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DISACCHARIDE UPTAKE BY BRUSH-BORDER MEMBRANE VESICLES LACKING THE CORRESPONDING HYDROLASES

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Intestinal disaccharide uptake was studied with isolated brush-border membrane vesicles lacking the corresponding hydrolase. Either 15-day-old chick intestine, lacking both trehalase and lactase, or newborn pig intestine, lacking sucrase, was used. Both animal species yielded osmotically active vesicles capable of D-glucose/Na^+ cotransport with a positive overshoot test. Vesicles from either origin gave quantitatively similar results in regard to both initial uptake rates and relative vesicle volumes. The nontransported analogs D-mannitol and L-glucose were used as diffusion markers. When tested with the appropriate disaccharidase-lacking vesicles, lactose, trehalose and sucrose exhibited uptake rates indistinguishable from those of D-mannitol and L-glucose. These uptakes were unaffected by the presence or absence of Na^+, phlorizin and Tris. Chromatographic analysis confirmed the lack of hydrolysis of each disaccharide after prolonged incubation. The inescapable conclusion seems to be that intact disaccharides are not transported through the brush-border membrane, their uptake occurring through simple diffusion.

Introduction

Miller and Crane [1,2] showed that glucose residues originating from disaccharide hydrolysis have a 'kinetic advantage' for transport across the brush-border membrane of the enterocyte. Similar conclusions were later reached by Parsons and Prichard [3], who proposed the equivalent concept of 'high probability of capture'.

Three hypotheses have been advanced to explain this kinetic advantage. (1) Some disaccharides cross the brush-border membrane intact and are hydrolyzed by intracellular disaccharidases [4]. (2) Disaccharide hydrolysis takes place at the outer face of the membrane but at a locus close to the transport carriers in such a way that nascent monosaccharides have a better chance of being transported into the cell than of being released into the external (incubation) medium [2,3]. (3) The membrane-bound disaccharidases act as vectorial enzymes, meaning that they take up the disaccharide from the outer face of the membrane and deliver the monosaccharide products to the cell interior [5–9].

The first hypothesis has been rejected [5,9] although some observations indicate that disaccharidase activity may exist on both sides of the brush-border membrane [4]. Since no direct evidence was available disproving the transport of intact disaccharides, we felt that this question had to be clarified before the hypothesis could be discarded. It is known, for instance, that certain phenylglycosides are actively transported intact by the small intestine of both hamster [10,11] and chicken [12]. Moreover, maltose seems to be taken up intact by human buccal mucosa [13].

However, work with disaccharides is hampered by the presence of specific hydrolases in the

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brush-border. Obviously, to study disaccharide transport in the intestine, the use of preparations lacking the corresponding enzyme is imperative. Some animal species, such as certain frogs, lack sucrase and lactase [14]; fowls lack trehalase, lactase and cellobiase [15,16]. In the present paper we summarize results concerning the uptake of trehalose and lactose by brush-border membrane vesicles from 15-day-old chicks, as well as results concerning the uptake of sucrose by newborn pig intestine that lacks sucrase activity [17].

Materials and Methods

Animals. Chicks and newborn pigs were supplied by the INRA breeding center of Jouy-en-Josas (France). The intestines were divided into three sections for freezing into separate lots: duodenum, jejunum and ileum. After rinsing with saline, the intestinal segments were everted, frozen and stored at −20 °C.

Materials. All reagents were of analytical grade. Radioactive compounds were: D-[U-^{14}C]glucose and [1-^{3}H]mannitol from New England Nuclear Corp.; α,α[U-^{14}C]trehalose and [U-^{14}C]sucrose from International Chemical Nuclear Pharmaceuticals Inc.; and [D-*glucose*-1-^{14}C]lactose from Amersham International. Radioactive purity was checked using either cellulose thin-layer chromatography (F 1440/LS 254, cellulose Avicel, Schleicher-Schüll, F.R.G.) with ethanol/butanol/water (33 : 52 : 15, v/v) as a solvent [18] or paper chromatography with water/*n*-butanol/ethyl acetate/isopropanol/acetic acid (30 : 35 : 100 : 60 : 35, v/v) [18].

Assays. Maltase, sucrase, trehalase and lactase were assayed by measuring glucose formation with glucose oxidase and peroxidase [19]. When very large amounts of membrane material were used for certain analyses, the incubation mixture was deproteinized [20] before analyzing for glucose. Protein was assayed by the procedure of Lowry et al. using bovine serum albumin as a standard [21].

