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Transepithelial Transport in Cell Culture: Stoichiometry of Na/Phlorizin Binding and Na/D-Glucose Cotransport. A Two-Step, Two-Sodium Model of Binding and Translocation

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Summary. The renal cell line LLC-PK_1 cultured on a membrane filter forms a functional epithelial tissue. This homogeneous cell population exhibits rheogenic Na-dependent D-glucose coupled transport. The short-circuit current (I_{sc}) was acccounted for by net apical-to-basolateral D-glucose coupled Na flux, which was $0.53 \pm 0.09(8)$ μeq $cm^{-2}hr^{-1}$, and I_{sc}, $0.50 \pm 0.50(8)$ μeq $cm^{-2}hr^{-1}$. A linear plot of concurrent net Na vs. net D-glucose apical-to-basolateral fluxes gave a regression coefficient of 2.08. As support for a 2:1 transepithelial stoichiometry, sodium was added in the presence of D-glucose and the response of I_{sc} analyzed by a Hill plot. A slope of $2.08 \pm 0.06(5)$ was obtained confirming a requirement of 2 Na for 1 D-glucose coupled transport. A Hill plot of I_{sc} increase to added D-glucose in the presence of Na gave a slope of $1.02 \pm 0.02(5)$. A direct determination of the initial rates of Na and D-glucose translocation across the apical membrane using phlorizin, a nontransported glycoside competitive inhibitor to identify the specific coupled uptake, gave a stoichiometry of 2.2. A coupling ratio of 2 for Na, D-glucose uptake, doubles the potential energy available for Na-gradient coupled D-glucose transport. In contrast to coupled uptake, the stoichiometry for Na-dependent-phlorizin binding was $1.1 \pm 0.1(8)$ from Hill plot analyses of Na-dependent-phlorizin binding as a function of [Na]. Although occurring at the same site the process of Na-dependent binding of phlorizin differs from the binding and translocation of D-glucose. Our results support a two-step, two-sodium model for Na-dependent D-glucose cotransport; the initial binding to the cotransporter requires a single Na and D-glucose, a second Na then binds to the ternary complex resulting in translocation.

Key Words kidney cell line · LLC-PK_1 · stoichiometry · phlorizin binding · coupled uptake · Hill plot · electrophysiology · I_{sc} · isotope flux · sodium gradient

Introduction

The retention of phenotypic qualities of transporting epithelia by primary cultures and established cell lines provide a simplified epithelial tissue when cultured on a membrane filter (Misfeldt, Hamamoto & Pitelka, 1976; Cereijido et al., 1978; Bisbee, Machen & Bern, 1979; Handler et al., 1979). We have reported for the renal cell line LLC-PK_1 rheogenic Na-dependent D-glucose cotransport, with short-circuit current (I_{sc}) due to D-glucose coupled net apical-to-basolateral Na flux (Misfeldt & Sanders 1981*a*). Prior reports (Mullin, Weibel, Diamond & Kleinzellar, 1980; Rabito & Ausiello, 1980) have identified Na-dependent α-methyl-D-glucose uptake by LLC-PK_1. This study examines the stoichiometry of Na phlorizin binding, Na/D-glucose uptake and transepithelial transport. The stoichiometry of coupled Na^+-dependent D-glucose transport is fundamental to the molecular mechanism of transport and to the thermodynamic adequacy of the Na^+ electrochemical gradient model for coupled uptake (Crane, 1977). A preliminary report of a portion of our data has been presented (Misfeldt & Sanders, 1981*b*).

Materials and Methods

Cell Filter Preparations

Pig kidney cell line LLC-PK_1 was obtained from the American Type Culture Collection (Rockville, Maryland) and for transepithelial measurements grown on collagen-coated Millipore filters (0.45 μm, type HAMK, 24 mm). Filters were prepared as previously described (Misfeldt & Sanders, 1981*a*) and LLC-PK_1 cells were plated onto culture dishes or filters and grown in F12:Minimal Essential Medium (GIBCO) (1:1) supplemented with 10% porcine serum, 10 μg ml^{-1} insulin. Cultures were kept in a 37 °C incubator, gassed with 5% CO_2 and 95% air and were ready for study in about a week.

