ISOLATION AND BIOCHEMICAL CHARACTERIZATION
OF BRUSH BORDERS FROM RABBIT KIDNEY

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ABSTRACT

A technique for the isolation of intact brush borders from rabbit renal cortex was evaluated. The procedure was monitored by phase and electron microscopy and marker enzymes, i.e. ATP-NMN adenylyl transferase, nuclear; cytochrome oxidase, mitochondrial; \( \beta \)-glucuronidase, lysosomal; and glucose-6-Pase, microsomal; and indicated an essentially pure preparation of brush borders. The disaccharidase, trehalase, previously reported in renal tubules, was localized uniquely in brush borders. Maltase was also found; the specific activities of the two enzymes in the brush borders were increased 10- to 20-fold. Other disaccharidases, such as sucrase, isomaltase, lactase, and cellobiase, were absent. It is suggested that trehalase and maltase are appropriate candidates for marker enzymes of the renal brush border. Isolated brush borders possessed a ouabain-sensitive \((Na^+ + K^+)\) ATPase, an oligomycin-insensitive \(Mg^{2+}\) ATPase, and a \(Ca^{2+}\)-activated ATPase. Alkaline phosphatases, dephosphorylating \(\beta\)-glycerophosphate, and trehalose-6-P were also present. The specific activities of these enzymes were increased three-to-five fold in the brush-border preparations; however, activities were found in other subcellular fractions of the renal cortex. Hexokinase, although evident in the isolated brush border, was found prominently associated with other membranous fractions. Phosphoglucomutase and UDPG pyrophosphorylase were localized in the soluble fraction of the renal cortex.

The renal brush border has a prominent role in determining the specificity and rate of reabsorption from the glomerular filtrate. A characterization of the biochemical properties of the isolated brush border, therefore, should provide significant information on the molecular mechanisms underlying tubular reabsorption. Brush borders from intestinal epithelial cells have been isolated as a discrete subcellular fraction, and several enzymes are now known to be associated with this membranous structure (1-4). In contrast, little has been done on the isolation or biochemical characterization of the renal brush border. Recently, however, Thuneberg and Rostgaard (5) described a procedure for isolating brush borders from rabbit kidney cortex. Binkley and King (7) prepared particulates enriched in membranes derived from brush borders of the rat kidney and noted the presence of a \((Na^+ + K^+)\)-stimulated ATPase (6) and alkaline phosphatase (7), as has been described earlier by histochemistry at the resolution of the electron microscope (8, 9). In this paper, isolated brush borders from rabbit kidney cortex are characterized by microscopy and also enzymatically. The presence of ATPases and alkaline phosphatases in the structurally intact, isolated brush border of this species is established. In

1 A preliminary account of part of this work was presented at the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1970 (10).
addition, the disaccharidase, trehalase, previously found uniquely in the particulate fraction of rabbit renal tubules (11, 12) and postulated to have a role in the transport of glucose (11, 13), is now localized exclusively in the brush border. The use of this disaccharidase, as well as maltase, for "marker" enzymes for rabbit renal brush-border preparations is suggested.

METHODS

Renal brush borders were isolated from New Zealand white male rabbits weighing 2-3 kg by a modification of the procedure of Thuneberg and Røtgaard (5). Kidneys were quickly removed from animals anesthetized with Nembutal, and were chilled in cold 0.5 M sucrose, decapsulated, and defatted. All subsequent steps in the procedure were carried out in the cold. The cortices were dissected, weighed, and then finely sliced. The tissue was homogenized by hand in 0.5 M sucrose with a Dounce homogenizer having a pestle clearance as given by Thuneberg and Røtgaard (5), with a ratio of 1 g of tissue to 6 ml of medium. As noted originally by these authors, an extremely gentle homogenization was necessary in order to obtain the large, intact brush borders; a longer or more drastic initial homogenization resulted in breaking the brush borders into fragments which were then sedimented with the microsomal fractions. The suspension was homogenized further with three complete strokes of a Potter-Elvehjem teflon pestle at 1000 rpm. Examination of the homogenate by phase-contrast microscopy revealed numerous intact, generally rounded brush borders. Each subsequent step of the isolation procedure was monitored morphologically by phase-contrast microscopy.

