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THE KINETIC ADVANTAGE FOR TRANSPORT INTO HAMSTER INTESTINE OF GLUCOSE GENERATED FROM PHLORIZIN BY BRUSH BORDER β-GLUCOSIDASE *

DAN W. HANKE, DEAN A. WARDEN, JAMES O. EVANS, FRANKLIN F. FANNIN and DONALD F. DIEDRICH

University of Kentucky, College of Medicine, Department of Pharmacology, Lexington, KY 40536 (U.S.A.)

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Summary

Phlorizin, labeled with tritium only in the glucose moiety, was used as substrate for the β-glucosidase present in brush border membranes from hamster intestine in order to study, simultaneously, the kinetics of hydrolysis and the fate of the [^{3}H]glucose liberated by the enzyme. The [^{3}H]glucose seems to experience the same hydrolase related transport into the intestinal villi as the hexoses liberated from the common disaccharides by their respective hydrolases. The released [^{3}H]glucose accumulation rate is only partially inhibited by unlabelled glucose added to the medium as either the free sugar or as the precursors sucrose, lactose or glucose 1-phosphate, and then only when these sugars are present at very high levels. Furthermore, glucose oxidase, added to the medium as a glucose scavenger, has no effect on the uptake rate of the phlorizin hydrolase-liberated sugar. These and other findings are presented as evidence that, under conditions where the Na^+-dependent glucose carrier is more than 97% inhibited by phlorizin, the glucose derived from the inhibitor, like the hexoses from disaccharides, has a kinetic advantage for transfer into the intestinal tissue.

Introduction

Despite scattered references to an aryl β-glucosidase activity in intestine, early workers studying phlorizin's inhibition of glucose transport failed to anticipate the hydrolysis of the inhibitor by this tissue. Two reports [1,2] finally drew special attention to the phlorizin hydrolase in jejunal brush borders which splits the inhibitor into phloretin and D-glucose. Since then, the

* This work is dedicated to T.Z. Csaky on the occasion of his 65th birthday in recognition of his contributions to the field of membrane transport and to our careers.

membrane enzyme has been purified and characterized [3—8]. Together with 'neutral' lactase, it constitutes a β-glycosidase aggregate, perhaps similar to the sucrase-isomaltase complex [9]. Like all the brush border disaccharidases except trehalase [10], this β-glycosidase complex can be released from the external surface of the mucosal cell by proteolytic enzymes [11,12]. Although the catalytic sites of the two sub-units are closely related and possibly exist as part of the same protein, they are at least partially independent; differences in their heat-lability and specificity for substrates and inhibitors have been reported [4—6,8,13]. Leese and Semenza [14] have provided evidence that phlorizin hydrolase corresponds to the glucosylceramidase activity of the intestine. With lactase, it enables the newborn mammal to digest and assimilate the carbohydrate and glycolipid in milk. Thus, it seems clear that phlorizin hydrolase cannot be viewed as a maverick enzyme. It is intimately related, both morphologically and functionally, to the better known brush border disaccharidases.

Crane [15] has summarized the long series of observations which led to his idea that the brush border glycosidases are directly involved in a 'hydrolase-related transport'. He proposed that disaccharidase-generated hexose has a 'kinetic advantage' for entry into cells over the same hexose added to the medium. All of the sugar liberated at the membrane is not simply released into the intestinal lumen; a portion is translocated into the epithelia in synchrony with glycosidic bond cleavage. This explained why the uptake rate of added free glucose is considerably less than that of sugar liberated into the surrounding fluid by the enzyme at an equivalent concentration. Arguments that this phenomenon cannot be attributed simply to differences in the distance the respective sugars must diffuse to be absorbed are presented in his review [15].

We wondered whether phlorizin hydrolase could also act as an apparent translocation enzyme with similar vectorial properties. Experiments were therefore designed to determine the fate of the glucose moiety after it is cleaved from phlorizin. The measurements were made while the glucoside simultaneously acts as an inhibitor of the Na^+-dependent, glucose carrier system. We have discovered that the hydrolase-generated glucose, like the hexose from disaccharides, experiences a kinetic advantage for accumulation by intestinal villi. In the following paper [16], more comprehensive kinetic data has led us to a conservative view of the mechanism of the process.

