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High-Affinity Phlorizin Binding to Brush Border Membranes From Small Intestine: Identity With (a Part of) the Glucose Transport System, Dependence on the Na^+-Gradient, Partial Purification

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In the presence of an NaSCN gradient phlorizin binds with a high affinity ($K_d \simeq$ 4.7 μM) to vesicles derived from brush border membranes of intestinal cells of rabbits. The value for K_d corresponds closely to that of K_i determined from phlorizin inhibition of sugar transport. The apparent affinity for phlorizin is decreased if NaCl is substituted for NaSCN and decreased substantially if the gradient of NaSCN is allowed to dissipate prior to the phlorizin binding. The number of high affinity binding sites is about 11 pmol/mg protein. Additional binding to low affinity sites can amount to as much as 600 pmol/mg protein after prolonged exposure to phlorizin (5 min). The high affinity sites are related to glucose transport based on the similarity of the K_d and K_i values under a variety of conditions and on the inhibition of the binding by D-glucose but not by D-fructose. The transport system and the high affinity phlorizin binding sites can be enriched by a factor of 2–3 by treatment of vesicles with papain, which does not affect the transport system, but considerably hydrolyzes nonrelevant protein.

Key words: **phlorizin binding, to intestinal membranes; D-glucose transport; sugar transport; sucrase, small intestinal; small intestine; membrane transport of D-glucose**

Phlorizin inhibition of monosaccharide transport across the brush border membrane of small intestine and of renal proximal tubuli has been extensively studied. In particular, the inhibition is known to be fully competitive with transport substrates (1, 2). Phlorizin, on this basis, may be assumed to bind to the sugar binding site of the transport system or to some closely associated site. The binding of phlorizin has been directly demonstrated in brush border membranes from renal tubuli by centrifugation (e.g., Refs. 3, 4) or membrane filtration (e.g., Refs. 5–7) techniques. In this paper we report some of the characteristics of phlorizin binding to vesicles derived from brush border membranes of rabbit small intestine. By using a rapid Millipore filtration technique, we could show that the

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binding consists of at least 2 components, a very rapid, low capacity, high affinity, element and a slower, high capacity, low affinity element. The former component is the one that is associated with the inhibition of sugar transport. In the presence of a Na^+ gradient its apparent affinity (K_d) is about the same as its apparent affinity as an inhibitor of transport (K_i), but in the absence of a Na^+ gradient, the binding affinity is considerably reduced. The high affinity binding is inhibited by D-glucose and the binding site is presumably identical with (a part of) the Na-dependent monosaccharide transport system(s). We also report a partial negative purification of this membrane component.

MATERIALS AND METHODS

Rabbit intestine was collected from freshly killed commercial rabbits at a local slaughterhouse. It was cleaned and frozen before being brought to the laboratory for storage. Vesicles were prepared from the frozen tissue on the day of each experiment according to the procedure of Schmitz et al. (8), as modified by M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Müller, and G. Semenza (Biochim Biophys Acta, in press). Briefly, 20 g of frozen tissue in 1–1.5-g pieces are allowed to thaw in 60 ml of 300 mM mannitol, 12 mM Tris-HCl, pH 7.5. The thawed tissue is mixed in solution with a Chemap Vibro-Mixer for 1.5 min to loosen the mucosal cell fragments from the connective tissue. The mixture is filtered through a Büchner funnel and the connective tissue discarded. The cell fragments are diluted sixfold with water, homogenized in a blender for 3 min at full speed, and then $CaCl_2$ is added to a final concentration of 10 mM. After standing for 20 min, the mixture is centrifuged at 3,000 X g for 15 min and the pellet discarded. The supernatant is then further centrifuged at 27,000 X g for 30 min. The pellet is resuspended in 40 ml of 100 mM mannitol, 10 mM Tris/Hepes, pH 7.5, homogenized in a Teflon Potter Elvehjem Homogenizer and centrifuged at 27,000 X g for 30 min. The pellet, containing the brush border vesicles, is suspended in 1 ml of the appropriate solution.

Uptake experiments were initiated by mixing equal volumes of vesicle suspension and incubation medium containing 100 mM mannitol and sodium and glucose at twice the desired final concentration. This incubation medium also contained the appropriately labeled radioactive compounds. At the desired times, 20-μl aliquots of reaction mixture were withdrawn, quickly pipetted into 1.5 ml ice cold 150 mM NaCl, filtered through a wet Sartorius microfilter (0.6 μm), washed with a further 5 ml of the cold 150 mM NaCl, dissolved in 10 ml of toluene, Triton X-100, butyl PBD, acetic acid scintillation solvent, and counted.

When influx or binding during short (10 sec or less) incubation periods were determined the procedure was slightly different. Ten microliters of vesicles were carefully placed in the bottom of a clear polystyrene test tube fitted into a vibration device controlled by an electric timer. Ten microliters of radioactive incubation medium was placed as a separate drop at the bottom of the tube. At the start of the timer the shaking of the vibrator rapidly mixed the 2 drops together (less than 80 msec). At the chosen time 2 ml of cold 150 mM NaCl were automatically injected into the incubation tube stopping the reaction. The sample was then treated in the usual manner.

The phlorizin binding in the presence of a sodium thiocyanate gradient was determined as described for the uptake studies. The sodium, phlorizin, and either glucose or fructose were all in the incubation medium. The vesicles were exposed to all 3 ligands simultaneously at the start of the incubation. When binding in the absence of a gradient was determined the vesicles were allowed to come to equilibrium (20 min at room temperature, followed by 1 h at 4°C) in 100 mM NaSCN before the phlorizin and either

glucose or fructose incubation was begun.

During stopping and washing (total time: 10 sec), undoubtedly some desorption of bound phlorizin did take place (the washout curves will be reported elsewhere). Therefore, the number of binding sites were certainly underestimated. However, since the washing procedure was identical for all test samples (which were incubated either with glucose or fructose present, and with or without a Na^+ gradient across the membrane), the general conclusions concerning the effect of glucose or the effect of a Na^+ gradient on high affinity phlorizin binding sites are valid.

