J. Membrane Biol. 67, 73–80 (1982)

The Journal of
Membrane Biology

Stoichiometric Studies of the Renal Outer Cortical Brush Border Membrane D-Glucose Transporter

R. James Turner and A. Moran
Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung and Blood Institute, Bethesda, Maryland 20205

Summary. The stoichiometric properties of the renal outer cortical brush-border membrane D-glucose transporter are studied. Experiments which establish the glucose/sodium, glucose/phlorizin and phlorizin/sodium stoichiometries are reported. Three independent methods of determining the substrate/activator (glucose/sodium) stoichiometry for coupled transport systems are presented and discussed. One of these, the "Static Head Method," is introduced here for the first time. This type of experiment appears to be more generally applicable than the usual procedure of directly measuring the coupled fluxes of substrate and activator to determine stoichiometric coupling ratios. The results presented in this paper demonstrate that the glucose/sodium/phlorizin stoichiometry of the renal outer cortical brush-border membrane D-glucose transport system is 1:1:1.

Key words vesicles · stoichiometry · brush-border membrane · glucose transport · coupled transport · phlorizin binding

Introduction

It is now well established that the active step in the vectorial transport of many solutes across mammalian epithelia occurs via cotransport of the substrate with sodium ions at one side of the cell (Crane, 1977). The stoichiometry of such a cotransport event is of particular interest since it figures heavily into the net concentrating ability of the epithelium for the substrate as well as into the energetic cost of the transepithelial substrate flux. More specifically, it can be shown that the concentrating ability of a cotransport system increases as the power of the number of activator ions transported per substrate molecule (Aronson, 1981). The energetic cost of coupled transport is essentially the work required to pump the cotransported sodium ions back out of the cell.

To date relatively few stoichiometric studies of sodium-coupled epithelial transport systems have been carried out. Most of these have concentrated on the glucose transporters of the proximal tubular and intestinal brush-border membrane (BBM). Beck and Sactor (1978) have reported a tentative sodium/glucose stoichiometry of 1:1 in the proximal tubule and several authors (Goldner, Schultz & Curran, 1969; Okada, 1979; Hopfer & Groseclose, 1980) have reported a 1:1 stoichiometry in the intestine. However, Kimmich has recently questioned the validity of several of these measurements arguing that the effects of the coupled flux of glucose and sodium on membrane potential have not been properly taken into account (Kimmich, 1981). Kimmich himself finds a sodium/glucose stoichiometry of 2:1 in isolated chick intestinal cells (Kimmich & Randles, 1980; Kimmich, 1981).

The determination of the sodium/glucose stoichiometry in the kidney is now complicated by recent demonstrations of glucose transport heterogeneity along the proximal tubule (Barfuss & Schafer, 1980; Turner & Moran, 1982). In our laboratory (Turner & Moran, 1982) we have shown that the sodium-dependent component of D-glucose transport in BBM vesicles prepared from the outer cortex (early proximal tubule) of rabbit kidney is characterized by a relatively low affinity system with $K_m = 6$ mM (as measured under zero trans conditions at 40 mM NaCl and 17 °C). Under the same experimental conditions in BBM vesicles prepared from outer medullary tissue this component of glucose flux is characterized by a high affinity system with $K_m = 0.35$ mM. The kinetic data from each of these two vesicle fractions are fit quite well by the Michaelis-Menten equation suggesting that a single transport system dominates in each preparation.

In this paper we present a series of detailed stoichiometric studies carried out on the low-affinity D-glucose transport system found in outer cortical BBM vesicles. Our objective here is twofold. First, we wish to take advantage of the apparent lack of significant glucose transport heterogeneity in this preparation to obtain more definitive results concerning the stoichiometric properties of the system. And secondly, we wish to compare and evaluate the usefulness of several methods for determining stoichiometric ratios. In this regard we present a new and novel method for measuring the substrate/activator stoichiometry in cou-

0022-2631/82/0067-0073 $01.60

pled transport systems. This procedure, which we refer to as the Static Head Method, is based directly on the thermodynamic implications of Crane's Gradient Hypothesis (Crane, 1977).

In addition to the determination of the sodium/glucose stoichiometry we also present a series of experiments which establish the stoichiometric relationships between phlorizin and glucose and phlorizin and sodium. Phlorizin is a well-known competitive inhibitor of renal D-glucose reabsorption (Silverman, 1981) and has been shown to compete with glucose for the outer cortical transport site (Turner & Moran, 1981; Turner & Moran, *manuscript in preparation*). The use of phlorizin as a probe of sodium-dependent D-glucose transport mechanisms is now well established (Turner & Silverman, 1980, 1981; Silverman, 1981).

The results presented in the present paper demonstrate that the phlorizin/glucose/sodium stoichiometry of the renal outer cortical D-glucose transport system is 1:1:1.

Materials and Methods

Vesicle Preparation and Characterization

BBM vesicles were prepared from outer cortical tissue obtained from the kidneys of White New Zealand rabbits as previously described (Turner & Moran, 1982). Relative to the starting tissue homogenate, the activity of maltase (a BBM marker) in the final vesicle fraction is enriched approximately 11 times while the activities of (Na, K)-ATPase (an antiluminal membrane marker), succinic dehydrogenase (a mitochondrial marker) and glucose-6-phosphatase (an endoplasmic reticulum marker) are reduced by factors of 5, 5 and 2.5, respectively.

