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# LEAKINESS OF BRUSH-BORDER VESICLES

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**From the water content of pelleted brush-border vesicles and from a comparison of the aqueous volume within the pellet that is available to [$^3$H]inulin (58%), inulin [$^{14}$C]carboxylic acid (34%, both approx. 5000 daltons), [$^3$H]raffinose (97%, 540 daltons) and [$^3$H]glucose (94%, 180 daltons) it is concluded that only 1 in 4 to 6 of the brush-border vesicles is sealed. The implication of this finding for labelling and transport studies and for vesicle formation is discussed.**

## Introduction

Brush-border vesicles prepared using a $CaCl_2$ or $MgCl_2$ purification step [1–4] are sealed in that they can accumulate D-glucose and L-amino acids against a concentration gradient [3,5–14]. The kinetics of uptake show an overshoot which decays with time towards an equilibrium value. From the equilibrium values shown in the figures of references [3,5–14] an internal aqueous volume of 1–2 $\mu$l per mg of protein can be calculated for brush-border vesicles prepared from mammalian microvilli and of 3 $\mu$l per mg of protein from fish microvilli [13,14]. The value for mammalian brush-border vesicles is independent of the substrate (D- or L-glucose, L-proline, L-leucine, L-phenylalanine, glycine, aminoisobutyric acid, L-ascorbate, choline, $Na^+$, $SO_4^{2-}$), the animal (mouse, rat, rabbit, dog, pig), the tissue (kidney or intestine), whether the tissue was fresh or had been stored frozen and also, importantly, of the minor variations that must occur from one laboratory to another in the preparation of the vesicles and in assaying substrate uptake. All these details indicate that the brush-border vesicles that are capable of accumulating substrates are relatively tightly sealed and that they do not, to any extent, lose these substrates when the uptake is stopped by diluting the vesicles in ice cold buffer and by filtering and washing them. Further, it has been concluded that the fraction of sealed vesicles is larger than 90%. This is based on the observation that trypsin only digests brush-border actin, which is enclosed inside the vesicles [15], in the presence of detergent [16].

In electron micrographs of thin sections of pelleted brush-border vesicles about half the area is apparently enclosed by the vesicles (e.g., Fig. 2c of Ref. 3). This would indicate, excluding gross artifacts arising during the preparation for microscopy, that within a pellet of brush-border vesicles at least 50% of the volume and presumably of the total water is inside the vesicles. This would give, if the value of 1–2 $\mu$l of water per mg protein is accepted as the internal volume of mammalian brush-border vesicles, a total water content for the pellet of 2–4 $\mu$l per mg protein. Here it is shown that this value is an underestimate, which arises from a substantial proportion of the vesicles being unsealed.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid.

## Materials and Methods

*Materials.* Chemical reagents were of analytical grade. The radioisotopes were from New England Nuclear, Boston, MA, U.S.A. (D-[$^3$H]glucose, [$^3$H]raffinose) and Amersham International, Bucks, U.K. ([$^3$H]inulin and inulin [$^{14}$C]carboxylic acid).

*Preparation of brush-border vesicles.* The source was rabbit small intestine that had been stored at −50°C. The method followed, essentially, those of Schmitz et al. [1], Kessler et al. [3] and Hauser et al. [4]. However the isolation medium, refered to as the buffer, consisted of 300 mM D-mannitol, 2 mM EGTA and 2 mM Hepes plus NaOH to pH 7.6; and 10 mM $MgCl_2$ was used in the precipitation step.

