contamination of Band 1–2, as has been found in brush borders prepared by others workers[1,2]. Levels found in the Band 1–2 preparations immediately after dialysis were approximately 0.09 μmole P_i formed/min per mg protein.

The fractions of the gradient indicated in Fig. 2 were pooled and pelleted for electron microscopy. Band 1–2 was found to consist of an array of small vesicles with a definite external fuzzy layer which was stained with ruthenium red (Fig. 3). Band 3–4 was almost entirely swollen mitochondria and Band 5 consisted of heterogeneous large membranes.

The lighter part of Band 1–2 was collected as indicated in Fig. 2 and dialyzed into cold Krebs–Ringer buffer. It was then centrifuged at 10300 × *g* for 15 min at 4° and the sediment resuspended in a known volume of the same buffer. The enzymic activities are reported in Table I. Specific activities of the disaccharidases have increased by about 1.7 times. Recoveries of enzymic activities at this stage were approx. 50 %. No β-glucuronidase activity was detected in Band 1–2. Assay for sucrase also failed to show any activity, *i.e.* spec. act. was < 0.002 μmole substrate hydrolyzed/min per mg protein.

As seen in Table I, recovery of the disaccharidase activity from the homogenate was about 25 %. Losses were due mainly to the gradient step where only a narrow cut of the gradient was taken (Fig. 2). This was deemed necessary to avoid contamination with mitochondria. Small losses of low specific activity occurred in the various supernatants.

Incubation with papain

Preparations of Band 1–2 were incubated with papain as indicated in Materials

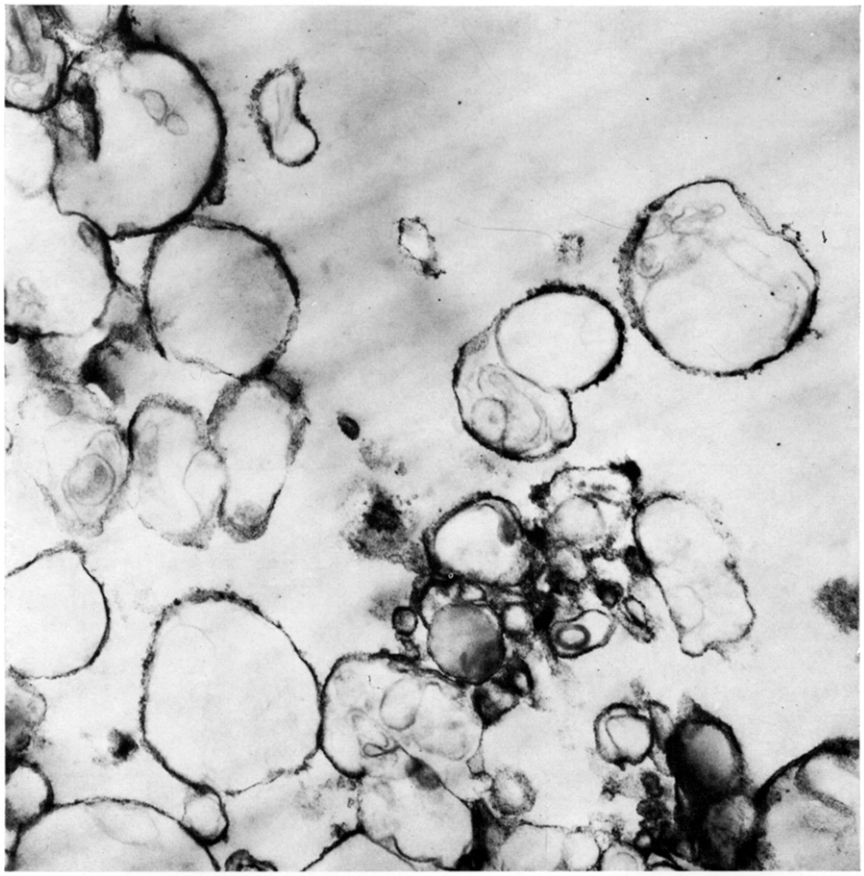

Fig. 3. Electron micrograph of the purified membrane fraction (Band 1–2) after fixation with glutaraldehyde and osmic acid in the presence of ruthenium red. Magnification × 56250.

and Methods. After the 5-min incubation the mixture was cooled in ice and centrifuged at 4° for 20 min at 10300 × *g*. The supernatant fraction, although clear, was decanted and centrifuged again to ensure complete removal of particulate matter. This second supernatant was then immediately applied to a column of Sephadex G-100 (50 cm × 1 cm) equilibrated with Krebs–Ringer buffer at room temperature, and 1.5-ml fractions collected. The effluent was monitored at a wavelength of 280 nm and the pattern is shown in Fig. 4. A substrate control and an enzyme control were treated in the same way and the results are also shown in Fig. 4. It is clear that the excluded peak has been split off the membranes by the papain. A very small peak is eluted in the substrate control, unfortunately insufficient to collect and analyze. The percentage of protein liberated by the papain was calculated as 7.4 % of total protein.