Uptake and Binding Measurements

Unless otherwise noted the procedure for uptake and binding measurements was as follows. A 50-µl aliquot of vesicles (2–4 mg/ml) was placed in a 12 × 75 mm glass test tube and at time zero a 100-µl aliquot of incubation medium containing radioactively labeled ligands and other constituents as required was added. 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 Turner and Moran (1982) 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. 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 (Turner & Moran, 1982). The filter, which retained the BBM vesicles, was 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 general Buffer AK (10 mM Tris-HEPES[1] containing 100 mM mannitol and 100 mM KSCN) was used as the basis for all media. In this way 100 mM KSCN was present in equilibrium across the vesicle membrane at all times. When appropriate, 12.5 µg valinomycin/mg vesicle protein was added as a stock solution of 25 mg/ml in ethanol. As shown in Turner and Moran (1982) 100 KSCN equilibrium with this concentration of valinomycin is sufficient to short-circuit transmembrane electrical potential differences. The Stop Solution was 10 mM Tris-HEPES with 300 mM NaCl, at least 300 µM phlorizin and sufficient mannitol to compensate for intravesicular osmolarity.

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. As previously reported (Turner & Moran, 1982) there is no detectable component of glucose binding in this preparation.

Unless otherwise noted all experiments were carried out in triplicate. 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. The errors quoted in the text on the least-squares parameters are 95% confidence intervals.

Phlorizin binding data were handled as previously described (Turner & Silverman, 1981). 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, ^{22}Na and ^{3}H-phlorizin were obtained from New England Nuclear Corp (Boston, MA). Unlabeled phlorizin and D- and L-glucose were from Sigma Chemical Co. (St. Louis MO). Choline chloride was obtained from Eastman Kodak Co. (Rochester NY) and was recrystalized from ethanol before use. Other chemicals were of highest purity available from commercial sources.

Results

The Phlorizin/Glucose Stoichiometry of the Transporter

Figure 1 shows the results of an experiment where the sodium-dependent component of equilibrium phlorizin binding to outer cortical BBM vesicles was measured as a function of D-glucose concentration. The data are presented in the form of a log-log (Hill-type) plot. Since glucose is a competitive inhibitor of phlori-

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

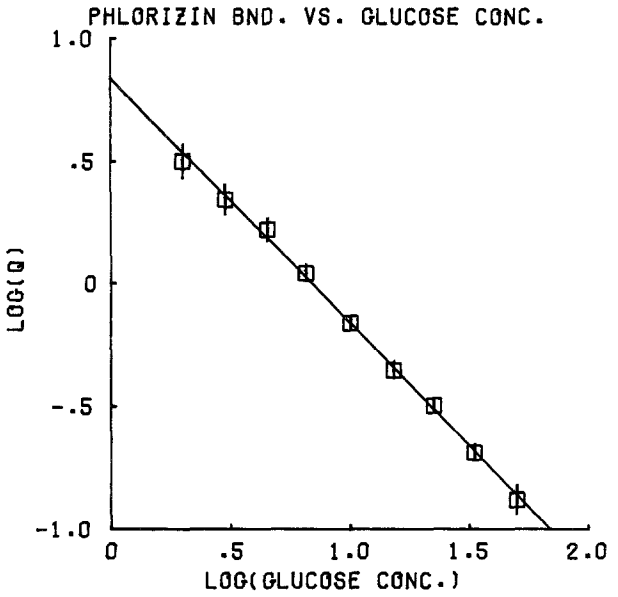

Fig. 1. The sodium-dependent component of equilibrium phlorizin binding measured as a function of glucose concentration (in mM). Vesicles were prepared in Buffer AK with 200 mM mannitol. The incubation media were Buffer AK containing 60 mM NaCl and 0.03 μM phlorizin (final concentrations) plus various concentrations of D-glucose. The glucose concentration range was 0–50 mM with mannitol replacing D-glucose isoosmotically. Binding was measured at 37 °C after 45 min of incubation. The quantity Q is given by $Q = B/(B_0 - B)$ where $B(B_0)$ is the sodium-dependent component of binding measured in the presence (absence) of glucose. The sodium-dependent component of phlorizin binding was obtained by subtracting away binding measured under identical conditions with choline replacing sodium. The least-squares fit to the data yields slope (stoichiometry) 1.00 ± 0.04 and x-intercept ($K_{0.5}$) 6.9 ± 0.7 mM, with $r = 0.999$