*Determination of the excluded water space in pelleted brush-border vesicles.* 50 μl of a brush-border vesicle suspension were diluted in 1 ml of the buffer and then centrifuged (25 000 × *g* for 30 min) in weighed tubes. The supernatants were discarded and the pellets were weighed. After equilibration either at 0°C or at room temperature (20 ± 2°C), the pellets were resuspended in, approximately, their own weight of a solution, at the same temperature, containing one of the following, dissolved in the buffer: D-[$^3$H]glucose, [$^3$H]raffinose, [$^3$H]inulin or inulin [$^{14}$C]carboxylic acid. After incubation, for either 10 or 90 min, the suspensions were centrifuged (as above) and the tubes were then reweighed to determine the total weight of the suspension ($S_{total}$) and the weight of the radioactive solution that had been added ($R_{added}$). For each determination the concentration of the added radioactive compound, relative to its initial concentration, ($C_{rel}$) was measured in six aliquots taken from each of eight separate supernatants. From the above the weight of the pellet that is not available to the added radioactive compound was calculated ($P_{unavailable} = S_{total} - (R_{added}/C_{rel})$). The remaining supernatant was then discarded and the tubes were reweighed to determine the pellet wet weight ($P_{total}$). To determine the dry weight of the pellets ($P_{dry}$), these were suspended in 2 mM Hepes plus NaOH to pH 7.6 and centrifuged (as above), after discarding the supernatant the pellets were dried for 16 h at 55°C. The dry weight was 1.7 ± 0.17 mg ($n = 128$) of which the contribution from the Hepes/NaOH is approx. 4 μg. The weight of available water in the pellet = $P_{total} - P_{unavailable}$. The weight of unavailable water = $P_{unavailable} - P_{dry}$. (Suspending brush-border vesicles in water does not release protein into solution, Gains, N. and Hauser, H., unpublished observation.)

## Results

The weight ratio of pellet water to brush-border vesicle dry weight is 9.1 mg/mg ($n = 128$), for vesicles prepared from rabbit small intestine that have been centrifuged at 25 000 × *g* for 30 min through approx. 3 mm of suspending medium. The fraction of the total pellet water that is available to D-[$^3$H]glucose, [$^3$H]raffinose, [$^3$H]inulin and inulin [$^{14}$C]carboxylic acid is given in Table I. For any one of these compounds, this fraction does not increase markedly with time (10 or 90 min) nor is it greatly affected by the temperature (0 or 20°C). Virtually all of the pellet water is available to D-[$^3$H]glucose (0.94 ± 0.06 mg/mg, $n = 32$) and [$^3$H]raffinose (0.97 ± 0.08 mg/mg, $n = 32$). In contrast, the fraction available to [$^3$H]inulin (0.58 ± 0.05 mg/mg, $n = 32$) and inulin [$^{14}$C]carboxylic acid (0.34 ± 0.06 mg/mg, $n = 32$) is much smaller. These values are also given in Table I as mg of unavailable pellet water per mg of dry weight.

## Discussion

The entrapped aqueous volume of brush-border vesicles from rabbit small intestine as determined from the equilibrium uptake values of a variety of low molecular weight compounds ($Na^+$, D- and L-glucose and choline) is 1–2 μl per mg of protein [3,6]. As the protein to lipid ratio of these brush border vesicles is approx. 2:1 [4,17], and if the carbohydrate content is ignored, then the above value can be rewritten as 0.7–1.3 mg of entrapped water per mg dry weight. The weight ratio of pellet water to brush-border dry weight is 9.1 ± 0.8 mg/mg ($n = 128$) (Table I). From these two sets of data the ratio of the internal to external aqueous spaces would lie between 0.08 : 0.92 and 0.17 : 0.83. These values are very different from the internal to external aqueous space determined from the [$^3$H]inulin and the inulin [$^{14}$C]carboxylic acid

TABLE I

COMPARTMENTATION OF D-[$^3$H]GLUCOSE, [$^3$H]RAFFINOSE, [$^3$H]INULIN AND INULIN [$^{14}$C]CARBOXYLIC ACID IN PELLETED BRUSH-BORDER VESICLES FROM RABBIT SMALL INTESTINE

Eight aliquots (approx. 35 mg) of a solution of one of the radioactive compounds in the buffer (300 mM mannitol, 2 mM EGTA, 2 mM Hepes plus NaOH to pH 7.6) were, each, mixed with approx. 30 mg of a suspension of brush-border vesicles (dry weight $1.70 \pm 0.17$ mg, $n = 128$) in the buffer. After incubation the suspension was centrifuged ($25000 \times g$ for 30 min) in order to separate a vesicle pellet (wet weight $17.1 \pm 1.7$ mg, $n = 128$; pellet water to dry weight $9.1 \pm 0.8$ mg/mg, $n = 128$) from a supernatant containing the radioactive compound. Radioactivity was measured in six aliquots of each supernatant. The volume (in mg) into which the radioactive compound had been diluted was calculated as is described in Materials and Methods.

