J. Membrane Biol. 58, 43–55 (1981)

The Journal of
Membrane Biology

Interaction of Phlorizin and Sodium with the Renal Brush-Border Membrane D-Glucose Transporter: Stoichiometry and Order of Binding

R. James Turner* and M. Silverman

Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20205 and Department of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A3

Summary. The order and stoichiometry of the binding of phlorizin and sodium to the renal brush-border membrane D-glucose transporter are studied. The experimental results are consistent with a random-binding scheme in which the ratio of phlorizin- to sodium-binding sites is one-to-one. When the kinetics of phlorizin binding are measured as a function of increasing sodium concentration no significant variation is found in the apparent number of binding sites; however, the apparent binding constant for phlorizin decreases rapidly from approximately 16 μM at [Na] $=0$ to 0.1 μM at [Na] $=100$ mM and approaches 0.05 μM as [Na] $\rightarrow \infty$. The experimental data are fit to a random carrier-type model of the coupled transport of sodium and D-glucose. A complete parameterization of the phlorizin binding properties of this model under sodium equilibrium conditions is given.

There is now a great deal of experimental evidence indicating that the transport of D-glucose across the renal proximal tubule brush-border membrane (BBM) occurs via a sodium coupled cotransport mechanism (Silverman & Turner, 1979). It has also been established that this transporter is competitively inhibited by the agent phlorizin which apparently competes with D-glucose for its binding site on the carrier but is not itself translocated (Stirling, 1967; Silverman, 1974).

A high-affinity, sodium-dependent, glucose-sensitive phlorizin-binding site with other properties consistent with those of the BBM D-glucose transporter has been observed in intact brush-border membrane fragments (Frasch, Frohnert, Bode, Baumann & Kinne, 1970; Bode, Baumann & Diedrich, 1972; Glossmann & Neville, 1972; Chesney, Sacktor & Kleinzeller, 1974; Silverman & Black, 1975) and more recently in brush-border vesicles (Aronson, 1978; Turner & Silverman, 1978*a*). Indeed both specificity and kinetic studies indicate that the BBM D-glucose transporter is preserved in a reasonably intact form, complete with its phlorizin-binding site, in brush-border vesicle preparations (Turner & Silverman, 1978*a*, *b*).

In a recent theoretical paper (Turner & Silverman, 1980) we have demonstrated that the binding properties of a nontransported competitive inhibitor such as phlorizin may be used to distinguish between and characterize various possible models of a coupled transport process. The advantage of studying inhibitor binding rather than substrate transport lies in the less complex interaction of the inhibitor with the carrier (binding for the inhibitor *vs.* association, translocation and dissociation for the substrate). This simplification is reflected in the kinetic equations describing the binding process. Although there are some limitations on what can be learned about the transporter from inhibitor binding studies we have shown that many features which are characteristic of the transport mechanism are retained in the kinetics of the binding events (Turner & Silverman, 1980).

In this paper we study the phlorizin-binding properties of the BBM D-glucose transporter in some detail using a versicle preparation from dog kidney proximal tubule. In particular we examine the order and stoichiometry of binding of phlorizin and sodium to the carrier. Our data suggest that phlorizin and sodium bind to the transporter in a random rather than an ordered fashion and that the ratio of phlorizin- to sodium-binding sites is one-to-one. The implications of these results for the glucose transporter are discussed and applications of some of our earlier theoretical results to the elucidation of the transport mechanism are given.

* *Address for reprint requests:* Building 10, Room 6N320, National Institutes of Health, Bethesda, Maryland 20205.

0022-2631/81/0058-0043 $02.60

Materials and Methods

Preparation of Brush-Border Membrane Vesicles

Dog renal brush-border membrane vesicles were prepared using the following procedure based on our previously published method (Turner & Silverman, 1978*a*) and the report of Malathi, Preiser, Fairclough, Mallett and Crane (1979). Unless otherwise noted all steps were carried out at 4 °C.

The renal cortex was removed by scraping with a razor blade, suspended in "isolation medium" (10 mM triethanolamine-HCl, 250 mM sucrose, pH 7.6 using NaOH) to a final dilution of 1 g cortex/10 ml and homogenized (5 strokes) using a tight-fitting Potter-Elvehjem tissue grinder (Kontes K886000). The resulting "initial homogenate" was centrifuged twice for 10 min at 190 × *g* and the pellets were discarded. The supernatant was centrifuged for 20 min at 16,000 × *g* and the resulting pellet was resuspended in fresh isolation medium (approximately 1.3 ml/g original cortex). This "crude membrane fraction" was then divided into 2-ml aliquots, snap frozen in ethanol and dry ice and stored above liquid nitrogen.