Brush-border membrane vesicle preparations. The calcium precipitation method as modified by Kessler et al. [22,23] was used with the following minor modifications: (i) the buffer was composed of 10 mM Hepes, 7 mM *n*-butylamine and maleic acid to adjust the pH to 7.4; (ii) the top-speed membrane homogenization procedure was performed in the presence of either 100 mM or 500 mM sorbitol and these sorbitol concentrations were maintained during the subsequent fractionation steps; (iii) the concentrated vesicle fractions (25 mg/ml) were often stored in liquid nitrogen. No difference was found in the transport behaviour of freshly prepared and frozen vesicles.

Uptake experiments. We determined substrate uptake by the membrane vesicles with the rapid filtration technique as described by Hopfer et al. [24] using the Short-time Incubation Apparatus of Innovative Labor AG (Switzerland). All incubations were performed at room temperature (about 25 °C) in a medium containing: (1) the Hepes/*n*-butylamine/maleic acid buffer, pH 7.4; (2) either NaCl or KCl to give concentration gradients (out)/(in) = 100/0 mM; and (3) the radioactive substrates consisting of a mixture of: (i) either ^{3}H-labeled mannitol or L-glucose and (ii) ^{14}C-labeled D-glucose, trehalose, sucrose or lactose. Within each experiment, osmolarity was kept constant with sorbitol (further details in the legends of the figures and tables). After a 1-min incubation period, the reaction was stopped with a 2 ml ice-cold stop solution containing the buffer, 100 mM sorbitol, 100 mM choline chloride, 25 mM $MgSO_4$ and 0.2 mM phlorizin. The diluted aliquots were rapidly filtered under vacuum through a cellulose nitrate membrane filter (0.65 μm pore size, 25 mm diameter, Sartorius, GmbH, Göttingen, F.R.G.) and rinsed twice with 5 ml of the ice-cold stop solution. The entire washing procedure lasted less than 15 s. The filters were then dissolved in 5 ml scintillation fluid (Aqualuma Plus, Lumac systems AG Basel, Switzerland). Radioactivity was measured in a B-2450 Packard Instruments Co. scintillation spectrometer.

Calculation and expression of the results. After correcting for unspecific marker retention on the filters, crude counts in each channel were corrected for window overlapping by the discriminator ratio method [25] and transformed into moles. The results are expressed either as the: (a) 'absolute uptake' (pmol per mg protein) or (b) 'relative uptake' or 'clearance' [26] where the absolute uptake is divided by the concentration (units: nl per mg protein). Use of relative uptakes has two advantages. First, they give concentration-inde-

pendent values that are constant when uptake involves only diffusion [27]. Second, they permit correlating initial uptake rates with the 'apparent vesicular volume' which is the relative uptake when measured at equilibrium (see the text). The results were evaluated statistically by a one-way analysis of variance [28,29]. All calculations were made using a Hewlett-Packard 9815-A desk calculator.

Results

Disaccharidase activities in the isolated brush-border membrane vesicle preparations

In agreement with earlier reports [15,16] trehalase and lactase activities were not detectable in any of the preparations made from the intestine of 15-day-old chicks. The absence of sucrase was also verified in the newborn pig intestine [17]. Sucrase and lactase were used as brush-border membrane markers for the chick and the piglet vesicle preparations, respectively. Both animal species yielded vesicles exhibiting purifications from 10- to 15-fold as compared with the initial homogenate.

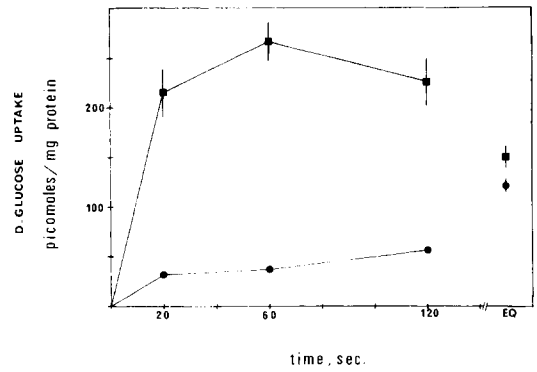

Fig. 1. Time-course of D-glucose uptake by brush-border membrane vesicles from chick intestine. The vesicles were prepared in the Hepes/*n*-butylamine/maleic acid buffer (pH 7.4), containing 100 mM sorbitol. Incubations were carried out in the same buffer supplemented with 0.1 mM D-[^{14}C]glucose, 0.1 mM D-[^{3}H]mannitol and either NaCl (■) or KCl (●) to give a cation gradient (out)/(in) = 100/0 mM. Absolute D-glucose uptakes are expressed in pmoles per mg protein ± S.E. The uptake of mannitol (not illustrated) was independent of the cation present in the medium and statistically indistinguishable from that of D-glucose in the presence of KCl. (*n*) was four or higher, the glucose overshoot data including up to 27 determinations per point.