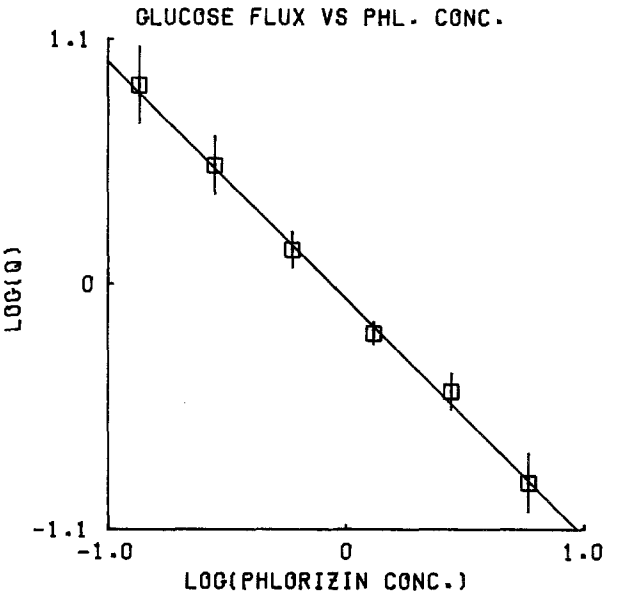

Fig. 2. The sodium-dependent equilibrium exchange flux of D-glucose measured as a function of phlorizin concentration (in μM). Vesicles were prepared in Buffer AK plus 100 mM mannitol, 60 mM NaCl or choline chloride, 1 mM D-glucose and various phlorizin concentrations (0–7.3 μM). The incubation medium was the same buffer containing tracer-labeled glucose. Uptake was measured after 2.5 sec of incubation at 20 °C. The quantity Q is given by $Q = F/(F_0 - F)$, where F is the sodium-dependent component of the initial flux rate at a given phlorizin concentration and F_0 is the same flux rate in the absence of phlorizin. No effect of phlorizin on glucose flux was observed in the absence of sodium. The least-squares fit to the data yields slope (stoichiometry) 1.06 ± 0.10 and x-intercept ($K_{0.5}$) 1.1 ± 0.1 μM with $r = 0.997$

zin binding in this preparation (Turner & Moran, 1981) the stoichiometry of interaction of phlorizin and glucose is given directly by the slope of the Hill plot (Segal, 1975). The slope of the least-squares fit to the points in Fig. 1 is 1.00 ± 0.04 (*see* Figure caption) indicating that phlorizin and glucose compete for the transporter in a 1:1 fashion.

The "sodium-dependent component" of phlorizin binding plotted in Fig. 1 is defined as the binding measured in the presence of sodium minus the binding measured in its absence (with choline replacing sodium). In fact this component of binding gives a slight underestimate of the total binding to the glucose carrier since a small amount of phlorizin binds to the transporter in the absence of sodium (Turner & Silverman, 1981). The magnitude of this error can be estimated from the K_d for phlorizin binding in the absence of sodium. We have measured the on and off constants for phlorizin binding to outer cortical BBM vesicles in the absence of sodium using techniques similar to those employed by Turner and Silverman (1981) in their Figs. 3 and 4. We find an on constant k_{on} of $0.0032\,(\text{sec}\,\mu\text{M})^{-1}$ and an off constant k_{off} of $0.221\,\text{sec}^{-1}$. For reasons discussed in Turner and Silverman (1981) these values represent an overestimate of the true k_{on} and an underestimate of the true k_{off} for the carrier. Thus $k_{on} < 0.0032\,(\text{sec}\,\mu\text{M})^{-1}$, $k_{off} > 0.221\,\text{sec}^{-1}$ and $K_d = k_{off}/k_{on} > 70$ μM. By contrast, $K_d = 0.2$ μM at 60 mM NaCl and 37 °C. Thus at 0.03 μM phlorizin and 60 mM NaCl (the experimental conditions of Fig. 1) the error associated with the above overcorrection for non-carrier related binding is $< 0.5\%$.

The Glucose/Phlorizin Stoichiometry of the Transporter

Figure 2 shows the results of an experiment complimentary to the one shown in Fig. 1. Here we have measured sodium-dependent glucose flux as a function of phlorizin concentration. The experiment was carried out at 20 °C under equilibrium exchange conditions. Fluxes were measured after 2.5 sec of incubation. In control experiments we have established that the influx of labeled tracer D-glucose added at time zero is linear with time for at least 4 sec under these