| | $n$ | Available water/total pellet water (mg/mg) | Unavailable water/dry weight (mg/mg) |
|---|---|---|---|
| D-[$^3$H]Glucose (+2 mM carrier) | | | |
| 10 min at 0°C | 8 | $0.93 \pm 0.06$ | $0.6 \pm 0.6$ |
| 90 min at 0°C | 8 | $0.91 \pm 0.04$ | $0.9 \pm 0.4$ |
| 10 min at 20°C | 8 | $0.93 \pm 0.05$ | $0.6 \pm 0.4$ |
| 90 min at 20°C | 8 | $1.01 \pm 0.05$ | $-0.1 \pm 0.5$ |
| Average of above | 32 | $0.94 \pm 0.06$ | $0.5 \pm 0.6$ |
| [$^3$H]Raffinose (+5 mM carrier) | | | |
| 10 min at 0°C | 8 | $0.94 \pm 0.08$ | $0.2 \pm 0.3$ |
| 90 min at 0°C | 8 | $0.93 \pm 0.04$ | $0.6 \pm 0.4$ |
| 10 min at 20°C | 8 | $0.94 \pm 0.05$ | $0.4 \pm 0.3$ |
| 90 min at 20°C | 8 | $1.05 \pm 0.10$ | $-0.4 \pm 0.9$ |
| Average of above | 32 | $0.97 \pm 0.08$ | $0.3 \pm 0.7$ |
| [$^3$H]Inulin | | | |
| 10 min at 0°C | 8 | $0.60 \pm 0.05$ | $3.6 \pm 0.5$ |
| 90 min at 0°C | 8 | $0.59 \pm 0.07$ | $3.7 \pm 0.7$ |
| 10 min at 20°C | 8 | $0.58 \pm 0.03$ | $3.8 \pm 0.3$ |
| 90 min at 20°C | 8 | $0.56 \pm 0.05$ | $4.0 \pm 0.5$ |
| Average of above | 32 | $0.58 \pm 0.05$ | $3.8 \pm 0.5$ |
| Inulin [$^{14}$C]carboxylic acid | | | |
| 10 min at 0°C | 8 | $0.38 \pm 0.03$ | $5.6 \pm 0.3$ |
| 90 min at 0°C | 8 | $0.39 \pm 0.06$ | $5.5 \pm 0.5$ |
| 10 min at 20°C | 8 | $0.29 \pm 0.03$ | $6.4 \pm 0.2$ |
| 90 min at 20°C | 8 | $0.29 \pm 0.05$ | $6.4 \pm 0.5$ |
| Average of above | 32 | $0.34 \pm 0.06$ | $6.0 \pm 0.6$ |

available space, these are 0.42 : 0.58 and 0.76 : 0.34, respectively (Table I). On both theoretical and empirical grounds the latter, and not the former, set of values would seem to be correct. For example, the internal to external volume of spheres having the same radius and at maximal packing is 0.76 : 0.24, of water equilibrated Sephadex and Sepharose is about 0.7 : 0.3 (from the void and total column volume), of pelleted red blood cells is 0.96 : 0.04 [18,19] of pelleted mitochondria is 0.6 : 0.4 (derived from data in Refs. 20 and 21) and calculated from electron micrographs of brush-border vesicles is 0.5 : 0.5 (see Introduction). The discrepancy between the fraction of the pellet water available to [$^3$H]inulin ($0.58 \pm 0.05$, $n = 32$) and that available to inulin [$^{14}$C]carboxylic acid ($0.34 \pm 0.06$, $n = 32$) could be that [$^3$H]inulin, like [$^3$H]methoxyinulin [3], is not totally excluded from the internal vesicular space. If this is the case then the available aqueous space will be greater than the external volume. It is also not certain that inulin [$^{14}$C]carboxylic acid is totally excluded.