Materials and Methods

Radioactive compounds. Phlorizin, labeled with ^3H on only the glucose moiety (on C_6) was prepared and analyzed earlier [17]. Its specific activity was 0.45 Ci/mol. D-[U-^{14}C]glucose (ICN Pharmaceuticals, Inc.) and D-[1-^{14}C]-mannitol (New England Nuclear) were used in our control experiments after adjusting their specific activities to a range of 0.04 to 6 Ci/mol.

Special compounds. Glucose oxidase (Type II, 15—20 U/mg), *N*-ethylmaleimide, *p*-chloromercuribenzoic acid (PCMB), *p*-chloromercuriphenylsulfonic acid (PCMPS) and iodoacetic acid (IOAA) were purchased from Sigma Chemical Co. Stock solutions of the sulfhydryl blockers and alkylating agents

were prepared just before each experiment. Non-radioactive phlorizin was obtained from Carl Roth OHG, Karlsruhe, F.R.G. Most phlorizin preparations are contaminated with phloretin which is difficult to remove by recrystallization from alcohol/water. However, phloretin is readily separated from the glucoside by Sephadex G-10 chromatography. While 20% ethanol/water elutes phlorizin, the aglycone remains tightly bound to the dextran matrix [18]. Alternatively, crude phlorizin can first be crystallized from ethyl acetate to remove small amounts of contaminants.

Incubation medium. Unless otherwise stated, all hydrolysis and transport experiments were conducted in conventional Krebs-Ringer phosphate buffer ('the buffer'), pH 7.4 (but Ca^{2+} at 1 mM) which contained the indicated concentration of phlorizin and/or sugar. Periodic measurement of medium pH following the 3 min incubation showed that no significant change occurred.

Tissue incubation. The villus technique, described and evaluated by Wright and co-workers [19,20], was used to simultaneously study phlorizin hydrolysis and the distribution of the liberated glucose. The original procedure was modified only slightly. Intestines from male, Golden Syrian hamsters were used and in order to minimize the age-dependent variation in phlorizin hydrolase activity [5,6,13], they were killed when they reached 63 ± 3 days. Jejunum and upper ileum were cut into twelve, 1-cm segments that were immediately placed into a jacketed, sintered-glass funnel filled with the buffer at 37 °C which served as the initial oxygenation and tissue randomization chamber (O_2 was delivered through the stem of the funnel). The total pre-incubation period lasted from 1 to 4 min. Incubation was started by adding two pieces of intestine to a 30 ml beaker containing 3 ml of the temperature- (37 °C) and oxygen-equilibrated medium in a metabolic shaker (100 oscillations/min). After 3 min, the tissue was blotted, quickly frozen in Krebs-Henseleit bicarbonate buffer and lyophilized. The dehydrated tissue was freed of adherent buffer salts and substrates and then transferred to a tared test tube. A thin steel rod was inserted and the tube was touched to the resilient cup of a Vortex mixer which caused the brittle tissue to shatter. By tilting and gently tapping the tube, the less dense sub-mucosa and muscle was easily separated from the villi. This step replaced the most inconvenient aspects of the original procedure, that of chipping off individual villi with a probe.

This in vitro method allowed us to compare the properties of phlorizin hydrolase in its native state with a transport system previously studied primarily in whole tissue. The technique offers distinct advantages including the important point that only those substances actually entering the villi space are measured; tissue not participating in transport (crypts, sub-mucosa and muscle) is separated from the villi before analysis. Insignificant amounts of sugar enter the villi from the direction of the serosal surface during our short incubation time.

Tissue analysis. 1 ml of nearly boiling water was added to the isolated dry villi (about 15 mg) and the tube was kept at 90 °C for 10 min. Contents were then chilled and transferred with water onto a 2 ml column of Dowex-1 anion exchange resin (X-8, 200—400 mesh, acetate form). The total eluate (5 ml) was concentrated to a small volume in a glass counting vial before the radioactive free glucose in this entire fraction was determined. The Dowex column was

then treated with a solution of 1.2 M KNO_3 which elutes all the anionic [^{3}H]-glucose metabolites.