On the day of the binding experiment a suitable number of aliquots of crude membrane fraction were thawed at room temperature and diluted to 40 ml/aliquot with ice cold Buffer A (10 mM Tris-HEPES[1], 100 mM D-mannitol, pH 7.4) containing 10 mM $CaCl_2$. The diluted crude membrane fraction was left on ice for 10 min then centrifuged for 15 min at 3000 × *g*. The supernatant from this spin was centrifuged for 20 min at 43,000 × *g*. The resulting pellet was resuspended in a few ml of an appropriate buffer for the experiment (*see below*), and recentrifuged for 20 min at 43,000 × *g*. The final pellet was resuspended in an appropriate buffer and passed once through a 25 gauge needle and twice through a 30 gauge needle. This "final vesicle fraction" was then incubated at 37 °C for approximately 30 min and stored on ice until used.

The "appropriate buffers" in which the vesicles were prepared as described above were typically Buffer A containing sodium and/or potassium salts in the same concentration found in the incubation media to which the vesicles were later exposed. The detailed compositions of the media for each experiment are given in the Figure captions.

All of the data presented here were obtained using tissue from NIH foxhounds; however, similar results were obtained in preliminary studies using our previous vesicle preparation procedure (Turner & Silverman, 1978*a*) and tissue from mongrel dogs.

Criteria of Purity

The purity of the final vesicle fraction was monitored by assaying the activity of enzymes known to be characteristic of brush-border microvilli (alkaline phosphatase), basal lateral membranes (Na^+, K^+-ATPase), mitochondria (succinic dehydrogenase) and endoplasmic reticulum (glucose-6-phosphatase).

Alkaline phosphatase was measured at pH 10.5 in a buffer containing 45 mM glycine, 2 mM $MgCl_2$, 2 mM $ZnSO_4$ and 6 mM *p*-nitrophenylphosphate as substrate. ATPase was measured at pH 7.5 in the presence of 40 mM Tris-HCl, 130 mM NaCl, 20 mM KCl, 3.33 mM $MgCl_2$ and 3.33 mM Na_2 ATP. The ATPase activity inhibited by 2.5 mM ouabain was taken to be that due to Na^+, K^+-ATPase. Succinic dehydrogenase and glucose-6-phosphatase were determined using the methods of Pennington (1961) and Baginski, Foa and Zak (1974), respectively. In the procedure of Baginski et al., however, 25 mM cacodylate buffer was replaced by 30 mM Tris-MES[2], pH 6.5. Inorganic phosphate was also measured according to Baginski et al. (1974). Protein was mesured by the method of Bradford (1976) using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine gamma globulin as the standard.

Binding Measurements

Unless otherwise noted the binding of phlorizin to BBM vesicles was measured using the following procedure. A 50-μliter aliquot of vesicles (containing approx. 1 mg protein/ml) was preequilibrated at 37 °C in a 12 × 75 mm glass test tube. At time zero a 100-μliter aliquot of incubation medium also at 37 °C and containing [^{3}H]-phlorizin and other constituents as required was added. The resulting mixture was kept at 37 °C for an appropriate time after which the reaction was terminated by the addition of a 10-fold dilution of ice-cold stop solution (*see below*). The vesicles were immediately applied to a millipore filter (HAWP 0.45 μ) under light suction which was then washed with a further 5.4 ml of stop solution. The filter, which retained the BBM vesicles, was then dissolved in liquid scintillation fluid and counted along with samples of the incubation medium and appropriate standards.

The stop solution was 10 mM Tris-HEPES, pH 7.4, containing an appropriate concentration of NaCl and unlabeled phlorizin; details for each experiment are given in figure captions. In control experiments in which the time between addition of the stop solution and filtration was prolonged we have established that less than 5% of bound phlorizin is lost during the stopping and washing procedure. Since the reproducibility of our filtration technique is 3–5% we have not attempted to make any correction for this effect.