The homogenate in 0.5 M sucrose was fractionated by layering the suspension on a discontinuous gradient comprising 8 ml each of 1.7 and 1.4 M sucrose. The tubes were centrifuged at 24,000 rpm (90,000 g, max.) for 60 min using a SB-110 rotor in an International Centrifuge. The bands resolved are shown in Fig. 1. A thin layer of dense cells was sedimented at the bottom of the tube, and a thicker band of predominantly whole cells (P1) was distributed in the 1.7 M sucrose zone. The brownish, mitochondrial-enriched fraction was localized in the 1.4 M sucrose band, with large cell fragments plus many nuclei (P2) at the interface of the 1.7 and 1.4 M sucrose zones. The pinkish fluffy layer containing the brush borders (P3) was distributed at the interface of the 1.4 M and 0.5 M sucrose zones. A supernatant (S1) with increasing turbidity towards the brush-border layer remained in the 0.5 M sucrose zone. The bands, thus obtained, were separated by careful aspiration from the top. With this modification of the procedure of Thuneberg and Røtgaard (5), mitochondria were dispersed in a relatively large volume of 1.4 M sucrose, thus facilitating the separation of most mitochondria from brush borders and avoiding the gross contamination of the brush borders at an early stage.

The brush-border–enriched fraction (P3) was subject to further purification according to the scheme outlined in Fig. 2. A centrifugation of the brush-border layer at 4,000 g, max., for 30 min largely separated the brush borders from the microsomal fraction, yet effectively prevented the loss of a great number of small brush borders to the supernatant.
An additional separation from mitochondrial contamination was obtained by centrifuging a suspension of P₃ at 32,000 g for 5 min. The small, tightly packed mitochondrial pellet (P₃) rarely contained brush borders, as viewed by phase microscopy. The light pink layer containing the brush borders could readily be removed by gentle agitation and careful aspiration without disturbing the mitochondrial pellet. The intact brush borders were further freed of membranous material and disrupted brush borders by repeated low-speed centrifugations for 10 min each. At each step in the procedure (P₃ through P₉), trace contamination by mitochondrial fragments could be detected at the bottom of the centrifuge tube, and these were discarded as described above. The P₁₀ fraction was essentially a pure preparation of intact brush borders; approximately 11 mg of protein (range of 10–13 mg) was obtained from about 1 g protein of renal cortex.

Trehalase was assayed by the “direct method” as described earlier (11), with slight modification. The reaction mixture contained 11 µmoles of NADP, 0.7 µmole of ATP, 0.7 µmole of MgCl₂, 15 µmoles of K phosphate, pH 6.3, 20 µmoles of trehalose, and excess crystalline glucose-6-P dehydrogenase and hexokinase in a final volume of 0.62 ml. Maltase, isomaltase, sucrase, lactase, and cellobiase were assayed similarly, except that 20 µmoles of the appropriate substrate were used. UDPG pyrophosphorylase, phosphoglucomutase, and hexokinase were measured as previously reported (11).

Cytochrome oxidase was determined by the method of Wharton and Tzagoloff (14). ATP:NMN adenylyl transferase was measured according to Kato and Kurokawa (15). β-Glucuronidase was assayed as described by Fishman and Bernfeld (16). Glucose-6-phosphatase was used to indicate nuclear contamination. Cytochrome oxidase served as the marker for mitochondria. Lysosomes were indicated by β-glucuronidase, and glucose-6-phosphatase was the marker for microsomes. The specific activities of these enzymes in the brush-border fraction were significantly lower than those in the unfractionated homogenate. Additionally, considerably less than 1% of each of the four enzymes was found in the P₁₀ fraction.

