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The Brush Border of Rabbit Kidney, a Cellular Compartment Free of Glycolytic Enzymes

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Activities of four enzymes of the glycolytic pathway, hexokinase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase, were determined in a vesicular brush-border preparation from rabbit kidneys. The specific activities of the enzymes were decreased several-hundredfold in the brush-border preparation compared with a kidney homogenate, but the enzymes were not totally absent. Density-gradient centrifugation of the brush-border preparation yielded brush border of even higher purity and also a characteristic pattern of distribution for each of the contaminating intracellular membranes. The presence of hexokinase in the brush-border preparation could be traced to contaminating mitochondria, and that of glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase to contaminating vesicles derived from the endoplasmic reticulum. The brush-border vesicles contained some ATP. An intravesicular concentration of 0.1 mM was estimated, indicating that the vesicles had retained at least a part of their original content. Experiments in which fluorescein isothiocyanate-dextran (mol.wt. 20000) was present during cell lysis revealed that much, but not all, of the brush-border contents had been exchanged with the medium. The complete absence of glycolytic enzymes from brush-border vesicles, which had retained part of their original content, indicates that the brush border does not contain glycolytic enzymes *in vivo* and can be thought of as a compartment of its own, somehow separated from the cytoplasm.

Most of the brush-border preparations described in the last 10 years have been well characterized as regards their contamination by intracellular membranes, but have not been checked for the activity of glycolytic enzymes, because the cytoplasmic enzymes were regarded as the cellular components most easily removable during brush-border isolation. However, when glycolytic enzymes were assayed in a brush-border preparation, quite diverging results were obtained. Hexokinase, for example, was found to a considerable extent in one preparation (Berger & Sacktor, 1970), but with one-seventh the relative specific activity in another preparation (May & McCarthy, 1976). Although May & McCarthy (1976) presumed that the hexokinase was brought into the brush-border preparation by contaminating mitochondria, they could not exclude the possibility that this enzyme might be genuinely bound to the brush-border membrane, as hexokinase and other glycolytic enzymes are bound to the erythrocyte membrane or to the plasma membrane of hepatoma cells (Tillmann *et al.*, 1975; McDaniel *et al.*, 1974; Emmelot & Bos, 1966). In the present study a density-gradient centrifugation of a brush-border preparation is described that gave a characteristic pattern of distribution for each contaminating intracellular membrane. By using this procedure the glycolytic enzymes present in the brush-border preparation could be traced to the contaminating mitochondria and endoplasmic reticulum. It has been shown that a part of the isolated brush border forms closed vesicles that could be used to study carrier-mediated transport (Busse *et al.*, 1972; Busse & Steinmaier, 1974). In a study on the transport of amino acids in isolated brush-border vesicles in our laboratory, the question arose as to whether the vesicles still contained ATP. In the present paper we give evidence that the isolated brush border indeed contains some ATP, which must represent at least a part of the original content. We also determined how much of the original brush-border content had been lost during cell lysis, and concluded that much, but not all, of the brush-border volume had been exchanged. The complete absence of glycolytic enzymes from brush-border vesicles, which had retained part of their original contents, offers new insights about the brush-border interior and its relation to the cytoplasm. A preliminary abstract of this work has been presented elsewhere (Busse *et al.*, 1976).

Abbreviations used: FITC-dextran, fluorescein isothiocyanate-dextran; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; ATPase, adenosine triphosphatase.

Materials and Methods

Animals and materials

Rabbits of both sexes (1.5–2kg body wt.) were purchased from a local dealer. Unlike in the previous studies (Busse *et al.*, 1972; Busse & Steinmaier, 1974), the rabbits were not of a particular strain. FITC-dextran (mol.wt. 20000) was purchased from Deutsche Pharmacia G.m.b.H., 6000 Frankfurt, Germany. Glucose oxidase (purity grade 1), peroxidase (purity grade 1) and collagenase (purity grade 2) were purchased from Boehringer Mannheim G.m.b.H., 6800 Mannheim, Germany. All other reagents were of the highest purity available.