Preliminary experiments showed that greater than 95% free [^{14}C]glucose, in buffer only or with boiled villi, was recoverable with this method. In other tests, glucose was first incubated with mucosal scrapings before the mixture was heated and applied to the column. After unmetabolized sugar was eluted with water, the radioactive metabolites were stripped from the resin with nitrate. Total recovery of ^{14}C was again 95%. Phlorizin (and phloretin) is quantitatively adsorbed on this resin and is not eluted under these conditions [17].

Medium analysis. The [^{3}H]glucose liberated into the medium during incubation was measured from 1.0 ml aliquots that had been processed through the Dowex column like the tissue extracts. In transport experiments with [^{14}C]-glucose and [^{14}C]mannitol, samples were analyzed directly since after 3 min, only traces of metabolites are found in the medium.

Calculations and experimental design

All experiments were performed as a set of six incubation conditions, always including a control, with randomized segments of intestine from a single animal. Calculation of glucose and glucose metabolite concentration in tissue water took the following form (dry villi weight was taken to be 20% of the total wet tissue weight):

$$\text{Villi sugar (nmol/ml)} = \frac{\text{nmol/mg dry villi}}{4\ \mu\text{l/mg dry villi}} \times \frac{1000\ \mu\text{l}}{\text{ml}}$$

Phlorizin hydrolysis rate was based on the total amount of free glucose plus metabolites found in the villi and medium. Less than 5% of the total liberated [^{3}H]glucose was present in the serosal fragment.

The term transport efficiency represents the tissue's capacity to capture the glucose released from phlorizin. It is the percent of liberated glucose trapped in villi measured as the sum of free and metabolized sugar. We have assumed that any radioactive metabolite present in the dried tissue was formed only after the free glucose entered the cell.

Results

The rates of phlorizin hydrolysis and tissue uptake of liberated glucose were measured with [^{3}H]phlorizin at 0.2 mM, which is about one-fifth the apparent K_m for hydrolysis under the present conditions [16]. Both rates are still linear over 3 min [16,21] and less than 10% phlorizin hydrolysis occurred in all experiments.

In this and our companion paper [16], it is important to distinguish between the glucose added to the medium as free sugar (always ^{14}C labeled) and the glucose enzymatically released from phlorizin which is always identified as [^{3}H]glucose.

Distribution of free glucose, mannitol and the glucose liberated from phlorizin

In Table I, the villi uptake rates of free [^{14}C]glucose and [^{14}C]mannitol

TABLE I

DISTRIBUTION AND UPTAKE RATES OF FREE GLUCOSE, MANNITOL AND GLUCOSE LIBERATED FROM PHLORIZIN INTO INTESTINAL VILLI

Rate values are given as nmol · min^{-1} · mg^{-1} dry villi (mean ± S.E.). n, number of observations. The villi/medium concentration ratio is based on the concentrations of unmetabolized sugar found in the tissue and medium following 3-min incubation. The tissue concentration was calculated as described in Methods.

Initial medium contains	n	Rate of uptake by villi		Villi/medium concentration ratio of free sugar
		Free sugar	Sugar plus metabolites	
[^{14}C]Glucose, 0.2 mM	88	2.56 ± 0.08	3.02 ± 0.09	15.0 ± 0.6
[^{14}C]Glucose + phlorizin both at 0.2 mM	30	0.118 ± 0.007	0.191 ± 0.009	0.45 ± 0.03
[^{14}C]Mannitol, 0.2 mM	30	0.060 ± 0.003	—	0.22 ± 0.01
[^{14}C]Mannitol + phlorizin both at 0.2 mM	18	0.051 ± 0.005	0.048 ± 0.003	0.19 ± 0.01
[^{3}H]Phlorizin, 0.2 mM	75	0.080 ± 0.003 *	0.124 ± 0.004 *	4.19 ± 0.17

* The uptake rate is that for [^{3}H]glucose liberated by phlorizin hydrolase. The mean (±S.E.) hydrolytic rate for these 75 observations was 1.158 ± 0.036 nmol · min^{-1} · mg^{-1} and the final concentration of [^{3}H]glucose liberated in the medium reached 0.014 mM.

added to the medium are compared to that of the glucose released by the action of phlorizin hydrolase. As expected, free [^{14}C]glucose was actively transported; the free sugar concentration in the tissue water is 15 times that remaining in the medium (about 0.15 mM). The uptake rate greatly exceeds the metabolic rate; only about 15% of the radioactivity associated with the villi is in the form of glucose metabolites, increasing to a maximum of 35% when sugar accumulation is low (e.g., when phlorizin is present).