Solutions

Hanks' salt solution (HSS), pH 7.2, used in most of the experiments had the following composition (mM): NaCl 140.0, $NaHCO_3$ 4.2, $MgCl_2$ 0.5, Na_2HPO_4 0.36, HEPES 10.0, KCL 5.4, $CaCl_2$ 1.3, KH_2PO_4 0.44, glucose 5.5. Na-free HSS was made with Tris. Adjustments in pH were made with NaOH or HCl with respective K salts replacing those of Na as necessary.

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Chemicals

Phlorizin was obtained from Sigma Chemical Company, radioisotopes from New England Nuclear. ^{3}H-phlorizin was a gift from Professor Robert K. Crane. Prior to use the purity was confirmed by thin-layer chromatography using chloroform/methanol/water (65:25:4, vol/vol). A single spot was identified which contained the amount of radioactivity placed at the origin with the same R_f as marker phlorizin.

All electrical and transepithelial flux measurements were carried out at 37 °C in an Ussing chamber as previously described (Misfeldt & Sanders 1981*a*). The filter preparation was carefully removed from the culture dish and placed between halves of the chamber sandwiched between two silicone washers. The exposed filter area was 2.54 cm^2. Each chamber half contained a well to accommodate a small magnetic stir bar; the fluid volume was 8 ml per side. Platinum-irridium wire was used to pass current through the chamber and potential difference measurements were made with a pair of Ag-AgCl electrodes contained in a microelectrode holder (W.P. Instruments). A 5-cm length of polyethylene tubing (PE50) filled with 3 M KCl in 3% agar was placed within 2 mm of the filter surface. Both current passing and potential measuring bridges were input to an automatic voltage clamp (D. Lee Instruments, Sunnyvale, Calif.). The output from the clamp was fed into a two-channel Varian recorder (G2000). In the voltage-clamp mode, the tissue is continuously short-circuited except for a 3-sec period. The Ussing chamber was immersed in a 37 °C water bath placed atop a magnetic stirrer.

Transepithelial Sugar and Sodium Fluxes

Simultaneous unidirectional Na and D-glucose fluxes were measured. Following a 3-min period for isotope equilibration, 50-µl bath samples were removed at 0, 15, 30 and 45 min. The samples were added to 8 ml of Biofluor (NEN) and counted on a Beckman LS100 liquid scintillation counter. The apical-to-basolateral flux was determined first, and after wash-out of the high-specific-activity bath, the basolateral-to-apical flux was determined with net flux being the difference between the two. The sequence was used to lessen rather than augment net apical-to-basolateral fluxes if there were deterioration of transport or increased permeability over time. Fluxes were calculated for three successive 15-min periods in both directions. To obtain a range of net fluxes, determinations were made at [D-glucose] 5.5 and 0.3 mM. Prior studies have determined that the radioactivity measured following epithelial transport is D-glucose (Misfeldt & Sanders 1981*a*).

Apical Membrane Uptake of Na and Glucose

Cells were grown to confluence (6–7 days) in 35 mm tissue culture dishes (Corning). Since cells form a polarized sheet only the apical membrane surface is exposed to the bathing solution. Cells were preincubated in a glucose-free solution of Tris-Hanks for 60 min. This solution was removed and replaced with Hanks containing 30 mM Na, 110 mM Tris and 2.5 mM glucose plus ^{22}Na and ^{3}H-D-glucose. At the end of the uptake period the isotope solution was aspirated and the dish vigorously washed with ice-cold phosphate-buffered saline from a squeeze bottle. The washing procedure dislodged all the cells in 1–3 sec and washed them over an 8 µm Millipore (SCWP) filter maintained under partial vacuum. An additional 15 ml of wash solution was poured over the filter. The filter was air dried, placed in a liquid scintillation vial containing 8 ml Bioflour (NEN), and radioactivity detected in a dual-channel scintillation counter, Beckman LS100. To determine uptake by coupled Na, D-glucose cotransport, 10^{-4} M phlorizin was added to the above solution with uptake due to the cotransport-specific pathway as being the difference between experiments with and without phlorizin present. From separate experiments the duration of initial linear uptake based on cellular protein was determined by the Biorad assay (Bradford, 1976). All experimental points were done in duplicate. For LLC-PK$_1$ under our culture conditions at steady-state cell density, cell protein per cm^2 is $0.05 \pm 0.012(24)$ mg protein.

