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Further Studies of Proximal Tubular Brush Border Membrane
D-Glucose Transport Heterogeneity

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Summary. The properties of two sodium-dependent D-glucose transporters previously identified in renal proximal tubule brush border membrane (BBM) vesicles are studied. The low-affinity system, found in BBM vesicles from the outer cortex (early proximal tubule), is shown to be associated with the high-affinity phlorizin binding site typically found in renal BBM preparations. The high-affinity system, found in BBM vesicles from the outer medulla (late proximal tubule), is almost two orders of magnitude less sensitive to inhibition by phlorizin and is apparently not associated with high-affinity phlorizin binding. The sodium/glucose stoichiometry of the outer medullary transporter is found to be 2:1 by two independent methods. Previous measurements have established that the stoichiometry of the outer cortical system is 1:1. It is suggested that this arrangement of transporters in series along the proximal tubule enables the kidney to reabsorb glucose from the urine in an energy-efficient fashion. The bulk of the glucose load is reabsorbed early in the proximal tubule at an energetic cost of one Na^+ per glucose molecule. Then in the late proximal tubule a larger coupling ratio and hence a larger driving force is employed to reabsorb the last traces of glucose from the urine.

Key Words coupled transport · glucose transport · phlorizin binding · brush border membrane · proximal tubule · vesicles

Introduction

In an earlier publication [18] we demonstrated the existence of two apparently distinct sodium-coupled D-glucose transport systems in the renal proximal tubule brush border membrane (BBM). A spatial separation of these transporters was obtained by preparing BBM vesicles from the outer cortex (early proximal tubule) and outer medulla (late proximal tubule). In the outer cortical preparation we observed a relatively low-affinity sodium-dependent D-glucose transport system with $K_m = 6$ mM and $V_{max} = 10$ nmol/min/mg protein as measured under zero trans sodium and glucose conditions at 40 mM NaCl and 17 °C. By contrast, in the outer medullary preparation this component of flux was characterized by a high-affinity system with $K_m = 0.35$ mM and $V_{max} = 4$ nmoles/min/mg protein. Differences in transport specificity between the two systems were also found. More recently we examined the stoichiometric properties of the outer cortical transporter and established that glucose, sodium and phlorizin interact with this system in a 1:1:1 fashion [19].

Owing to its kinetic properties and its location in the early proximal tubule we have suggested [18] that the outer cortical BBM D-glucose transporter is responsible for the reabsorption of the bulk of the filtered load of D-glucose from the urine. Thus it represents the "classical" D-glucose transporter identified, for example, in clearance studies. The high-affinity system, on the other hand, would seem to be responsible for the recovery of the last traces of glucose from the urine in the late proximal tubule. Since this represents only a small fraction of the total glucose reabsorbed, the presence of this transporter may be masked by the low-affinity system in many experimental protocols.

In the present paper we present the results of a series of experiments which give strong support to the above suggestions regarding the roles of the outer cortical and outer medullary transport sites. At the same time we establish a physiologic rationale for the existence of this observed transport heterogeneity along the length of the proximal tubule. Briefly stated we demonstrate that the outer cortical transporter exhibits the mutually competitive interaction between phlorizin and glucose usually associated with renal D-glucose reabsorption [15]. Also it is this transporter which is associated with the high-affinity sodium-dependent phlorizin binding site commonly found in renal BBM preparations [1, 5, 7, 9, 10, 16, 21, 23]. The outer medullary transporter is almost two orders of magnitude less sensitive to inhibition by phlorizin than the outer cortical one and is apparently not associated with the high-affinity phlorizin

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binding site. In contrast to our earlier finding of a 1:1 interaction of sodium and glucose with the outer cortical transporter [19], we find a coupling ratio of (approx.) 2 sodium ions per glucose molecule in the outer medullary preparation. As we discuss later in the paper this arrangement of transporters in series along the proximal tubule apparently enables the kidney to reabsorb glucose from the urine in an energy-efficient fashion.

Materials and Methods

Vesicle Preparation and Characterization

BBM vesicles were prepared from outer cortical and outer medullary tissue obtained from the kidneys of White New Zealand rabbits as previously described [18]. In both final vesicle fractions the activity of the BBM enzyme marker maltase was enriched approximately 12 times relative to the starting tissue homogenate, while the activity of enzymatic markers for antiluminal membranes and intracellular organelles was <1 times that of the homogenate.

The sidedness of the vesicle preparations was investigated by measuring latency of maltase activity upon digestion of the membranes with Triton X-100. No difference in maltase activity could be detected between intact and solubilized vesicles in either preparation. These results indicate that within the limits of accuracy of our measurements (~10%) no maltase catalytic sites are oriented toward the intravesicular space. Thus both the outer cortical and outer medullary vesicle preparations are oriented right-side-out (i.e. urine side out). These results are consistent with a number of earlier studies which indicate that renal BBM vesicles are typically oriented right-side-out [6, 11, 12].