Papain digestion of vesicles, usually with 0.4 or 0.8 units papain/mg protein was carried out at 37°C for 20 min. The mixture also contained 1 mM EDTA; 5 mM cysteine, 15 mM Tris/Hepes, pH 7.5, 50 μM $MgCl_2$; 50 μM $CaCl_2$. After the incubation, the mixture was diluted 20-fold with cold 15 mM Tris/Hepes, pH 7.5, and centrifuged at 27,000 × g for 30 min. The pellet then was washed in 10 mM Hepes/Tris, 100 mM mannitol, pH 7.5, and again centrifuged. The treated vesicles were then used for the determination of either glucose transport or phlorizin binding activity.

Protein (9), sucrase (10), and lactase (10) activities were determined by routine procedures.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in Tris/glycine buffer, pH 8.8, in slab gels, according to Maestracci et al. (11) with minor modifications. D-[1-^{3}H]Glucose was from Amersham, Buks. (5.2 Ci/mmol). [^{3}H(G)] Phlorizin, lot No. 929-221, was obtained from New England Nuclear (Boston, Massachusetts). The radioactive phlorizin yielded with cold phlorizin a single spot by both UV and radioactive detection. It had the same R_f value of Whatman 3MM, using water as the chromatographic solvent at room temperature (J. S. Cook, personal communication, 1977), as authentic recrystallized phlorizin from ICN Pharmaceuticals, Inc., Life Science Group, Plainview, New York. The authenticity of the nonradioactive sample was checked by NMR. Papain was obtained from Boehringer, Mannheim, GFR. All other chemicals were reagent grade.

RESULTS

Kinetics of Phlorizin Inhibition of D-Glucose Uptake

The vesicle preparation yields itself particularly well to the kinetic studies, due to the high and relatively persistant substrate overshoot that occurs when a Na^+ gradient is imposed in the presence of the permeant anion SCN^- [M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Müller, and G. Semenza (Biochim Biophys Acta, in press) and also Fig. 6 below]. The prolonged overshoot is presumably indicative of the dissipation of the Na^+ gradient being slow. Under the conditions employed, the uptake of D-glucose is linear with time up to 2–3 sec, deviates slightly from linearity by 4 sec, and significantly after 8 sec (Fig. 1). Other conditions and lower D-glucose concentrations also yielded essentially linear uptake up to 2 sec, our standard error on the time scale being approximately 2%. We, therefore, used 2-sec incubation periods when studying phlorizin inhibition of D-glucose uptake into these vesicles. The K_i value determined from Dixon plots (Fig. 2) was 7 μM.

Phlorizin Binding

Initial attempts to determine phlorizin binding were carried out by centrifugation techniques after relatively prolonged exposure of the vesicles to phlorizin (up to 30 min). In each case a large low-affinity binding was evident, Scatchard plots providing little or no

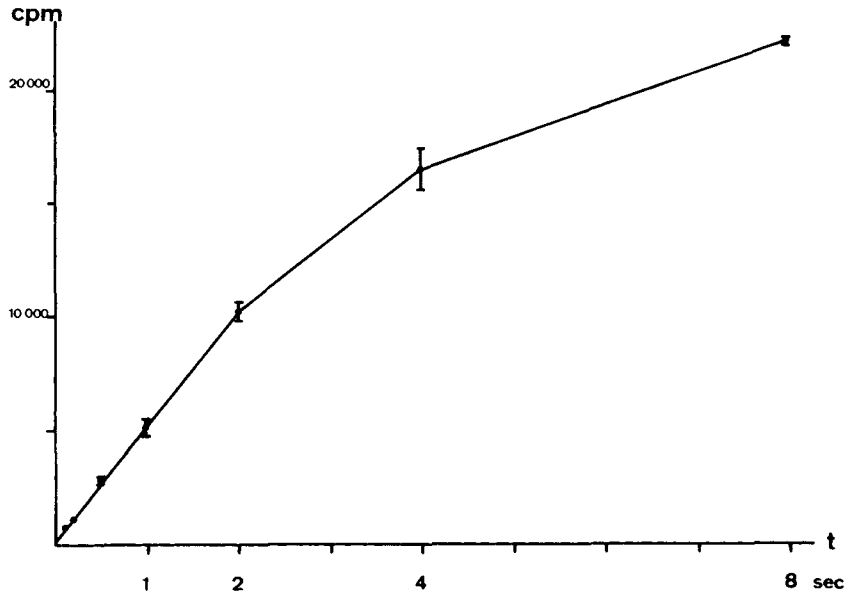

Fig. 1. Time course of D-glucose uptake into membrane vesicles from rabbit small intestinal brush borders. The uptake of D-[^{3}H]glucose (0.1 mM) was measured at room temperature in the presence of a NaSCN gradient, at the start Na^+_{out} = 100 mM, Na^+_{in} = 0 mM. All solutions contained also 100 mM mannitol and 10 mM Hepes/Tris, pH 7.5. The values were obtained from measurements in triplicates, the bars indicate the standard deviations. The figure reports only the initial rising part of the overshooting glucose uptake; after a few minutes the radioactivity in the vesicles declines to approximately 3,000 cpm, corresponding to the equilibration of the inner and outer substrate and Na^+ concentrations. Specific radioactivity of the D-glucose used: approximately 250 cpm per pmol glucose.

indication of any high-affinity binding (i.e., no K_d in the range corresponding to the K_i for inhibition). From the time course of phlorizin binding (Fig. 3), it is apparent that a steady state of binding is not reached within the first minute of incubation. Even after 5 min the level of binding still increases. The amount bound at 5 min using 1 μM phlorizin is approximately 5 pmol/mg protein but at 200 μM phlorizin it is over 100 times as high, 600 pmol/mg protein. It is very probable, therefore, that most of the binding at high phlorizin concentrations is to nonspecific sites, unrelated to glucose transport.