With an equal concentration of phlorizin present, the [^{14}C]glucose uptake rate (Table I) is greatly inhibited and failed to even equilibrate in the tissue water (its villi/medium distribution ratio was only 0.45). Nevertheless, its entry was still twice that found for [^{14}C]mannitol, a material presumed to penetrate the tissue by passive means. This indicates that the Na^+-dependent glucose carrier is profoundly, but incompletely, inhibited by 0.2 mM phlorizin, the concentration we have used to study its hydrolysis. This fraction, f, of the carier which remains operational can be calculated:

$$f = \frac{\text{glucose uptake minus mannital uptake, both with phlorizin present}}{\text{glucose uptake minus mannitol uptake, no phlorizin present}}$$

$$f = \frac{0.118 - 0.051}{2.56 - 0.06} \times 100 = 2.7\%$$

The corresponding theoretical activity is predictable using Eqn. 1, which describes competitive inhibition:

$$\frac{1}{f} - 1 = \frac{K_m}{K_m + C_s} \times \frac{C_i}{K_i} \qquad (1)$$

Under our conditions, concentrations of [^{14}C]glucose (C_s) and phlorizin (C_i) are both 0.2 mM; K_m for glucose transport is 1.6 mM [16] and phlorizin's inhibition constant, K_i, is between 0.03 and 0.008 mM [22]. Solution of

Eqn. 1 gives values for f between 1.5 and 4.2%, which matches the experimentally observed level (2.7%).

This is an important point. Our interpretation of the results to be described later hinges on the recognition that about half of the uptake rate for the glucose which escapes 0.2 mM phlorizin blockade is attributable to this uninhibited glucose carrier activity (see Ref. 16, the experiments with sodium-free medium).

A final inspection of the data in Table I reveals that [^{3}H]glucose liberated from tritiated phlorizin is also accumulated by the villi. However, the total uptake rate (0.124 nmol/min) represents only approximately one-tenth of the amount generated by the membrane enzyme and this rate is comparable to that of [^{14}C]glucose uptake when inhibited by 0.2 mM unlabeled phlorizin. Therefore, since hydrolysis greatly exceeds tissue uptake, the [^{3}H]glucose generated at the membrane can be expected to approach its theoretical maximum of 0.2 mM, the concentration of its source. However, it is clear that the [^{3}H]-glucose experiences a vastly different apparent distribution from free [^{14}C]-glucose (with phlorizin present). The villi/medium concentration ratio for free sugar under these otherwise similar conditions differs by a factor of 10. Glucose generated by phlorizin hydrolase attains a concentration in the villi which is at least four times that found in the final incubation fluid. Simply stated, this glucose appears to be preferentially transferred into the tissue and has a kinetic advantage for entry over the free hexose added to the medium. This finding resembles those made by Crane and his colleagues [15,23], Parsons and Pritchard [24] and Grey and Ingelfinger [25] for the hexose set free from disaccharides by other brush border glycosidases. However, this computation must be interpreted cautiously. A villi/medium ratio greater than unity does not mean that an active transport mechanism is operational in our system even though this is the cited criterion. It must be recognized that the enzyme-liberated sugar enters the tissue directly from its site of origin, the membrane surface, where its concentration must be greater than that in the medium.

Consequence of anaerobic conditions

In some experiments, intestinal segments were gassed with nitrogen at all stages. We reasoned that pre-incubation of the tissue in the absence of oxygen would compromise any energy dependent component of the phlorizin hydrolase related transport mechanism. The results of control experiments with [^{14}C]glucose and [^{14}C]mannitol are informative (Table II). Without O_2, the tissue is able to accumulate free glucose at only 25% of the normal rate and the level of [^{14}C]glucose metabolites in the anoxic tissue is almost doubled. On the other hand, the uptake of exogenous glucose by the fraction of the carrier which escapes phlorizin blockade appeared to be unaffected by the lack of O_2. This was an unexpected finding since the energy-dependent carrier uptake should have been inhibited. However, the experiments with [^{14}C]mannitol revealed that this passively diffusing material has a tissue entry rate which is increased by 35% under N_2. It appears, therefore, that the portion of [^{14}C]-glucose which enters the villi space by a mannitol-type uptake is similarly exaggerated. Increased passive uptake could offset the decrease which occurs when the energy-dependent carrier system fails to operate. The net result