The results shown in Fig. 4 were obtained using a fast sampling apparatus which will be described in detail in a future publication. Briefly, the apparatus consists of a pneumatic pump (Hamilton, 77002) controlled by a photographic timer which automatically dispenses the stop solution after the required period of incubation.

Unless otherwise noted all experiments were carried out in triplicate. The error bars shown in the figures are the standard deviations on the points. If no error bars are shown the standard deviations are smaller than the symbols. The results of representative experiments are shown.

The sodium concentration of all incubation and preincubation media was routinely checked using a Model 143 Flame Photometer (Instrumentation Laboratory Inc., Boston, Mass.).

Chromatography

The purity of [^{3}H]-phlorizin (>96%) was regularly verified by thin-layer chromatography using the solvent system chloroform methanol/water (65:25:4, v/v). The solvent system *n*-butanol/ethanol/water (50:32:18, v/v) was used to chromatograph monosaccarides.

Calculations

Phlorizin concentrations in the incubation media and the amount of phlorizin bound to the BBM vesicles were calculated using the known specific activity of [^{3}H]-phlorizin. The calculated phlorizin concentrations were corrected for the phlorizin bound to the

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

[2] 30 mM Tris-MES: 30 mM MES (2-(N-morpholino)ethanesulfonic acid) buffered with Tris to pH 6.5.

vesicles to obtain the "free" phlorizin concentration in the final incubation mixture.[3] In experiments where it was desirable to compare binding at equal phlorizin concentrations results were normalized to correct for changes in free phlorizin concentration resulting from binding to the membranes.

In least-squares fits the data points were weighted in proportion to the inverse of their relative experiment errors. Nonlinear least-squares fits were carried out using the nonlinear regression routine "P3R" (BMD, Biomedical Computer Programs, University of California, Los Angeles). The errors quoted in the text on the least-squares parameters are the 95% confidence intervals.

Materials

[^{3}H]-phlorizin was obtained from New England Nuclear Corp. (Boston, Mass.). Unlabeled phlorizin and all monosaccarides were from Sigma Chemical Co. (St. Louis, Mo.). Other chemicals were of highest purity available from commercial sources.

Results

Enzymatic Characterization of the Membranes

Relative to the initial homogenate the final vesicle fraction described here typically shows a 12- to 13-fold enrichment in alkaline phosphatase activity, a two-fold reduction in Na^+, K^+-ATPase activity, no change in glucose-6-phosphatase activity and no detectable succinic dehydrogenase activity. This preparation represents a considerable improvement over our previous BBM vesicle fraction (Turner & Silverman, 1978*a*) particularly with regard to antiluminal membrane contamination.

General Observations Concerning Phlorizin Binding to BBM Vesicles

In general we find that the rate of phlorizin binding to BBM vesicles varies with experimental conditions; however, for all situations considered here the half-time for binding is $\leq$20 sec. Accordingly all *equilibrium* binding measurements were carried out after 6 min of incubation to ensure for complete equilibration of phlorizin with its receptor.

Fig. 1 shows a Scatchard plot of equilibrium phlorizin binding to BBM vesicles in presence of 100 mM NaCl over the concentration range 0.04 to 37 μM. The data have been corrected for unspecific binding as