The onset of phlorizin inhibition of sugar transport is very rapid both in the small intestine (2) and kidney (12). In the case of brush border membrane vesicles, the inhibitory effect is completed within 2 sec after the addition of phlorizin, with no increase after more prolonged exposure times. It can be concluded, therefore, that the binding to the inhibitory site is virtually complete in 2 sec and that the large binding component after 2 sec in Fig. 3 is to sites that are not associated with inhibition. Furthermore, the inhibition of glucose uptake was determined in the presence of a NaSCN gradient across

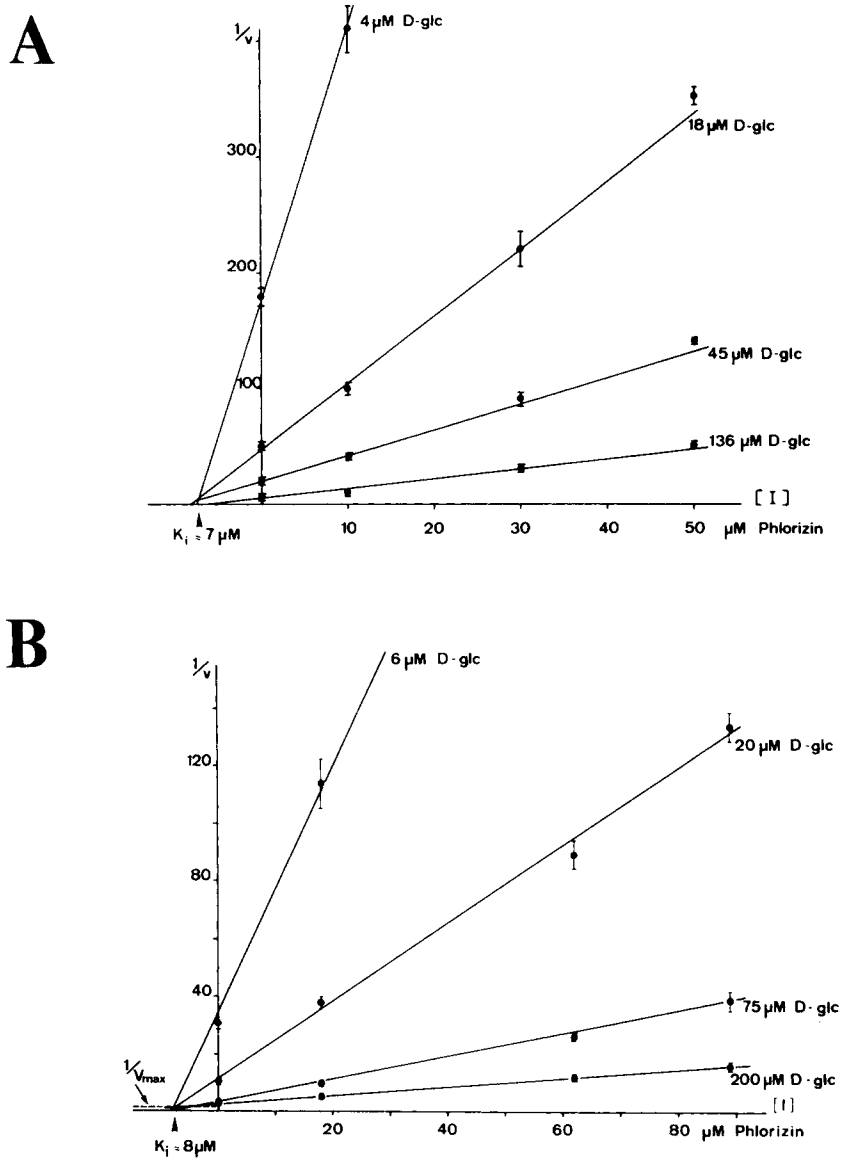

Fig. 2. Dixon plot of phlorizin inhibition of D-glucose uptake into brush border membrane vesicles, at room temperature. A) Incubation time, 2 sec. Phlorizin, D-[^{3}H]glucose and NaSCN were added simultaneously to the membrane vesicles. Final concentrations were: NaSCN (out), 100 mM; mannitol, 100 mM; Hepes/Tris, pH 7.5, 10 mM. The concentrations of D-glucose and phlorizin are indicated in the figure. The uptake values were obtained from measurements in triplicates. The bars indicate the standard deviations, the lines were calculated by linear regression. The velocity v is expressed as nmol of glucose taken up per 176 μg of protein in 2 sec. B) As in A, but with incubations lasting 7 sec. The velocity v is expressed as nmol og glucose taken up per 290 μg of protein in 7 sec. [From G. Toggenburger, M. Kessler, A. Rothstein, G. Semenza, and C. Tannenbaum (in preparation).]

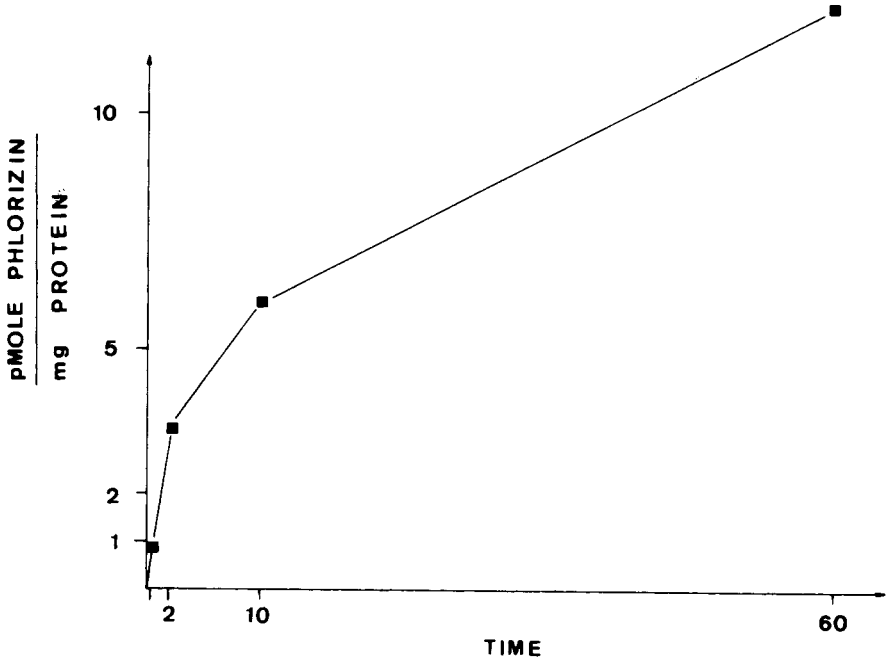

Fig. 3. Time course of phlorizin binding to brush border membrane vesicles. Phlorizin, to a final concentration of 2 μM, was added to membranes which had been previously equilibrated in 150 mM NaCl. Incubation was run at room temperature and was stopped by addition of an excess of ice cold saline, followed by immediate filtration. The time needed for diluting, filtering, and washing was about 10 sec.