TABLE II

EFFECT OF ANAEROBIOSIS ON THE DISTRIBUTION OF [^{3}H]GLUCOSE AND OF GLUCOSE AND MANNITOL ADDED TO THE MEDIUM

The values are expressed as percent of control and represent the mean of at least 6, and as many as 24 observations for each measurement under each condition (O_2 vs N_2). In the anaerobic experiments, nitrogen replaced oxygen at all stages including the pre-incubation period. Total time of anoxia, 4—7 min.

Measurement	Anaerobiosis causes:
Villi accumulation of 0.2 mM free [^{14}C]glucose	75% decrease
Fraction of [^{14}C]glucose appearing in villi as metabolites	90% increase
Villi accumulation of free [^{14}C]glucose at 0.2 mM which is insensitive to 0.2 mM phlorizin blockade	7% increase
Villi accumulation of 0.2 mM [^{14}C]mannitol	35% increase
Rate of 0.2 mM phlorizin hydrolysis	40% decrease
Villi accumulation of [^{3}H]glucose liberated from [^{3}H]phlorizin	25% increase
Transport efficiency of the liberated [^{3}H]glucose	120% increase

appears to be an unchanged overall transport rate. This interpretation is supported by the work of Baker et al. [26,27] who found that mucosal anaerobiosis caused a decreased uphill galactose transport into jejunum. The effect was due to an increase in galactose efflux from the tissue and/or an increase in its phlorizin-insensitive uptake. Anaerobiosis apparently makes the brush border more 'leaky'.

When the tissue is O_2 deficient, the [^{3}H]glucose liberated from phlorizin enters the villi space at a faster rate than normal. This occurs even though the glycoside hydrolysis rate is sharply decreased under N_2, for reasons that escape us. Thus, even short periods of anoxia compromise the luminal membrane; although less free glucose is generated by the hydrolase, those molecules which are set free face less of a barrier for passive entry and the transport efficiency under this condition is more than doubled.

Effect of adding unlabeled glucose to medium

The 'hydrolase related transport' hypothesis indicates that the hexose released from a disaccharide enters the tissue from a compartment which is not readily accessible to the same hexose added in the medium. The phlorizin hydrolase system behaves similarly. An effect on the uptake of [^{3}H]glucose is seen only when free glucose is added to the medium at very high levels (10 and 30 mM; see Table IIIA); transport efficiency is affected and not the rate of phlorizin hydrolysis. Since 30 mM mannitol had no influence on [^{3}H]glucose accumulation, the inhibition caused by the glucose at this level cannot be attributed to osmotic changes. On the other hand, these findings are readily described in terms of conventional competitive type inhibition for a single, phlorizin-inhibitable transport system *.

* $$v_i = V \times \frac{[Glc]}{[Glc] + K_t\left(1 + \frac{[I]}{K_i}\right)} + m[Glc]$$

where, v_i = inhibited total glucose transport velocity, V = 25.3 nmol/min/mg villi (Fig. 1, Ref. 16), K_t for glucose transport = 1.6 mM (Fig. 1, Ref. 16), $[I]$ = phlorizin concentration; 0.2 mM, K_i = phlorizin inhibition constant; 0.005 mM (Ref. 22), m = constant for passive diffusion of sugar; 0.29 nmol/min/mg

TABLE III

EFFECT OF EXOGENOUS UNLABELED GLUCOSE AND GLUCOSE PRECURSORS ADDED TO THE MEDIUM ON THE UPTAKE OF [^{3}H]GLUCOSE DERIVED FROM [^{3}H]PHLORIZIN

Rates are as those in Table I and were determined from a separate series of experiments from those reported there, *n*, number of observations. Transport efficiency is the fraction of the total [^{3}H]glucose liberated from phlorizin which appears in the villi.