Uptake and Binding Measurements

The procedure for uptake and binding measurements was as previously described [18, 23]. Briefly, a 50-µl aliquot of vesicles (2–4 mg/ml) was incubated with 100 µl of "incubation medium" containing radioactively labeled ligands and other constituents as required. After an appropriate time the reaction was terminated by the addition of a 10-fold dilution of ice-cold stop solution (*see below*). For incubation times less than 10 sec the fast sampling apparatus described in reference [18] was used. After addition of the stop solution the vesicles were applied to a Millipore filter (HAWP 0.45 µ) under light suction. The filter was then washed by a further 4.5 ml of stop solution, dissolved in scintillation fluid and counted along with samples of the incubation medium and appropriate standards.

The detailed composition of the various media used in each experiment are given in the Figure captions. In most cases Buffer AK (10 mM Tris-HEPES[1] containing 100 mM mannitol and 100 mM KSCN) was used as the basis for all preequilibration and test media. In this way 100 mM KSCN was present in equilibrium across the vesicle membrane at all times. When appropriate, valinomycin at a concentration of 12.5 µg/mg vesicle protein was added as a stock solution of 25 mg/ml in ethanol. As shown previously [18] 100 KSCN equilibrium plus this concentration of valinomycin is sufficient to short-circuit transmembrane electrical potential differences.

[1] 10 mM Tris-HEPES: 10 mM HEPES (*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffered with Tris to pH 7.4.

The stop solution was 10 mM Tris-HEPES with 300 mM NaCl, 1000 µM phlorizin and sufficient mannitol to compensate for intravesicular osmolarity. From control experiments in which the time between addition of the stop solution and filtration was prolonged we have established that no significant loss of D-glucose occurs during the stopping and washing procedure in either vesicle preparation [18].

Tracer D-^{14}C-glucose and L-^{3}H-glucose were used at concentrations of 10–20 µCi/ml and 50–100 µCi/ml, respectively. The simultaneously measured "uptake" of L-glucose was used to correct D-glucose uptake for nonspecific binding and trapping by the membranes and filters. The resulting difference is referred to as the stereospecific component of D-glucose flux. Relative to the uptake of D-glucose by the membranes the actual transport of L-glucose (as distinct from binding and trapping) is so small as to be negligible in this system. All experiments were carried out in the presence and absence of sodium with choline replacing sodium isosmotically. The sodium-dependent component of glucose flux or phlorizin binding, due to the BBM transporter, was calculated by subtraction. As previously reported [18] there is no detectable component of D-glucose binding in either vesicle preparation.

All experimental points were carried out in triplicate. Unless otherwise noted the errors quoted and error bars shown in the Figures (provided these are large enough to illustrate) are the standard deviations on the points. The results of representative experiments are shown.

Criteria of Purity

The purity of ^{3}H-phlorizin (>97%) was regularly verified by thin-layer chromatography using the solvent system chloroform/methanol/water (65/24/4 vol/vol).

Calculations

In least-squares fits to the data, points were weighted according to the inverse of their relative experimental errors. Nonlinear least-squares fits were carried out using the program "P3R" (BMD, Biomedical Computer Programs, University of California, Los Angeles). The errors quoted in the text on the least-squares parameters are standard deviations.

Phlorizin binding data were handled as previously described [23]. The Student's *t*-test was used in the statistical evaluation of data and P values <0.05 were taken to indicate statistically significant differences.

Materials

L-^{3}H-glucose, D-^{14}C-glucose and ^{3}H-phlorizin were obtained from New England Nuclear Corp. (Boston, Mass.). Unlabeled phlorizin and D- and L-glucose were from Sigma Chemical Co. (St. Louis, Mo.). Choline chloride was obtained from Eastman Kodak Co. (Rochester, N.Y.) and was recrystalized from ethanol before use. Other chemicals were of highest purity available from commercial sources.

Results

Phlorizin Binding Kinetics

Figure 1 illustrates the kinetic properties of the sodium-dependent component of phlorizin binding to outer cortical and outer medullary BBM vesi-