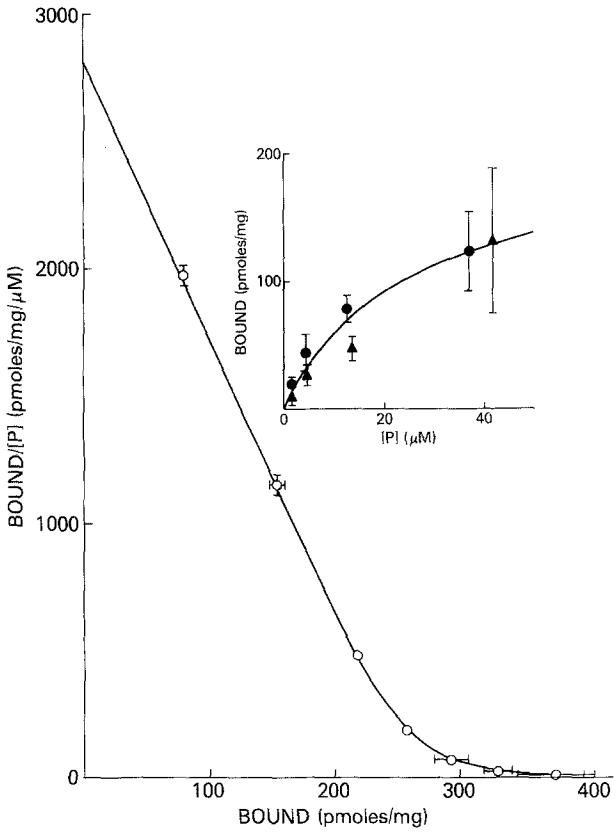

Fig. 1. Scatchard plot of equilibrium phlorizin binding as a function of concentration ([P] = 0.04 to 37 μM). Vesicles were prepared in Buffer A containing 100 mM NaCl. The incubation medium was the same buffer with appropriate concentrations of labeled phlorizin. The stop solution was 10 mM Tris-HEPES containing 200 mM NaCl. The data have been corrected for unspecific binding as described in the text. The results shown in the inset represent the low-affinity (high [P]) region of the plot and were obtained as described in the text. The points were determined in triplicate except for those representing the two highest phlorizin concentrations which were determined in sextuplicate

measured in the presence of 2.5 mM unlabeled phlorizin. Unspecific binding measured in this way is consistently found to be a linear function of concentration in our preparation. For the experiment of Fig. 1 this correction was given by:

$$\text{unspecific binding} = (10.1 \pm 0.7)\,[P]\ \text{pmoles/mg protein} \quad (1)$$

where [P] is the concentration of labeled phlorizin in μmoles/liter. The Scatchard plot clearly shows the existence of at least two components of phlorizin binding, a high affinity component which is the site previously associated with the BBM D-glucose transporter (Silverman & Black, 1975; Turner & Silverman, 1978*a*) and a much lower affinity site (or sites) to be discussed below. Fitting the data by the method

[3] This correction was not taken into account in our earlier report of phlorizin binding to BBM vesicles where we estimated that the apparent K_d for phlorizin binding to the BBM D-glucose transporter was $\approx$0.3 μM at 37 °C and 100 mM NaCl. This omission together with the scatter of our previous results and slightly different experimental conditions account for the discrepancy between this value and the value of 0.1 μM which we report here.

of least-squares to the sum of two Michaelis-Menten type terms we find that the high affinity component is characterized by 258 ± 17 pmoles of binding sites/mg protein and an apparent binding constant, $K_d^{app} = 0.093 \pm 0.009$ μM.

We have found that the low affinity site(s) cannot be reliably or reproducibly fit by nonlinear regression techniques. This is primarily due to the relatively large uncertainties on the experimental points at high phlorizin concentrations. In the inset to Fig. 1 we have plotted this component of binding versus concentration. The data have been obtained by subtracting away the high-affinity component (as characterized by the parameters derived from the nonlinear least-squares fit) from the total binding shown in the figure proper. Results from two experiments have been combined. The curve drawn through the points was obtained by trial and error. It is of Michaelis-Menten form and is given by:

$$\text{low affinity binding} \simeq \frac{210[P]}{26+[P]} \text{ pmole/mg protein} \quad (2a)$$

$$\simeq 8.1[P] \text{ pmoles/mg protien} \quad (2b)$$
$$\text{for } [P] \ll 26\ \mu M.$$

At the present time neither the origin nor the significance of this low-affinity binding is clear; however, it is obvious that at phlorizin concentrations ~ 1 μM where proximal tubule D-glucose transport is strongly inhibited *in vivo* (Silverman, 1976) relatively little binding would occur at this site.

In the kinetic studies of the high-affinity site presented later in this paper it was desirable to correct for both unspecific and low-affinity binding. However, it was impractical to carry out a complete kinetic curve such as the one shown in Fig. 1 in each case. Hence the following procedure was employed. Unspecific binding in the presence of 2.5 mM unlabeled phlorizin was measured as described above. It was then assumed that the low-affinity component of binding was approximately equal to 80% of the unspecific binding [cf. Eqs. (1) and (2b)]. Thus the total correction for unspecific plus low-affinity binding was given by 1.8 times the unspecific binding. In most cases this correction represents a very small fraction of the total binding observed.