Na-Dependent Phlorizin Binding

The procedure for assaying phlorizin binding was similar to that used for cellular uptake studies. Cells grown in 35 mm dishes were preincubated in glucose-free Tris-Hanks for 60 min. ^{3}H-phlorizin was added to solutions containing 0, 3.5, 7, 14, 28, 56, 100 and 140 mM Na. After a 60-sec incubation, cells were washed free of the dish with ice-cold Hanks with the same Na concentration used in the incubation. For the zero [Na] solution Tris-Hanks was used as a control for nonspecific, Na-independent phlorizin binding to the filter and cells. Each point was performed in triplicate.

Results

I_{sc} Equals J^{Net}_{Na}, Apical-to-Basolateral

Na fluxes were measured in the apical-to-basolateral and basolateral-to-apical direction under short-circuited conditions. For 3-sec periods each minute, the electrical potential and resistance were monitored under open-circuit conditions; the I_{sc}, $0.50 \pm 0.05(8)$, and net apical-to-basolateral Na flux, $0.53 \pm 0.09(8)$, were not significantly different ($\bar{x} \pm$ SEM, $p > 0.05$ Student's t test) expressed as µeq cm^{-2}hr^{-1}.

Transepithelial Net Na and D-glucose Ratio was 2:1

Bidirectional transepithelial fluxes for ^{22}Na and ^{3}H-D-glucose allowed determination of net apical-to-basolateral flux (Table 1). When the net Na vs. net D-glucose were plotted (Fig. 1), a resultant relationship (slope) of 2.08 was obtained and linear $p < 0.01$. The change in flux over three successive 15-min periods did not on average ($n = 7$) exceed 20% of the maximal flux for Na or D-glucose. We have previously reported that in the presence of phlorizin (2.5×10^{-5} M), the net ^{3}H-D-glucose apical-to-basolateral flux, and I_{sc} were abolished (Misfeldt & Sanders 1981*a*).

I_{sc} as a Function of [Na] was 2:1; as a Function of [D-glucose] was 1:1

The relation of [Na] to I_{sc} was studied by increasing the [Na] in the presence of 20 mM D-glucose,

Table 1. Transepithelial unidirectional fluxes

D-glucose in bath (mM)	D-glucose (μmoles $cm^{-2}hr^{-1}$			Sodium (μmoles $cm^{-2}hr^{-1}$)			$J^{Na}_{net}/J^{D\text{-}glc}_{net}$
	$J_{ap\to bl}$	$J_{bl\to ap}$	J_{net}	$J_{ap\to bl}$	$J_{bl\to ap}$	J_{net}	
5.5 ($n=6$)	0.364±0.035[a]	0.102±0.013	0.262±0.03	1.44±0.16	0.85±0.08	0.58±0.08	2.2
0.3 ($n=3$)	0.189±0.040	0.015±0.004	0.174±0.04	1.24±0.18	0.81±0.08	0.41±0.13	2.3