Preparation of brush border

Rabbits, which had free access to water and commercial laboratory food, were killed by decapitation. After injection of collagenase (10–15ml of a 2mg/ml solution) into the arteries and preparation of isolated tubule segments from the kidney cortex (Busse *et al.*, 1972), the isolation of brush border from these tubule segments was performed as previously described (Busse *et al.*, 1975). Only the composition of the hypo-osmotic solution for cell lysis was modified in the present study by the addition of 23mM-KCl and the omission of *N*-acetyl-D-glucosamine.

Density-gradient centrifugation

After differential centrifugation the brush-border preparation was resuspended in 200mM-mannitol, 1.4mM-$MgCl_2$, 1.4mM-Hepes/Tris, pH7.5, and immediately placed on top of a linear gradient of solutions between densities of 1.2 and 1.05g/ml and centrifuged for 160min, at 25000rev./min (70000$g_{av.}$) in a SW 25.1 rotor in a Beckman Spinco L50 ultracentrifuge. The density gradient was prepared by a Beckman density-gradient former from the following two solutions: (A) 41% (w/v) sucrose and 10% (w/v) Ficoll in 10mM-Tris/HCl, pH7.5; (B) 12% (w/v) sucrose and 3% (w/v) Ficoll in 10mM-Tris/HCl, pH7.5. After centrifugation the tube was pierced through the bottom with a hypodermic needle, and ten fractions of different volume were usually collected. The fractions, numbered from the top to the bottom of the tube, contained 2, 2, 2, 2, 3, 3, 4, 5, 5 and 2ml of the density gradient respectively.

Marker enzymes and protein

Trehalase (EC 3.2.1.28), a brush-border enzyme, was assayed as previously described (Sacktor, 1968; Busse & Steinmaier, 1974), except for the determination of glucose. The Boehringer test kit for blood sugar, which was used previously, was found to contain traces of trehalase recently. Therefore glucose oxidase (EC 1.1.3.4) and peroxidase (EC 1.11.1.7) of a higher purity were used and the reagent was prepared as described by Bergmeyer (1970). Alkaline phosphatase (EC 3.1.3.1) and $(Na^+ + K^+)$-stimulated Mg^{2+}-dependent ATPase (EC 3.6.1.3) were assayed as previously described (Busse & Steinmaier, 1974). Acid phosphatase (EC 3.1.3.2) was assayed as described by Hübscher & West (1965). The KF-sensitive hydrolysis of glycerol 2-phosphate was taken as acid phosphatase activity. Succinate–cytochrome *c* reductase (EC 1.3.99.1) was assayed as described by Sottocasa *et al.* (1967), with the modification of the final volume from 3ml to 0.75ml. Protein was determined as described by Lowry *et al.* (1951).

Glycolytic enzymes

All glycolytic enzymes were assayed on the basis of the redox reaction of NAD^+ or $NADP^+$. At high concentrations of the enzymes the increase or decrease of NADH or NADPH was observed spectrophotometrically and fluorimetrically; at low enzyme concentrations only the fluorimetric method was used (Rodbell, 1966). Substrates were added as the last component in the assay mixture only when no increase or decrease of absorbance or fluorescence had been observed. Hexokinase (EC 2.7.1.1) was assayed as described by Walker (1963), by using 0.1M-glucose. In a kidney homogenate an exact measurement of hexokinase was not possible, because the homogenate reduced $NADP^+$ at a high rate in the absence of the substrate. This problem, probably caused by endogenous substrates, was also encountered by Walker (1963), who abolished this kind of reduction by dialysis. In our hands, dialysis of the kidney homogenate before the assay did not decrease reduction by endogenous substrates substantially. Therefore hexokinase was assayed in the membrane fractions only. When glucose was replaced by 6-phosphogluconate, no reduction of $NADP^+$ occurred, indicating that the brush-border preparation does not contain 6-phosphogluconate dehydrogenase (EC 1.1.1.44). For the calculation of hexokinase activity it could therefore be assumed that the formation of glucose 6-phosphate is equimolar to the reduction of $NADP^+$ (Walker, 1963). Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) were assayed by the methods of McDaniel *et al.* (1974), Bücher & Pfleiderer (1955) and Rodbell (1966) respectively.