Uptake studies

(1) D-Glucose. Similar to mammalian vesicle preparations [30–32] and in agreement with Lerner's work with adult chickens [33], vesicle preparations from both chick and piglet intestine exhibited the typical D-glucose 'overshoot' phenomenon in the presence of an inward-directed Na^+ gradient (illustrated with chick intestine in Fig. 1). Quantitatively similar overshoots were observed with adult chickens (8 weeks; results not illustrated), thus confirming the tenet that the

TABLE I

LACTOSE, SUCROSE AND MANNITOL UPTAKE BY BRUSH-BORDER MEMBRANE VESICLES FROM CHICK AND PIGLET INTESTINE

Vesicles were prepared and resuspended in the Hepes/*n*-butylamine/maleic acid buffer (pH 7.4), containing 100 mM sorbitol. Incubation media contained 0.1 mM [^{3}H]mannitol and 0.1 mM [^{14}C]lactose or sucrose. Either NaCl or KCl were added to give (out)/(in) = 100/0 mM cation concentration gradients. The total osmolarity gradient (out → in) was therefore 200 mosM. Results are expressed as relative uptakes ± S.E. All the data obtained were found to be homogeneous according to a one-way analysis of variance.

	Relative uptake (1 min) (nl per mg protein)							
	Chicken ($n = 6$)				Piglet ($n = 5$)			
	Lactose		Mannitol		Sucrose		Mannitol	
	NaCl	KCl	NaCl	KCl	NaCl	KCl	NaCl	KCl
Control	213 ± 21	187 ± 30	248 ± 26	225 ± 30	222 ± 19	194 ± 40	258 ± 23	244 ± 43
50 mM Tris	160 ± 33	183 ± 24	178 ± 34	217 ± 29	174 ± 16	184 ± 13	170 ± 18	168 ± 7
1 mM phlorizin	172 ± 18	167 ± 23	187 ± 13	183 ± 21	–	–	–	–
Glucose oxidase	162 ± 24	147 ± 23	197 ± 25	182 ± 26	–	–	–	–

sugar/Na^+ cotransport system in chicken intestine is fully developed at hatching [12]. When Na^+ was replaced by either K^+ (Fig. 1), Li^+, Cs^+ or choline (results not shown), the D-glucose uptake level fell to that of L-glucose or D-mannitol which we use as diffusion markers (see next section). This indicates that in chicken intestine Na^+ is an obligatory activator for D-glucose transport, as we have proposed for mammalian intestine [34].

(2) Diffusion marker uptake. Mannitol is generally used as a model nontransported sugar analogue for determining the extracellular space in intestinal experiments. Its inertness (it is neither a substrate nor an inhibitor of intestinal sugar transport [35]) justifies our using it as a marker for passive diffusion in the present vesicle work. The same reasoning applies to L-glucose [36], as demonstrated here. First, the uptake rate of both D-mannitol and L-glucose is a linear function of their concentration in the 1000-fold range from 0.1 to 100 mM. These results are illustrated only for L-glucose (Table II) in the form of relative uptake which, according to the definition (Methods and Ref. 27) exhibit constant, concentration-independent values. Second, relative uptake of both D-mannitol (Table I) and L-glucose (Table II) is unaffected by the cation composition of the incubation medium or by the presence of effectors such as phlorizin and Tris. Although these results cannot exclude the possibility that mannitol or L-glucose uptakes involve some very low-affinity carrier, the simplest interpretation remains that their uptake occurs by simple diffusion.