[a] $\bar{X}\pm$SD

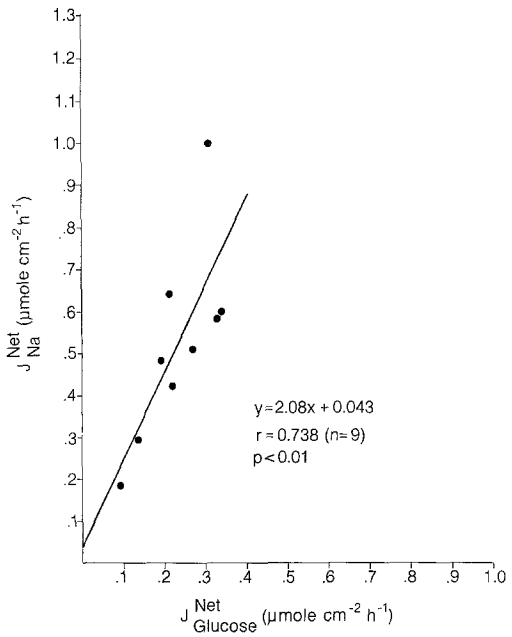

Fig. 1. Relationship of simultaneous net ^{22}Na and ^{3}H-D-glucose fluxes across LLC-PK_1 pig kidney epithelial cells. The slope was obtained by least-squares analysis of each pair of flux determinations. Details of the experimental methods are in the text

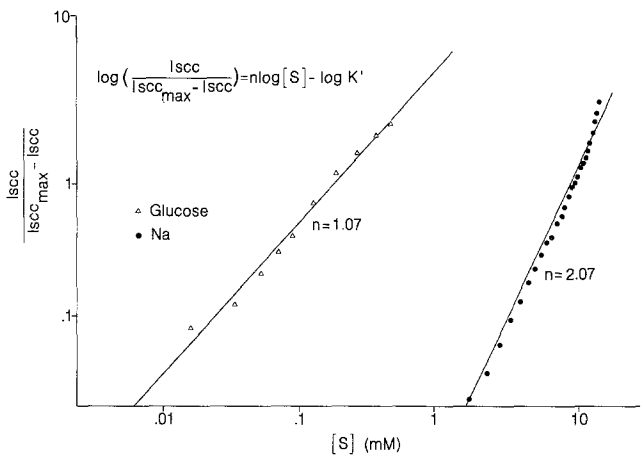

Fig. 2. Representative experiments: Hill plots of I_{sc} varying [D-glucose] with constant [Na] 140 mM, and varying [Na] with constant [D-glucose] 20 mM. The slope was calculated from least-squares analysis of a log-log plot. The slope indicates the minimum number of molecules translocated for each unit of I_{sc}

in excess of the apparent $K_{1/2}$ for D-glucose of 1.3 mM at 20 mM Na. Similarly, the relation for [D-glucose] to I_{sc} was analyzed. The $K_{1/2}$ for D-glucose was 0.27±0.02(6) mM and for Na was 14.2±0.88(5) mM. A typical experiment is plotted in Fig. 2. The Hill plot allows an estimate of the minimum number of ligands interacting with the receptor (Segal, 1975). For the I_{sc} response to added D-glucose with [Na] held constant (140 mM), the slope calculated by linear regression was 1.02±0.02(5), correlation coefficients > 0.99, and the I_{sc}-max was 16.4±0.7(5)μA cm^{-2}. The I_{sc} response to added Na, [D-glucose] held constant was 2.08±0.06(5), $r=0.90$–0.98, and the I_{sc}-max was 15.9±1.1(5) μA cm^{-2}. The maximum I_{sc} responses were determined at substrate concentrations taken twofold greater than the I_{sc} plateau to insure an accurate maximum value.

