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; β-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^{++} ATPase, and a Ca^{++}-activated ATPase. Alkaline phosphatases, dephosphorylating β-glycero-P, 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, but no biochemical correlates were made, and Kinne and Kinne-Saffran (6) and 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,[1] 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 Rostgaard (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 Rostgaard (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 phage-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 (P_1) 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 (P_1') at the interface of the 1.7 and 1.4 M sucrose zones. The pinkish fluffy layer containing the brush borders (P_2) was distributed at the interface of the 1.4 M and 0.5 M sucrose zones. A supernatant (S_1) 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 Rostgaard (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 (P_2) 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

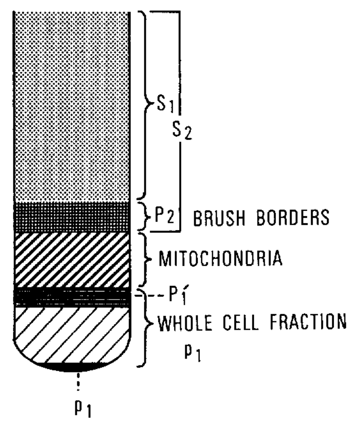


Figure 1 Initial separation of brush-border fraction from rabbit renal cortex homogenate by a sucrose density gradient. The brush borders are concentrated in the P_2 region, the interface of the 1.4 and 0.5 M sucrose zone, after centrifugation at 90,000 *g* for 60 min. See text for details.

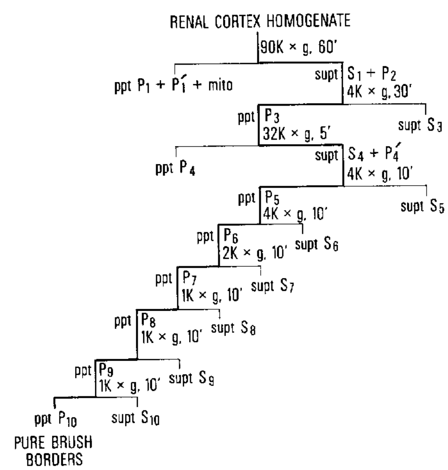


Figure 2 Schematic representation of the procedure for isolating and purifying the brush-border fraction from kidney cortex. The heavy lines indicate the distributions of the brush borders.

(S_3). An additional separation from mitochondrial contamination was obtained by centrifuging a suspension of P_3 at 32,000 *g* for 5 min. The small, tightly packed mitochondrial pellet (P_4) 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 (P_5 through P_9), trace contamination by mitochondrial fragments could be detected at the bottom of the centrifuge tube, and these were discarded as described above. The P_{10} 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 $MgCl_2$, 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-Pase was determined by the method of Hubscher and West (17), except that the reaction contained 0.01 M glucose-6-P and 0.1 M maleic acid buffered at pH 6.5 in a total volume of 0.4 ml. The reaction was incubated at 37°C for 10 min and stopped with 0.1 ml of 12% $HClO_4$. Total ATPase ($Na^+ + K^+ + Mg^{++}$), ouabain-sensitive ($Na^+ + K^+$) ATPase, and oligomycin-sensitive and -insensitive Mg^{++} ATPase were assayed as detailed by Quigley and Gotterer (18). Ca^{++} ATPase was measured according to Martin et al. (19). Alkaline phosphatase and trehalose-6-Pase were determined using a reaction containing 5 mM β-glycero-P or 5 mM trehalose-6-P as substrate, 5 mM $MgCl_2$ and 50 mM *N*-amino methyl propanol, pH 9.6, in a total volume of 0.2 ml. The reactions were incubated at room temperature for 10 min and were stopped with 0.1 ml of 12% $HClO_4$. The inorganic phosphate liberated in the various enzyme assays was determined by the method of Chen et al. (20). Protein was analyzed according to Layne (21).

