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A Two Sodium Ion/D-Glucose Symport Mechanism: Membrane Potential Effects on Phlorizin Binding†

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ABSTRACT: Apical membrane vesicles isolated from a continuous renal cell line, LLC-PK$_1$, catalyze electrogenic Na^+-stimulated hexose transport and Na^+-dependent binding of ^{3}H-labeled 1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone ([^{3}H]phlorizin), a competitive ligand of this transport system. Phlorizin was not itself transported across the membrane and thus can serve as a probe of the binding step. The stoichiometry of Na^+-dependent phlorizin binding in vesicles was 1:1, whereas Na^+/hexose cotransport in vesicles exhibited a 2:1 stoichiometry. Na^+ increased the affinity of phlorizin binding without affecting the total number of binding sites. An increased number of Na^+-dependent phlorizin binding sites was observed under conditions of interior-negative membrane potential. These results are consistent with a model of the Na^+/glucose cotransport cycle in which the unloaded transporter is negatively charged and its orientation influenced by membrane potential. Glucose and one sodium ion interact with the transporter, resulting in an uncharged complex. Binding of a second sodium ion triggers translocation of glucose and both sodium ions via formation of a loaded carrier complex bearing a single positive charge.

Electrogenic Na^+-stimulated hexose cotransport is expressed in apical membrane vesicles isolated from confluent cultures of LLC-PK$_1$, an established kidney epithelial line (Lever, 1982; Moran et al., 1982). The Na^+/hexose symporter expressed in this cell line exhibits a sugar and inhibitor specificity similar to that of the corresponding renal cortical proximal tubule transporter but differs in one important aspect. The stoichiometry of Na^+/glucose cotransport in LLC-PK$_1$ cells is 2:1 on the basis of studies using apical membrane vesicles (Lever, 1982; Moran et al., 1982) and short-circuit current measurements of intact cell monolayers grown on filters (Misfeldt & Sanders, 1981, 1982). By contrast, a Na^+/glucose stoichiometry of 1:1 has been estimated for the renal cortical proximal tubule symporter (Beck & Sacktor, 1978; Turner & Moran, 1982a).

The stoichiometry of the coupled species in a symport mechanism is of fundamental importance in determining the energetics of the transport mechanism, since the ability to accumulate substrate is proportional to the power of the number of sodium ions cotransported per substrate. Evaluation of the individual contributions of membrane potential and the coupled ionic species to the binding step must also be considered in order to understand the energetics and mechanism of the transport cycle.

1-[2-(β-D-Glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone (phlorizin)[1] is a competitive inhibitor of Na^+/glucose symport across the renal proximal tubule brush border membrane (Frasch et al., 1970). The binding of phlorizin to the glucose carrier is Na^+ dependent, but several lines of evidence indicate this inhibitor is not translocated across the membrane (Toggenburger et al., 1982). As a consequence, this nonpenetrating ligand has been used as a probe in studies of the mechanism of Na^+/glucose symport in brush border preparations from intestine and renal cortex

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[1] Abbreviations: αMeGlc, methyl α-D-glucopyranoside; $TPMP^+$, triphenylmethylphosphonium ion; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; phlorizin, 1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone; phloretin, 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; BES, 2-[bis(2-hydroxyethyl)amino]-ethanesulfonic acid.

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Table I: Fractionation of Na^+-Dependent [^{3}H]Phlorizin Binding Activity

	[^{3}H]phlorizin binding (pmol/mg)		specific activity[a]				
fraction	total	Na^+ dependent	(Na^+,K^+)-ATPase	γ-glutamyl transpeptidase	trehalase	NADH oxidase	succinate:cytochrome *c* reductase
homogenate	1.96 (100)[b]	ND	0.048 (100)	0.381 (100)	1.25 (100)	47.2 (100)	23.1 (100)
P1	2.94 (6.4)	ND	0.052 (38.7)	0.225 (21.1)	0.89 (25.6)	78.2 (59.1)	46.8 (69.3)
P2	5.11 (71)	0.44	0.050 (27.8)	0.447 (31.9)	1.47 (32.1)	102.9 (59.1)	29.7 (33.4)
P3	4.74 (12.7)	1.32	0.081 (8.8)	1.507 (20.9)	6.84 (28.8)	38.3 (4.3)	5.0 (1.1)
10^5g supernatant	ND[c]	ND	0 (0)	0.004 (1.0)	0.014 (1.1)	25.5 (13.6)	0.1 (0.1)

[a] Units of specific activity are defined as follows: γ-glutamyl transpeptidase and (Na^+,K^+)-ATPase, micromoles of substrate hydrolyzed per minute per milligram of protein; trehalase, nanomoles of substrate hydrolyzed per minute per milligram of protein; NADH oxidase and succinate:cytochrome *c* reductase, change in optical density in 60 min per milligram of protein. [b] Numbers in parentheses refer to the percent recovery of total enzyme activity. [c] ND, not detectable.