the membrane, i.e., NaSCN, glucose, and phlorizin, were all added simultaneously, whereas phlorizin binding mentioned above was measured (Fig. 3) after NaCl had time to equilibrate across the membrane. We therefore remeasured phlorizin binding after 2 sec and under the same conditions as in the case of phlorizin inhibition of glucose transport, i.e., in the presence of a gradient of NaSCN, phlorizin, and glucose (or fructose) added simultaneously. The results of Fig. 4 were obtained. The lower curve (glucose present) represents the glucose nonprotectable phlorizin binding sites. The upper curve (with an equal concentration of fructose as a control) represents the glucose protectable binding sites. When the difference is plotted according to Scatchard we get the results of Fig. 5. Under these conditions (i.e., before the dissipation of the gradients of both NaSCN and glucose, at a time when the glucose uptake is still linear) a glucose-protectable, high-affinity site of phlorizin binding is apparent, with K_d = 4.7 μM and total binding of 11 pmol/mg protein. (based on extrapolation of the high affinity component of the Scatchard plot).

In contrast, if the vesicles are preequilibrated with 100 mM NaSCN prior to the addition of phlorizin, to dissipate the Na^+ and SCN^- gradients, the binding affinity is substantially reduced. In the presence of 25 mM D-glucose, with or without a Na^+ gradient, no high affinity binding is apparent, and indeed the binding is the same in either case.

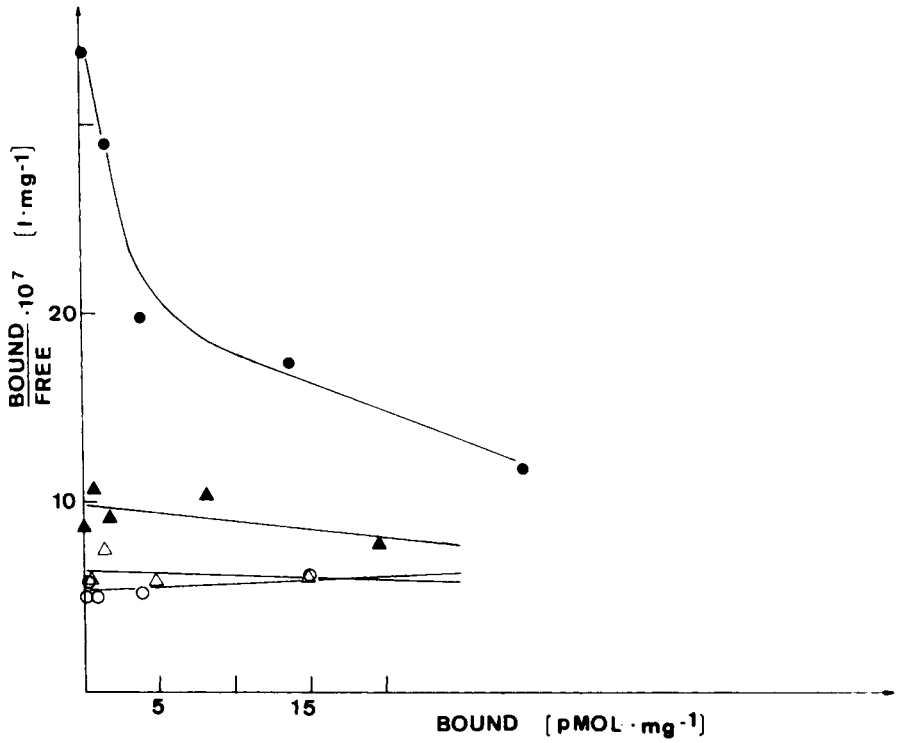

Fig. 4. Phlorizin binding to intestinal brush border membrane vesicles: effect of a NaSCN gradient. Conditions as in the glucose uptake experiments of Fig. 2A (i.e., 2-sec incubations at room temperature in 10 mM Hepes/Tris, pH 7.5, and 100 mM mannitol; phlorizin concentrations from 0.2 to 25 μM). • and ○) 100 mM NaSCN, phlorizin, and either 25 mM D-fructose (•) or 25 mM D-glucose (○) added simultaneously at time zero. ▲ and △) the vesicles had been equilibrated in 100 mM NaSCN (+ buffer and mannitol) before the addition, at time zero, of phlorizin and either 25 mM D-fructose (▲) or 25 mM D-glucose (△).

Table I compares the K_d values of the D-glucose protectable high-affinity binding sites (as estimated from the steeper, more linear portion of the plot) with the K_i values for inibition of D-glucose uptake obtained under comparable conditions. The K_d and K_i values agree reasonably well and change according to the experimental conditions in the same way: when a NaCl gradient is substituted for the NaSCN gradient, K_d and K_i values both increase (and the initial velocity of D-glucose uptake decreases to approximately one fourth).

Hydrolysis of Phlorizin (13, 14)

Brush border vesicles were incubated for 30 min at room temperature in the standard incubation medium containing phlorizin. They were then extracted with hot ethanol. Thin layer chromatographic analysis of the extract indicated that less than 10% of the phlorizin had been hydrolyzed. The amount of hydrolysis in the 2-sec duration of the binding and inhibition experiments must, therefore, be insignificant.