(3) Disaccharide uptake. The availability of brush-border membrane vesicles naturally lacking the corresponding hydrolases permited us to study the uptake of intact trehalose, lactose and sucrose. With our vesicle preparations, any role of the disaccharidases (either directly or indirectly) can be excluded in the uptake of the disaccharides. Nevertheless, in order to make sure that not even a trace of hydrolysis took place in our experiments, these were performed in the presence of either Tris (a well known disaccharidase inhibitor [37,38]) or phlorizin which is known for its specific inhibitory effect on both rat trehalase [39] and the Na^+-dependent monosaccharide transport system [40]. Phlorizin should prevent any trace of D-glucose, if formed, from entering the vesicles. For similar reasons glucose oxidase was also tested, since this enzyme should destroy any glucose traces present in the incubation media before or during the incubation with the vesicles. The 1-min uptake of trehalose (Table II), lactose and sucrose (Table I) was the same in both a Na^+ or a K^+ buffer. Disaccharide uptake levels were indistinguishable from those of L-glucose and D-mannitol or from the uptake of D-glucose in K^+ buffer. Among all the inhibitors we used, none had any significant effect on the uptake level of the disaccharides, either in Na^+ or in K^+ buffers. Such results suggest that the uptake of radioactive label by these vesicles reflects diffusion of the intact disaccharides. The hypothesis that some specific transport system for disaccharides exists can therefore be rejected.

It should be noted that both the disaccharide and the diffusion marker uptakes are quantitatively identical in the two widely different animal species used (Table I).

TABLE II

TREHALOSE, D-GLUCOSE AND L-GLUCOSE UPTAKE BY BRUSH-BORDER MEMBRANE VESICLES FROM CHICK INTESTINE

Conditions similar to those in Table I, except that the substrates were either [^{14}C]trehalose or D-glucose and L-[^{3}H]glucose at the indicated concentrations. Cation gradients (out)/(in) = 100/0 mM were obtained with either NaCl or KCl. The total osmolarity of the buffers is indicated in Table III. Expression of results and statistical analysis as in Table I (all data are statistically homogeneous). A control with 0.5 mM D-glucose in the presence of NaCl gave a relative uptake = 260 ± 40 (n = 10).

Substrate	mM	n	Relative uptake (1 min) (nl per mg protein)		
			NaCl		KCl
			Control	+0.7 mM phlorizin	
Trehalose	0.1	5	21 ± 1	20 ± 1	25 ± 2
L-Glucose	0.1	5	29 ± 5		
Trehalose	10.0	10	30 ± 3	35 ± 3	33 ± 5
L-Glucose	10.0	10	27 ± 2		32 ± 3
D-Glucose	10.0	10			23 ± 2
L-Glucose	100.0	5	34 ± 7		

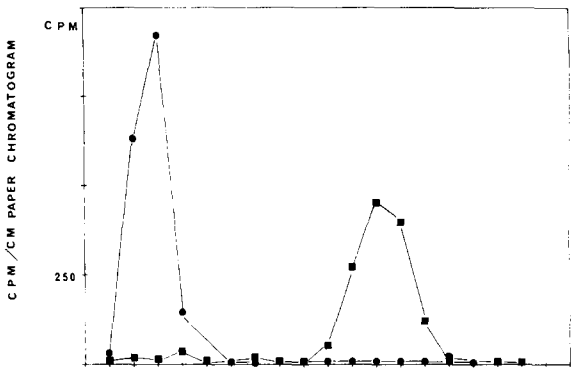

Fig. 2. Paper chromatography of [^{14}C]lactose (●) and D-[^{3}H]glucose (■) after a two hour incubation with chicken brush-border membrane vesicles.

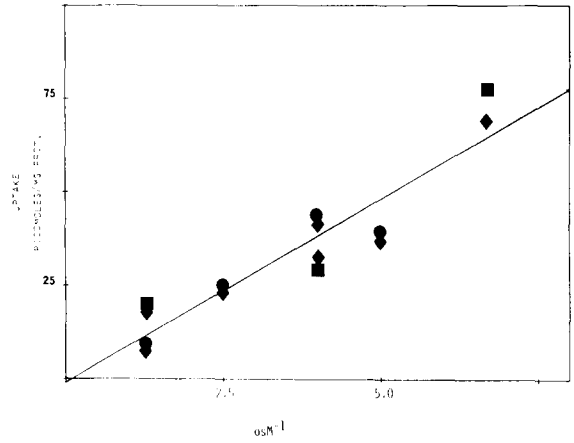

Fig. 3. Effect of medium osmolarity on lactose (●), D-glucose (■) and mannitol (◆) uptake by chicken brush-border membrane vesicles. The incubation media contained: (1) 0.1 mM of either ^{14}C-labeled lactose or D-glucose and ^{3}H-labeled mannitol; (2) NaCl to give a concentration gradient (out)/(in) = 100/0 mM; (3) the Hepes/*n*-butylamine/maleic acid buffer (pH 7.4); and (4) sufficient sorbitol to give osmolar differences [(out)−(in)] ranging from 0.15 to 0.80 osM. Equilibrium uptakes are expressed as picomoles per mg protein. The straight line calculated by the least-squares method gave an intercept indistinguishable from zero and a correlation coefficient = 0.934 ($n = 14$).