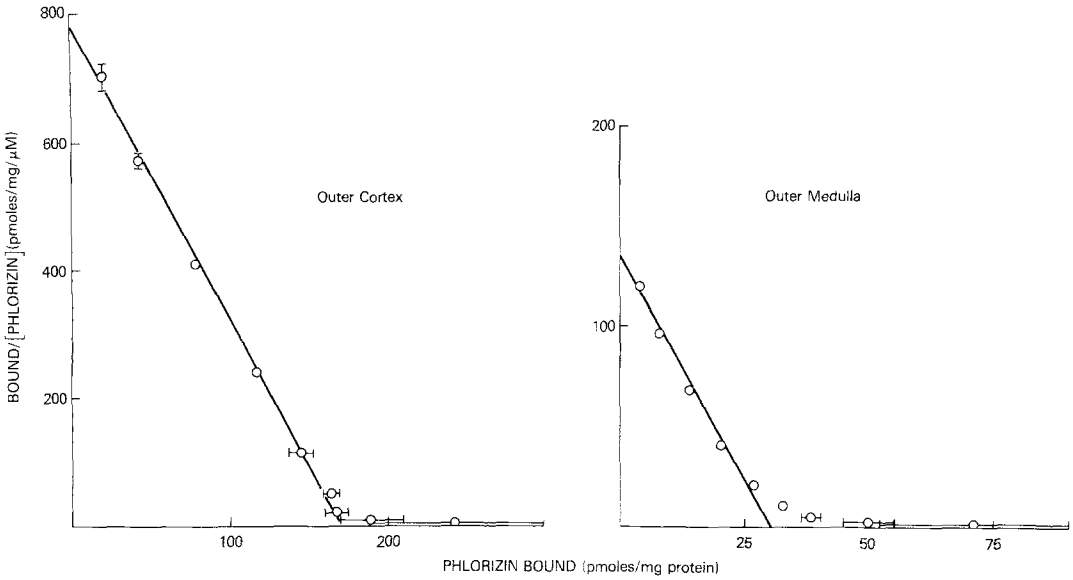

Fig. 1. Kinetics of the sodium-dependent component of phlorizin binding to outer cortical and outer medullary BBM vesicles. Vesicles were prepared in 10 mM Tris-HEPES containing 100 mM mannitol. The incubation media were the same buffer containing ^{3}H-phlorizin (0.03 to 50 μM) and 60 mM NaCl or 60 mM choline chloride (final concentrations). Binding was measured at 37 °C after 40 min of incubation. Nonlinear least-squares analysis of the outer cortical data yield $K_d = 0.21 \pm 0.01$ μM and $N_o = 166 \pm 3$ pmol/mg protein for the high-affinity site. The least-squares program was unable to generate reliable estimates of the kinetic parameters for the low-affinity component owing to its small contribution to the total binding. Nonlinear least-squares analysis of the outer medullary data yield $K_d = 0.24 \pm 0.02$ μM and $N_o = 30 \pm 1$ pmol/mg protein for the high-affinity site and $K_d = 102 \pm 34$ μM and $N_o = 131 \pm 31$ for the low-affinity component. The lines drawn through the points correspond to K_d values of 0.22 μM

cles. The results are presented as Scatchard plots. Except for the number of binding sites per mg protein the phlorizin binding properties of the two preparations are quite similar (*see* Figure caption). In each case we observe a high-affinity component of binding with $K_d = 0.2$ μM together with a much lower affinity component (estimated $K_d \gg 1$ μM) whose presence is only detectable at phlorizin concentrations > 3 μM in the outer cortex and > 1 μM in the outer medulla.

Specificity of Phlorizin Binding

In the Table we examine the effects of D-glucose and D-galactose on the high-affinity components of sodium-dependent phlorizin binding observed in the two preparations. In our previous studies [18] we found that D-galactose was considerably more effective in inhibiting D-glucose flux in the outer medullary preparation than in the outer cortex, indicating that it was a preferred substrate for the former system. (From these earlier data we estimate that D-galactose is ~15 times less effective than D-glucose in inhibiting the outer cortical transporter and ~4 times less effective than D-glucose in the outer medulla.) The data in the Table demonstrate that the outer cortical and outer medullary high-affinity phlorizin binding sites

Table Effects of D-glucose and D-galactose on the high-affinity sodium-dependent component of phlorizin binding to BBM vesicles[a]

Experimental conditions	K_d (μM)	Number of sites (pmol/mg protein)	K_I (mM)
Outer cortex:			
Control	0.182 ± 0.002	158 ± 1	–
7.5 mM glucose	0.368 ± 0.003	155 ± 1	7.3 ± 0.2
75 mM galactose	0.257 ± 0.004	156 ± 1	182 ± 11
Outer medulla:			
Control	0.183 ± 0.005	31.0 ± 0.5	–
7.5 mM glucose	0.375 ± 0.015	29.3 ± 0.9	7.1 ± 0.6
75 mM galactose	0.246 ± 0.011	30.1 ± 0.8	218 ± 42

[a] The experimental details are similar to those of Fig. 1 except that 100 mM KSCN was present in all buffers. The "control" incubation medium contained 75 mM mannitol (final concentration) which was replaced isosmotically by glucose or galactose in the other runs. Least-squares parameters were calculated by linear regression analysis; in all cases $r > 0.997$. The phlorizin concentration range was 0.015 to 1.6 μM.

are both competitively inhibited by D-glucose and D-galactose with no significant difference in K_I's between preparations in either case. The ratio of D-galactose to D-glucose K_I's is ≈24. Thus the behavior of the high-affinity phlorizin binding site