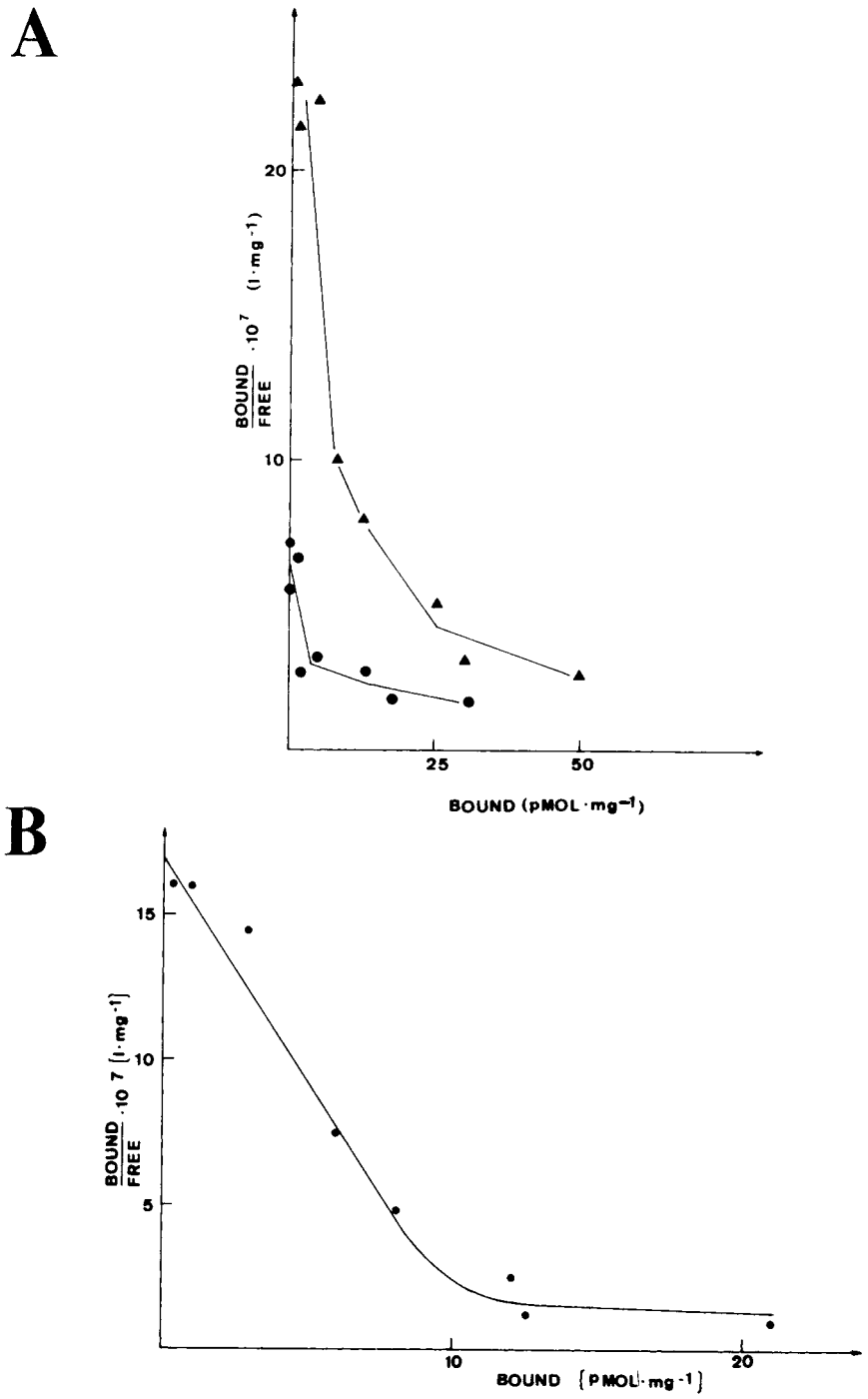

Fig. 5. A) Phlorizin binding to intestinal brush border membrane vesicles: effect of D-glucose. Conditions as in Fig. 4. 100 mM NaSCN, phlorizin (from 0.2 to 100 μM) and either 25 mM D-glucose (•) or D-fructose (▲) were added simultaneously at the start. B) • is a Scatchard plot of the glucose-protectable, NaSCN gradient-dependent phlorizin binding (calculated as the difference between the values in fructose minus the values in glucose of A). [From G. Toggenburger, M. Kessler, A. Rothstein, G. Semenza, and C. Tannenbaum (in preparation).]

TABLE I. Interaction of Phlorizin With Membrane Vesicles From Rabbit Small Intestinal Brush Borders: K_i Values From Phlorizin Inhibition of Initial D-Glucose Uptake and K_d Values for the High-Affinity, D-Glucose-Protectable Phlorizin Binding Sites.

	Phlorizin inhibition of D-glucose uptake	Phlorizin binding[a]	
	K_i (μM) $\bar{x} \pm$ S.D. (range)	K_d (μM) $\bar{x} \pm$ S.D. (range)	Approximate concentration of high-affinity, glucose-protectable binding sites (pmol per mg membrane protein) (range)
Intact vesicles			
with NaSCN gradient	7.8 ± 1.4 (6–10)	4.7 ± 2.4 (2–7)	9.6 ± 3.2 pmol per mg (5–13)
with NaCl gradient	25–35	23–33	approx. 55–70 pmol per mg
with neither NaSCN nor NaCl gradient	not determined	> 100	
Papain digested vesicles			
with NaSCN gradient	not determined	6.3–16	29–34 pmol per mg

[a]Due to the curvature of some Scatchard plots, quantitative comparision can only be made among experiments carried out under strictly identical conditions.