(Aronson, 1978; Turner & Silverman, 1980, 1981; Toggenburger et al., 1982). However, these previous characterizations of phlorizin binding have described symport systems exhibiting 1:1 Na^+/glucose stoichiometries. Two forms of the intestinal (Kaunitz et al., 1982; Kaunitz & Wright, 1983) and renal (Turner & Moran, 1982b) Na^+/D-glucose symporter have recently been described which differ in Na^+ stoichiometry. This heterogeneity complicates the interpretation of phlorizin binding data from tissue preparations which may consist of multiple cell types expressing forms of the transporter which differ in the energetics of the transport mechanism.

The present study characterizes the interaction between Na^+, membrane potential, hexose transport, and phlorizin binding in apical membrane vesicles from the clonal cell line LLC-PK_1 as a more homogeneous source of the Na^+/D-glucose symporter from a single cell type. Results from this approach are interpreted in terms of a two sodium ion/D-glucose symport mechanism.

Materials and Methods

Materials. [*phenyl*-3,3′,5,5′-^{3}H, *propyl*-3-^{3}H]Phlorizin (42.5 Ci/mmol), [^{14}C]methyl α-D-glucopyranoside (275 mCi/mmol), and [^{3}H]triphenylmethylphosphonium ion (3.59 Ci/mmol) were purchased from New England Nuclear. Phlorizin, phloretin, valinomycin, and sugars were purchased from Sigma. Monensin was from Calbiochem. The purity of [^{3}H]phlorizin was verified by thin-layer chromatography in chloroform/methanol/water (65:25:4 v/v). [^{3}H]Phlorizin was not metabolized after incubation with vesicles, as verified by thin-layer chromatography.

Cell Culture. The LLC-PK_1 (Hull et al., 1976) pig kidney epithelial cell line (ATCC CL101) was obtained through the courtesy of Dr. Joseph Handler, National Institutes of Health, Bethesda, MD. Cell growth was in a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F12 supplemented with 10% fetal calf serum (Sterile Systems, Inc., Logan, UT), 2 mM L-glutamine, and 3.7 g/L $NaHCO_3$.

Membrane Vesicle Isolation and Marker Enzyme Assay. Apical membrane vesicles (P3 fraction) were isolated from densely confluent cultures of LLC-PK_1 by a method based on nitrogen cavitation and selective membrane precipitation by Mg^{2+} (Lever, 1982). Preparations were analyzed for purity by marker enzyme analysis. Assay of succinate:cytochrome *c* reductase and NADH oxidase was as described previously (Lever, 1980, 1982). (Na^+,K^+)-ATPase activity was assayed by the method of Forbush (1982). γ-Glutamyl transpeptidase activity was determined by the method of Glossman & Neville (1972). Trehalase activity was assayed by the method of Dalqvist (1968). Protein was determined by the method of Lowry et al. (1951). Vesicles were stored frozen in 1-mL aliquots suspended in 0.25 M sucrose/0.01 M Tris-HCl, pH 7.5. For some experiments, vesicles were obtained by a modification of this procedure in which mannitol isoosmotically replaced sucrose.

Assay of Transport Activity in Vesicles. A nonmetabolizable glucose analogue, methyl α-D-glucopyranoside (αMeGlc), was used as a substrate relatively specific for the Na^+/hexose transport system. Transport was assayed at 21 °C in 100-μL volumes containing 0.125 M sucrose, 5 mM $MgCl_2$, and 10 mM K-HEPES, pH 7.2 (standard incubation mixture), with other additions as indicated. Termination and filtration steps were as described previously (Lever, 1982). Uptake of the lipophilic cation [^{3}H]-$TPMP^+$ was used to measure interior-negative membrane potentials as described previously (Lever, 1977, 1982).