Assay of ATP

The brush border was extracted as described by Greengard & Straub (1959) and assayed for ATP as described by Bergmeyer (1970) by using the phosphoglycerate kinase method. The oxidation of NADH was followed fluorimetrically.

Measurement of intravesicular space

The intravesicular space was determined as previ-

ously described (Busse & Steinmaier, 1974). The difference between the ^3H_2O space and the poly-([^{14}C]ethylene glycol) space of the brush-border sediment was taken as intravesicular space.

Measurement of exchange of vesicular content during cell lysis

The exchange of vesicular content during cell lysis was estimated by the measurement of vesicular uptake of FITC-dextran (mol.wt. 20000). This compound was present in the hypo-osmotic lysis solution at a concentration of 0.4mg/ml. After differential and density-gradient centrifugation the FITC-dextran left in the fractions of the gradient was determined in 10mM-Tris/HCl buffer, pH8.5, with a Farrand Ratio Fluorometer-2 (excitation wavelength 490nm). Emitted light was filtered by a narrow-band interference filter of 504nm and a sharp-cut yellow filter (no. 3-69).

Results

Isolation of brush border by density-gradient centrifugation

The method of brush-border isolation by differential centrifugation was described by Busse & Steinmaier (1974), but since the lysis of the tubule segments in a hypo-osmotic medium had been modified in the present study (see the Materials and Methods section), the marker enzymes for various cell organelles had to be checked again. The upper part of Table 1 shows the specific activities of the enzymes in the brush-border preparation and compares them with the activities in a kidney homogenate.

A density-gradient centrifugation as an additional step of purification yielded brush border almost free of contaminating basal–lateral plasma membrane and mitochondria, as shown by the distribution of their markers $(Na^+ + K^+)$-stimulated Mg^{2+}-dependent ATPase and succinate–cytochrome *c* reductase respectively (Fig. 1). In the central part of the density gradient the only significant contamination was by endoplasmic reticulum, as revealed by its marker enzyme glucose 6-phosphatase. Also, a characteristic distribution for each marker enzyme was achieved by the density-gradient centrifugation. This feature has helped us to localize the membrane-bound glycolytic enzymes, as shown below.

Stimulation of enzyme activity by Triton X-100

The latency of lactate dehydrogenase activity in the brush-border preparation was known to us from previous work (Busse & Steinmaier, 1974). Pretreatment with mild sonication, repeated freeze–thawing or toluene (Tillmann *et al.*, 1975), and the presence of Triton X-100 during the assay, gave a similar stimulation of lactate dehydrogenase activity. Triton X-100 was consistent in its effect and was therefore used for the measurement of the total activity in the present study. Lactate dehydrogenase was stimulated

Table 1. *Specific activities of marker enzymes and glycolytic enzymes in a kidney homogenate and in the brush-border preparation*

Specific activities are expressed as nmol of product formed/min per mg of protein. For details of the enzyme assays, see the Materials and Methods section. Each value is the mean for at least three observations. 'Kidney homogenate' refers to a homogenate of decapsulated whole kidney. Relative specific activity is obtained by the division of the specific enzyme activity in the brush-border preparation by that of a kidney homogenate. Hexokinase could not be assayed in the kidney homogenate (see also the Materials and Methods section). –, Not measured.