Partial Negative Purification of the High-Affinity Phlorizin Binding Sites and of the Glucose Transport Agency(ies); the Location of Small Intestinal Sucrase-Isomaltase Complex[1]

Papain treatment has been extensively used to solubilize the sucrase-isomaltase complex and other intestinal hydrolases (e.g., see Refs. 16–19) Since the sucrase-related sugar transport system is not identical with the Na-dependent monosaccharide transport system(s) (20), it seemed appropriate to attempt to enrich the phlorizin high-affinity binding sites and the Na-dependent glucose transport system(s), by controlled papain digestion. In Fig. 6A the data are presented as pmol D-glucose uptake/mg protein, where the protein content is that actually measured in each incubation medium; in B, the results are normalized to the amount of protein present in the vesicles prior to papain solubilization.

It is apparent that papain treatment under the conditions used did not reduce the function of the Na-dependent D-glucose transport system(s) – as shown by the "normal" initial velocity of uptake – nor did it make the vesicles leaky – as shown by the retention of equilibrium concentrations of the substrate in the vesicles after washing with substrate-free media. Thus, the recovery of the transport activity after papain treatment was quantitative or nearly so. However, 30–60% of the proteins – apparently not associated with this transport system(s) – had been removed from the vesicles by papain. This resulted in a twofold increase of specific activity (Fig. 6A and Table II). In addition, the treated vesicles retain the ability to discriminate between D- and L-glucose. There is no indication that papain exposes cryptic transport sites or that it degrades membrane proteins extensively: if SDS-PAGE patterns of the control vesicles, of the treated vesicles, and of the supernatant are compared (Fig. 7), then we see that those proteins that have been removed from the vesicles by papain treatment appear in the supernatant. Only 2 new bands appear in either gel pattern. None of the bands found in the control is absent from the patterns after papain treatment, and there is no increase in bands found in the low molecular weight region of the gel.

[1] Some of these data have already appeared in a preliminary torm (15).

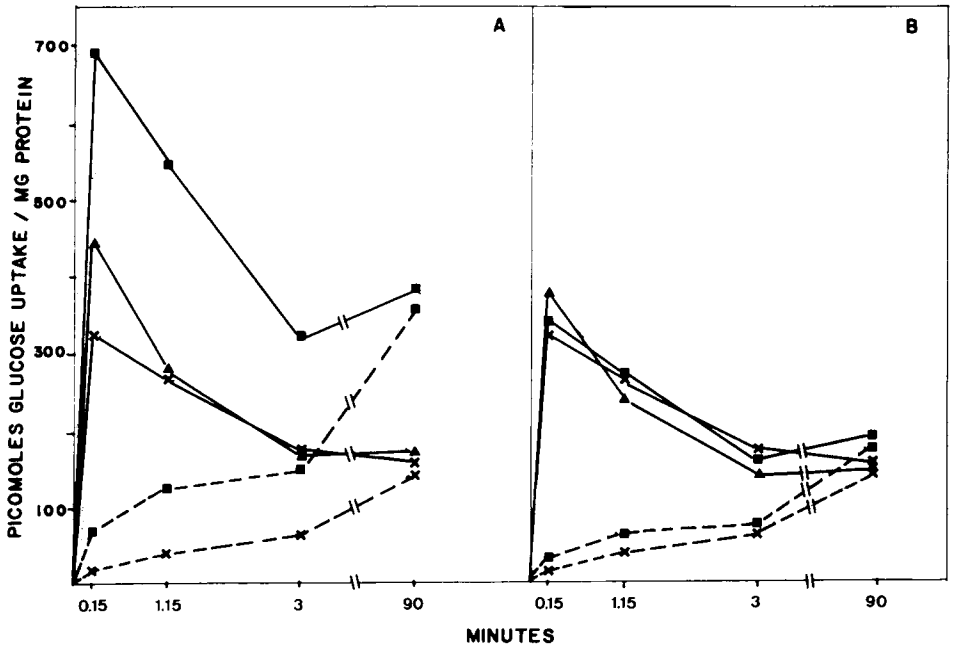

Fig. 6. Uptake of D- and L-glucose by papain (+EDTA) treated brush border membrane vesicles. A) The uptake values are given in terms of amounts of protein remaining in the membrane vesicles after papain treatment. B) in terms of amount of protein present in the original vesicle preparation prior to papain treatment. - - -) L-glucose uptake; ——) D-glucose uptake; x——x) control; ■——■) vesicles treated with 0.8 U papain mg^{-1} protein in 1 mM EDTA; ▲——▲) vesicles treated with 1 mM EDTA alone.

TABLE II. Effect of Papain Treatment on Glucose Uptake by Vesicles of Brush Border Membranes

	Uptake[a]		Sucrase[b]
	D	L	
Control	215	11	1.08
After EDTA treatment (1 mM)	295	34	1.11
After EDTA + papain (0.8 U/mg protein)	460	44	< 0.05

[a]Uptake (of D- or L-glucose, respectively) in picomoles • mg^{-1} protein • 10 sec^{-1} at room temp.

[b]Sucrase activity left in the vesicles in units • mg^{-1} protein. The total yield of sucrase activity during the papain treatment (supernatant + pellet) was essentially quantitative.