Assay of Phlorizin Binding to Vesicles. Phlorizin binding activity of membrane vesicles was assayed in triplicate at 21 °C in 100-μL volumes containing 0.125 M sucrose, 5 mM $MgCl_2$, and 10 mM K-HEPES, pH 7.2 (standard incubation mixture), with other additions as indicated. Binding was terminated by addition of 3 mL of ice-cold 0.8 M NaCl/10 mM Tris-HCl, pH 7.5 (wash buffer), and filtration through a 0.45-μm pore size nitrocellulose filter (Schleicher & Schuell) followed by a second wash with 5 mL of wash buffer. Radioactivity of dried filters was measured by scintillation counting in toluene/Liquifluor.

Due to the relatively low phlorizin binding activity expressed in vesicles from LLC-PK_1 cells compared with those reported for vesicles from dog kidney (Turner & Silverman, 1981), it is difficult to detect low-affinity phlorizin binding sites. The present study focuses on the more readily determined high-affinity Na^+-dependent phlorizin binding component. At Na^+ concentrations of 100 mM or lower, utilized in this study, low-affinity Na^+-dependent phlorizin binding makes a negligible contribution to total binding. Unless noted otherwise, all data points are corrected by subtraction of Na^+-free binding, which may represent contributions from low-affinity states of the Na^+/glucose symporter or from nonspecific sites or binding to residual amounts of the basolateral glucose transporter which may contaminate apical preparations.

Results

Na^+-Stimulated Phlorizin Binding Activity Accompanies Apical Membrane Markers and Na^+-Stimulated Glucose Transport Activity during Subcellular Fractionation. Table I illustrates the distribution of phlorizin binding activity in subcellular fractions prepared and defined as described previously (Lever, 1982). Total phlorizin binding activity in the presence of Na^+ is shown. The Na^+-dependent component was determined by subtraction of binding observed when Na^+ was replaced by choline. Over 90% recovery of total phlorizin binding activity was observed among all fractions, but the

Na^+-dependent component, presumably attributable to specific interaction with the Na^+-glucose symporter (Silverman, 1974), was only detectable in the P2 and P3 fractions. As reported previously (Lever, 1982), the P3 fraction is enriched in specific activities of apical marker enzymes such as γ-glutamyl transpeptidase and trehalase (Table I) and essentially devoid of the mitochondrial marker succinate:cytochrome *c* reductase. The P3 fraction contained 9% of the basolateral marker (Mills et al., 1979) (Na^+,K^+)-ATPase and 4% of the endoplasmic reticulum marker NADH oxidase. The distribution of Na^+-stimulated glucose transport activity, measured by using the analogue [^{14}C]αMeGlc, also paralleled that observed for apical marker enzymes (Lever, 1982).

Na^+ Increases the Affinity of Phlorizin Binding. The apparent binding constant of Na^+-dependent phlorizin binding, K_d^{app} was decreased as a function of increasing Na^+ concentration with no effect on the apparent number of binding sites. From least-squares fits to Lineweaver–Burk plots (not shown) of the Na^+-dependent component of phlorizin binding to 0.2-mg aliquots of vesicles at phlorizin concentrations over the range 2.5–50 nM, values of K_d^{app} were calculated as 161 nM at 10 mM NaCl/90 mM choline chloride and 33 nM at 100 mM NaCl. The number of Na^+-dependent phlorizin binding sites was estimated as 0.38 pmol/mg at 10 mM Na^+ and 0.67 pmol/mg at 100 mM Na^+. Na^+-free binding observed in parallel incubations containing 100 mM choline chloride was subtracted to obtain the Na^+-dependent binding component. Qualitatively similar effects of Na^+ on phlorizin binding in brush border vesicles have been noted previously (Frasch et al., 1970; Turner & Silverman, 1981). Optimal stimulation of binding required Na^+ and was observed after a 5-min incubation. Na^+ could not be replaced by K^+, Li^+, Rb^+, Tris, or choline (not shown). Over 90% of the phlorizin binding observed in the presence of Na^+ was prevented if 1 mM unlabeled phlorizin was added to the incubation mixture (not shown). The same Na^+-dependent phlorizin binding component was observed in the presence of either Cl^-, NO_3^-, or SCN^- although chloride stimulated a higher total phlorizin binding.