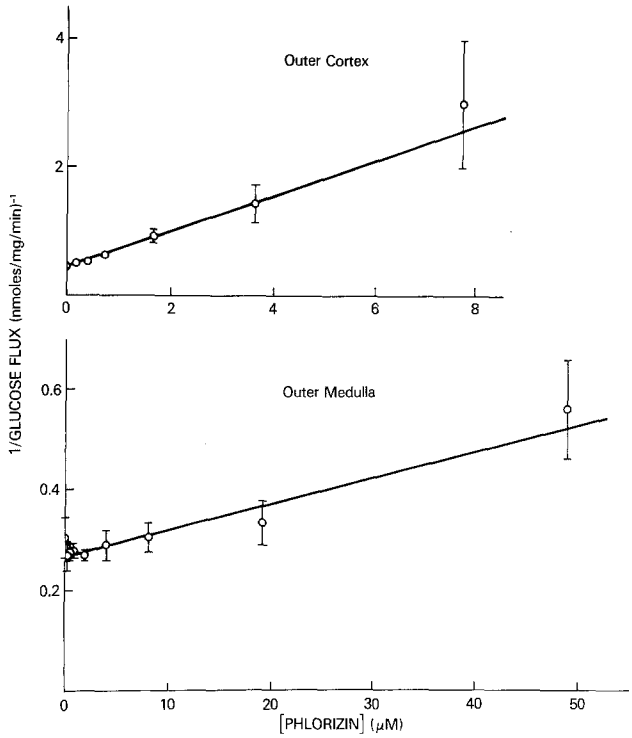

Fig. 2. The relationship between sodium-dependent phlorizin binding and sodium-dependent equilibrium exchange D-glucose flux in outer cortical and outer medullary BBM vesicles. Only the glucose flux data are illustrated (as Dixon plots). The experiment was carried out at 17 °C in buffer AK containing 100 mM mannitol, 1 mM D-glucose, ^{3}H-phlorizin (0–50 μM) and 60 mM NaCl or choline chloride. Vesicles were preincubated in the appropriate buffer for 30 min at 37 °C and for 2 hr at 17 °C before the flux and binding measurements were made. At time zero the same buffer containing tracer D-^{14}C-glucose was added and 2.25- sec points were taken. In control experiments (*not shown*) we have established that equilibrium exchange fluxes are linear with time for at least 3 sec under the conditions of this experiment. Glucose fluxes and equilibrium phlorizin binding were measured on the same sample. The lines drawn through the points are linear least-squares fits. Nonlinear least-squares analysis of the sodium-dependent component of phlorizin binding (*data not shown*) yielded $K_{0.5} = 1.67 \pm 0.16$ μM and $N_o = 143 \pm 7$ pmol/mg protein for the high-affinity site in the outer cortical preparation and $K_{0.5} = 1.9 \pm 0.3$ μM and $N_o = 29 \pm 3$ pmol/mg protein for the high-affinity site in the outer medulla. The least-squares program was unable to generate reliable estimates of the kinetic parameters for the low-affinity component of binding in either preparation

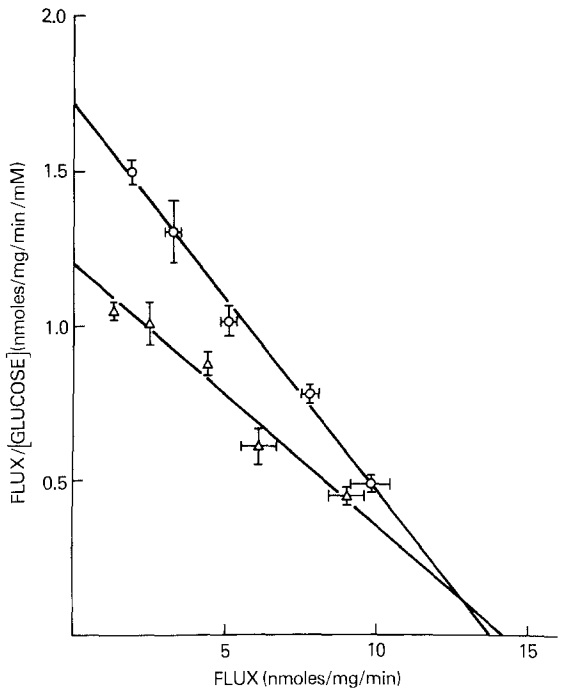

Fig. 3. Effect of phlorizin on the kinetics of stereospecific sodium-dependent D-glucose equilibrium exchange flux in outer cortical BBM vesicles. The data are shown as an Eadie-Hofstee plot. The experiment was carried out at 17 °C in buffer AK containing 100 mM mannitol, D- and L-glucose (1.26–20 mM) and 60 mM NaCl or choline chloride. Vesicles were preincubated in the appropriate buffer with (△) or without (○) 1 μM phlorizin for 30 min at 37 °C and for 2 hr at 17 °C before flux measurements were made. At time zero the same buffer containing tracer labeled D- and L-glucose was added and 2-sec points were taken. In control experiments (*not shown*) we have established that equilibrium exchange fluxes are linear with time for at least 3 sec under the conditions of this experiment. Linear least-squares analysis of the data yield $K_m = 7.9 \pm 0.4$ mM and $V_{max} = 13.8 \pm 0.5$ nmol/min/mg protein in the absence of phlorizin and $K_m = 11.9 \pm 1.2$ mM and $V_{max} = 14.2 \pm 1.1$ nmol/min/mg protein in the presence of phlorizin

is more consistent with the expected properties of the outer cortical transporter.

Relationship between Phlorizin Binding and Glucose Flux

In Fig. 2 we show the results of an experiment which directly examines the relationship between phlorizin binding and glucose flux in the two preparations. Here we have simultaneously measured the sodium-dependent components of phlorizin binding and D-glucose equilibrium exchange flux as functions of increasing phlorizin concentration. Only the glucose flux data are illustrated in the Figure since the phlorizin binding results are qualitatively very similar to those shown in Fig. 1 (least-squares fits to the phlorizin binding data are given in the Figure caption).