The excluded peak from the digest was collected in three tubes and these were assayed separately for protein and enzymic activities. The particulate residues from both digest and control were each collected and quickly washed twice with cold Krebs–Ringer buffer with centrifugation, in order to remove any residual enzyme. The sediment was then resuspended in 1 ml cold Krebs–Ringer buffer and

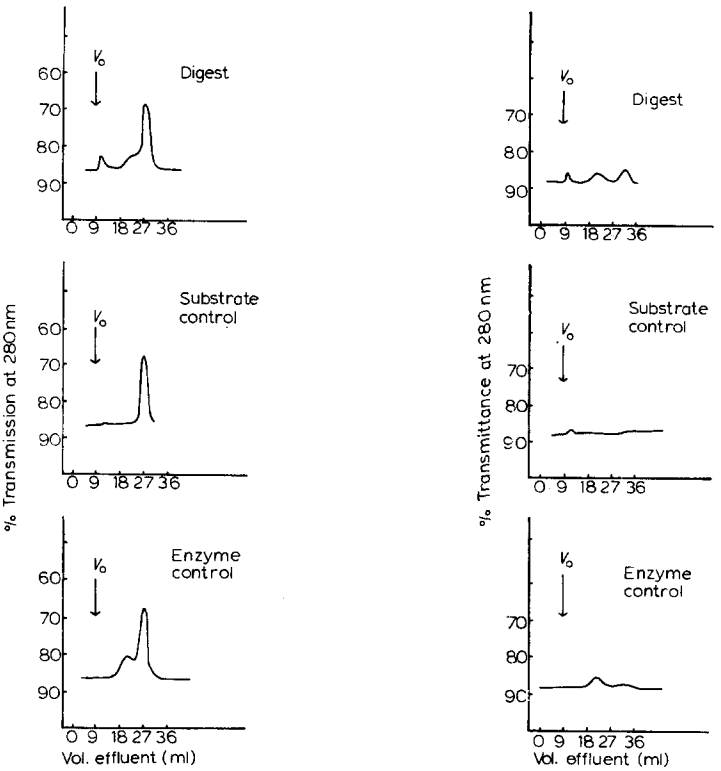

Fig. 4. Gel filtration of the soluble product of papain digestion of Band 1–2 on Sephadex G-100 (50 cm × 1 cm) in Krebs–Ringer buffer, pH 7.4. Fractions (1.5 ml) were collected at a flow rate of 22 ml/h. The elution patterns of supernatants from the substrate control and the enzyme control are also shown.

Fig. 5. Gel filtration of the soluble product of trypsin digestion of Band 1–2 on Sephadex G-100 (50 cm × 1 cm) in Krebs–Ringer buffer, pH 7.4. Fractions (1.5 ml) were collected at a flow rate of 22 ml/h. The elution patterns of supernatants from the substrate control and the enzyme control are also shown.

analyzed together with the solubilized fractions. The results of a typical experiment are shown in Table II and have been found to be quite reproducible over a range of preparations.

The release of maltase activity is about 90 % with an increase of specific activity in the soluble fraction of 10–20 times. Virtually no trehalase activity is released and the specific activity in the residue is slightly increased above the original. The substrate control retains very similar specific activities of the disaccharidases to the original material, although there are some losses in total activity due to the manipulations involved. Recovery of total protein in the substrate control was 72 % and in the enzyme-treated residue *plus* soluble fraction 56 %. The pH optimum of the soluble maltase was found to be fairly broad, between pH 6 and 7, activities at pH 5 and pH 8 being 73 % and 88 %, respectively of that at pH 6. The pH optimum of the particulate trehalase was 6, activities at pH 5 and 7 being 62 % and 79 %, respectively of that at 6. One puzzling feature is the dramatic loss in alkaline phosphatase activity in both control and digest, only 16 % of the original activity being