Na^+-Stimulated Phlorizin Binding Is Inhibited by Sugar Substrates of Na^+ Cotransport. Na^+-dependent phlorizin binding to vesicles was inhibited by sugars such as glucose and galactose which serve as substrates of Na^+-dependent sugar transport in kidney proximal tubule (Silverman, 1976) and compete with labeled αMeGlc for Na^+-stimulated uptake into similar apical vesicles from LLC-PK_1 cells (Lever, 1982). Equilibrium Na^+-dependent phlorizin binding to vesicles was measured at 70 nM [^{3}H]phlorizin in the presence of 100 mM NaSCN as a function of glucose concentrations in the range 1–50 mM. The data were analyzed by using a Hill plot (not shown) after subtraction of the Na^+-independent component obtained in the presence of 100 mM KSCN. A straight line with a slope of 0.91 was obtained, indicating a 1:1 stoichiometry between phlorizin and glucose for interaction with the transporter. From the *x* intercept, a value of $K_{0.5}$ for glucose of 3.8 mM in the presence of 100 mM Na^+ was calculated. This value agrees well with the K_m value of 10 mM reported for αMeGlc transport in LLC-PK_1 vesicles (Lever, 1982) but is higher than the K_m of 0.27 mM reported for glucose transepithelial transport in LLC-PK_1 cells (Misfeldt & Sanders, 1981). Binding of 0.24 μM phlorizin was also inhibited by 50 mM galactose but was not significantly reduced after addition of 50 mM fructose or mannose, which do not serve as substrates of this system (not shown). Addition of glucose or excess unlabeled phlorizin to the suspension medium accelerated the rate of release of bound [^{3}H]phlorizin (not shown).

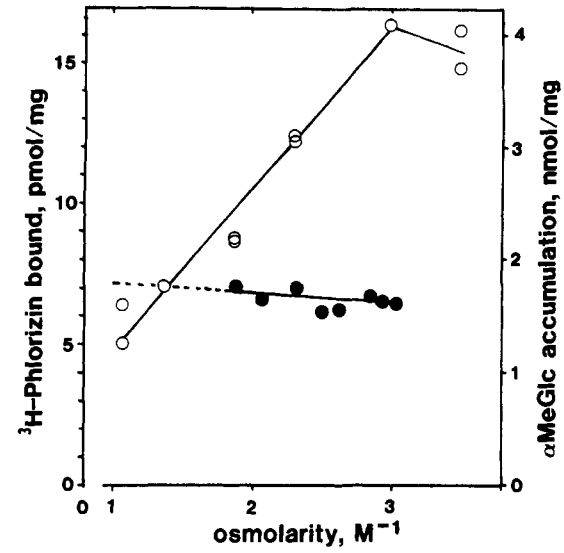

FIGURE 1: Effect of osmolarity on phlorizin binding. Vesicles were incubated 5 min in standard mixtures containing 70 nM [^{3}H]phlorizin and 100 mM NaCl plus sufficient mannitol to achieve the indicated osmolarity (●). Accumulation of 5 mM [^{14}C]αMeGlc in similar mixtures containing 100 mM NaSCN was assayed after a 15-min incubation (○).

Unlabeled phloretin had no significant effect on binding or release of phlorizin.

The inhibition of Na^+-stimulated αMeGlc transport in vesicles at 50 mM NaCl as a function of phlorizin concentration was analyzed by a Hill plot (not shown). Na^+-free uptake, estimated in the presence of 50 mM choline chloride, was subtracted from total uptake. From the slope of 0.99, a 1:1 stoichiometry between αMeGlc and phlorizin for interaction with the transporter was estimated, in agreement with the estimate based on glucose inhibition of [^{3}H]phlorizin binding. An apparent $K_{0.5}$ for phlorizin of 1.9 μM was estimated from the *x* intercept. It should be noted that αMeGlc transport and phlorizin binding were assayed in vesicles under the same temperature and ionic conditions. From the previously reported maximal velocity of αMeGlc transport in this vesicle preparation (Lever, 1982) of 5 nmol min^{-1} mg^{-1} and the value of 0.67 pmol of phlorizin bound at saturation per mg of protein observed at 100 mM Na^+ as an estimate of the concentration of the transporter, a turnover number of 7500 can be calculated for the glucose transporter.

Phlorizin Is Not Transported into Vesicles. If phlorizin is to serve as a valid probe for the binding step in sugar/Na^+ cotransport, it is important to establish that it is not transported. Figure 1 shows that equilibrium phlorizin binding (shown as closed circles) is not affected by vesicle size changes caused by changes in osmolarity of the vesicle suspension medium. In comparison, αMeGlc accumulation (shown as open circles) was markedly sensitive to changes in osmolarity over the same range, as expected for a solute which has been transported into an osmotically sensitive intravesicular space. This experiment suggests that phlorizin is bound to fixed sites and is not contained in solution within the intravesicular space. Estimates based on an intravesicular volume of 1.75 μL/mg for these vesicle preparations (Lever, 1982) for the amount of [^{3}H]phlorizin which could potentially be retained in an intravesicular space rather than bound give a value of 0.12 pmol/mg. This cannot account for the significantly higher values observed in the experiment shown in Figure 1.