From the flux data of Fig. 2 we calculate $K_{0.5}$ values for phlorizin inhibition of equilibrium exchange D-glucose flux of 1.53 ± 0.05 μM in the outer cortical preparation and 51 ± 6 μM in the outer medulla. From the simultaneously measured phlorizin binding data (*see* Fig. 2 caption) we find that the $K_{0.5}$ values for the high-affinity sodium-dependent component of binding are 1.67 ± 0.16 μM in the outer cortex and 1.9 ± 0.3 μM in the outer medulla. Thus in the outer cortex there

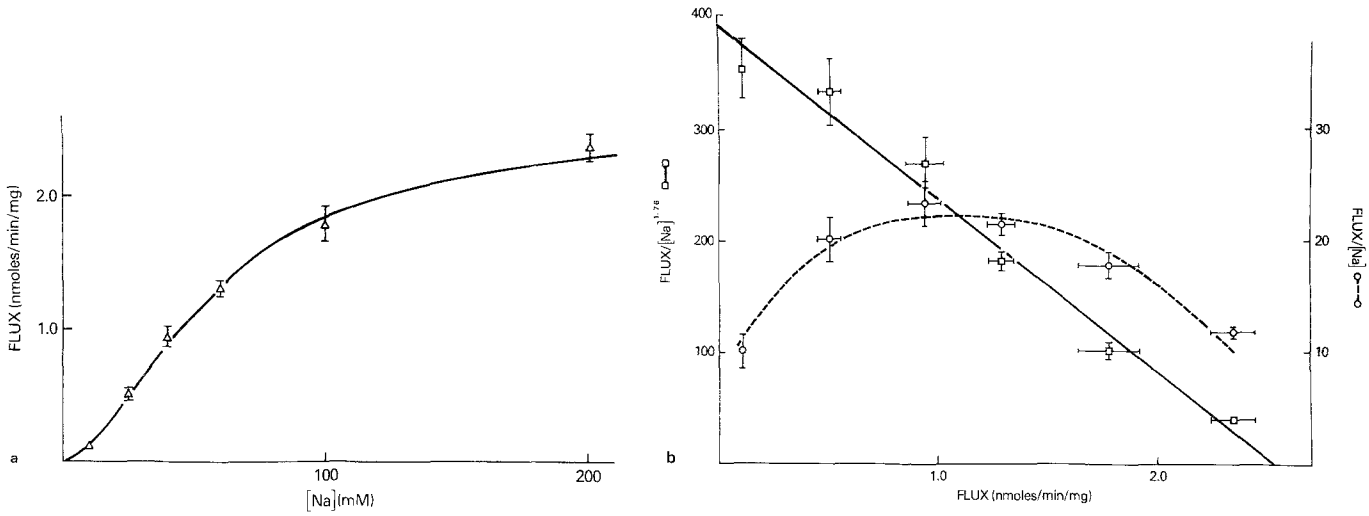

Fig. 4. Sodium-dependent stereospecific D-glucose flux as a function of sodium concentration in the outer medullary preparation. Vesicles were prepared in buffer *AK* containing 600 mM mannitol plus valinomycin. The incubation medium was buffer *AK* containing sufficient glucose and NaCl to give final concentrations of 0.1 mM and 0–200 mM, respectively. Choline replaced sodium isosmotically to obtain the various sodium concentrations studied. Uptake was measured after 3 sec of incubation at 17 °C. (*a*) A plot of flux vs. sodium concentration. (*b*): Plots of flux/[Na] vs. flux (o and dashed line) and flux/$[Na]^{1.76}$ vs. flux (□ and solid line). The linearity of the latter plot is indicative of the involvement of approximately 1.8 sodium ions per glucose transport event (*see text*). The data were fit to Eq. (1) by nonlinear least-squares analysis. The result was $V_{max} = 2.53 \pm 0.18$ nmol/min/mg protein, $K_{0.5} = 57 \pm 7$ mM and $n = 1.76 \pm 0.08$. The lines through the data points were all calculated from this theoretical fit. In panel (*b*) of the Figure the units of [Na] are M

is an excellent correlation between the phlorizin concentration which inhibits 50% of the sodium-dependent D-glucose flux and the phlorizin concentration at which 50% of the high-affinity binding sites are occupied. This observation leaves little doubt that the high-affinity component of phlorizin binding observed in the outer cortical preparation is intimately associated with the outer cortical sodium-dependent D-glucose transporter. A similar association is not apparent in the outer medullary preparation.

In Fig. 3 we investigate the inhibitory effect of phlorizin on the kinetics of the sodium-dependent component of D-glucose equilibrium exchange flux in outer cortical BBM vesicles. The behavior of phlorizin is consistent with that of a competitive inhibitor with $K_I = 2.0 \pm 0.7$ μM. Using the K_m for D-glucose calculated from Fig. 3 (7.9 ± 0.4 mM), the K_I for phlorizin inhibition of D-glucose flux and the K_d for phlorizin binding can be calculated from the outer cortical experiment shown in Fig. 2. The results are $K_I = 1.36 \pm 0.05$ μM (from the $K_{0.5}$ for phlorizin inhibition of D-glucose flux) and $K_d = 1.48 \pm 0.16$ μM (from the $K_{0.5}$ for phlorizin binding). Thus the phlorizin kinetic parameters from Figs. 2 and 3 are in good agreement with one another. Taken together, the data presented in the Table and Figs. 2 and 3 convincingly demonstrate that the outer cortical transporter is the "classical" sodium-coupled BBM D-glucose carrier usually associated with high-affinity phlorizin binding in the proximal tubule.