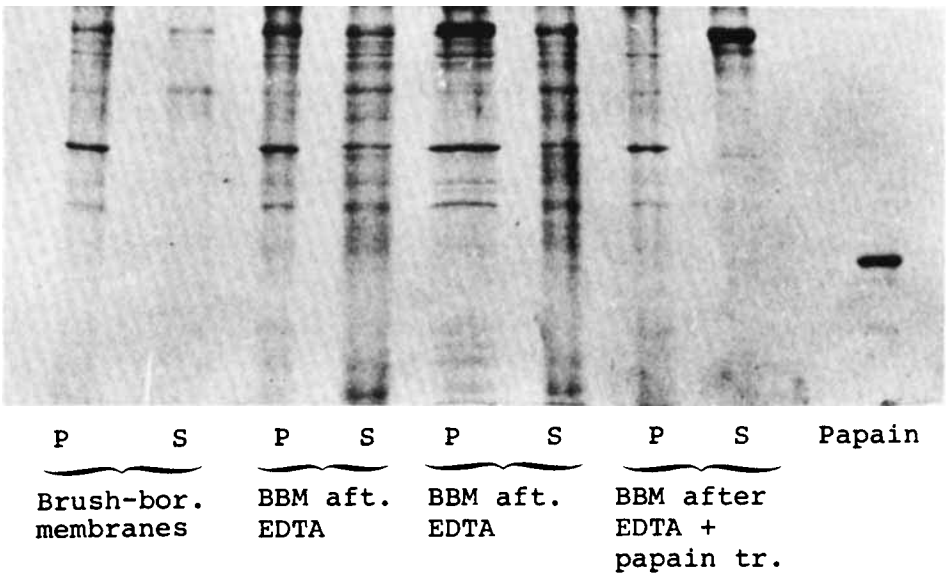

Fig. 7. SDS-PAGE (start from the top) of small intestinal rabbit brush border membranes (BBM) with no treatment (left), after EDTA treatment (center), or after EDTA + papain treatment (right). After each treatment, the membrane vesicles were spun down (P, pellet; S, supernatant).

The vesicles were assayed for sucrase and lactase activity before and after treatment with papain. Treatment of the vesicles with EDTA resulted in the liberation of only about 4% of sucrase activity; treatment with papain liberated 95–98% of this activity (Table II) and also of lactase activity (data not shown). As to the high-affinity phlorizin binding sites, papain treatment of the vesicles resulted in a slight (or no) increase in the apparent K_d value, with a doubling or tripling of the amount of binding sites per mg protein (Fig. 8), as a result of the removal of other proteins from membrane.

It should be emphasized that the increase in specific transport activity reported in Table II was obtained in 3 out of 6 experiments. In the others papain treatment failed to increase the specific transport activity, in spite of differential solubilization of membrane proteins identical to those of the "successful" experiments (as revealed by the SDS-PAGE patterns). We have failed as yet to identify the reason why papain treatment is not quantitatively reproducible. Neither the segment of the intestine, nor the age or the breed of the rabbit nor the contamination by variable amounts of serine-proteases were found to be of relevance.

An important corollary of the results of Fig. 6 and Table II is the information as to the location of small intestinal sucrase. It has already been shown by functional (21, 22), immunologic (23), and morphologic (24, 25) criteria that (at least some) sucrase activity is located at the luminal surface of the brush border membrane. The quantitative solubilization of intestinal sucrase (Table II) under conditions which do not alter the permeability properties of the membrane (Fig. 6), demonstrates that all sucrase is available to a reagent (papain) which was added to the luminal side of the vesicles and which is very unlikely to cross the membrane. Likewise, addition of lytic concentrations of Triton X-100 to native vesicles did not lead to any apparent increase in sucrase activity [M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Müller, and G. Semenza (Biochim Biophys Acta, in press)].

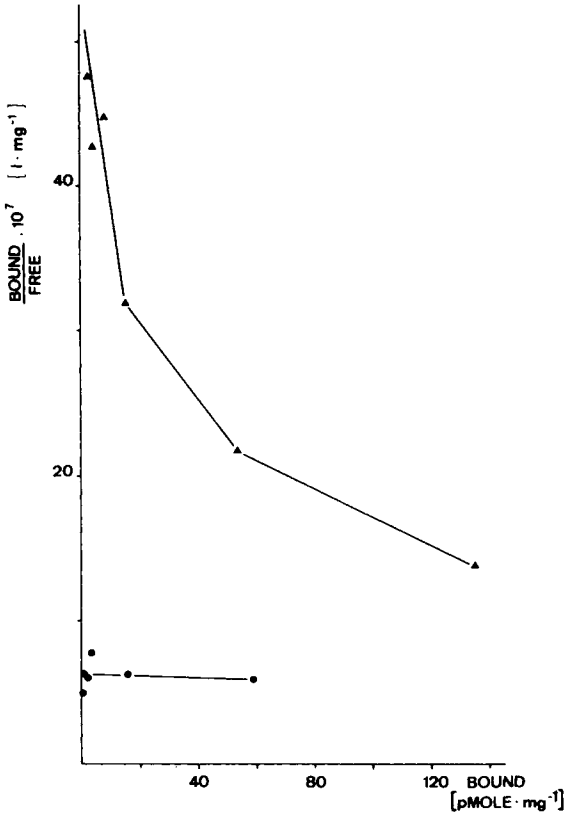

Fig. 8. Phlorizin binding to papain-digested brush border membrane vesicles. Conditions as in Figs. 4 and 5A. ▲) in the presence of 25 mM D-fructose; ●) in the presence of 25 mM D-glucose.

DISCUSSION

Kinetics of D-Glucose Uptake Into Vesicles From Brush Border Membranes and of its Inhibition by Phlorizin

The question may arise as to the validity of measuring parameters of transport systems under nonsteady-state conditions. Specifically, the experiments are performed under conditions of an initial NaSCN gradient, so that during the course of the experiment the gradient dissipates and all parameters dependent on the gradient will change with time. In addition, the rate of Na gradient dissipation, due to the glucose-Na-coupled cotransport, might depend upon the initial external glucose concentration. An effect of glucose on the rate of dissipation of the Na^+ gradient is unlikely, however, since the percentage of Na^+ that can enter the vesicles through the glucose-dependent pathway is only a small portion of the Na^+ entering the vesicles by all pathways [M. Kessler, V. Tannenbaum, and C. Tannenbaum (submitted to Biochim Biophys Acta)]. The complete dissipation of the Na^+ gradient (as shown by reversion of glucose flux and by Na^+-uptake measurements) takes up to 2 to 3 min, but the measurements of glucose uptake are carried out for only 2 sec. In this short time the flux of Na^+ would be relatively small and only a small fraction of the

NaSCN gradient would dissipate, so that it would be a relative constant factor with respect to its effect on transport or binding parameters. This relative constancy is suggested by the finding that the glucose uptake is linear for up to almost 4 sec, and by the finding that the measured "initial" velocity of glucose uptake gives a linear kinetic plot (Lineweaver-Burk, Eadie, Dixon). Substantial dissipation of the Na^+ gradient would be expected to cause some curvature in Fig. 2. In addition, the K_m values for D-glucose uptake measured at 7 sec (i.e., shortly after deviation from linearity) did not differ significantly from the K_m values obtained from 2-sec incubations despite continued dissipation of the gradient. Furthermore the K_i for phlorizin inhibition is also not substantially altered between 2 and 7 sec (Fig. 2). These findings suggest that the reported K_i and K_d values represent minimal values associated with relatively large NaSCN gradients that are not substantially dissipated in the 2 sec required for measurement. A theoretical argument further supports this view. In electrogenic transport systems, Geck and Heinz (26) have shown that kinetic parameters such as K_m and V_{max} may be relatively independent of changes in the membrane potential (such as those that might occur as a result of a partial dissipation of the NaSCN gradient), as long as the membrane potential is very high. Finally, measuring uptake velocities at almost zero-trans concentrations of substrates has the obvious advantage of minimizing possible trans effects.