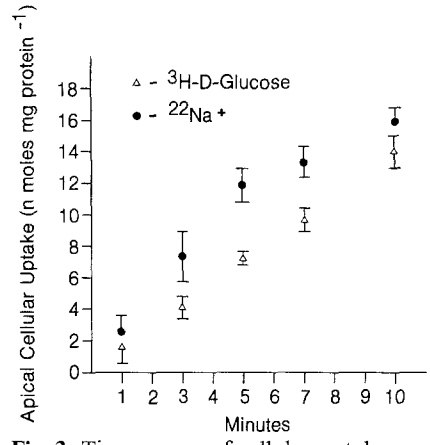

Fig. 3. Time course of cellular uptake across the apical membrane for ^{22}Na and ^{3}H-D-glucose. This was linear over the first 5 min and represents all routes of uptake including the coupled, phlorizin-sensitive cotransport

Phlorizin-Inhibited Na and D-glucose Cellular Uptake across the Apical Membrane was 2:1

The time course of apical membrane uptake was determined simultaneously for ^{22}Na and ^{3}H-D-glucose to establish the duration of linear uptake

Table 2. Coupled cellular uptake of ^{22}Na and ^{3}H-D-glucose across the apical membrane

	A. Control[a]	*B*. Control with phlorizin (10^{-4} M)	$(A-B)$	Δ Na/Δ D-glucose
	(nmoles min^{-1} mg protein^{-1})			
^{3}H-D-glucose	2.53±0.24(8)[b]	1.90±0.14(8)	0.63±0.28	2.2±0.57[c]
^{22}Na	4.86±0.25(8)	3.44±0.28(8)	1.42±0.38	

[a] Medium composition: 30 mM Na, 110 mM Tris, 2.5 mM D-glucose.
[b] $\bar{X}\pm$SEM (n).
[c] $\bar{X}\pm$SD calculated by the "jacknife" method (Mostosteller & Tukey, 1977).

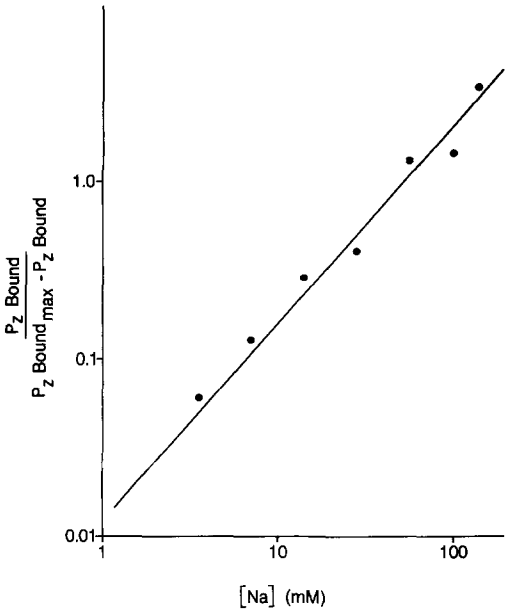

Fig. 4. Hill plot analysis of Na-dependent ^{3}H-phlorizin binding as a function of [Na]. The slope of 1 indicates minimum sodium binding for each bound phlorizin. The analysis of this experiment is representative of 8 separate experiments. *See* Results for further details

(Fig. 3). For ^{22}Na the uptake was linear for the first 5 min; uptake of ^{3}H-D-glucose was linear over the first 10 min. Because of low cellular concentration of Na, the specific activity of ^{22}Na was increased by lowering the [Na] in the external bath to 30 mM. Simultaneous uptake of Na and D-glucose was measured at 2 min with and without phlorizin (10^{-4} M). The uptake which was inhibited by phlorizin is presented in Table 2 and the ratio of phlorizin-inhibited Na /D-glucose was 2.2.

The significance of the unstirred layer was calculated for D-glucose and Na:

$$\frac{1}{P_{obs}}=\frac{1}{P}+\frac{\delta}{D}.$$

P_{obs} was the uptake in moles cm^{-2} sec^{-1} across the apical membrane; D, the diffusion constant at 25 °C for 0.02 M D-glucose and 0.1 M Na; δ, the thickness of the unstirred layer, and P, the calculated permeability. The thickness of the unstirred layer was determined by the time to half-maximal ($T_{1/2}$ I_{sc} response, about 1 sec, when D-glucose was added to a stirred apical bath from the following relationship:

$$T_{1/2}=\frac{(0.38)\,(\delta)^2}{D}.$$

For Na and D-glucose the difference in P was less than 1% when calculated with and without an estimated 50 μm unstirred layer. This negligible effect was disregarded.

