DEMONSTRATION OF HYDROLASE-RELATED GLUCOSE TRANSPORT IN BRUSH BORDER MEMBRANE VESICLES PREPARED FROM GUINEA PIG SMALL INTESTINE

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Summary Osmotically active brush border-membrane vesicles were prepared from guinea pig small intestine. Transport into these vesicles of glucose released by the action of the membrane enzymes, sucrase and alkaline phosphatase, was measured. At comparable hydrolytic rates, several fold more glucose was transferred into the intravesicular space from sucrose than from G-1-P, thus confirming the existence of hydrolase-related transport in the brush border membrane.

Introduction Hydrolase-related transport (HRT) was first identified in vitro by finding a major component of glucose uptake in addition to Na^+-dependent uptake when disaccharides that are substrates of the various brush border membrane enzymes of hamster small intestine were used (1,2). HRT appears to be a direct transfer of the products of hydrolysis across the cell membrane. This pathway is easily distinguished from free glucose transport in that it does not require Na^+, it is not available to free glucose and sugars to be transported must be presented as glycosides susceptible to hydrolysis by brush border membrane enzymes (2,3). HRT has been found in the several species tested so far, viz: rat, mouse, guinea pig and rabbit (4). The phenomenon of HRT has been reported to be reconstituted by the incorporation of purified sucrase-isomaltase complex into artificial black-lipid membranes (5).

Following on the pioneering work of Kaback (6) with purified membrane vesicles from bacteria, similar preparations in which transport properties are preserved have been prepared from intestinal brush border membranes and used to provide a means of studying events occurring in the membrane

exclusive of contributions of other cellular organelles or cytoplasmic events (7). Along these lines we have taken steps to further characterize HRT in purified membrane vesicles prepared from guinea pig brush border membranes.

Materials and Methods D-[U-^{14}C] glucose, α-D-[U^{14}C]-glucose-1-phosphate, [^{14}C] Sucrose-([U-^{14}C] glucose) and D-[1-^{3}H] Mannitol were purchased from New England Nuclear Corp. Radioactivity was measured in a Beckman LS counter. All other chemicals were from commercial sources.

Adult male guinea pigs (Perfection Hartley, 300-350 g.) were obtained from Perfection Breeders, Inc., Douglassville, Pennsylvania.

Purified brush border membrane vesicles were prepared according to Sigrist-Nelson et al. (8) and suspended in a final medium containing 60 mM mannitol-20 mM KCl-1 mM Hepes-Tris pH 7.5. Uptake of the compounds tested was measured essentially as described previously by Hopfer et al. (7). Membrane filters (HAWP 02500) were obtained from the Millipore Corp. Protein was assayed by the method of Lowry et al. (9).

Results and Discussion The guinea pig was chosen as the test animal because in this species omission of Na^+ in order to inactivate Na^+-dependent free glucose transport had little effect on the rate of glucose uptake by HRT as measured with in vitro tissue preparations (4). However, intestinal sucrases generally are activated by Na^+ and the reduction of sucrase activity by its omission turned out to be important when working with vesicles. Fortunately, guinea pig intestinal sucrase is activated by K^+ as well as Na^+ (10). Thus, it is possible to have maximal sucrase activity even in the necessary complete absence of Na^+ by addition of relatively small concentrations of K^+.

Control for the diffusion of free glucose into the vesicles:

Purified membrane vesicles from guinea pig small intestine as prepared in this laboratory exhibited the characteristics of Na^+-dependent glucose transport described by Hopfer et al. (7) for rat membrane vesicles; that is, an imposed Na^+-gradient enhanced the entry of glucose into the vesicles and the dissipation of the sodium gradient with time coincided with equilibration of glucose across the vesicular membrane. However, some glucose diffuses nonspecifically into these vesicles, whether from rat

or guinea pig, and it is a considerable component of the total that enters. In studies with sucrose, free glucose is, of course, not added but the total glucose released by sucrase action always exceeds HRT in tissue preparations and a substantial amount appears extracellularly (2,3). The same may be expected in vesicles. In the simplest terms this means that glucose will always be found to accumulate in the suspending medium as well as within the vesicles, and it is not possible to distinguish by available analytical methods that portion of glucose which is transferred into the vesicles by HRT from that which enters by diffusion. With tissue preparations this was not a serious problem because free glucose was almost entirely excluded in the absence of Na^+. With vesicles this is not the case, and the essence of a proper control for the passive diffusion component of glucose released at the membrane by sucrase action would be to have glucose released at the same location and at the same rate by a membrane enzyme which contributes little or nothing to HRT. As it turns out, alkaline phosphatase has the required properties and can release glucose rapidly from glucose-1-phosphate (G-1-P) (11).

The phosphatase and sucrase activities of guinea pig brush border membrane vesicles were measured and compared to determine the substrate concentrations to be used for the transport experiments. Alkaline phosphatase is several fold more active than sucrase and even at 1 mM G-1-P the rate of glucose released is higher than that from sucrose at 10 mM (Table II). However, this rate differential favors the entry of glucose released from G-1-P as compared to that released from sucrose and serves to increase the reliability of G-1-P as a control for glucose diffusion.