Medium contains 0.2 mM [^{3}H]phlorizin plus:	Rates of			
	n	Phlorizin hydrolysis	Villi accumulation, [^{3}H]glucose + metabolites	Transport efficiency (%)
Part A				
No addition	(6)	1.49 ± 0.16	0.174 ± 0.021	11.8 ± 0.3 *
Glucose, unlabeled				
0.2 mM	(5)	1.40 ± 0.09	0.163 ± 0.012	11.7 ± 0.4
1.0 mM	(6)	1.44 ± 0.15	0.167 ± 0.016	11.7 ± 0.5
10.0 mM	(6)	1.33 ± 0.09	0.109 ± 0.010 **	8.2 ± 0.7
30.0 mM	(6)	1.43 ± 0.14	0.083 ± 0.005 ***	6.0 ± 0.3
Mannitol				
30.0 mM	(12)	1.52 ± 0.15	0.164 ± 0.016	12.6 ± 1.1
Part B				
Sucrose				
30.0 mM	(6)	1.44 ± 0.14	0.084 ± 0.005	5.8 ± 0.4
Lactose				
30.0 mM	(6)	1.54 ± 0.15	0.129 ± 0.008	8.3 ± 0.6
Glucose 1-phosphate				
5.0 mM	(6)	1.30 ± 0.13	0.109 ± 0.011	8.2 ± 0.7
30.0 mM	(6)	1.40 ± 0.14	0.093 ± 0.008	6.7 ± 0.7

* Transport efficiency from 75 observations over the course of this study was 11.03 ± 0.33.
,* Significantly different from control; $P < 0.01$ and < 0.005, respectively.

Effect of adding disaccharides and glucose-phosphate to medium

Substrates of other membrane hydrolases which should liberate unlabeled glucose very near the [^{3}H]glucose release site were also tested (Table IIIB). Sucrose, lactose and glucose 1-phosphate (Glc-1-*P*) had no effect on phlorizin hydrolysis rate; even lactose, at 150 times the phlorizin concentration, fails to inhibit phlorizin hydrolase, which confirms a previous report [5]. However, the villi accumulation rate of the liberated [^{3}H]glucose is inhibited. At 30 mM, sucrose is more potent than lactose, while the activity of Glc-1-*P* is intermediate but the inhibition is seen only when these very large amounts of sugars are used. We found in preliminary experiments [20] that lower levels of sucrose and lactose (twice the phlorizin concentration) had no effect. Presumably, when a sufficiently high level of glucose is generated from these glycosides, it is able to mix with the [^{3}H]glucose liberated from phlorizin and compete for the entry process. This mechanism would explain why lactose is least potent; phlorizin is a competitive inhibitor of lactase (Ref. 4, and Evans, J.O. and

villi/mM (Fig. 1, Ref. 16), [Glc] = added glucose plus membrane concentration of [^{3}H]glucose split from 0.2 mM phlorizin; 30 mM + 0.155 mM (Table II, Ref. 16) then, v_i (for total Glc) = 16.7 nmol/min/mg villi, v_i (for [^{3}H]Glc) = v_i (for total Glc) × [[^{3}H]glucose]/[glucose]$_{total}$ = 0.086 nmol/min/mg villi. This theoretical value for the uptake rate of split glucose, inhibited by the phlorizin substrate and the 30 mM added free glucose, is equal to the observed value of 0.083 nmol/min/mg villi (Table IIIA).

Diedrich, D.F., unpublished data) and thus, less free glucose from lactose would be generated to compete than that from sucrose.

Effect of adding glucose oxidase to medium

The proposal of a hydrolase related transport was based in part on early studies with glucose oxidase by Miller and Crane [28]. This enzyme did not appear to have access to the glucose generated from sucrose by sucrase. Our system behaves identically (Table IV). Large amounts of scavenger enzyme hardly affected the villi accumulation rate of hydrolase-generated [^{3}H]glucose. And yet, the oxidase readily metabolizes the fraction of [^{3}H]glucose which diffuses into the medium from the membrane site, reducing its concentration 10-fold compared to that attained in the control medium (from 13.1 to 1.29 μM). The large villi/medium ratio differences under the two conditions reflect these changes. The data support our earlier view [21] that the glucose split from phlorizin is released at a site inaccessible to glucose oxidase added to the medium.