	Triton X-100 in assay (%)	Specific activities in Kidney homogenate	Specific activities in Brush-border preparation	Relative specific activity
Trehalase	0	43	729	17.0
Alkaline phosphatase	0	135	1470	10.9
$(Na^+ + K^+)$-stimulated, Mg^{2+}-dependent ATPase	0	125	690	5.5
Glucose 6-phosphatase	0	81	33	0.41
Succinate–cytochrome *c* reductase	0	20	5.0	0.25
Acid phosphatase	0	3.0	4.3	1.4
Hexokinase	0	–	9.7	–
	0.1	–	13.4	–
Glyceraldehyde 3-phosphate dehydrogenase	0	10510	7.5	
	0.2	14050	27	0.002
Pyruvate kinase	0	257	1.1	
	0.1	264	1.5	0.006
Lactate dehydrogenase	0	8100	6.2	
	0.2	8230	15.6	0.002

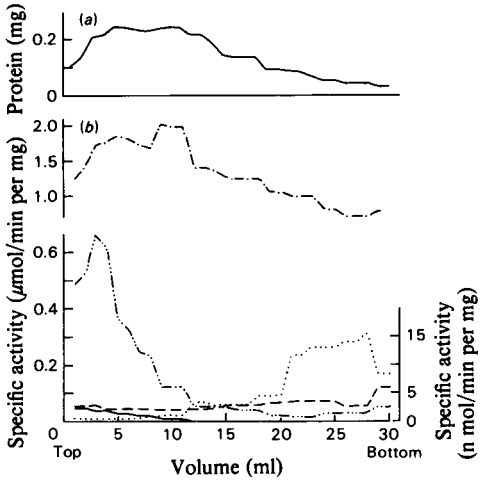

Fig. 1. *Distribution of protein (a) and specific activities of marker enzymes (b) after density-gradient centrifugation*
For details of the centrifugation, see the Materials and Methods section. For the assay of succinate–cytochrome *c* reductase the fractions had to be sedimented (100000*g* for 1 h after 3-fold dilution with water) and taken up in a smaller volume (1 ml) of 200 mM-mannitol/1.4 mM-$MgCl_2$/1.4 mM-Hepes/Tris, pH 7.5. In (*b*) (left-hand ordinate): –·–·–, alkaline phosphatase; –···–···–, $(Na^+ + K^+)$-stimulated, Mg^{2+}-dependent adenosine triphosphatase; – – – –, glucose 6-phosphatase; ——, acid phosphatase; (right-hand ordinate): ····, succinate–cytochrome *c* reductase. All enzymes were determined after the same density-gradient centrifugation. Results shown shown are the means of data obtained by three centrifugations.

10-fold, and glyceraldehyde 3-phosphate dehydrogenase 4–5-fold. Hexokinase and pyruvate kinase showed only little stimulation (1.8- and 1.4-fold respectively). At high concentrations of Triton X-100 (0.4%), even this small stimulation of hexokinase activity was not observed. The stimulation of the other three enzyme activities was not decreased by 0.4% Triton X-100. The concentrations of Triton X-100 used in subsequent experiments were those giving maximum stimulation (for lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase, 0.2%, and for hexokinase and pyruvate kinase, 0.1%). Triton X-100 had only a very small stimulating effect on alkaline phosphatase and trehalase, and decreased the activity of succinate–cytochrome *c* reductase and of glucose 6-phosphatase. For this reason the detergent was only used for the assay of glycolytic enzymes. The omission of Triton X-100 could have been highly misleading. When lactate dehydrogenase was assayed in the fractions obtained by density-gradient centrifugation with and without Triton X-100, a more-than-10-fold difference of activity was observed in the central part of the density gradient, whereas at the top and the bottom the difference was only 2–3-fold. This observation suggests that more than 90% of the activity is cryptic in the central part and is either bound to the inner surface or dissolved in the contents of the lumen of vesicles. At the bottom and top, less of the enzyme activity is cryptic, which suggests that more of it is either bound to membranes but accessible to substrates or has been released from the membranes during centrifugation. In the presence of Triton X-100 the distribution of lactate dehydrogenase activity was similar to the distribution of glucose 6-phosphatase; in the absence of the detergent no correlation with any of the marker enzymes was possible.