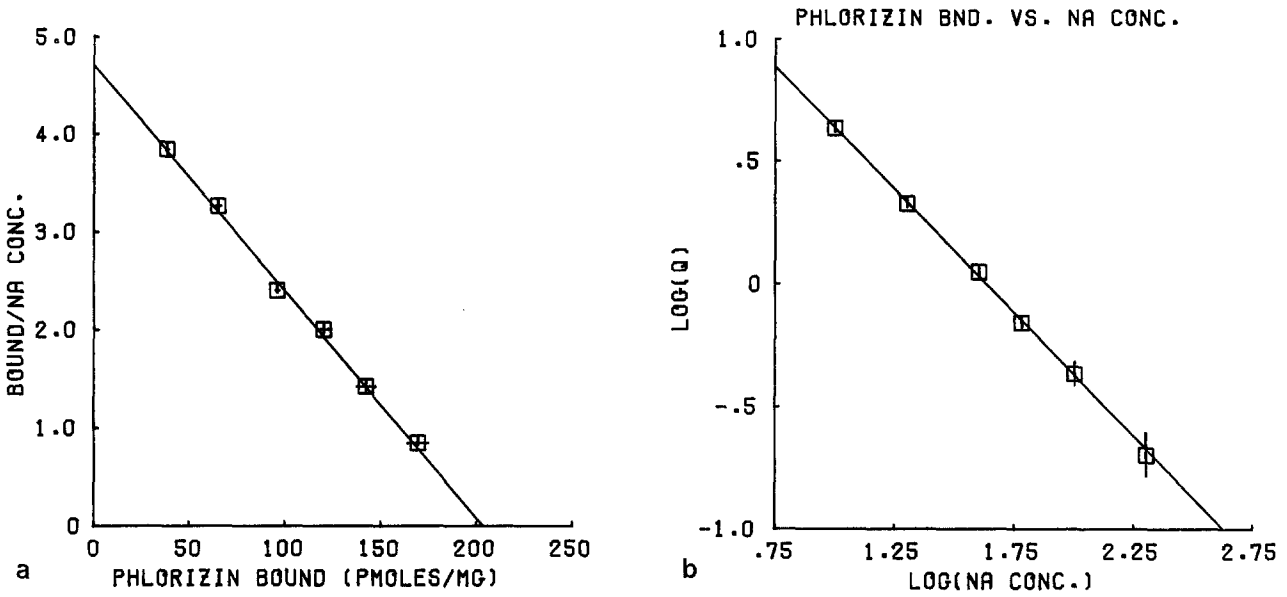

Fig. 3. The sodium-dependent component of phlorizin binding measured as a function of sodium concentration (in mM). Vesicles were prepared in Buffer AK containing 300 mM choline chloride. The incubation media were Buffer AK containing 1.4 μM phlorizin plus 0–200 mM NaCl (final concentrations) with choline replacing sodium isoosmotically. Binding was measured at 37 °C after 30 min of incubation. (*a*) Scatchard-type plot. The least-squares fit to the data yields slope ($K_{0.5}$) 43 ± 4 mM and *x*-intercept (B_∞, the binding at saturating sodium concentrations) 203 ± 11 pmol/mg with $r = 0.998$. (*b*) Log-log (Hill-type) plot. The quantity Q is given by $Q = (B_\infty - B)/B$ where B is the phlorizin bound at a given sodium concentration and B_∞ has been taken from the least-squares fit to Fig. 2*a*. The least-squares fit to Fig. 2*b* yields slope (stoichiometry) 1.01 ± 0.04 and *x*-intercept ($K_{0.5}$) 44 ± 7 mM with $r = 0.999$

conditions (*data not shown*). The slope of the log-log (Hill-type) plot of these data is 1.06 ± 0.10 again indicating a 1:1 stoichiometry.

The Phlorizin/Sodium Stoichiometry of the Transporter

It has been previously demonstrated in BBM vesicles prepared from the whole cortex of dog kidney that the phlorizin/sodium stoichiometry of the BBM D-glucose transporter is 1:1 (Turner & Silverman, 1981). This was shown by measuring equilibrium phlorizin binding as a function of sodium concentration and analyzing the resulting data on Scatchard- and Hill-type plots (*see below*). These plots were in fact found to be slightly curvilinear owing to the ability of phlorizin to bind to the transporter in the absence of sodium (Turner & Silverman, 1981). The results of a similar experiment in outer cortical BBM vesicles from the rabbit are shown in Fig. 3. Here, however, we have analyzed the data in a slightly modified way. Whereas Turner and Silverman (1981) explicitly determine and subtract away the nontransporter related binding we have again simply used the sodium-dependent component of binding as the basis for our analysis. As discussed above this component of binding is a good approximation to the actual binding to the transporter. Moreover, it can be easily demonstrated for carrier-type models where the phlorizin/sodium stoichiometry is 1:1 that this component of binding is expected to yield a Michaelis-Menten-type dependence on sodium concentration regardless of the ability of phlorizin to bind in the absence of sodium[2]. It is clear that the Scatchard-type plot of the phlorizin binding *vs.* sodium concentration data shown in Fig. 3*a* is linear in accord with the above prediction of the carrier model. That the results shown in Fig. 3*a* indicate a phlorizin/sodium stoichiometry of 1:1 can be seen more clearly in Fig. 3*b* were the data have been reanalyzed on a log-log (Hill-type) plot. The slope of this plot gives the stoichiometry of the phlorizin/sodium interaction directly. The slope of the least-squares fit to the data in Fig. 3*b* is 1.01 ± 0.04 indicating that phlorizin and sodium interact with the transporter in a 1:1 fashion.

The Glucose/Sodium Stoichiometry of the Transporter

In this subsection we present the results of three independent methods for determining the glucose/sodi-

[2] In carrier models of cotransport which allow a random 1:1 binding of inhibitor (phlorizin) and activator (sodium) the expression for total binding to the carrier can be written in the form $(a + bA)/(c + dA)$ where a, b, c and d are constants depending on the parameters of the model and A is the activator concentration (Turner & Silverman, 1980, 1981). Thus the "activator-dependent" component of binding (binding in the presence of activator minus binding in its absence) is given by $(b - da/c)A/(c + dA)$ which has Michaelis-Menten form. A similar result can be obtained for the activator-dependent component of substrate flux for rapid equilibrium carrier models (Turner, 1981).