Although there is uncertainty about the absolute values of the kinetic and binding parameters in the present experiments, the comparison of the K_i for phlorizin inhibition of sugar transport and K_d for phlorizin binding is certainly valid because the size of the gradient and the extent of its dissipation was the same in each case. It is also certain that if the less permeant species, Cl^-, is substituted for SCN^- as the accompanying anion, the apparent affinity of phlorizin measured by K_i or K_d is significantly reduced, and if the gradient is allowed to dissipate completely it is substantially reduced (Table I).

Identity of the High-Affinity Phlorizin Binding Sites With (a part of) the Na-Dependent Glucose Transport Agency(ies).

This identity is borne out by the following observations: i) the K_d values of the high-affinity phlorizin binding sites match the K_i values of phlorizin inhibition of D-glucose transport (Table I); ii) the K_d values and the K_i values change with the experimental conditions in parallel fashion (Table I); iii) high-affinity phlorizin binding is inhibited by D-glucose but not by D-fructose (Figs. 4,5).

From the data in Figs. 4 and 5 there appear to be 10–12 pmol of high-affinity phlorizin binding sites per mg membrane proteins in these vesicles from small intestinal brush borders. Assuming a 1:1 stoichiometric relation with the transport agency(ies) for D-glucose, the "transport turnover number" at 1 mM D-glucose, 20°C, in buffer pH 7.5 can be calculated to be of the order of 20 sec^{-1} in the presence of a starting NaSCN gradient of 100 mM (out) vs 0 mM (in). The 10–12 pmols of high-affinity phlorizin binding sites found in the vesicles of small intestinal brush border membranes are in very close agreement with the corresponding figure reported for the high-affinity phlorizin binding sites of renal proximal tubulin membranes (3,5). Assuming a molecular weight of 80,000-100,000 daltons for glucose transport agency(ies), this number of high-affinity phlorizin binding sites would indicate that the glucose transport involves only about 0.1% of the membrane proteins.

Our determination of the high-affinity binding sites corresponds rather well to similar values reported for kidney brush border fragments [rat, by Bode et al., (4) and by Glossmann and Neville, (5); dog, by Silverman and Black, (27)]. Others have reported

larger numbers for high affinity binding sites [Bode et al., (28), Chesney et al., (7)] in similar preparations from kidney. Due to the different ways of expressing the data, it is more difficult to make a comparison with the results of Diedrich (13) and of Stirling (29, 30) for the small intestine.

At any rate, all quantitative calculations of this sort, including ours, must be taken with caution, due to the critical effect of the incubation conditions (see for example, Table I), the different washing times, the curvature sometimes present in Scatchard plot (see below), and so on.

Elsewhere (M. Kessler, A. Rothstein, G. Semenza, G. Toggenburger, and C. Tannenbaum, in preparation) we present that phlorizin, as generally accepted, is indeed a fully competitive inhibitor (also in the presence of a NaSCN gradient), and not a poorly transported substrate.

Dependence of the High-Affinity Binding Sites Upon the Na^+ Gradient

As pointed out under Results, we were unable to show any high-affinity phlorizin binding in membrane vesicles of rabbit small intestinal brush borders unless a NaSCN (or NaCl) gradient had been imposed on them (Fig. 4). To the best of our knowledge no such dependence upon such a gradient has even been reported for phlorizin binding to kidney tubuli membranes [although the K_d value has been reported to depend on the presence of Na^+ (3)], or to small-intestinal membranes. Since it is not always clear whether the conditions employed by other authors have generated a Na^+ gradient during binding measurements, we do not know whether the need for the Na^+ gradient is limited to the biological preparation used by us.

The dependence of the size of the transient D-glucose overshoot in the presence of a highly permeant anion (31, 32), and the apparent reduction in affinity for phlorizin binding when Cl^- is substituted for SCN^- (Table I), suggests that the membrane potential may modulate the interaction of the carrier with phlorizin as it has been shown with glucose (31, 32). Such findings can be accomodated by a mobile cotransport carrier model in which at least one form of the carrier is charged, for which the kinetics have been developed by Geck and Heinz (26), but a detailed discussion of a mechanism would be inappropriate until more data are collected concerning the individual effects of Na^+ concentrations and of membrane potentials on phlorizin binding.

Scatchard Plots

Another point which is presently being investigated is the curvature of the Scatchard plots of high-affinity, glucose protectable phlorizin binding sites (Figs. 5, 8). The extent of the curvature varied somewhat from preparation to preparation of the vesicles. A number of reasons can be envisaged. One of the possibilities is the existence of two, rather than one, transport agencies for D-glucose, having different K_d values. This would agree with the conclusions reached for hamster small intestine on the basis of kinetic investigations of monosaccharide uptake (33).

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NOTE ADDED IN PROOF

Subsequent, extensive experimentation has shown that although there is a significant increase in the amount of phlorizin bound by the vesicles when a Na-gradient is imposed across the vesicle membrane, the amount of glucose-protectable phlorizin binding in the absence of a Na-gradient is not insignificant. [See G. Toggenburger, M. Kessler, A. Rothstein, G. Semenza, and C. Tannenbaum (in preparation).]

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