Activity of the glycolytic enzymes in the brush-border preparation and distribution after density-gradient centrifugation

The lower part of Table 1 shows the specific activities of four glycolytic enzymes in the brush-border preparation and compares them with the activities of a kidney homogenate. Again, the effect of Triton X-100 is striking in the brush-border preparation, but small in the kidney homogenate, consistent with the vesicular nature of the membranes containing these enzymes. The specific activities of glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase were decreased 500-fold

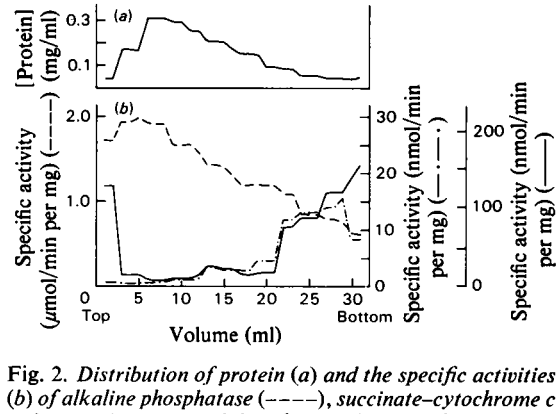

Fig. 2. *Distribution of protein (a) and the specific activities (b) of alkaline phosphatase (– – –), succinate–cytochrome c reductase (–·–·–) and hexokinase (——) after density-gradient centrifugation*
For details of the centrifugation, see the Materials and Methods section. All protein and enzyme assays were performed after the ten fractions collected as described in the Materials and Methods section had been sedimented (100000*g* for 1 h after 3-fold dilution with water) and taken up in a smaller volume (1 ml) of 200 mM-mannitol/1.4 mM-$MgCl_2$/1.4 mM-Hepes/Tris, pH 7.5. Results shown are the means for three observations for each enzyme obtained from three density-gradient centrifugations.

and that of pyruvate kinase was decreased 170-fold in the brush-border preparation compared with the kidney homogenate. Since we were not able to determine the hexokinase in the kidney homogenate (see also the Materials and Methods section), we could not obtain a dilution factor in this case. Are these small amounts of glycolytic enzymes a genuine part of the brush border or do they derive from other membranes still contaminating the brush-border fraction? To answer this question we looked for the pattern of enzyme distribution after density-gradient centrifugation.

Fig. 2 shows the distribution of hexokinase activity after density-gradient centrifugation. Except for the uppermost 3 ml and the bottom 1 ml of the tube it shows a close resemblance to the distribution of succinate–cytochrome *c* reductase, indicating that hexokinase present in the brush-border preparation is localized in contaminating mitochondria. The deviation of distribution pattern of the two enzymes at the top is most likely due to solubilized hexokinase. At the bottom of the gradient the small amount of membrane material did not allow an exact measurement of both enzymes.

Fig. 3 shows that the distribution of the activities of glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase is very like that of glucose 6-phosphatase, indicating that these enzymes are localized in contaminating endoplasmic reticulum.

Estimation of exchange of vesicular content during cell lysis

The experiments depicted in Figs. 2 and 3 show that the glycolytic enzymes present in the preparation are not bound to the brush border, but came into this preparation by contaminating intracellular membranes. Since most of the brush-border microvilli in this preparation are closed vesicles (Busse & Steinmaier, 1974), they should have retained glycolytic enzymes within their lumen, if the brush-border organelles contained glycolytic enzymes and if the contents had not been exchanged during cell lysis and vesicle formation. The presence of small amounts of ATP in the brush-border preparation (Fig. 4) suggests that not all of the original content of the brush border had been lost during the isolation.

The exchange of vesicular content during cell lysis can be estimated quantitatively by the measurement of vesicular uptake of a substance of high molecular weight. For this purpose FITC-dextran (mol.wt. 20000) was added to the hypo-osmotic lysis solution and the amount recovered after the density-gradient centrifugation was determined. When FITC-dextran was dissolved in the membrane suspension just before density-gradient centrifugation, it did not move into the density gradient, but remained in the uppermost 2 ml. This contrasted with FITC-dextran added during