Owing to technical difficulties (low flux rates and high sodium independent background) we have not been able to carry out reliable equilibrium exchange kinetic experiments on outer medullary vesicles. Thus the nature of the interaction between phlorizin and glucose in this system remains to be clarified.

Stoichiometry of the Outer Medullary Transporter

The coupling stoichiometry of a brush border cotransport system is of obvious physiological significance since it figures heavily into the concentration gradient achieved by the epithelium as a whole [2]. We have previously demonstrated that the sodium/glucose stoichiometry of the outer cortical BBM D-glucose transporter is 1:1 [19]. This ratio is determined here in the outer medullary preparation by two independent procedures, the "activation method" and the "static head method" [19].

Activation Method. The results of an "activation method" experiment on outer medullary BBM vesicles is illustrated in Fig. 4. Here we have measured the initial flux of 0.1 mM D-glucose as a function of sodium concentration over the range 0 to 200 mM. The experiment was carried out at 17 °C

under zero trans sodium and glucose conditions. Uptake was measured after 3 sec of incubation. In control experiments we have established that the flux of 0.1 mM D-glucose is linear with time for at least 4 sec over the entire range of sodium concentrations used in this experiment (*data not shown*).

Sodium-dependent D-glucose flux is plotted vs. sodium concentration in Fig. 4*a*. It is clear from this Figure that the flux data show a marked sigmoidal dependence on sodium concentration indicative of multiple activator (sodium) binding sites [14]. The stoichiometry of the system is examined more quantitatively in Fig. 4*b*. In the case of a sodium/glucose stoichiometry of 1:1 it is expected that the sodium-dependent component of glucose flux will show a Michaelis-Menten type dependence on sodium concentration [17, 19], i.e., that a plot of flux/[Na] vs. flux will be linear. We have previously demonstrated this linearity for the outer cortical system [19]. Such a plot of the outer medullary data is represented by the open circles and dashed line in Fig. 4*b*. The points clearly do not lie on a straight line. The dependence of flux on activator concentrations for transport models which involve activator/substrate stoichiometries greater than 1 can be quite complex. However, by analogy with enzyme kinetics [14] one might expect the following Hill-type equation to hold as a first approximation:

$$\text{flux} = V_{\max}[\text{Na}]^n/(K_{0.5}{}^n + (\text{Na}]^n). \quad (1)$$

This equation assumes the existence of n essential cooperative sodium binding sites per glucose site [14]. A non linear least-squares fit of the data to Eq. (1) (*see* Fig. 4 caption) gives $n = 1.76 \pm 0.08$ indicating that a plot of flux/$[\text{Na}]^{1.76}$ vs. flux should be reasonably linear. That this is indeed the case is demonstrated by the closed circles and solid line in Fig. 4*b*. Further discussion of this result is given later in the paper; however, it is useful at this point to mention that the stoichiometry determined from the activation method is the number of sodium ions involved per glucose transport event and not necessarily the actual coupling ratio (the number of sodium ions translocated by the carrier per glucose molecule). In other words, the stimulation of glucose flux produced by sodium in Fig. 4 could be a result of sodium-glucose cotransport or of sodium-induced facilitation of glucose entry without concomitant sodium transport, or a combination of both.

Static Head Method. We have developed the static head method as an alternative means of measuring stoichiometric coupling ratios of cotransport mechanisms [19]. This method is much less dependent on the $V_{\max}$ of the system under consideration than the usual procedure of measuring the ratio of activator-stimulated substrate flux to substrate-stimulated activator flux. The latter method is very difficult to employ for low $V_{\max}$ systems where signal-to-noise ratios may be low. The static head method is based on the following argument. Consider a tightly coupled cotransport system where n activator ions (A) are transported per substrate molecule (S). (By tightly coupled transport we mean that translocation of either glucose or sodium alone via the transporter is not possible; i.e., that the partially loaded forms of the carrier (carrier plus glucose and carrier plus sodium) are immobile. Additional considerations for nontightly coupled systems are mentioned in the Discussion.) The thermodynamic condition that there is no net flux of either substance via the transporter (or equivalently that the driving forces for activator and substrate fluxes via the transporter are balanced) is given by

$$\ln(S_i/S_o) = n[\ln(A_o/A_i) + F\Delta\Psi/RT]. \quad (2)$$

Here the subscripts i and o label intravesicular and extravesicular concentrations, respectively, $\Delta\Psi$ is the transmembrane potential and F,R and T have their usual thermodynamic interpretation. We further assume that $\Delta\Psi = 0$ in Eq. (2) since membrane potentials are short-circuited in our experiments[2]

[2] By neglecting the $\Delta\Psi$ term in Eq. (2) we are also assuming that any effects of Donnan potentials due to impermeant intravesicular polyelectrolytes are negligible. The value of such a Donnan potential would be given by

$$V = \frac{RT}{F}\sinh^{-1}(zC_p/2C_o)$$

where C_p is the intravesicular polyelectrolyte concentration, z is the average charge per polyelectrolyte molecule and C_o is the total permeant extravesicular salt concentration. Since the static head experiment involves balancing thermodynamic driving forces between the bulk phases of the extravesicular and intravesicular spaces, it is the Donnan potential between these bulk phases which is of concern here. Thus only polyelectrolytes in the bulk phase of the intravesicular space contribute to V. Trapped cytoplasmic proteins and microvillar core material are two possible sources of intravesicular polyelectrolyte.