Order of Binding of Phlorizin and Sodium

In Fig. 2 we illustrate three possible schemes for the sequence of binding of phlorizin and sodium to their respective sites on the BBM D-glucose transporter. In the AS and SA Schemes the binding is ordered with

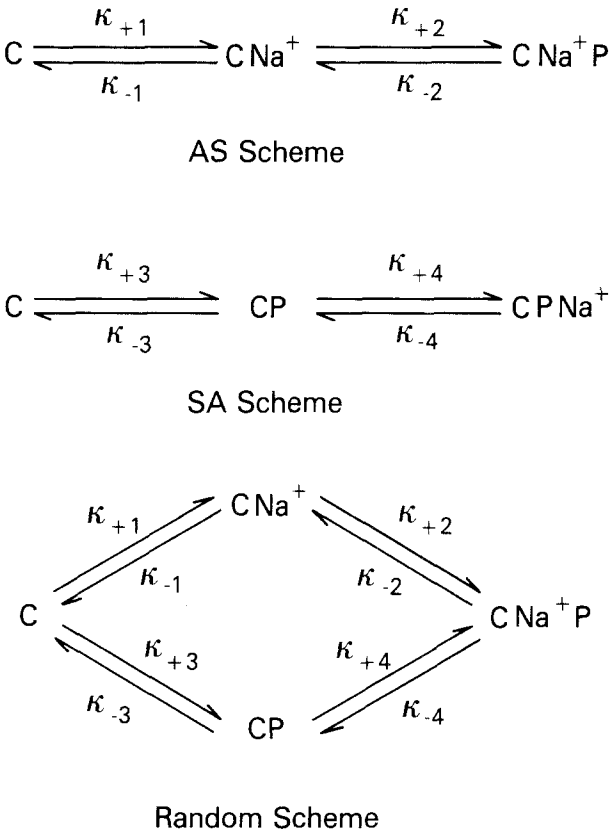

Fig. 2. Three possible representations of the binding sequence of phlorizin (P) and sodium (Na^+) to the BBM D-glucose carrier (C)

Na^+ necessarily preceding P in the former and P necessarily preceding Na^+ in the latter. In the Random Scheme either order of binding is allowed. The rate constants for the various on and off reactions are denoted by $\kappa_{\pm n}$ ($n = 1, 2, 3, 4$). The following series of experiments indicate that neither the AS nor the SA scheme is an adequate representation of phorizin binding to BBM vesicles.

Evidence Against the AS Scheme. In Fig. 3 we examine the rate of dissociation of phlorizin from vesicles preequilibrated with 0.036 μM [^{3}H]-phlorizin in the presence of 100 mM NaCl and subsequently diluted 1:100 into [^{3}H]-phlorizin-free media with and without sodium. From the figure we calculate that the dissociation rate constants for phlorizin at 100 and 1 mM NaCl are 6.0×10^{-3} sec^{-1} and 3.1×10^{-2} sec^{-1}, respectively. Control runs in which 100 μM unlabeled phlorizin was added to the dilution medium superimpose with the experimental points and thus rule out the possibility that the slower dissociation rate observed in the presence of 100 mM sodium is due to rebinding of labeled phlorizin.