As the internal volume derived from equilibrium uptake is 0.7–1.3 mg water per mg dry weight [3,6] and that from the pellet water space that is unavailable to [$^3$H]inulin and inulin [$^{14}$C]carboxylic acid is 3.8–6.0 mg water per mg dry weight, it must be concluded that only one in four to six vesicles is sealed. One possible counter argument is that the internal water space as measured by the exclusion of macromolecules contains water that is not available, perhaps because it is bound to lipids and proteins, to small molecules taken up into the vesicles. This argument is contradicted by the results in Table I that show that more than 94% of the total pellet water is available to [$^3$H]glucose and [$^3$H]raffinose. A second possible counter argument is that the internal water space measured by equilibrium uptake (0.7–1.3 mg per mg dry weight) is the correct value and that measured by inulin and inulin [$^{14}$C]carboxylic acid (3.8–6.0 mg per mg dry weight) includes a water space contained in an externally situated glyco- or mucocalyx from which these macromolecules are excluded. This however seems unlikely. Firstly, it is known from their disruptive activity that papain (23000 daltons [22]), cholesterol oxidase (56000 daltons [23]) and phospholipase $A_2$ (14000 daltons [24]) have access to the membrane surface. Secondly, assuming that the glyco- plus mucocalyx is one-third of the dry weight then it must sequester and render 9- to 14-times its own weight of water unavailable to these macromolecules. Thirdly, if

the brush-border vesicle radius is taken as 69 nm (see below) then the glyco- plus mucocalyx must be 150 to 120 nm thick; giving a closest approach between two vesicles of 230 to 240 nm. The closest approach found in electron micrographs of brush border vesicles (e.g., Fig. 2c of Ref. 3) is about 20 nm and of transverse sections through microvilli attached to enterocytes (e.g., Fig. 10.2 of Ref. 25) is about 25 nm.

Results that indicate the possibility of sealed and unsealed vesicles have been obtained from labelling brush-border vesicles with ATP. Hammerman and Hruska [26] showed that two proteins (62000 and 98000 daltons) were labelled with ATP only in the presence of cyclic AMP and only if the brush-border vesicles were osmotically shocked. However, actin was labelled in the controls that were not osmotically shocked (compare Figs. 4 and 1 in Ref. 26), this indicates that the two proteins are only present in a subpopulation of the vesicles that are normally sealed with respect to ATP and cyclic AMP. These proteins could be unique to the subpopulation of vesicles or, merely, have been lost from the less well sealed vesicles at some point during their isolation.

*Labelling of brush-border vesicles with amino reagents*

This also indicates that a sizeable proportion of brush-border vesicles is unsealed. Although a variety of low molecular weight ($< 700$) labels, including some that have been considered to be impermeant, has been used, actin, which is taken as a cytoplasmic marker [15], is always labelled. Semenza and his co-workers [27,28] have used various photolabile derivatives of phlorizin. Although these specifically inhibit the $Na^+$-dependent glucose transporter they also label actin. Similarly Norén and his co-workers [29,30] showed that azido[$^3$H]phenylalanine inhibited the neutral amino acid carrier, however, only actin and other Triton X-100-insoluble proteins were labelled. Bürgi et al. [31] have used the slowly permeating 3-(dimethyl-2-([$^3$H]acetimidoxyethyl)-ammonio) propanesulphonate and the relatively rapidly permeating ethyl acetimidate to show that the $NH_2$-terminus of sucrase · isomaltase is on the luminal surface of brush-border vesicles. Both label actin, but the luminal position of the $NH_2$-terminus was derived from their relative rates of permeation and of labelling. Booth and Kenny [32], using brush border vesicles from pig kidney showed that 3,5-diiodo-4-azidobenzenesulphonate labelled actin, whereas membrane proteins, but not actin, were labelled after treatment with lactoperoxidase and $^{125}$I. It has also been found that various amino reactive fluorochromes label actin (cycloheptaamylose complexes of dansyl chloride and fluorescamine, fluorescein isothiocyanate, lissamine rhodamine sulphonyl chloride and lucifer yellow); a time-course showed that some of these labels were first detectable in actin and then in sucrase and isomaltase (Gains, N. and Hauser, H., unpublished observations).

Labelling studies seem to be hampered by two factors: firstly, low molecular weight, charged, hydrophilic compounds penetrate the membrane; and secondly, the reaction rate of actin with many of these probes is faster than that of the externally situated sucrase and isomaltase.