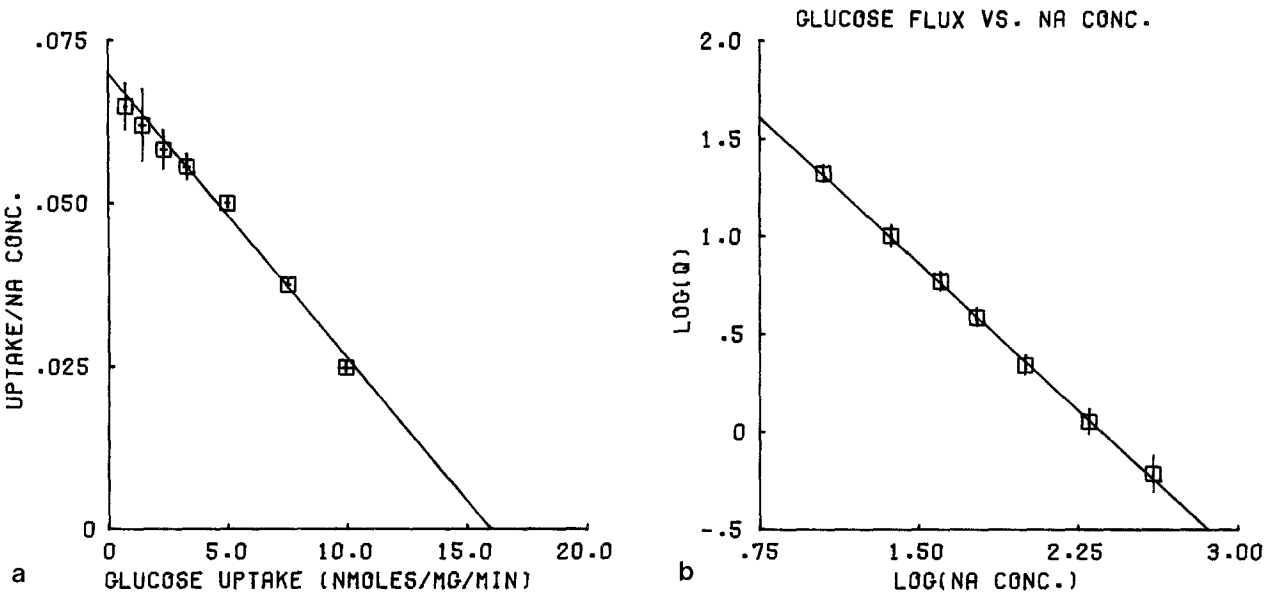

Fig. 4. The Activation Method. The stereospecific sodium-dependent component of initial D-glucose flux measured as a function of sodium concentration (in mM). Vesicles were prepared in Buffer AK with 200 mM mannitol plus valinomycin. The incubation media were Buffer AK containing 1 mM D-glucose plus 0–400 mM NaCl (final concentrations) with choline replacing sodium isoosmotically. Uptake was measured after 3 sec of incubation at 17 °C. (*a*) Scatchard-type plot. The least-squares fit to the data yields slope ($K_{0.5}$) 228 ± 26 mM and *x*-intercept (F_∞, the initial flux at saturating sodium concentrations) 16.0 ± 1.2 nmol/mg protein/min with $r = 0.993$. (*b*) Log-log (Hill-type) plot. The quantity Q is given by $Q = (F_\infty - F)/F$ where F is the initial flux rate at a given sodium concentration and F_∞ has been taken from the least-squares fit to Fig. 4*a*. The least-squares fit to Fig. 4*b* yields slope (stoichiometry) 1.00 ± 0.03 and *x*-intercept ($K_{0.5}$) 228 ± 35 mM with $r = 0.999$

um stoichiometry of the outer cortical BBM D-glucose transporter.

The Activation Method. In this method one measures the stimulation of substrate (glucose) flux by increasing concentrations of activator (sodium); it is analogous to the method of measuring the phlorizin/sodium stoichiometry shown in Fig. 2. Figure 4 shows the results of an experiment where the initial flux of 1 mM D-glucose was measured as a function of sodium concentration over the range 0 to 400 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 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*).

The quantity plotted in Fig. 4*a* is the sodium-dependent component of flux, i.e. the flux observed in the presence of sodium minus the flux observed in its absence. In analogy to the case of inhibitor binding discussed above it can be shown that for carrier-type models where the glucose/sodium stoichiometry is 1:1 this component of flux is expected to show a Michaelis-Menten-type dependence on sodium concentration regardless of the ability of the carrier to transport glucose in the absence of sodium (footnote 2 and Turner, 1981). It is clear that the Scatchard-type plot of the data shown in Fig. 4*a* is linear in accord with this prediction. The data have been reanalyzed in Fig. 4*b* on a log-log (Hill-type) plot. The slope of the least-squares fit to these points which gives the glucose/sodium stoichiometry is 1.00 ± 0.03, again indicating a 1:1 interaction.

Further discussion of this method of measuring stoichiometry will be given later in the paper, however, it is useful at this point to mention that the Activation Method does not distinguish between "energetic" and "catalytic" coupling mechanisms. In other words, the data shown in Fig. 4 indicate that one sodium ion is involved per glucose transport event but it does not indicate whether the stimulation produced by sodium is a result of its being cotransported with glucose (energetic coupling) or its interacting with the carrier in some other way which leads to a stimulation of glucose flux without concommitant sodium transport (catalytic coupling).