The action of non-specific inhibitors on the system

In order to examine whether the transport of [^{3}H]glucose is coupled directly to the hydrolysis mechanism, the effects of some non-specific protein modifiers were tested. If uptake and cleavage were intimately paired, then inhibition of phlorizin hydrolase by any of these agents should lead to a corresponding decrease in transport of liberated sugar. The results of our attempts to perturb the system are shown in Table V. *N*-ethylmaleimide, inorganic Hg^{2+} and iodoacetic acid all had profound inhibitory action on the transport of free [^{14}C]-glucose added to the medium, whereas *p*-chloromercuribenzoic acid and *p*-chloromercuriphenylsulfonic acid were without statistically significant effect. None of the agents influenced the uptake rate of mannitol nor of [^{14}C]-glucose when unlabeled phlorizin was present.

The only noteworthy effect of these inhibitors on the phlorizin hydrolase system was that of $HgCl_2$. As previously reported by Malathi and Crane [1],

TABLE IV

INFLUENCE OF GLUCOSE OXIDASE ON PHLORIZIN HYDROLASE RELATED TRANSPORT

Rate values are as those in Table I and represent the mean ± S.E. of six observations.

Initial medium contains	Rate of		Concentration (μM) of liberated [^{3}H]glucose		Villi/medium concentration ratio of [^{3}H]glucose
	[^{3}H]Phlorizin hydrolysis	Villi accumulation, [^{3}H]glucose + metabolites	in medium	in villi	
0.2 mM [^{3}H]phlorizin	1.18 ± 0.12	0.126 ± 0.012	13.10 ± 0.99	62.8 ± 8.3	4.8
0.2 mM [^{3}H]phlorizin + 0.3% glucose oxidase	1.46 ± 0.15 *	0.104 ± 0.008	1.29 ± 0.16	35.0 ± 3.2	27.1

* Hydrolysis rate is not statistically different from the control value ($P > 0.1$).

TABLE V

EFFECTS OF NON-SPECIFIC INHIBITORS ON THE UPTAKE OF FREE [^{14}C]GLUCOSE AND [^{14}C]-MANNITOL, PHLORIZIN HYDROLYSIS AND PHLORIZIN-DERIVED [^{3}H]GLUCOSE ACCUMULATION RATE

Rates are as those given in the previous tables. The number of observations are given in parentheses. All of the protein modifier reagents were added to both the pre-incubation chamber and the incubation beaker. The accumulation rates are a measure of both free sugar plus the labeled metabolites. NEM, *N*-ethylmaleimide; IOAA, iodoacetic acid; PCMB, *p*-chloromercuribenzoic acid; PCMPS, *p*-chloromercuriphenylsulfonic acid.

Villi accumulation rate of	Incubation in the presence of					
	No additions	$HgCl_2$ 0.1 mM	NEM 1.0 mM	IOAA 10.0 mM	PCMB 0.2 mM	PCMPS 0.2 mM
[^{14}C]Glucose at 0.2 mM	3.37 ± 0.16 (17)	1.04 ± 0.11 [a] (12)	1.44 ± 0.09 [a] (18)	2.45 ± 0.12 [a] (12)	3.11 ± 0.12 (18)	3.19 ± 0.13 (18)
[^{14}C]Glucose at 0.2 mM which escapes 0.2 mM phlorizin blockade	0.24 ± 0.01 (11)	0.22 ± 0.01 (6)	0.21 ± 0.01 (11)	0.26 ± 0.01 (11)	0.29 ± 0.02 (6)	0.29 ± 0.02 (6)
[^{14}C]Mannitol at 0.2 mM	0.06 ± 0.005 (12)	0.08 ± 0.004 [b] (6)	0.06 ± 0.005 (12)	0.06 ± 0.003 (12)	0.06 ± 0.003 (6)	0.05 ± 0.04 (6)
[^{14}C]Glucose liberated from phlorizin at 0.2 mM	0.12 ± 0.01 (18)	0.13 ± 0.01 (12)	0.14 ± 0.01 (18)	0.11 ± 0.01 (18)	0.14 ± 0.01 (18)	0.14 ± 0.01 (18)
Rate of 0.2 mM phlorizin hydrolysis	1.29 ± 0.07 (18)	0.92 ± 0.06 [c] (12)	1.21 ± 0.05 (18)	1.24 ± 0.07 (18)	1.46 ± 0.09 (18)	1.40 ± 0.06 (18)
Transport efficiency of the liberated [^{3}H]glucose (%)	9.3	14.1 [d]	11.6	9.0	9.6	10.0