*Formation of brush-border vesicles from microvilli*

If only one in four to six brush-border vesicles are sealed then the question arises as to whether the sealed vesicles are, otherwise, representative of the whole population or whether they form a distinct subpopulation. From this point of view it is interesting to consider the physical limitations that could determine the formation of vesicles and what the possible outcome would be. For the sake of the argument it has been assumed that when the microvilli break up to form vesicles the structural organisation although altered does not lead to a change in the ratio of the surface area to the enclosed volume. In which case, the following two equations relate the surface area and volume of the microvillus (assuming it is a cylinder with two flat ends) to those of the vesicles formed (assuming they are spherical):

$$\pi 2 r_m l = n 4 \pi r_v^2$$

$$\pi r_m^2 l = n 4 \pi r_v^3 / 3$$

where $r_m$ is the radius and $l$ the length of a typical microvillus and $n$ is the number and $r_v$ the radius of the vesicles formed from it. Solving these equations shows that $r_v = 3r_m/2$ and $n = 2l/9r_m$. If the length of a typical microvillus is taken as 1120 nm

and its radius as 58 nm (from Fig. 2a of Ref. 33) then from each microvillus 4.6 vesicles of 86 nm radius will be formed. Although these values are hypothetical, the calculated vesicle radius is similar to that found in electron micrographs of brush-border vesicles (e.g., Fig. 2b of Ref. 3, where $r = 69 \pm 11$ nm (8)). Further the value of 4.6 vesicles per microvillus could be relevant to the results in Table I, which indicate that only one in four to six vesicles are sealed. The possibility arises that the maximum number of sealed vesicles that can form from a microvillus is one. A broken off microvillus is a cylinder of which the luminal end is naturally sealed and the proximal end may or may not be resealed; Bretscher [34] has reported that some of the cytoskeleton can be liberated from microvilli without disrupting the membrane. When the microvillus breaks up to form vesicles the one that forms from the naturally sealed luminal end will have only one break in the membrane, whereas the other three or four vesicles will have two breaks in their membranes. It may not, therefore, be unrealistic to assume that if only one from these four or five vesicles subsequently seals it will be that from the luminal end, which has the least damaged membrane. In this case the sealed vesicles would form a distinct subpopulation, the protein composition and the enzymatic and transport activities of which may not be typical of the microvillus as a whole.

There is some evidence from electron microscopy of chicken intestinal microvilli that partially supports the above [34,35]. $CaCl_2$ treatment of isolated microvilli causes them to adopt a beaded appearance, which may well be an intermediary stage in vesiculation. In Fig. 4a of Ref. 34 the microvilli are between 1.5 and 2.8 $\mu$ long with a radius of approx. 0.05 $\mu$m and from the above argument would be predicted to give rise to 7 to 12 vesicles. Treatment with 5 mM $CaCl_2$ produces microvilli with 6 to 10 beads. Similarly, it can be predicted that the microvillus in Fig. 1b of Ref. 35 would break up to give 8 or 9 vesicles. It is compared with a microvillus, shown in Fig. 1a of the same reference, which after $CaCl_2$ treatment has formed 9 beads.

*Transport studies*

If one in four to six vesicles is sealed and if a particular transport function within these vesicles is representative of that in the vesicles as a whole then $V_{max}$ and $n$, the number of carrier molecules per unit weight of protein, will be underestimated by a factor of four to six. If the sealed vesicles contain less transporter than on average then $V_{max}$ and $n$ will be further underestimated. However, even a combination of these factors would seem unlikely to account for the difference between the maximal rate of glucose production via sucrase, isomaltase and maltase (90, 180 and 184 nmol/s per mg brush-border protein, respectively [36]) and the maximum rate of glucose transport by the $Na^+$-dependent glucose transporter (200 pmol/s per mg brush-border vesicle protein [3,37]).

*Detection of holes by electron microscopy*

There is no evidence to be found in electron micrographs for the existence of holes in brush-border vesicle membranes. However, this may not be surprising. If it assumed that only molecules of 5000 daltons or less are permeable, that they are spherical and have a density of 1, then their radius will be 1.3 nm or less. They should therefore pass freely through a hole of radius 3 nm. If the holes arise from imperfections in resealing then a maximum of two holes per vesicle would be expected. For either reason their detection in electron micrographs is improbable. Lieber and Steck [38,39] have shown that there is one, haemolysis induced, hole in each red blood cell ghost. These holes are stable and have a radius (0.7–100 nm) that is dependent on the experimental conditions used in resealing.

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