The Direct Method. The Direct Method of measuring stoichiometry relies on the actual determination and comparison of the activator-dependent substrate flux and the substrate-dependent activator flux. The results of such an experiment using ^{14}C – D-glucose and ^{22}Na in outer cortical BBM vesicles are shown in Fig. 5. It is clear from the Figure that the fluxes of glucose and sodium in this preparation are coupled in a 1:1 fashion.

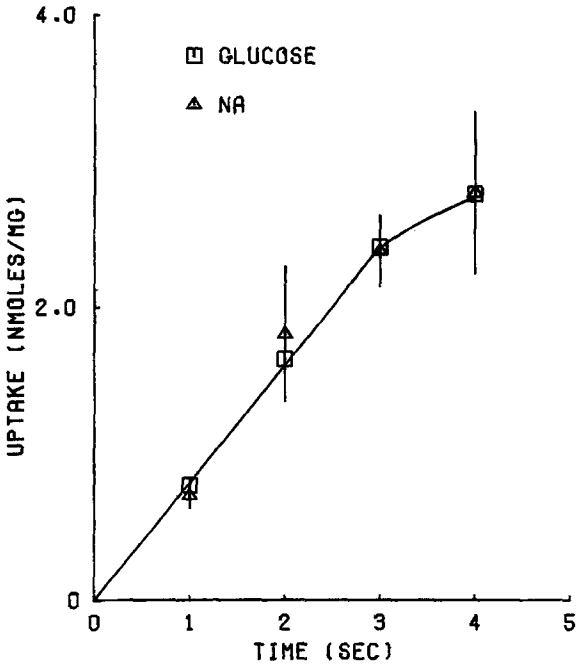

Fig. 5. The Direct Method. Comparison of the sodium-dependent stereospecific D-glucose flux and the glucose-dependent sodium flux in outer cortical BBM vesicles. Vesicles were prepared in Buffer AK with 200 mM mannitol plus valinomycin. The incubation media were Buffer AK containing 30 mM NaCl plus 7.5 mM D-glucose (*A*), 30 mM NaCl plus 7.5 mM mannitol (*B*) or 30 mM choline chloride plus 7.5 mM D-glucose (*C*); all are final concentrations. Sodium-22 fluxes were measured with incubation media *A* and *B* and glucose fluxes with incubation media *A* and *C*. The glucose-dependent sodium flux and the sodium-dependent glucose flux were calculated by subtraction. The experiment was carried out at 28 °C. Note that the fluxes are linear within experimental error for 3 sec

Although the direct method of measuring stoichiometry has an advantage over the activation method in that it allows one to demonstrate the direct and therefore energetic coupling of substrate and activator fluxes it suffers from a potentially serious practical limitation. Briefly stated, the problem is that in order to simultaneously stimulate glucose and sodium fluxes via the transporter above the non-BBM-transporter related background one is restricted (in our experience) to a somewhat narrow range of glucose (>5 mM, <20 mM) and sodium (>10 mM, <100 mM) concentrations. Fortunately the outer cortical BBM D-glucose transporter has a sufficiently high capacity that these measurements can be carried out with some degree of accuracy albeit over this somewhat restricted range of concentrations. However, it is easy to see that similar measurements on other lower capacity transport systems might be impossible owing to low signal-to-noise ratios.

The Static Head Method. We introduce the Static Head Method as a possible means of circumventing some of the limitations of the Direct Method discussed above. The Static Head Method is based on the following argument. Consider a tightly coupled transport system where n activator (A) ions are transported per substrate (S) molecule. (By "tightly coupled" transport we mean that translocation of either substrate or activator in the absence of the other is not possible via the carrier, i.e., that the partially loaded forms of the carrier (carrier plus substrate and carrier plus activator) are immobile. Complications arising from nontightly coupled systems are considered 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

$$\ln(S_i/S_o) = n[\ln(A_o/A_i) + F\Delta\psi/RT] \quad (1)$$

where $\Delta\psi$ is the transmembrane potential and F, R and T have their usual thermodynamic interpretation. We refer to the situation where Eq. (1) holds as a "static head" condition (we have taken the term "static head" from the work of Kedem and Caplan (1965)). Simply stated, the Static Head Method for determining stoichiometry consists of establishing a fixed substrate (activator) transmembrane gradient and then searching for an activator (substrate) gradient which will balance it, i.e., to search for static head conditions. Once this is achieved the stoichiometry can be calculated from the known activator and substrate gradients using Eq. (1). Practical details are given below.

We restrict ourselves here to experimental conditions where $\Delta\psi = 0$ (we also exclude the trivial case of activator and substrate equilibrium). Although under "static head" conditions there is no net transport via the carrier, in any real system there will always be fluxes occurring via other unrelated pathways. Thus the general procedure for the static head experiment as applied here is: 1) to pre-equilibrate vesicles with given glucose and sodium concentrations, 2) to dilute an aliquot of these vesicles 1:6 into appropriate glucose-free media thus establishing an intravesicular-to-extravesicular glucose gradient of 6:1, and 3) to measure the glucose retained in the 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.