Na^+ Stoichiometry of Phlorizin Binding. Figure 2 shows the relationship between Na^+-dependent phlorizin binding and external Na^+ concentration. These data were analyzed by

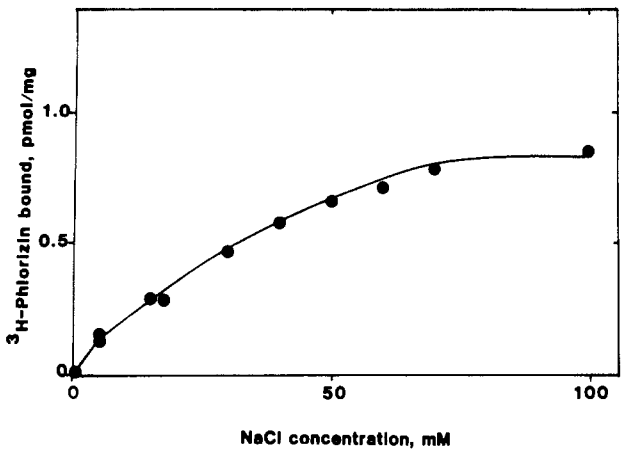

FIGURE 2: Na^+ dependence of phlorizin binding. Vesicles (0.2 mg) were incubated 5 min in the standard mixture containing 0.24 μM [^{3}H]phlorizin and the indicated concentration of NaCl. Total chloride concentration was maintained constant by the addition of choline chloride. Data averaged from duplicates with an SE of ±10% are shown after subtraction of binding observed in the absence of Na^+.

Table II: Effect of Membrane Potential on Phlorizin Binding[a]

K^+_o (mM)	K^+_i (mM)	phlorizin binding (pmol/mg)	
		Na^+ dependent	Na^+ independent
5	50	2.11 ± 0.33	2.02 ± 0.20
50	5	0.93 ± 0.14	2.49 ± 0.19
50	50	1.34 ± 0.25	2.60 ± 0.37

[a] Vesicles (99 μg/aliquot) were preincubated with KCl and/or choline chloride and 100 μM valinomycin for 15 min to achieve the indicated internal K^+ concentration (K^+_i) with the total chloride concentration maintained constant at 50 mM. Then aliquots were diluted 10-fold into standard incubation mixtures containing 0.24 μM [^{3}H]-phlorizin and either 50 mM NaCl or 50 mM choline chloride, and binding was terminated after 30 s. Na^+-dependent binding was obtained by subtraction of Na^+-independent binding from total binding observed in the presence of Na^+. Values are means ± SE from triplicate determinations.

using a Hill plot (not shown), after subtraction of the Na^+-independent component. From the slope of 1.04, a value of 1:1 was estimated for the stoichiometry of phlorizin and Na^+ interaction with the glucose transporter. The apparent $K_{0.5}$ for Na^+ estimated from the x intercept was 46 mM. By contrast, a stoichiometry of 2:1 Na^+/glucose was estimated for Na^+-stimulated glucose transport in similar vesicle preparations (Lever, 1982).

Na^+-Stimulated Phlorizin Binding Is Stimulated by Interior-Negative Membrane Potentials. Table II shows the effect of electrical membrane potential on the rate of Na^+-dependent phlorizin binding. In this experiment, membrane potentials were artificially generated by means of K^+ diffusion potentials in the presence of valinomycin. Creation of an interior-negative membrane potential by means of a K^+ diffusion potential ($K^+_i > K^+_o$) resulted in a significant stimulation of the rate of phlorizin binding compared to control samples in which electrical potential was abolished by making internal and external K^+ concentrations equal ($K^+_i = K^+_o$). The expected generation of membrane potentials under the conditions of this experiment was confirmed in parallel incubations (not shown) in which 20 μM [^{3}H]TPMP$^+$ ion replaced [^{3}H]phlorizin. The distribution of this lipophilic cation has been previously shown to monitor interior-negative membrane potentials generated by these experimental manipulations (Lever, 1977). By contrast, the rate of Na^+-dependent phlorizin dissociation was not affected by membrane potential (not shown). In the absence of Na^+, phlorizin binding was not appreciably affected by