Control for the diffusion of unhydrolyzed sucrose:

Since guinea pig brush border membrane vesicles are 'leaky' to free glucose, it was necessary to determine whether they may be leaky also to sucrose and G-1-P. Measurement of sucrose and G-1-P diffusion was done

as described in Table 1. Sucrose was tested in the presence and absence of $Tris^+$. $Tris^+$ is a strong competitive inhibitor of sucrase (12) and its use can help to identify the extent of sucrose permeation. At the end of incubation, the membranes were collected on Millipore filters and transferred to 4 ml of 80% ethanol and allowed to extract overnight at 0-5°. The extracts were evaporated to a small volume (< 100 µl.) and chromatographed on Silica gel plates using 2-Butanone-Acetic acid (6:2) as the solvent system, which separates sucrose and G-1-P from glucose. The plates were assayed for radioactivity and the data obtained used to calculate the results in Table 1. With sucrose in the absence of $Tris^+$, 72-81% of the measured radioactivity associated with the vesicles was due to glucose and the rest was unhydrolyzed sucrose. With sucrose in the presence of $Tris^+$, total uptake was reduced to less than 40% and the proportions of glucose and sucrose were reversed. These results show that unhydrolyzed disaccharide can occupy some portion of the vesicular space. The tests with G-1-P indicate no detectable entry of the

TABLE 1

Comparison of the Uptake of Glucose and Sucrose in Membrane Vesicles Following Incubation with Sucrose

	Time	Uptake by Vesicles pmoles/mg. protein Glucose	Disaccharide
Sucrose	1 min.	913	362
	2 min.	1,882	441
Sucrose and Tris	1 min.	130	338
	2 min.	155	585

Incubation was at room temperature (∿ 25°) in a total volume of 100 µl containing 260 mM mannitol, 20 mM KCl, 0.1 mM $MgSO_4$, 1 mM Hepes-Tris (pH 7.5) and membranes (∿ 1 mg protein). Sucrose was at 10 mM. $Tris^+$ was added by replacing 200 mM mannitol with 100 mM Tris Cl, pH 7.5. At the end of incubation, 50 µl was removed and the membrane vesicles collected on Millipore filters as described by Hopfer *et al.* (7). Chromatography of the extracts from filtered membrane vesicles is described in the text.

unhydrolyzed compound. The data of Table I can be used to correct for the diffusion of intact sucrose.

Differential uptake of glucose from sucrose compared to G-1-P:

Based on the above considerations, experiments were designed to study the transport of glucose, derived from either sucrose or G-1-P, across the brush border membrane. The results of these experiments are given in Table II. Measurements of transport into the intravesicular space were made between 5 sec. and 5 min. of incubation at 20 sec. intervals. Transport from sucrose at 10 mM concentration was compared to that from 1 and 10 mM G-1-P. When G-1-P was used at 1 mM, incubation volumes were

TABLE II

Differential Uptake by Membrane Vesicles of Glucose Released from Sucrose and Glucose-1-Phosphate

	Time Sec.	Relative Amounts of Glucose Released	Glucose Taken Up pmoles/mg Protein	
			^{+}Na	$-Na^{+}$
Sucrose (10 mM) (0.181)	60	1	2,570*	800**
G-1-P (1 mM) (0.268	40	1	548	119
	60	1.49	663	168
G-1-P (10 mM) (0.565)	20	1.04	887	212
	60	3.14	1,667	487

The average of two experiments are given here. The experimental conditions are described in Table II except that incubation volume was increased. Na^{+} was added by replacing 200 mM mannitol with 100 mM NaCl. 20 µl aliquots were removed at appropriate time intervals diluted with ice-cold buffer and washed as described in Table I. The filters were then transferred to counting vials and the radioactivity measured. Figures in parenthesis are specific activities of sucrase and phosphatase expressed as µmoles substrate hydrolysed per min. per mg. protein, assayed as described earlier (3).

*includes unhydrolyzed sucrose.

**corrected for unhydrolyzed sucrose

increased suitably in order to provide enough substrate to maintain constant hydrolytic rates during the experimental period. The amount of glucose released from 10 mM sucrose at the end of 1 min. incubation is comparable to that released from 1 mM G-1-P at 40 sec. and from 10 mM G-1-P at 20 sec. Comparing vesicular uptake in the presence of Na^+ at these respective time points, that from sucrose was 4.7 and 2.9 times greater than that from 1 mM and 10 mM G-1-P respectively. In the absence of sodium, the corresponding values were 9.4 and 5.3. Even when the amount of glucose released was 3 fold greater from G-1-P (10 mM G-1-P at 1 min.) uptake of glucose from sucrose (10 mM at 1 min.) predominated, being 1.5 times greater in the presence of Na^+ and 2.3 times in its absence. In the absence of sodium, which abolishes Na^+-dependent free glucose transport, total sugar uptake from 10 mM sucrose is 1117 pmoles/mg protein at the end of 1 min. incubation; correcting for intact sucrose entry from Table 1 (317 pmoles of sucrose), it is seen that considerably more of glucose from sucrose is transferred into the vesicles than from G-1-P. This observation appears to demonstrate glucose transfer attributable to HRT.

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