The results of a static head experiment on outer cortical BBM vesicles is shown in Fig. 6. It is clear

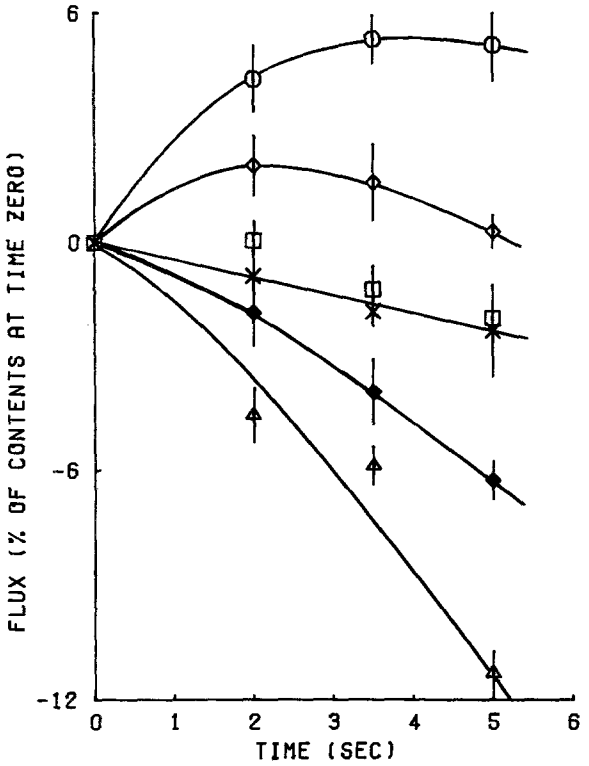

Fig. 6. The Static Head Method. Vesicles were prepared in Buffer AK containing 0.5 mM glucose, valinomycin and 20 mM NaCl plus 180 mM choline chloride (test) or 200 mM choline chloride (control). The incubation media for the "test" points were Buffer AK containing sufficient NaCl to give final extravesicular concentrations of 148 mM ($n=0.90$, ○), 132 mM ($n=0,95$, ◇), 120 mM ($n=1.00$, □), 104 mM ($n=1.09$, ◆) or 91 mM ($n=1.18$, △) and sufficient choline chloride to give a total salt concentration of 200 mM. The n values given above are the stoichiometries which would be predicted [*cf.* Eq. (1)] if that sodium concentration were to result in static head conditions (*see text*). The incubation medium for the "control" points (x) was Buffer AK containing 200 mM choline chloride. The experiment was carried out at 28 °C; 100 µl of incubation medium was added to 20 µl of vesicles. The stereospecific efflux (or influx) of D-glucose has been expressed as a percent of the total (equilibrium) intravesicular glucose at time zero (0.6 nmol/mg protein). Standard errors rather than standard deviations have been illustrated in this Figure for clarity. With the exception of the 120 mM NaCl points and the first 104 mM point all of the measurements are statistically significantly different from the control ($p<0.05$). Thus the experiment is consistent with a sodium/glucose stoichiometry of 1:1 with an (estimated) tolerance of ±0.04

from the data that stoichiometry of the transporter as measured by this method is 1:1 within experimental error (*see* Figure caption).

Discussion

This paper presents a series of experiments which demonstrates that the outer cortical BBM D-glucose transporter possesses a phlorizin/glucose/sodium stoichiometry of 1:1:1. The interpretation of all these results is necessarily somewhat concept and/or model dependent. A brief discussion of these points is included below for each experiment.

The interpretation of Figs. 1 and 2 (the determinations of the phlorizin/glucose and glucose/phlorizin stoichiometries) rests on classical enzyme kinetics (Segal, 1975). In fact the Hill plot yields the stoichiometry of a substrate/inhibitor interaction regardless of the type of inhibition (competitive, noncompetitive or uncompetitive).

In Fig. 3 we confirm the measurement of the phlorizin/sodium stoichiometry carried out by Turner and Silverman (1981) on BBM vesicles prepared from the whole cortex of the dog. The analysis of this experiment relies on the assumption that the dependence of binding on activator concentration is of the form $(a+bA)/(c+dA)$ for 1:1 systems (*see* footnote 2). This result holds rigorously for carrier-type models of cotransport (Turner & Silverman, 1980, 1981) and is almost certainly more general; it is difficult to imagine a 1:1 inhibitor/activator binding scheme which would result in the introduction of higher powers of A into this expression.

The analysis of the determination of the sodium/glucose stoichiometry by the Activation Method (Fig. 4) rests on similar assumptions to those just discussed in the preceding paragraph. Again, this treatment holds rigorously for rapid equilibrium carrier-type models (Turner, 1981). Similar activation experiments have been attempted several times in the past (Kinne, Murer, Kinne-Saffran, Thees & Sachs, 1975; Turner & Silverman, 1977, 1978). However, in these early experiments it is not clear that true initial transport rates were being measured or that membrane potentials were being adequately controlled (Turner & Moran, 1982). In one instance a non-Michaelis-Menton type dependence on sodium concentration was found (Turner & Silverman, 1978). This may well have been due to the presence of multiple sodium-dependent glucose transport sites in this BBM vesicle preparation (Turner & Moran, 1982). As mentioned earlier the Activation Method does not distinguish between energetic and catalytic coupling. Thus taken together with the results of a Direct or Static Head experiment the Activation Method provides a powerful tool for evaluating the role or roles of the activator in coupled transport systems.