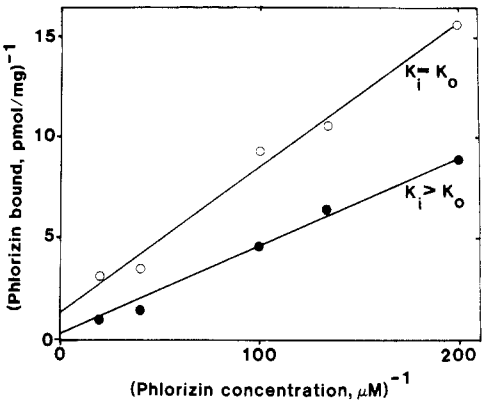

FIGURE 3: Effect of interior-negative membrane potential on kinetics of Na^+-dependent phlorizin binding. Aliquots (0.13 mg) of vesicles were preincubated 15 min with 50 mM KCl, 50 mM choline chloride, and 100 μg/mL valinomycin and then diluted 10-fold into standard incubation mixtures containing 50 mM NaCl, the indicated concentration of [^{3}H]phlorizin, and either 50 mM choline chloride (●) or 50 mM KCl (○). Binding was terminated by filtration after 30 s. Data averaged from duplicate determinations with an SE of ±15% are shown analyzed by a Lineweaver–Burk plot. From the least-squares lines, under conditions of interior-negative membrane potential ($K^+_i > K^+_o$), maximal phlorizin binding of 5.0 pmol/mg and a K_d^{app} of 1.66 μM can be calculated. At zero membrane potential ($K^+_i = K^+_o$) maximal phlorizin binding of 0.76 pmol/mg and a K_d^{app} of 0.06 μM were observed.

membrane potential (Table II).

Effects of interior-negative membrane potential on the kinetics of Na^+-dependent phlorizin binding were manifest as an apparent increase in the number of phlorizin binding sites (Figure 3). In the presence of an interior-negative membrane potential ($K^+_i > K^+_o$), 5.0 pmol of phlorizin bound/mg of protein was observed at saturation. When the membrane potential was abolished ($K^+_i = K^+_o$), 0.76 pmol of phlorizin bound/mg of protein was observed. An increase in the K_d^{app} of phlorizin was associated with interior-negative membrane potentials and thus cannot explain increased phlorizin binding induced by charge effects. These data suggest that interior-negative membrane potentials result in the exposure of an increased number of phlorizin binding sites on the vesicle exterior.

An alternate explanation of these results is that the primary effect of electrical asymmetry is on the Na^+ microenvironment in the membrane. In this view, phlorizin binding enhancement driven by an electrical gradient would be due to increased accessibility of Na^+ to glucose transporter sites within the membrane. This model predicts that membrane potential effects on phlorizin binding should not be observed under conditions where Na^+ is fully equilibrated across the membrane. Results shown in Table III indicate that this explanation is unlikely. Equilibration of Na^+ across the membrane, either by preincubation of vesicles with NaCl or by addition of monensin, did not prevent stimulation of phlorizin binding by interior-negative membrane potentials. Monensin is an electrochemical Na^+/H^+-exchanging ionophore which dissipates a Na^+ gradient but does not affect the membrane potential (Pressman, 1976). Under conditions of zero membrane potential, conditions expected to elevate internal Na^+ concentration inhibited phlorizin binding by 50% (Table III) as similarly noted by Toggenburger et al. (1982). We interpret this as due to increased inward orientation of the phlorizin binding site trapped as the electroneutral Na^+/transporter complex in the presence of intravesicular Na^+.

pH Dependence of Phlorizin Binding. Figure 4 shows that the pH dependence of Na^+-stimulated [^{3}H]phlorizin binding

Table III: Na^+ Gradient Dissipation Does Not Prevent Stimulation of Phlorizin Binding by Interior-Negative Membrane Potentials

condition	Na^+-dependent phlorizin binding (pmol/mg)	
	$K^+_i > K^+_o$	$K^+_i = K^+_o$
control ($[Na^+_o] > [Na^+_i]$)	1.49 ± 0.10	0.80 ± 0.17
Na^+ preincubation	1.54 ± 0.10	0.47 ± 0.10
+monensin (100 μg/mL)	1.15 ± 0.17	0.37 ± 0.10

[a] Vesicles (0.18 mg/aliquot) were incubated 15 min with 100 μg/mL valinomycin and 50 mM KCl and then diluted 10-fold into standard incubation mixtures containing 0.24 μM [^{3}H]phlorizin, 50 mM NaCl, and either 50 mM choline chloride ($K^+_i > K^+_o$ or 50 mM KCl ($K^+_i = K^+_o$). Binding was terminated at 30 s after dilution. Na^+-dependent binding was calculated after subtraction of Na^+-free binding observed when 50 mM choline chloride was substituted for NaCl. 50 mM NaCl was added during the 15-min preincubation step in the case of Na^+ preincubation samples. Values are means ± SE from triplicate determinations.