The condition that the Donnan potential is negligible in the static head experiment is $\ln(A_o/A_i) \ll FV/RT$ [cf. Eq. (2)]. Since $\ln(A_o/A_i) \approx 1$ (*see* Fig. 5 caption) this means that $V \ll RT/F = 25.8$ mV.

We have verified that increasing the concentration of permeant salt (C_o) from 170 mM (Fig. 5) to 300 mM does not significantly alter the results of the static head stoichiometry determination in the outer medullary preparation (*data not shown*). Since this increase in C_o would result in an approximate twofold decrease in V we can safely conclude that the effects of Donnan potentials are negligible in this experiment. Similar control experiments have been carried out with the outer cortical BBM vesicle preparation.

(*see* Materials and Methods). The principle of the static head experiment is to search for activator and substrate conditions for which Eq. (2) holds. The coupling stoichiometry can then be simply calculated from the known concentration gradients.

The procedure we employ here is to dilute vesicles preloaded with given glucose and sodium concentrations into appropriate glucose-free media, thus establishing an outwardly directed (intravesicular to extravesicular) glucose gradient. We then measure the glucose retained in these vesicles as a function of time and as a function of extravesicular sodium concentration. A control run is also carried out in the absence of sodium to measure efflux via unrelated sodium-independent pathways. The static head condition is characterized by that external sodium concentration which causes the test points to superimpose on the control points. Here the inwardly directed sodium gradient exactly balances the outwardly directed glucose gradient.

In Fig. 5 we show the results of a static head experiment on outer medullary BBM vesicles. The sodium concentrations used in the extravesicular "test" solutions (*see* Figure caption) would be expected to produce static head conditions for stoichiometries (n) of 1.6, 2.0 and 2.4. It is clear from Fig. 5 that static head conditions will occur at a value of n slightly less than 2.0 but significantly greater than 1.6. Further discussion of this result is given in the next section.

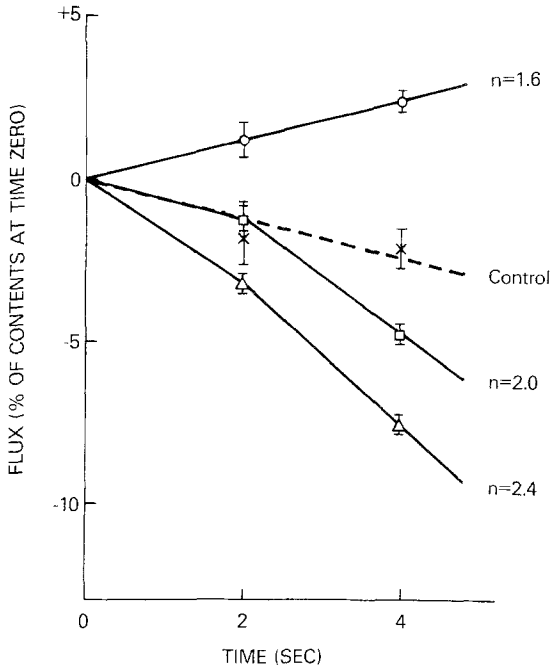

Fig. 5. Static head determination of the sodium/glucose coupling ratio in outer medullary BBM vesicles. Vesicles were prepared in buffer AK containing 0.25 mM labeled glucose, valinomycin and 20 mM NaCl plus 50 mM choline chloride (test) or 70 mM choline chloride (control). One hundred μl of incubation medium was added to 20 μl of vesicles giving a final extravesicular glucose concentration of 0.0417 mM. The incubation media for the test points were buffer AK containing sufficient NaCl to give final extravesicular concentrations of 62 mM (○), 49 mM (□) or 42 mM (△) and sufficient choline chloride to give a total sodium plus choline salt concentration of 70 mM (equiosmotic with the intravesicular medium). The values of n indicated on the Figure are the stoichiometries which would be calculated if that sodium concentration were to result in static head conditions (*see text*). The incubation medium for the control points (×) was buffer AK containing 70 mM choline chloride. The experiment was carried out at 28 °C. The stereospecific efflux (or influx) of D-glucose has been expressed as a percent of the total (equilibrium) intravesicular glucose at time zero (0.3 nmol/mg protein). Standard errors rather than standard deviations have been illustrated for clarity

Discussion

In this paper we have extended earlier observations [4, 18] regarding renal proximal tubular D-glucose transport heterogeneity. We had previously established that two distinct sodium-dependent D-glucose transport systems could be identified in proximal tubule BBM vesicles, a low-affinity system found in vesicles prepared from the outer cortex (early proximal tubule), and a high-affinity system in vesicles from the outer medulla (late proximal tubule). Complementary results have been obtained by Barfuss and Schafer [4] using the isolated perfused tubule technique.

The experiments presented in Figs. 1 and 2 and in the Table show that the outer cortical BBM preparation contains a high-affinity, sodium-dependent phlorizin binding site which is competitively inhibited by D-glucose and D-galactose. The K_d for this site is in good agreement with the K_I for phlorizin inhibition of sodium-dependent D-glucose equilibrium exchange flux measured simultaneously in the same preparation (Fig. 2). Taken together with Fig. 3, which illustrates the competitive nature of phlorizin inhibition of D-glucose flux, these results clearly indicate that phlorizin and glucose interact with the outer cortical transporter in a mutually competitive fashion.