Disaccharides remain intact after moving into the intravesicular space

To make sure that in experiments with radioactive disaccharides the label taken up corresponds to the intact substrate, not to a monosaccharide product of its hydrolysis, we performed a paper-chromatographic analysis of the entire incubation mixtures after a 2-h incubation. In Fig. 2, we show a typical profile of the chromatography of [^{14}C]lactose and D-[^{3}H]glucose after their incubation with chick vesicles. It is clear that no D-[^{14}C]glucose was formed during the incubation. Equivalent results (not illustrated) were obtained when this test was applied to trehalose (chick) or to sucrose (piglet).

In order to know whether the intact disaccharide enters the vesicular space or simply binds to the outside of the vesicles, we looked at the effect of increasing medium osmolarity on the equilibrium uptake. D-Glucose, lactose and mannitol uptake were found to be proportional to the reciprocal of the osmolarity of the medium and extrapolation to infinite medium osmolarity (zero on the osM^{-1} scale; Fig. 3) yielded no uptake. Therefore, under the conditions of our experiments, all uptakes observed can be attributed to marker transfer across the vesicle wall into an osmotically active space.

Relationship between absolute uptakes and the apparent vesicle volume

The present work was realized as two separate series of chicken vesicle experiments performed two years apart. The corresponding results (Tables I and II, respectively) reveal the existence of a 10-fold difference in the relative uptakes (1 min) for the various nontransported sugars used. Parallel work on D-glucose transport in the presence of sodium (Table III) indicates that the difference between the two sets of experiments reduces to one of apparent vesicle volume.

As shown in Table III, the D-glucose absolute uptakes cannot be compared directly since the two groups of experiments involved different substrate concentrations. However, both sets of vesicles seem to transport D-glucose equally efficiently because, at 1 min, both gave similar peak/equilibrium concentration ratios indicating that the respective overshoots are proportional. Use of relative uptakes permits a direct comparison of the two sets of experiments and shows that, both at 1 min and at equilibrium, their corresponding results differ by a factor of 10, similar to those obtained with diffusion markers (Tables I and II).

The apparent vesicle volume can be interpreted as a measure of yield, i.e., of the effective transport

TABLE III

RELATIONSHIP BETWEEN APPARENT VESICLE VOLUME AND RELATIVE UPTAKES

Data correspond to the two different populations of chick brush-border membrane vesicles described in the text. Experiments I and II were performed with the vesicle populations used in Table I and II, respectively. Buffer osmolarities indicate the sum of all electrolytes and non-electrolytes present either during the preparation of the vesicles or in the extravesicular medium at the start of incubation (time zero).

		Expt. I	Expt. II
D-Glucose/Na^+ cotransport		($n = 27$)	($n = 10$)
Glucose concentration (mM)		0.1	0.5
Absolute uptake (pmol per mg protein)			
	1 min	268	130
	Equilibrium	120	45
Peak/equilibrium ratio (per cent)		223	288
Relative uptake (nl per mg protein)			
	1 min	2680	260
	Equilibrium	1200	90
Buffer osmolarity (mosM)			
Preparation buffer		100	500
Incubation buffer		300	600
Osmotic gradient (out → in)		200	100

capacity of a given vesicle population. This is why the time-course of substrate uptake is in proportion to the apparent vesicle volume, both when it is expressed as relative uptake (Table III) and as absolute uptake, except that here the initial substrate concentration would need to be taken into account. This conclusion applies regardless of whether uptake involves transport or simple diffusion.

The preceding statements aim at calling attention towards a neglected and unresolved deficiency in current vesicle research. To the best of our knowledge, no one has thus far found the conditions necessary for an optimal, quantitatively reproducible yield of closed, effectively transporting vesicles.

Table III lists some of the differences in the experimental conditions used for the preparation and test of the two vesicle populations. In experiment I where the osmotic gradient is double that in experiment II, the apparent volume should have been smaller (the vesicles do behave as true osmometers as shown in Fig. 3). We have no explanation for the result found, that the relative volumes of the two populations are the opposite of what one would expect.

Nevertheless, in spite of the quantitative differences discussed here, it should be clear that the two sets of results obtained are qualitatively identical, independent of the difference in yield.

Acknowledgements

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