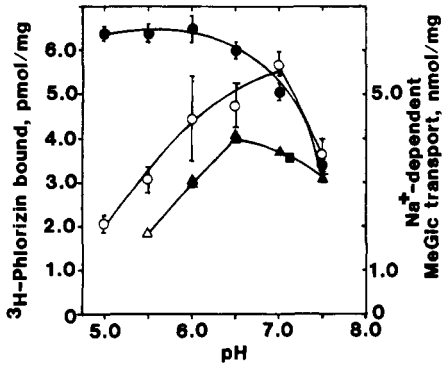

FIGURE 4: Effect of external pH on Na^+-dependent phlorizin binding and αMeGlc transport. [^{3}H]Phlorizin binding was assayed by incubation of 0.24 mg of vesicles for 5 min in the standard incubation mixture containing 0.24 μM [^{3}H]phlorizin, 100 mM either of NaCl or of choline chloride, and 10 mM MES (used in the pH range 5.0–6.5), BES, pH 7.0, or HEPES, pH 7.5. (○) Na^+-dependent phlorizin binding, after subtraction of Na^+-free binding. (●) Na^+-free phlorizin binding. Transport of αMeGlc was assayed by incubating 0.5 mg of vesicles for 5 min in the standard incubation mixture containing 5 mM [^{14}C]αMeGlc, 100 mM NaSCN, and 10 mM either of MES (△), of BES (▲), or of HEPES (■) at the indicated pH.

paralleled that observed for Na^+-stimulated [^{14}C]αMeGlc transport in vesicles but differed by 0.5 pH unit in the pH optimum. By contrast, Na^+-independent phlorizin binding (shown by closed circles) differed in pH dependence from the Na^+-dependent component.

Discussion

In this paper, phlorizin binding properties of apical membrane vesicles isolated from the LLC-PK_1 kidney epithelial cell line are presented. It is assumed that specific phlorizin binding is a probe for the binding step in glucose transport, on the basis of evidence that phlorizin is not itself transported into vesicles. Also, specific phlorizin binding shows Na^+ dependence, competitive interactions among sugars, and a pH profile similar to those described for substrates of this system.

The properties of the Na^+/glucose symporter expressed in this long-term, differentiated cell line are of particular interest since this transport system exhibits a Na^+/glucose stoichiometry of 2:1 whether measured in vesicles (Lever, 1982) or intact cells (Sanders & Misfeldt, 1981). A stoichiometry of 2:1 has also been reported for Na^+/glucose symport in isolated chick intestinal cells (Kimmich & Randles, 1980), rabbit intestinal brush border vesicles (Kaunitz et al., 1982), and renal outer medulla (Turner & Moran, 1982b) and for Na^+/glycine symport in pigeon red cells (Vidaver, 1964) and hepatocytes

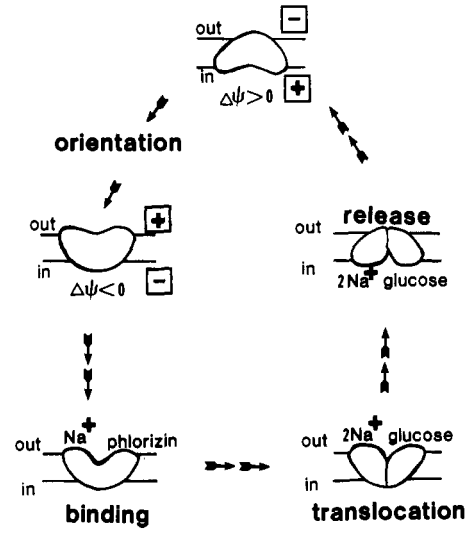

FIGURE 5: Model to explain Na^+/sugar symport and phlorizin binding to the hexose transporter in the apical membrane of LLC-PK_1 cells. The depiction of transporter forms is purely schematic and not meant to imply structural features, specific conformations, or rotation in the membrane.