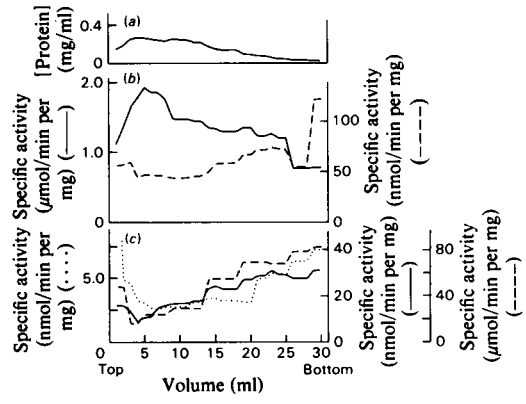

Fig. 3. *Distribution of protein (a) and of the specific activities of alkaline phosphatase* (——), *glucose 6-phosphatase* (----) *(b) and of lactate dehydrogenase* (——), *glyceraldehyde 3-phosphate dehydrogenase* (----) *and pyruvate kinase* (····) *(c) after density-gradient centrifugation*

For details of the centrifugation and of the enzyme assays, see the Materials and Methods section. Values represent the means of data obtained by three centrifugations.

cell lysis, which sedimented, indicating that it was enclosed by vesicles and not bound to the outside of membranes. From the concentration of FITC-dextran in the lysis solution the intravesicular volume exchanged was calculated, as shown in Fig. 5. In addition, the intravesicular volume was estimated in the fractions obtained by density-gradient centrifugation and can be compared with the volume exchanged during hypo-osmotic lysis. The distribution of FITC-dextran, i.e. the exchanged vesicular volume, is similar to the distribution of alkaline phosphatase, indicating that FITC-dextran is taken up preferentially by plasma-membrane vesicles. However, the distribution of actual intravesicular volume resembles more the distribution of protein, indicating that vesicles are present that had been derived from membranes other than plasma membranes. The exchanged volume is smaller than the actual intravesicular volume by about one-third. We will discuss below whether these data imply that the brush border has retained one-third of its soluble content.

Discussion

Glycolytic enzymes are not bound to the brush border

The data presented clearly indicate that the enzymes hexokinase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase are not bound to the brush border. Their specific activity in the brush-border preparation is decreased several-hundredfold compared with a

kidney homogenate (Table 1). Similar results have been obtained by May & McCarthy (1976), who reported a 15-fold decrease of hexokinase specific activity in an intestinal brush-border preparation.

Density-gradient centrifugation yielded brush border of even higher purity and also gave a characteristic pattern of distribution for each of the contaminating intracellular membranes (Fig. 1). The glycolytic enzymes in the preparation could be attributed to the contaminating mitochondria and endoplasmic reticulum (Figs. 2 and 3). The use of Triton X-100 in the enzyme assays was essential for the release of cryptic glycolytic enzymes, so that they could be correlated with the marker enzymes in the density gradient. It is also very unlikely that glycolytic enzymes originally bound to the brush border had been lost or inactivated during hypo-osmotic lysis and differential centrifugation, because contaminating intracellular membranes, which had gone through the same procedure, have retained at least a part of the glycolytic enzymes originally bound to them.

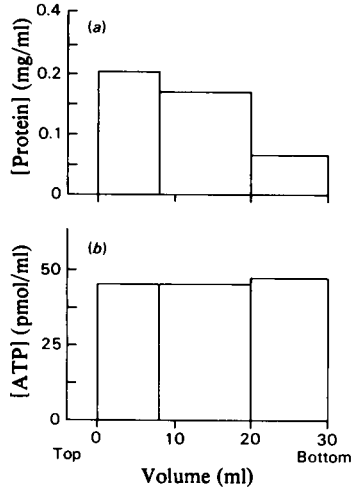

Fig. 4. *Distribution of protein (a) and ATP (b) after density-gradient centrifugation*

Since the assay of ATP was not sensitive enough to measure the ATP content of a 1 ml fraction, the 30 ml of the density gradient were divided into three fractions only. The membranes of the three fractions were sedimented (100000*g* for 1 h after 3-fold dilution with water), resuspended in 0.6 ml of 200 mM-mannitol/1.4 mM-Hepes/Tris, pH 7.5. Samples were taken for the determination of protein. The remainder was processed for the ATP assay as described in the Materials and Methods section. For a better comparison with the other Figures, the values are depicted as if protein and ATP had been assayed per ml of fraction.