Na-Dependent ^{3}H-Phlorizin Binding as a Function of [Na] was 1:1

The amount of ^{3}H-phlorizin binding increased with the [Na]. A Hill plot analysis of the relationship (Fig. 4) provided from the slope an estimate of the minimum number of sodium ions for each phlorizin molecule bound. As calculated by linear regression the slope is 1.1±0.1(8), correlation coefficients = 0.93–0.99. The maximum phlorizin binding was 1.24±0.18(8) pmoles mg protein^{-1} at 140 mM [Na].

Discussion

Net transcellular epithelial transport of Na and D-glucose is dependent on the function of a phlorizin-sensitive mechanism of apical uptake. Phlorizin, a competitive inhibitor of Na, D-glucose coupled uptake, blocks both apical-to-basolateral net isotopic D-glucose flux (Misfeldt & Sanders, 1981*a*) and I_{sc}, a presumed result of net Na flux. The 2:1 Na/D-glucose stoichiometry for both uptake and transepithelial transport implies that parallel paths of Na and D-glucose uptake and egress at either apical or basolateral membrane are unaffected by function of the coupled process.

Although I_{sc} is a composite of conjugate driving forces and resistance to transepithelial Na

transport, it is a sensitive indicator of interaction among Na, D-glucose and the apical membrane cotransport mechanism. The assumption that apical membrane interactions are the rate-limiting step for I_{sc} is supported by concordance between I_{sc} and the following observations:

1. The sugar analogs that stimulate I_{sc} are identical to those actively transported (Ullrich, Rumrich & Kloss, 1974) and associated with apical membrane depolarization (Frömter & Luer, 1973), and their hierarchy of effectiveness is the same.

2. From the I_{sc} response, our $K_{1/2}$ for D-glucose in 140 mM [Na] is 0.27 mM compared to a range of 0.2 to 1.0 mM for dog kidney brush border vesicles (Turner & Silverman, 1978*b*), rabbit proximal tubule brush border membrane (Fairclough, Malathi, Preiser & Crane, 1979), and in rat proximal tubule (Frömter & Luer, 1973). The $K_{1/2}$ for Na from the I_{sc} response is $14.2 \pm 0.88(5)$ mM and from 3-0-methylglucose uptake by rabbit ileum cells it is 18.2 mM (Goldner, Schultz & Curran, 1969).

3. The reduction of Na from 140 to 12.5 mM is associated with a fourfold increase in $K_{1/2}$ for D-glucose calculated from zero net flux transtubular concentration difference (Ullrich et al., 1974) compared to our fourfold increase when calculated from I_{sc} (Misfeldt & Sanders, 1981*a*).

4. The absence of Na or addition of phlorizin in the apical bath inhibits I_{sc} (Misfeldt & Sanders, 1981*a*) as well as apical vesicle uptake (Murer et al. 1979; Aronson & Sacktor, 1974).

Both Na and D-glucose interact with apical cotransporter to effect I_{sc}. As a function of [D-glucose] the D-glucose/Na-cotransporter interaction was a slope of one for the effect on I_{sc}, i.e., a single D-glucose for each cotransport translocation. In contrast for sodium, the slope of two indicates that at least two sodiums must interact with the cotransport site and D-glucose before there is translocation.