(Christensen & Handlogten, 1981) as well as succinate (Wright et al., 1982) and phenylalanine (Evers et al., 1976) uptake in renal brush border vesicles.

The rate of phlorizin binding is stimulated by Na^+, arguing against an ordered mechanism in which phlorizin (or sugar) obligatorily binds to the symporter first, followed by the binding of Na^+. Na^+ increases the affinity of the carrier for phlorizin but does not affect the maximal number of binding sites. Hill plot analysis of the Na^+ dependence of [^{3}H]phlorizin binding in vesicles yielded a 1:1 stoichiometry for the binding step. A 1:1 Na^+/phlorizin stoichiometry has been reported for binding to intact LLC-PK_1 cell monolayers attached to plastic dishes (Misfeldt & Saunders, 1982); however, the use of intact cells did not permit the estimation of Na^+-independent binding or the contribution of membrane potential.

Our results provide the first demonstration that Na^+-dependent phlorizin binding to a symporter exhibiting a 2:1 Na^+/glucose stoichiometry is stimulated by interior-negative potentials. This property is also characteristic of Na^+/D-glucose symporters exhibiting 1:1 stoichiometry (Aronson, 1978; Toggenburger et al., 1978). Given that phlorizin itself binds as its uncharged form and is not translocated, and that phlorizin binding does not result in Na^+ translocation (Schultz & Zalusky, 1964), this result implies that electrical asymmetry across the membrane affects the orientation of the symporter (Figure 5). Furthermore, this observation is consistent with the hypothesis that the uncomplexed glucose transporter bears a net single negative charge. The substrate binding site of the unloaded transporter is negatively charged and thus would undergo a conformational change to preferentially face inward under the attractive force of interior-positive membrane potentials and would face outward when repelled by interior-negative membrane potentials. This transition is unlikely to involve carrier rotation or large conformational change due to thermodynamic constraints but need only involve alternate exposure of binding sites accessible from the interior or exterior. Phlorizin (or the sugar substrate) and Na^+ bind to the outward-facing substrate site in a 1:1 stoichiometry, converting the symporter to a form with no net charge. Binding of a second Na^+ in the case of a complex with a sugar substrate converts the complex to a productively translocating complex with a single positive charge. The sugar and two Na^+ ions move inward and are released to the interior. The total driving

force available for sugar cotransport would consist of a factor proportional to the square of the chemical Na^+ gradient plus an electrical contribution. The latter contribution due to membrane potential would consist of an energetic contribution due to movement outward of the negatively charged substrate binding site of the unloaded carrier plus a contribution due to movement inward of the positively charged loaded carrier during productive substrate translocation.

An alternate explanation, that the membrane potential acts by facilitating the access of Na^+ to transporter sites in channels within the membrane rather than by affecting its orientation, is unlikely, since conditions designed to fully equilibrate Na^+ across the membrane did not prevent stimulation of phlorizin binding by an interior-negative membrane potential. Results of kinetic analysis of Na^+-coupled glucose fluxes in intestinal brush border vesicles (Kessler & Semenza, 1983) were compatible with a negatively charged transporter.

As predicted by this model, Na^+-stimulated αMeGlc transport in these vesicle preparations is electrogenic, stimulated by interior-negative membrane potentials (Lever, 1982). Also, the dissociation of bound phlorizin is not affected by membrane potential, as predicted if the phlorizin/Na^+ carrier complex is uncharged.

Phlorizin, but not glucose, strongly inhibits efflux of glucose via this cotransporter (Rabito & Ausiello, 1980). This observation can be explained within the context of this model since external Na^+ and phlorizin would lock the symporter in an uncharged, nontranslocating form which could not bind Na^+ and glucose on the inner side of the membrane.

We have not specifically addressed the question of the order of binding of Na^+ and phlorizin. Earlier kinetic models (Schultz & Curran, 1970; Stein, 1967; Turner, 1981) based on 1:1 Na^+/glucose symport must now be reformulated to describe 2:1 Na^+/glucose symport. A random Bi-Bi mechanism for Na^+/glucose cotransport has been proposed for the intestinal transport system (Lyon & Crane, 1966) and the renal cortical transporter (Turner & Silverman, 1981) in contrast with the iso-ordered Bi-Bi kinetic model proposed by Hopfer & Groseclose (1980).

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Registry No. αMeGlc, 97-30-3; Na, 7440-23-5; D-glucose, 50-99-7; phlorizin, 60-81-1.

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