The isolated brush borders were fixed for electron microscopy in 2.5% glutaraldehyde and 0.3 M sucrose in 0.05 M cacodylate buffer, pH 7.4. The pellet was postfixed in 2% osmium tetroxide and 4.5% sucrose in 0.029 M veronal acetate buffer, pH 7.4, dehydrated in ethanol, and embedded in Epon 812. Thin sections were cut with a diamond knife on an LKB Ultratome 3 and subsequently stained with 3% uranyl acetate in 50% ethanol and lead citrate. For negative staining of the brush borders, 2% potassium phosphotungstate, pH 6.5, was used.

RESULTS

A biochemical evaluation of the procedure for isolating renal brush borders (fraction P_{10}), demonstrating the effectiveness of the separation of brush borders from other subcellular components, is shown in Table I. ATP:NMN adenylyl transferase 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 P_{10} 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 in the P_1 and P_1' fraction, which contained whole cells, large cellular fragments, and nuclei. An additional 10% was located in the P_4 fraction. An undetermined part of the total activity was probably associated with that portion of the P_5–P_9 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 preparation was only one-twentieth that in the original homogenate. In contrast, fraction P_4, 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

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	0.65	63	0.5

The specific activity for trehalase is expressed as μmoles of glucose formed $\times$ min^{-1} $\times$ mg^{-1} protein $\times$ 10^{-2}; for ATP:NMN adenylyl transferase as μmoles of NAD formed $\times$ min^{-1} $\times$ mg^{-1} protein $\times$ 10^{-2}; for cytochrome oxidase as $k = 2.3 \log \frac{A\ (\text{time}_0)}{A\ (\text{time}_0 + 1\ \text{min})} \times \text{min}^{-1} \times \text{mg}^{-1}$ protein $\times$ 10^{-2}; for β-glucuronidase as μmoles of phenolphthalein formed $\times$ hr^{-1} $\times$ mg^{-1} protein $\times$ 10^{-3}; for glucose-6-Pase as μmoles of Pi $\times$ min^{-1} $\times$ mg^{-1} protein $\times$ 10^{-1}; and for hexokinase as μmoles of glucose-6-P formed $\times$ min^{-1} $\times$ mg^{-1} protein $\times$ 10^{-2}.

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

in fraction P_{10}. Fractions P_4 and $P_1 + P_1'$ 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 P_5–P_9 pellets. Approximately 90% of the total β-glucuronidase activity was recovered, with about 60 and 30% being found in the S_3 and $P_1 + P_1'$ fractions, respectively. The specific activity of the enzyme in the S_3 fraction was 100-fold that in the brush-border fraction. The S_3 fraction also contained significant (about 25%) glucose-6-phosphatase activity. 58% of the enzyme remained in the $P_1 + P_1'$ fraction, and an additional 20–30% of the total glucose-6-phosphatase activity was found in the supernatants (S_3 through S_8). 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 P_{10}. Some trehalase (21%) of low specific activity, 0.029 μmoles $\times$ min^{-1} $\times$ mg^{-1} protein, was found in the S_3 fraction. This is likely caused by broken pieces of the brush border which failed to sediment at 4,000 *g*. When fraction S_3 was centrifuged at a higher speed (35,000 *g* for 15 min), approximately 75% of the trehalase present in fraction S_3 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 $P_1 + P_1'$ and S_3 fractions, respectively. The specific activities of the maltase in the $P_1 + P_1'$ and S_3 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

TABLE II

Disaccharidase and Phosphatase Activities of Brush Borders

Enzyme	Specific activity		% Recovery	
	Homogenate	Brush borders	All fractions	Brush borders
Maltase	2.8	39.5	94	18
Alkaline phosphatase	1.3	5.8	120	5
Trehalose-6-P-phosphatase	1.7	7.6	94	5
Total ATPase	17	47	—	2

The specific activities of maltase are expressed as μmoles of glucose formed $\times$ min^{-1} $\times$ mg^{-1} protein $\times$ 10^{-2}, and that of alkaline phosphatase and trehalose-6-P-phosphatase and total (Na^+, K^+, Mg^{++}) ATPase as μmoles of Pi $\times$ min^{-1} $\times$ mg^{-1} protein $\times$ 10^{-2}.