Note also that the $K_{0.5}$ values reported in the captions of Figs. 3 and 4 are the apparent affinities of the transporter for sodium under the experimental conditions stated. In general, these apparent affinities are expected to be functions of the phlorizin (Turner & Silverman, 1981) or glucose (Turner, 1981) concentrations used and thus cannot be compared to one another directly.

The interpretation of the Direct stoichiometry experiment (Fig. 5) can be complicated by more complex behavior of the transporter. For example, in a nontightly coupled system (*see above*) it is possible for the carrier-sodium complex to recycle repeatedly across the membrane so that one sodium ion acts to catalyze the transport of several glucose molecules. This type of behavior would lead to an underestimate of the number of sodium ions translocated per glucose molecule. The activation method does not suffer from this potential problem. Thus the combination of these two experiments strongly supports a 1:1 tightly coupled sodium/glucose cotransport system.

The Static Head Method is based on thermodynamic arguments. However, in our derivation of the static head condition [Eq. (1)] we have assumed a tightly coupled transport system. In nontightly coupled systems Eq. (1) becomes quite complex even for simple cotransport models (R.J. Turner, *unpublished observations*). This is due to the complications of "internal leaks", i.e., uncoupled fluxes of activator and/or substrate via the transporter. Note that "external leaks", i.e., fluxes not related to the sodium-dependent carrier, do not affect the reliability of the technique since these are corrected for by the control run carried out in the absence of sodium. Since both the Direct and Static Head Methods give a 1:1 stoichiometry in agreement with the Activation Method we conclude that there is little if any transport of the partially loaded carrier species. The Static Head Method like the Direct Method provides a direct confirmation of Crane's gradient hypothesis (Crane, 1977).

We would like to thank Drs. Maurice Burg and Joseph Handler for continuing discussions and encouragement as well as for critical readings of the manuscript. We also thank Janet N. George and Douglas Tang for their technical assistance.

References

Aronson, P.S. 1981. Identifying secondary active transport in epithelia. *Am. J. Physiol.* **240**:F1–F11

Beck, J., Sacktor, B. 1978. The sodium electrochemical potential mediated transport of D-glucose in renal brush border membrane vesicles. *J. Biol. Chem.* **253**:5531–5535

Barfuss, D.W., Schafer, J.A. 1981. Differences in active and passive glucose transport along the proximal nephron. *Am. J. Physiol.* **240**:F322–F332

Crane, R.K. 1977. The gradient hypothesis and other models of carrier mediated active transport. *Rev. Physiol. Biochem. Pharmacol.* **78**:99–159

Goldner, A.M., Schultz, S.G., Curran, P.F. 1969. Sodium and sugar fluxes across the mucosal border of rabbit ileum. *J. Gen. Physiol.* **53**:362–383

Hopfer, U., Groseclose, R. 1980. The mechanism of sodium dependent D-glucose transport. *J. Biol. Chem.* **255**:4453–4462

Kedem, O., Caplan, S.R. 1965. Degree of coupling and its relation to efficiency of energy conversion. *Trans. Faraday Soc.* **61**:1897–1911

Kimmich, G. 1981. Gradient coupling in isolated intestinal cells. *Fed. Proc.* **40**:2474–2479

Kimmich, G., Randles, J. 1980. Evidence for an intestinal Na:sugar transport coupling stoichiometry of 2.0. *Biochim. Biophys. Acta* **596**:439–444

Kinne, R., Murer, H., Kinne-Saffran, E., Thees, M., Sachs, G. 1975. Sugar transport by renal plasma membrane vesicles. *J. Membrane Biol.* **21**:375–395

Okada, Y. 1979. Solute transport process in intestinal epithelial cells. *Membr. Biochem.* **2**:339–365

Segel, I.H. 1975. Enzyme Kinetics. John Wiley and Sons, New York

Silverman, M. 1981. Glucose reabsorption in the kidney. *Can. J. Physiol. Pharmacol.* **59**:209–224

Turner, R.J. 1981. Kinetic analysis of a family of cotransport models. *Biochim. Biophys. Acta* **649**:269–280

Turner, R.J., Moran, A. 1981. Heterogeneity of glucose transport along the proximal tubule – Evidence from vesicle studies. *Fed. Proc.* **40**:371 (*Abstr.*)

Turner, R.J., Moran, A. 1982. Heterogeneity of sodium dependent D-glucose transport sites along the proximal tubule: Evidence from vesicle studies. *Am. J. Physiol.* (*in press*)

Turner, R.J., Silverman, M. 1977. Sugar uptake into normal human renal brush border membrane vesicles. *Proc. Natl. Acad. Sci. USA* **75**:2825–2829

Turner, R.J., Silverman, M. 1978. Sugar uptake into brush border vesicles from dog kidney. II. Kinetics. *Biochim. Biophys. Acta* **511**:470–486

Turner, R.J., Silverman, M. 1980. Testing carrier models of cotransport using the binding kinetics of non-transported competitive inhibitors. *Biochim. Biophys. Acta* **596**:272–291

Turner, R.J., Silverman, M. 1981. Interaction of phlorizin and sodium with the renal brush border membrane D-glucose transporter: Stoichiometry and order of binding. *J. Membrane Biol.* **58**:43–55

Received 5 October 1981; revised 4 December 1981