ATP:NMN adenylyl transferase could not be detected in the brush-border fraction, a finding suggesting the complete absence of nuclear contamination. Summation of the activities found in the numerous P and S fractions (Fig. 2) showed that approximately 67% of the activity in the unfractionated homogenate was recovered. As was to be expected from the very gentle homogenization of the renal cortex, a large percentage of the enzymatic activity, almost 50%, was found in the P₁ and P₁′ fraction, which contained whole cells, large cellular fragments, and nuclei. An additional 10% was located in the P₄ fraction. An undetermined part of the total activity was probably associated with that portion of the P₃–P₉ pellets that was discarded, as described above in the method of isolation, and was not assayed. The specific activity of cytochrome oxidase in the brush-border fraction was only one-twentieth that in the original homogenate. In contrast, fraction P₄, which comprised mainly the mitochondrial contamination of the brush-border band after the sucrose gradient separation step, showed a twofold increase in specific activity as compared to that in the unfractionated homogenate. Moreover, merely 0.2% of the cytochrome oxidase was

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TABLE I
Biochemical Evaluation of the Isolated Brush-Border Preparation

| Enzyme                      | Specific activity | % Recovery |
|-----------------------------|-------------------|------------|
|                            | Homogenate        | Brush borders | All fractions | Brush borders |
| Trehalase                   | 4.0               | 48.5        | 97            | 13           |
| ATP:NMN adenylyl transferase| 1.95              | 0           | 67            | 0            |
| Cytochrome oxidase          | 0.78              | 0.04        | 50            | 0.2          |
| β-Glucuronidase             | 0.80              | 0.15        | 90            | 0.6          |
| Glucose-6-phosphatase       | 3.75              | 1.30        | 88            | 0.1          |
| Hexokinase                  | 0.74              | 0.63        | 63            | 0.5          |

The specific activity for trehalase is expressed as µmoles of glucose formed × min⁻¹ × mg⁻¹ protein × 10⁻²; for ATP:NMN adenylyl transferase as µmoles of NAD formed × min⁻¹ × mg⁻¹ protein × 10⁻²; for cytochrome oxidase as k = 2.3 log \( \frac{A \text{ (time)}}{A \text{ (time} + 1 \text{ min)}} \) × min⁻¹ × mg⁻¹ protein × 10⁻²; for β-glucuronidase as µmoles of phenolphthalein formed × hr⁻¹ × mg⁻¹ protein × 10⁻²; for glucose-6-Pase as µmoles of Pi × min⁻¹ × mg⁻¹ protein × 10⁻¹; and for hexokinase as µmoles of glucose-6-P formed × min⁻¹ × mg⁻¹ protein × 10⁻².

The percentage recovery is based on the activity in the homogenate, set at 100%.

in fraction P10. Fractions P4 and P1 + P1' contained 6 and 30% respectively, of the total activity. Only 50% of the enzyme was recovered in the fractions that were measured, however. Undoubtedly, some of the unaccounted for cytochrome oxidase activity would have been found in the discarded portions of the P4-P5 pellets. Approximately 90% of the total β-glucuronidase activity was recovered, with about 60 and 30% being found in the S3 and P1 + P1' fractions, respectively. The specific activity of the enzyme in the S3 fraction was 100-fold that in the brush-border fraction. The S3 fraction also contained significant (about 25%) glucose-6-phosphatase activity. 58% of the enzyme remained in the P1 + P1' fraction, and an additional 20-30% of the total glucose-6-phosphatase activity was found in the supernatants (S4 through S9). Only 0.1% of the enzymatic activity was found with the brush borders.

In clear contradistinction to the distribution of these enzymes, which all showed marked decreases in their specific activities in brush borders, the disaccharidase, trehalase, showed an increased specific activity of 12-fold as compared to that of the original homogenate. Some preparations had increases in specific activity as great as 20-fold. The pronounced localization was evidenced further by a continuous increase in specific activity at each step during the purification procedure leading to fraction P15. Some trehalase (21%) of low specific activity, 0.029 µmoles × min⁻¹ × mg⁻¹ protein, was found in the S3 fraction. This is likely caused by broken pieces of the brush border which failed to sediment at 4,000 g. When fraction S3 was centrifuged at a higher speed (35,000 g for 15 min), approximately 75% of the trehalase present in fraction S3 was sedimented. However, because this pellet also contained some microsomal material, it was discarded. Compared to the trehalase activity in the homogenate, the recovery of activity in all P and S fractions totalled to 93%; 31% was in the fraction containing whole cells and large cellular fragments.