TABLE II

ENZYMIC ACTIVITY OF FRACTIONS TO DEMONSTRATE THE EFFECT OF PAPAIN

Fraction	*Enzymic activity (μmoles substrate hydrolyzed/min per mg protein)*								
	Maltase			*Trehalase*			*Alkaline phosphatase*		
	Spec. act.	*Total act.*	*Recovery of act. (%)*	*Spec. act.*	*Total act.*	*Recovery of act. (%)*	*Spec. act.*	*Total act.*	*Recovery of act. (%)*
Band 1–2 from glycerol gradient	0.43	0.70		0.51	1.58		0.25	0.77	
Supernatant from digestion: Leading	1.05	0.04		0.06	0.002		N.A.	N.A.	
excluded peak from G-100 Middle	2.60	0.16	47	0.04	0.002		0.42	0.03	
Trailing	1.75	0.10		N.A.	N.A.		0.76	0.04	25
Particulate residue from digestion	0.02	0.03		0.71	1.12	71	0.07	0.12	
Particulate residue from control	0.22	0.48	69	0.47	1.05	67	0.06	0.12	16

N.A. = no activity detected.

TABLE III

ENZYMIC ACTIVITY OF FRACTIONS TO DEMONSTRATE THE EFFECT OF TRYPSIN

Fraction	*Enzymic activity (μmoles substrate hydrolyzed/min per mg protein)*								
	Maltase			*Trehalase*			*Alkaline phosphatase*		
	Spec. act.	*Total act.*	*Recovery of act. (%)*	*Spec. act.*	*Total act.*	*Recovery of act. (%)*	*Spec. act.*	*Total act.*	*Recovery of act. (%)*
Band 1–2 from glycerol gradient	0.10	0.21		0.59	1.25		0.23	0.48	
Supernatant from digestion: Leading	N.A.	N.A.		0.15			N.A.	N.A.	
excluded peak from G-100 Trailing	N.A.	N.A.		0.05			N.A.	N.A.	
Particulate residue from digestion	0.11	0.16	76	0.62	0.89	71	0.21	0.30	63
Particulate residue from control	0.10	0.20	95	0.60	1.25	100	0.18	0.38	79

N.A. = no activity detected.

recovered even in the control. It is evident from Table II that papain has solubilized some alkaline phosphatase activity and that the peak of maximum activity is slightly behind that of maltase. However, recovery in this case is still low.

Incubation with trypsin

This was carried out as described in Material and Methods and the ratio of enzyme to substrate protein was similar to that for papain. The elution pattern of the soluble fraction on Sephadex G-100 is shown in Fig. 5, together with that of supernatants from substrate alone and enzyme alone.

The elution pattern shows that there is only a small amount of material liberated by trypsin, calculated as approx. 3 % of the total. Analysis of the excluded peak from the digest and the resuspended residues from both digest and control in a typical experiment gave the results shown in Table III.

It is evident that no maltase activity has been released, and that it can in fact be recovered in the particulate residue. A very small amount of trehalase activity is eluted but this shows low specific activity, the majority of the total being recovered in the particulate fraction. Alkaline phosphatase shows a similar failure to solubilize and is recovered in good yield in the sediment.

DISCUSSION

The preparative methods used in this investigation have produced a particulate, membranous fraction (Band 1-2) from the rabbit renal cortex with no detectable lysomal contamination. This fraction consists of an array of small vesicles which carry at least three enzymic activities: maltase, trehalase and alkaline phosphatase. The vesicles also have an outer coat which reacts with ruthenium red. By means of an elegant series of fractionations in cold sucrose, Berger and Sacktor[2] were able to demonstrate that morphologically identifiable brush borders from the rabbit renal cortex carried with them high activities of maltase and trehalase, and these workers suggested that these enzymes may be used as markers for the renal brush border. In this case, the vesicles obtained in the present study must also represent a brush border-enriched fraction. Morphological identification however is not possible for this preparation as the microvilli are unstable under the conditions used[5].

Cleavage by proteolytic enzymes has been used by other workers to solubilize disaccharidases from intestinal brush borders. Papain has been shown to solubilize maltase from intestinal brush borders of the hamster[12,13,14] and of man[15]. Trehalase was not liberated in these species.

The present work has demonstrated that with rabbit renal brush borders, solubilization of maltase occurs rapidly and almost completely with papain, whereas trehalase remains firmly bound to the membrane. Evidently there is considerable analogy between the kidney and the intestine with regard to accessibility of the disaccharidases to papain. Trypsin fails to release either enzyme from the renal membranes although it does solubilize a small fraction (approx. 3 %) of total protein. The contrasts with the results of Dahlqvist[16] on the hog intestinal brush borders, where trehalase was solubilized by trypsin treatment, although in this case the exposure to trypsin was prolonged.

Further work is now in progress to characterize chemically the material

solubilized from renal membranes by papain and to discover its association, if any, with the fuzzy coat.

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