The confirmation that I_{sc} is an accurate but indirect indication of apical membrane, Na/D-glucose stoichiometry was determined directly by initial rates of isotopic uptake. The ratio of the phlorizin-inhibited uptake was 2.2:1, Na/D-glucose. Kimmich and Randles (1980) reported for ATP-depleted, valinomycin-treated chicken enterocytes that the coupled phlorizin-inhibited portion of Na:3-0-methylglucoside uptake also has a 2:1 stoichiometry. By this stoichiometry the electrochemical potential from the Na gradient can explain the 70-fold bath-to-cell concentration gradient for sugar they have measured. In two species of cestodes the coupling coefficients for Na/D-glucose cotransport is at least 2:1 (Fisher & Read, 1971; Read, Stewart & Pappas, 1974). A stoichiometry of at least 2:1 or greater, Na D-glucose, has also been measured for rabbit intestinal brush border vesicle uptake (Kaunitz, Gunther & Wright, 1982). The stoichiometry for other Na-dependent organic solute uptake of 2:1 is not unknown, having been described for Na-coupled glycine uptake by pigeon red cells (Vidaver, 1964), in renal brush border vesicles for succinate (Wright et al., 1982) and phenylalanine (Evers, Murer & Kinne, 1976) uptake.

A 1:1 stoichiometry, Na:3-0-methylglucoside, (3-MG), reported by Goldner et al. (1969) for the rabbit ileum did not estimate the stoichiometry of uptake for that portion which was phlorizin-inhibited. Although there was an 86% reduction in 3-MG uptake in presence of phlorizin, the inhibition of Na uptake by phlorizin was not reported. Despite that limitation, Na and 3-MG influxes were nearly 1:1 at three concentrations of Na ranging from 21–130 mM. Hopfer and Groseclose (1980) determined the effect of [Na] on the time for half-maximal uptake under equilibrium exchange conditions and reported a slope of 1 by log-log plot for [Na] from 75 to 1000 mM; and, for [Na] below 75, the slope was less than 1.

The apical uptake stoichiometry of 2:1, Na/D-glucose, doubles the Na electrochemical potential for coupled uptake as the gradient is a force on each Na^+. Thus, as a consequence of doubling the electrochemical potential, the concentration ratio possible for D-glucose is increased to the second power.

Our results describing a 1:1 ratio for Na/Na-dependent phlorizin binding and a 2:1 ratio for Na/Na-coupled D-glucose apical uptake, suggest different mechanisms of coupled Na interaction. The stoichiometry of the initial ternary complex of Na, phlorizin and cotransporter implies that phlorizin binding does not require a second Na for binding. As [Na] is increased the apparent binding constant for phlorizin is decreased, yet the number of binding sites is unchanged (Frasch et al., 1970; Glossman & Neville, 1972; Chesney, Sacktor & Kleinzeller, 1974; Turner & Silverman, 1981). The weak (0–5%) inhibitory potency of the phlorizin aglycone, phloretin, and Na independence (Diedrich, 1966; Vick, Diedrich & Baumann, 1973) indicates that phlorizin binding requires interaction between the glycoside of phlorizin and Na at the cotransporter site. The Na-dependent transport rates of D-glucose analogs follow the same hierarchy (Ullrich et al., 1974; Misfeldt & Sanders, 1981*a*) as the potency for D-glucose transport inhibition by phloretin-2′-β-D-glucose (Bode,

Baumann & Diedrich, 1972; Vick et al., 1973). Thus, the Na/phlorizin interaction with the cotransporter is likely to involve the glycoside moiety of phlorizin.

A 1:1 stoichiometry of Na/Na-dependent phlorizin binding for dog kidney brush border has also been determined by Turner and Silverman (1981). They and Crane and Dorando (1979) also provide evidence that the binding process is random. In contrast, from measurement of D-glucose and Na equilibrium exchange flux, Hopfer and Groseclose (1980) argue that their data are consistent with an ordered mechanism of binding and translocation. Although our results do not directly address this question, the difference in stoichiometry between Na and phlorizin binding and Na and D-glucose uptake caution against the assumption that phlorizin binding is identical to Na, D-glucose and cotransporter interaction.