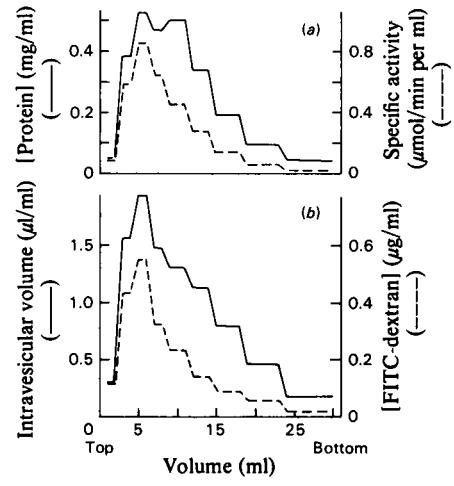

Fig. 5. *Distribution of protein, alkaline phosphatase (a), intravesicular volume and FITC-dextran (b) after density-gradient centrifugation*

For details of the centrifugation, see the Materials and Methods section. The brush-border preparation used was obtained by hypo-osmotic lysis in the presence of 0.4 mg of FITC-dextran/ml. After density-gradient centrifugation the fractions obtained were sedimented (100000*g* for 1 h after 3-fold dilution with water) and resuspended in 1.5 ml of 140 mM-mannitol/1.4 mM-$MgCl_2$/1.4 mM-Hepes/Tris, pH 7.5. A portion (1 ml) of this membrane suspension was taken for the measurement of intravesicular volume as previously described (Busse & Steinmaier, 1974), and up to 0.1 ml for the measurement of alkaline phosphatase. In a 0.3 ml sample FITC-dextran was determined by its fluorescence after the addition of 0.9 ml of 10 mM-Tris/HCl, pH 8.5, as described in the Materials and Methods section. After the measurement of FITC-dextran the same samples were used for the protein assay. All values are expressed per ml of fraction. The scale for the amount (in μg) of FITC-dextran was adapted such that the amount (in μg) taken up by the vesicles can easily be transformed into μl of exchanged vesicular volume on the left-hand scale. Results shown represent the means of data obtained by three density-gradient centrifugations.

Is the interior of the brush border free of glycolytic enzymes?

Electron-microscopic studies demonstrated that the brush-border microvillus is not just a simple tube closed at one end, but contains a skeleton of contractile proteins (Rostgaard & Thuneberg, 1972; Rodewald *et al.*, 1976). Since glycolytic enzymes have not been found in isolated brush-border vesicles, the question arises as to whether glycolytic enzymes ever were present in the brush-border interior. Fig. 4 shows that the brush-border prepara-

tion contains some ATP. From the amount of ATP (0.27 nmol/mg of protein) found in the central part of the density gradient (this part contains brush border of the highest purity), an intravesicular concentration of 0.1 mM was calculated. If we are correct in our conclusion that the isolated brush-border vesicles retain ATP, they should have also retained glycolytic enzymes, if they too are normally present in the brush-border interior. Since glycolytic enzymes are not associated with the isolated brush border, we suggest that the brush border does not contain glycolytic enzymes *in vivo*. If this is so, a barrier for large molecules between brush-border lumen and the cytoplasm is suggested.

The experiments depicted in Fig. 5 indicate that the brush border had been open and accessible for larger molecules like FITC-dextran for a certain time. Therefore the loss of large molecules from the interior of the brush border during hypo-osmotic lysis is also possible. However, the FITC-dextran space is about two-thirds of the intravesicular volume, suggesting that one-third of the contents had not been exchanged and lost. But we should be cautious about this interpretation, because the intravesicular volume had not been measured under the same osmotic condition as the vesicular uptake of FITC-dextran. Also, when the uptake of FITC-dextran occurred, the vesicles were leaky, and at that time the vesicles might have had a smaller volume than after they became resealed. Therefore this determination of vesicular exchange can be regarded only as a rough estimate and provides only tentative evidence that not all the brush-border contents had been lost during the isolation procedure.

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