Table II reports other enzymes whose specific activities in the brush border were markedly enhanced above the activities found in the homogenate. The disaccharidase maltase, like trehalase, showed a progressive increase in specific activity during the purification of the brush border, resulting in a specific activity approximately 14-fold that in the homogenate. The distribution pattern of maltase was almost identical with that of trehalase. Of the 94% of the total activity recovered, 18% was found in the purified brush borders, while 30 and 18% were associated with the P1 + P1' and S3 fractions, respectively. The specific activities of the maltase in the P1 + P1' and S3 fractions were only one-tenth that in the brush border fraction. Thus, both trehalase and maltase appeared to be localized in the brush border. In contrast to intestinal brush borders, other disaccharidases, such as sucrase, isomaltase, lactase, and cellobiase, which presumably serve
Enzyme Maltase Alkaline phosphatase Trehalose-6-P-phosphatase Total ATPase in the intestine exclusively as digestive enzymes, were completely absent from the renal brush border or from any other subcellular fraction of the kidney. Other disaccharidases and α-glucosidase activities not present in the kidney were reported previously (11).

Alkaline phosphatase, closely associated with cell membranes and transport processes, and found in rat kidney brush borders (6, 7), showed a four- to fivefold increase in specific activity in the rabbit renal brush border (Pl). About 5% of the total alkaline phosphatase activity resided in this fraction. However, fraction S3, containing the microsomal membranes, was also considerably enriched in activity, having an increase in specific activity of 2.6-fold and possessing 78% of the total cortical enzyme. An additional 37% of the alkaline phosphatase was localized in the Pi + Pl' fraction. The total recovery of the enzyme was approximately 120%. The apparent increase in activity, when the activity in each subfraction is added, above that in the unfractionated homogenate is presently not understood. The dephosphorylation of trehalose-6-P, a possible intermediate in trehalose synthesis and having a pH optimum of 9.3 (11), was also found in the brush border with a four- to fivefold increase in specific activity. As with alkaline phosphatase, 80% of the total trehalose-6-Pase activity was found in fraction S3; however, the percentage recovered in all fractions summed to about 95%.

The specific activity of total ATPase, measured in the presence of Na+, K+, and Mg++, was increased almost threefold in fraction P4, although the brush borders contained only 2% of the total activity of the homogenate. Other cellular fractions, i.e. the mitochondria in P6, also showed an enhanced specific activity. The ATPases in the brush borders were characterized additionally, as shown in Table III. A (Na+ + K+)-dependent ATPase constituted 37% of the total ATPase. This value corresponded closely to the percentage of the total ATPase that was sensitive to ouabain. In other brush border preparations, as much as 52% of the total ATPase was activated by Na+ + K+ and sensitive to ouabain. In contrast, ouabain had little, if any, effect on the ATPases in other subcellular fractions. For example, only 3-8% of the total ATPase in several preparations of fraction P4, which was enriched in mitochondria, was inhibited by ouabain. On the other hand, oligomycin inhibited about 80% of the total ATPase in fraction P4, whereas it inhibited less than 20% of the total ATPase in the brush border, clearly distinguishing between the Mg++-dependent ATPase in brush borders from the enzyme characteristic of phosphorylating mitochondria. To be noted from Table III is the consistent observation that the inhibition of the total ATPase in the brush border by ouabain plus oligomycin.
was not additive. In fact, less inhibition was found with the combination of inhibitors than with ouabain alone, suggesting an oligomycin antagonism of the ouabain inhibition by a mechanism as yet unexplained. Ca++ (5 mM) stimulated the dephosphorylation of ATP approximately threefold when compared to the ATPase in the brush border without added divalent cation. However, this was less than the fourfold enhancement in ATPase obtained with exogenous Mg++. The addition of both Mg++ and Ca++, at 5 mM each, with a constant ATP concentration of 5 mM, resulted in a 20-30% inhibition of the brush-border ATPases relative to the activity found with Mg++ alone. Higher concentrations of Ca++ gave greater inhibitions.