It is worth pointing out that, although considerable experimental evidence has been gathered to support the conjecture that glucose and phlorizin compete for the renal BBM transporter [15], a detailed quantitative confirmation such as the one presented here has never before been given. Turner and Silverman [21, 22] have reported preliminary experiments of this type on BBM vesicles prepared from the whole cortex of dog kidney. But it is now clear [18] that their vesicle preparation contained an admixture of high-affinity and low-affin-

ity glucose transport sites presumably arising from early and late nephron segments. Also the results of these authors indicate that it is the high-affinity transport site which is associated with high-affinity phlorizin binding, a finding apparently contradicted by the results presented here. Different species were examined in the two studies (dog vs. rabbit), however, and further experiments are needed to be certain of the situation.

In our previous studies of the outer cortical transporter [18] we found that under zero trans glucose, sodium and phlorizin conditions, 10 μM phlorizin inhibited approximately 50% of sodium-dependent D-glucose flux measured at 28 °C. This result seems to be at variance with the data presented here since it indicates a K_I for phlorizin of approximately 10 μM. The explanation is that phlorizin binding at 28 °C is a relatively slow process and the flux measurements referred to above were made after only 10 sec of incubation. Phlorizin binding is far from equilibrium under these experimental conditions (*data not shown*). Thus the K_I values so calculated are not representative of the actual affinity of phlorizin for the transporter. This difficulty has been overlooked in most earlier studies of the kinetics of phlorizin inhibition of BBM D-glucose flux [3, 13, 20, 22]. The problem is avoided in the experiments presented here by the use of equilibrium exchange conditions where both glucose and phlorizin have been pre-equilibrated with the membranes.

The K_I for phlorizin inhibition of sodium-dependent D-glucose flux in the outer medullary preparation is approximately 50 μM. Thus there is clearly no compelling reason to associate the high-affinity phlorizin binding site observed in this preparation with the high-affinity glucose transporter. This high-affinity binding site may represent contamination by outer cortical transporters. Arguing against this possibility is the fact that kinetic analysis of glucose flux data from the outer medullary preparation shows little if any evidence of outer cortical contamination [18]. It is tempting to associate the low-affinity sodium-dependent component of phlorizin binding seen in Fig. 1 with the outer medullary transport site. However, there is little evidence for this assignment at the present time.

We have previously demonstrated that the sodium/glucose coupling stoichiometry of the outer cortical BBM transporter is 1:1 [19]. The results presented here indicate that this ratio is approximately 2:1 in the outer medulla. This stoichiometric ratio was measured by two independent methods.

The sigmoidal dependence of glucose flux on sodium concentration seen in Fig. 4*a* is clearly indicative of a sodium/glucose stoichiometry >1. The quantitative interpretation of these activation method data relies on knowledge of the form of the kinetic equation for the transport process. Equation (1) represents a commonly used first approximation for the sodium dependence of substrate flux in tightly coupled systems. A nonlinear least-squares fit of the data to this equation yields a stoichiometry (n) of 1.76 ± 0.08. It should be pointed out that this analysis is completely equivalent to analyzing the data on a Hill plot [14]. Nonintegral values of n can be interpreted as being the result of contributions from lower order terms in [Na] which have been neglected in the derivation of Eq. (1). In our case this might occur if a portion of the glucose flux was characterized by a 1:1 sodium/glucose stoichiometry while the bulk of the flux was 2:1. This situation could be due to a nontightly coupled transporter or to contamination by a 1:1 system such as the outer cortical one.

The static head method of measuring stoichiometric coupling ratios is based on the thermodynamic implications of Crane's gradient hypothesis [8]. The estimated stoichiometry from this experiment (1.8–1.9) is in good agreement with the value obtained from the activation method. Since the former method is only sensitive to cotransported sodium while the latter detects all sodium involved in activation of the transport event we conclude that there is no evidence for the involvement of nontransported sodium ions. In other words, all of the sodium ions associated with the transport event are actually cotransported with glucose.

We suggest that the arrangement of glucose transporters we have observed along the length of the proximal nephron leads to the following reabsorptive sequence. The bulk of the glucose load is reabsorbed early in the proximal tubule by the outer cortical transporter at an energetic cost of one sodium ion per glucose molecule. (The energy involved is essentially that required to pump the cotransported sodium ion out of the cell.) As glucose is reabsorbed, and the urinary glucose concentration falls, a point is reached where the extracellular to intracellular electrochemical gradient for sodium is too low for a 1:1 system to drive any more glucose out of the urine. By this point, however, the 2:1 system is present. Since the concentrating ability of a cotransport system increases as the power of the coupling ratio [2] this transporter can use the same $\Delta\mu_{Na}$ to further lower the concentration of glucose in the urine in the late proximal tubule. These last traces of glucose are

pumped from the urine at the higher energetic cost of two sodium ions per glucose molecule. This arrangement of transporters in series along the proximal tubule leads to a more energy-efficient reabsorptive mechanism for D-glucose than could be achieved by either of the transporters acting alone.

It remains to be determined whether the scheme suggested above applies only to D-glucose or is a common feature of other renal reabsorptive cotransport systems.

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