[a] $P < 0.001$ and [b] $P < 0.01$ when compared to the control of 12 values.
[c] $P < 0.005$. [d] The increase over control is not significant ($P < 0.05$).

the inorganic heavy metal at 0.1 mM inhibits phlorizin hydrolase. All the other agents were inactive. Furthermore, none of these compounds had any significant effect on the uptake rate of [^{3}H]glucose released by the enzyme. Thus, even though Hg^{2+} blocks the hydrolase by 30%, the transport of the liberated [^{3}H]glucose was not influenced. This finding tends to rule out the idea that transport is coupled to hydrolysis.

Discussion

The main purpose of this report is to present evidence that phlorizin hydrolase functions like the better known intestinal disaccharides, such as sucrase and maltase. Our supporting observations are: (1) About 10% of the total glucose liberated from phlorizin is generated at the membrane surface under circumstances that give it an apparent kinetic advantage for entry into the tissue. (2) The phlorizin-derived glucose attains a tissue/medium concentration ratio which is 10 times greater than that attained by free glucose added to the medium under similar conditions. (3) Glucose oxidase in the medium attacks only that fraction of the liberated glucose which diffuses away from the membrane and does not affect the tissue uptake rate. (4) Glucose added to the

medium is relatively ineffective in blocking the uptake of the enzyme-generated hexose. (5) Substrates of other hydrolases which liberate glucose at the membrane surface must be added in very large concentrations before the uptake of glucose released from phlorizin is inhibited.

Hydrolase related transport was previously demonstrated as a Na^+-independent process using disaccharides as substrates. These reports [15,23—25] require careful interpretation before ascribing a mechanism to the process since the Na^+-depleted glucose carrier system is still able to transport sugar, although at a lower rate. We have employed an alternate in vitro method to demonstrate this phenomenon by using phlorizin as the substrate for a typical membrane glycosidase. The use of the glycoside offers a distinct advantage to study the events occurring at the membrane surface since phlorizin acts concurrently as a potent inhibitor of the Na^+-dependent glucose transporter. All of our experiments in the present study were conducted at a single concentration of 0.2 mM ^3H-labeled phlorizin and under conditions previously used to study its inhibition of free glucose transport. Since our experiments were performed for only short periods when less than 10% of the phlorizin was hydrolyzed, we can make accurate estimates of the activity of the free glucose carrier system which escapes phlorizin blockade and the contribution this pathway makes to the overall uptake process. It amounts to about 50% at this substrate concentration (Table I). How does the remainder of the enzyme-generated glucose enter the tissue? Does the hydrolase function as a vectorial enzyme and carrier system to transfer its sugar product directly across the membrane independent of the normal glucose transport process? Some of our present observations are not consistent with this idea: (1) Even though, under anoxic conditions (i.e. under N_2), phlorizin hydrolysis rate is decreased, the glucose which does become liberated by hydrolase action is accumulated at an increased rate, apparently because of an exaggerated passive diffusion (Table II). (2) Mercuric ion inhibits the glucosidase but has no influence on the transport of the fraction of glucose which is set free (Table V). (3) Although high levels are necessary, when exogenous glucose does reach these concentrations at the membrane, it can interfere with the transport efficiency of the hydrolase-generated hexose (Table III).

All of these points rule out any likelihood that phlorizin hydrolase directly transfers glucose into the cell by a mechanism tightly coupled to the cleavage process. Rather, we can account for our results with an alternate explanation, similar to the view held earlier by Crane [15], that the glucose derived from the glycoside reaches a high concentration in the aqueous compartment adjacent to the microvilli membrane. Because of the close spatial organization between the hydrolytic enzyme, the glucose transport carriers and the passive entry portals, the enzyme products are specially localized and have priority over exogenous hexose for entry into the tissue. Evidence for this conclusion is presented in the following report [16].

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