Sodium is required for high-affinity phlorizin binding (Frasch et al., 1970; Glossman & Neville, 1972; Chesney, Sacktor & Kleinzeller, 1974; Rabito, 1981) and phlorizin-inhibitable D-glucose uptake (Aronson & Sacktor 1975; Kinne et al., 1975; Toggenburger et al., 1978; Turner & Silverman, 1978*a*). In the presence of Na but absence of a chemical gradient, increased vesicle electrical negativity inside is associated with enhanced D-glucose uptake (Murer & Hopfer, 1974; Beck & Sacktor, 1978) and an increased number of phlorizin binding sites without effect on the dissociation of bound phlorizin (Aronson, 1978; Toggenburger et al., 1978). The effect of intravesicular electronegativity on the number of phlorizin binding sites is consistent with an electrostatic effect favoring the apparent translocation or exposure of unloaded cotransporter to the exterior membrane surface. "Apparent translocation" may be a conformational change in the carrier which vectorally directs the binding and dissociation resulting in ligand translocation.

Considering the effect of intravesicular electrical negativity promoting the exterior location for binding to an unloaded cotransporter, the enchanced Na-dependent D-glucose uptake and our stoichiometry results, we proposed a two-step, two-sodium mechanism of cotransport (*see* Fig. 5). First, a single Na and D-glucose bind randomly (Crane & Dorando, 1979; Turner & Silverman, 1981) to a negatively charged unloaded cotransporter forming a ternary complex that is neutral in net charge. To the ternary complex a second Na binds conferring net positive charge to the quarternary complex which results in translocation

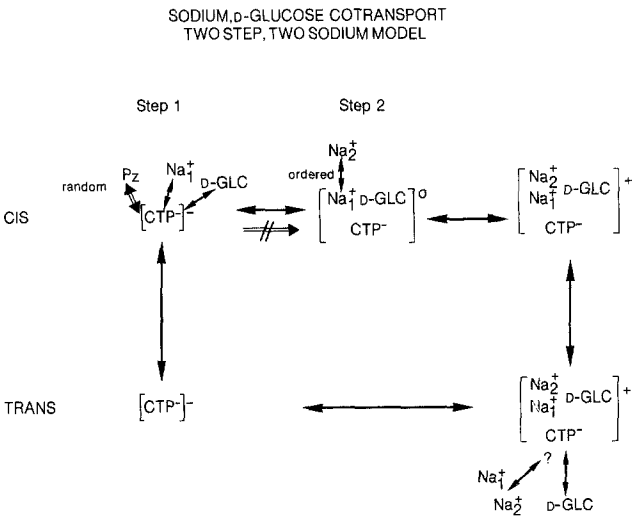

Fig. 5. Two-step two-sodium model of Na-dependent D-glucose cotransport. *See* Discussion for explanation

to the interior of two Na ions and D-glucose. The negative unloaded cotransporter is then translocated to the exterior of the membrane. For phlorizin there is binding with a single sodium at the cotransporter without further binding of a second sodium or translocation.

This model is consistent with the *trans* effect of Na-inhibited phlorizin binding (Aronson, 1978) and D-glucose uptake (Hopfer, Nelson, Perrotto & Isselbacker, 1973; Aronson & Sacktor, 1975; Kinne et al., 1975) if it can be assumed that prevention of Na release at the interior would maintain a neutral or positive charge on the cotransporter and inhibit apparent translocation of the negatively charged unloaded cotransporter to the exterior of the membrane. The *trans* effect of D-glucose, however, is not inhibitory, and there is enhanced coupled uptake by preloading vesicles with D-glucose (Kinne et al., 1975) or LLC-PK_1 cells with α-methyl-D-glucose (Rabito & Ausiello, 1980). The mechanism of the effect may relate to a more rapid translocation and exchange of unlabeled for labeled D-glucose driven by separate chemical gradients, an inward-directed Na gradient, and outward-directed D-glucose gradient as well as an electrical gradient effecting both a positively charged loaded and negatively charged unloaded cotransporter.

The validity of this model will be supported by measurements of a fixed 2:1 stoichiometry of coupled uptake under conditions of varying electrochemical gradients.

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