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 (P_{10}). About 5% of the total alkaline phosphatase activity resided in this fraction. However, fraction S_3, 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 $P_1 + P_1'$ 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 S_3; 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 P_{10}, although the brush borders contained only 2% of the total activity of the homogenate. Other cellular fractions, i.e. the mitochondria in P_4, 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 P_4, which was enriched in mitochondria, was inhibited by ouabain. On the other hand, oligomycin inhibited about 80% of the total ATPase in fraction P_4, 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 oubain plus oligomycin

TABLE III

ATPases in Renal Brush Border

ATPase	Specific activity
Na^+, K^+, Mg^{++}-dependent	18.9
Mg^{++}-dependent	11.8
Ouabain-sensitive	5.3
Oligomycin-sensitive	3.5
Ouabain + Oligomycin-sensitive	3.8

Specific activity is expressed as μmoles of P_i/min per mg protein $\times$ 10^{-2}. The oligomycin concentration was 2 μg/ml, equal to 65 μg/mg protein. The concentration of ouabain used was 10 μM. The Tris salt of ATP was used as the substrate.

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.

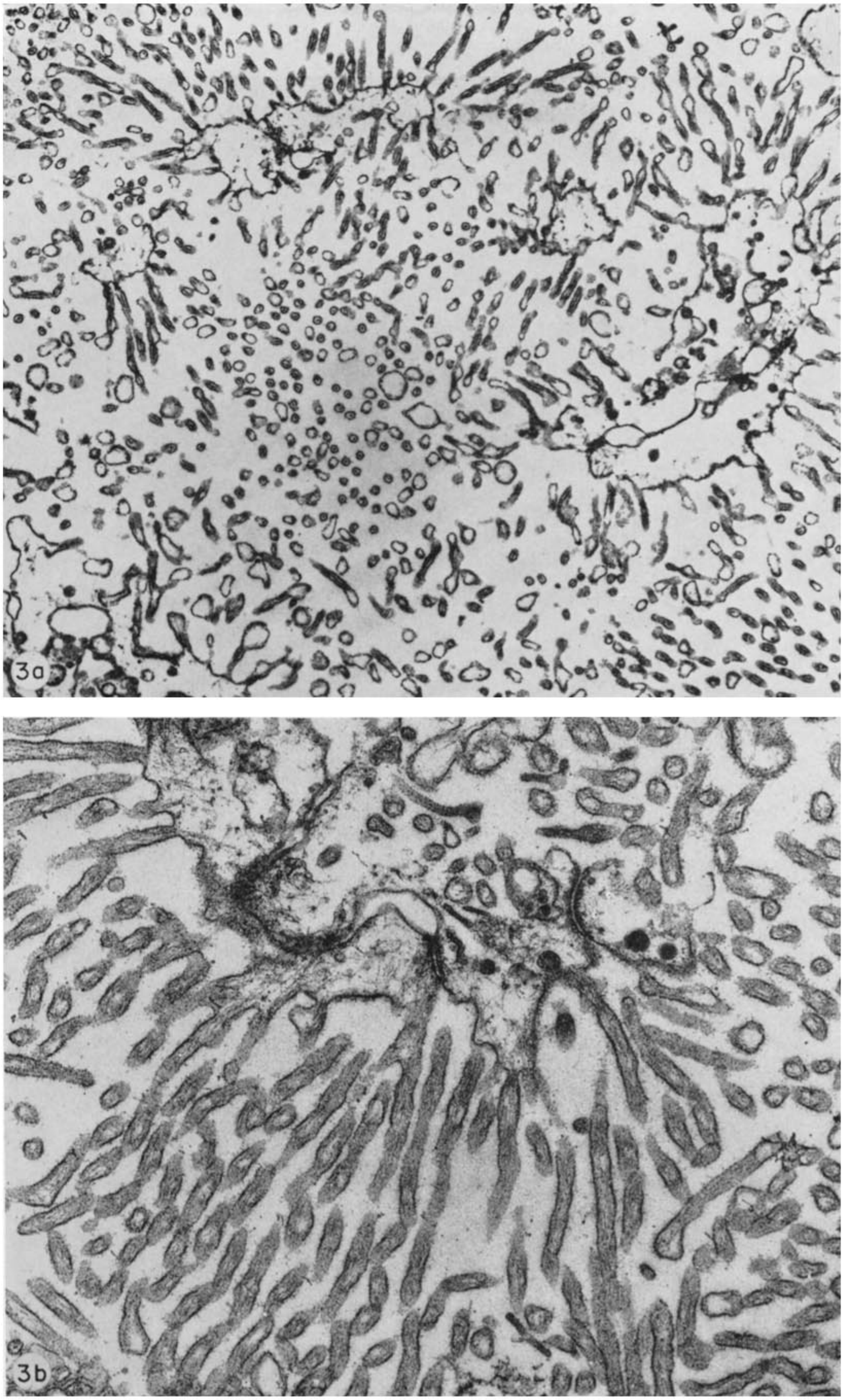
Several enzymes, including hexokinase, phosphoglucomutase, and UDPG pyrophosphorylase, which were shown to participate in the synthesis of trehalose from glucose in yeast and insects, were found previously in the renal cortex (11). Hexokinase, which was predominantly membrane bound (11), was now found to be associated, in part, with the mitochondrial fraction. The specific activity of the enzyme in fraction P_4 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 S_3, 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 P_{10} 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-glycero-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 P_{10} 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).