Several enzymes, including hexokinase, phosphoglucomutase, and UDPG pyrophosphorylase, which were shown to participate in the synthesis of trehalose from glucose in yeast and insects, were found previously in the renal cortex (11). Hexokinase, which was predominately membrane bound (11), was now found to be associated, in part, with the mitochondrial fraction. The specific activity of the enzyme in fraction P4 was four- to fivefold that in the homogenate. The presence of some hexokinase in the brush border was indicated, however. As shown in Table I, the specific activity of hexokinase in brush borders was the same or slightly less than that in the homogenate; on the other hand, the specific activity of the mitochondrial marker, cytochrome oxidase, in the brush-border fraction was only one-twentieth that in the homogenate. Phosphoglucomutase and UDPG pyrophosphorylase were found almost exclusively in fraction S3, in accord with their localization earlier in the cytosol of the renal cortex (11).

In addition to evaluating biochemically the procedure for isolating the renal brush borders, correlated morphological examinations by phase and electron microscopy were performed. As illustrated in Fig. 3, fraction P9 was found to be a homogeneous preparation of brush borders with only rare contamination with mitochondrial fragments. The microvilli were well preserved. A less opaque central core was seen in each microvillus. Preparations of the isolated brush borders, negatively stained, appeared as clusters of intertwining finger-like projections (Fig. 4). Significantly, the surface of the microvillar membrane was relatively smooth. In contrast, intestinal brush borders from hamster are studded with 60-90 A knoblike projections (22). Johnson (22) has claimed that the disaccharidase activities of the intestinal brush border are associated with these knobs. Clearly, no correlation between the presence of knobs and the disaccharidases, trehalase and maltase, is evident, at least in the rabbit kidney brush border.

**DISCUSSION**

A procedure for the isolation of brush borders from the rabbit renal cortex has been evaluated. The disaccharidases, trehalase and maltase, alkaline phosphatases (β-glycer-P and trehalose-6-P), a ouabain-sensitive (Na+ + K+) ATPase, an oligomycin-insensitive Mg++ ATPase, a Ca++-activated ATPase, and, perhaps, hexokinase were found in isolated brush borders. The presence of alkaline phosphatase and the (Na+ + K+) ATPase in brush borders was suggested previously (6-9), and the present findings of a three- to fivefold increase in specific activities of the enzymes in fraction P9 relative to the homogenate confirm these earlier suggestions. However, other evidence indicates that alkaline phosphatase and (Na+ + K+) ATPase activities may not be localized exclusively in the renal cortical brush border. Reale and Luciano (23) have shown histochemically that alkaline phosphatase was not restricted to the brush border but was found on the entire tubular cell surface. A (Na+ + K+) ATPase of high activity was recently described from the outer medulla of the rabbit kidney, and activity in the “microsomal” fraction of the cortex was noted (24). Our results also show ATPase activities in subcellular fractions of the kidney cortex in addition to that in the renal brush border. Analogously, a (Na+ + K+) ATPase of high specific activity was reported in the plasma membrane of rat intestinal mucosal cells, with the enzyme in the brush-border fraction having a lower specific activity (18).

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**Figure 3** Electron micrographs of the isolated brush-border fraction from rabbit renal cortex. a, Microvilli seen in longitudinal and cross-section. X 13,000. b, Microvilli showing central core. X 31,000.
The 10- to 20-fold increases in specific activity of the disaccharidases, trehalase and maltase, uniquely in the brush border, plus their complete absence from other areas of the kidney (11, 12), suggests that these enzymes may be better candidates as marker enzymes for the renal brush border. The finding of trehalase in the isolated brush border also provides evidence on the fine localization of the enzyme beyond that resolvable by light-microscope histochemistry (12). In some species, such as the rat, trehalase activity in the kidney was low (11), but maltase was extremely active (Sacktor and Balakir, unpublished). The failure of Binkley and King (7) to find disaccharidase activities in renal brush-border preparations is clearly attributed to their use of deoxycholate in their procedure without taking cognizance of an earlier report that detergents, including deoxycholate, remove disaccharidases from renal cortex membranes (11).

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