FIGURE 3 Electron micrographs of the isolated brush-border fraction from rabbit renal cortex. *a*, Microvilli seen in longitudinal and cross-section. × 13,050. *b*, Microvilli showing central core. × 31,000.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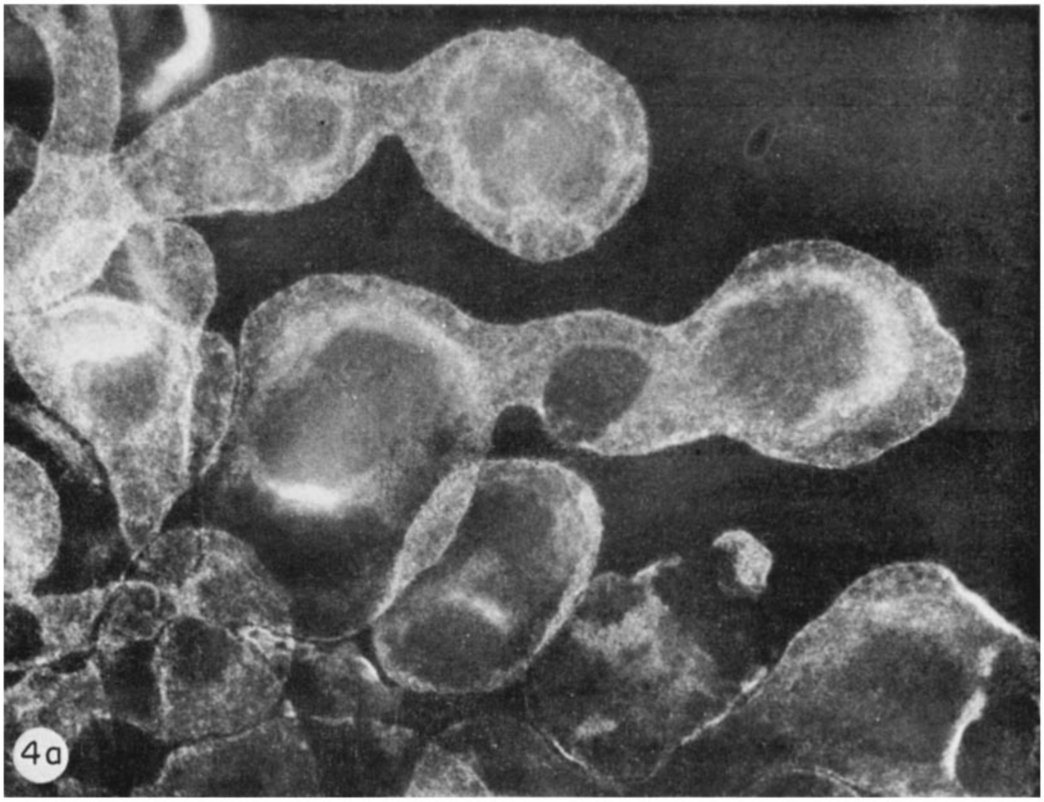
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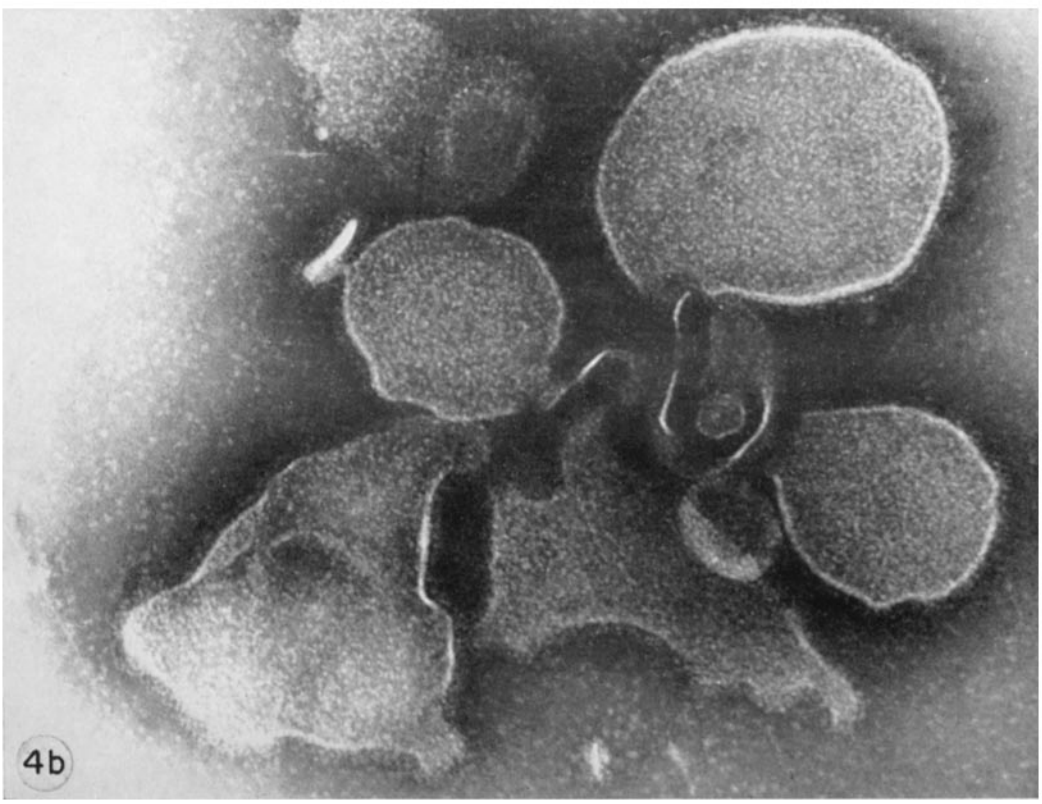
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The 10- to 20-fold increases in specific activity of the disaccharidases, trehalase and maltase, uniquely in the brush border, plus their complete absence from other areas of the kidney (11, 12), suggests that these enzymes may be better candidates 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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Figure 4 Microvilli negatively stained with phosphotungstate. *a*, Microvilli from rabbit renal cortex brush border showing smooth surface. × 115,500. *b*, For comparison, Microvilli of hamster intestinal brush border showing 60–90 A knobs covering the surface. × 115,500.