In additional experiments (data not shown) we

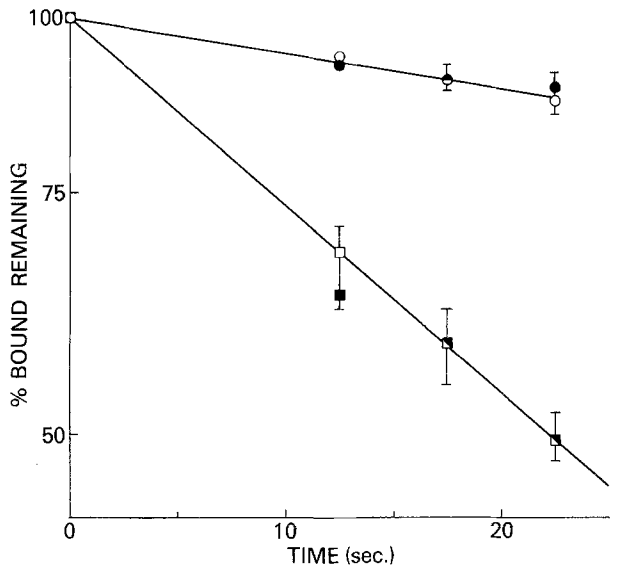

Fig. 3. Dissociation of high-affinity phlorizin binding in the presence of 100 mM and 1 mM NaCl. Vesicles were prepared in Buffer A containing 100 mM NaCl then preequilibrated at 37 °C in the same buffer with 0.036 μM [^{3}H]-phlorizin. At time zero 15 μliters of preequilibrated vesicles were diluted 100-fold into Buffer A plus 100 mM NaCl (○), Buffer A plus 100 mM NaCl and 100 μM unlabeled phlorizin (●), Buffer A plus 100 mM KCl (□) or Buffer A plus 100 mM KCl and 100 μM phlorizin (■) all at 37 °C. At the times indicated the mixture of vesicles plus dilution medium was filtered through a Millipore filter which was subsequently washed with 5.4 ml of 10 mM Tris-HEPES containing 200 mM NaCl. Binding at time zero was determined as described above but using ice cold dilution media. The points were done in quadruplicate

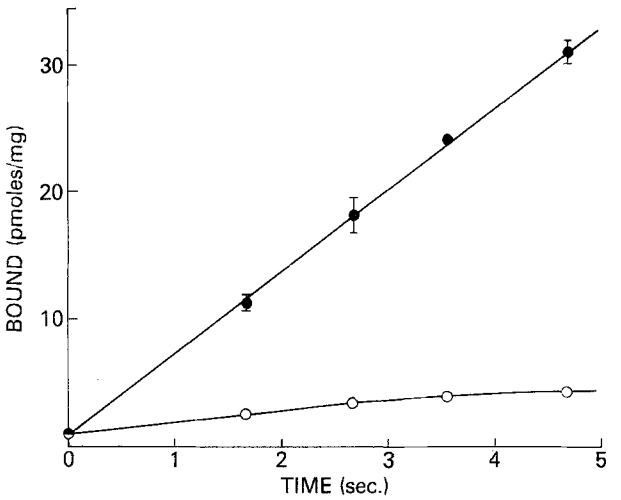

Fig. 4. Initial phlorizin binding in the presence and absence of sodium. Vesicles were prepared in Buffer A containing either 100 mM NaCl (●) or 100 mM KCl (○). The incubation medium was the same buffer containing 0.37 μM [^{3}H]-phlorizin (final concentration). The stop solution was 10 mM Tris-HEPES containing 200 mM NaCl and 300 μM unlabeled phlorizin. The zero time point was obtained by adding the stop solution to the vesicles before the addition of the incubation medium

have confirmed the observation of Aronson (1978) that the rate of dissociation of bound phlorizin is independent of membrane potential. These results eliminate the further possibility that the difference in dissociation rates observed in Fig. 3 is due to a diffusion potential resulting from the sodium and potassium gradients present when the vesicles are diluted into a sodium-free medium.

In the AS Scheme of Fig. 2 the rate of dissociation of phlorizin from the complex CNa^+P is given by $\kappa_{-2}[CNa^+P]$ and thus is necessarily independent of $[Na^+]$. Thus, this binding scheme is in apparent disagreement with the experimental observations in Fig. 3. Fig. 3 is, however, consistent with both the SA and Random Schemes.

Evidence Against the SA Scheme. In Fig. 4 we show the results of an experiment in which the rate of binding of 0.37 μM phlorizin is measured over the time interval 0 to 5 sec under 100 mM sodium or potassium equilibrium conditions. The initial rate of phlorizin binding as estimated from the figure is almost an order of magnitude faster in the presence of sodium than in its absence. This observation is inconsistent with the SA Scheme which predicts that the initial rate of binding is given by $\kappa_{+3}[C][P]$ and thus should be independent of $[Na^+]$.

The experiments illustrated in Fig. 3 and 4 indicate that both the association and dissociation rates of phlorizin for BBM vesicles and thus apparently for the BBM D-glucose transporter are dependent on sodium concentration. Related experiments with similar results have been carried out by Aronson (1978). Of those binding sequences shown in Fig. 2 only the Random Scheme is consistent with both of these experiments. Further evidence against the AS and SA Schemes is presented later in this paper.

Glucose Inhibition of Phlorizin Binding

The inhibition of phlorizin binding by D-glucose and its analogs in the presence of sodium has been documented repeatedly in the past (Frasch et al., 1970; Glossman & Neville, 1972; Chesney et al., 1974; Silverman & Black, 1975; Turner & Silverman, 1978*a*). However, since the Random Scheme of Fig. 2 predicts that phlorizin can bind to the BBM D-

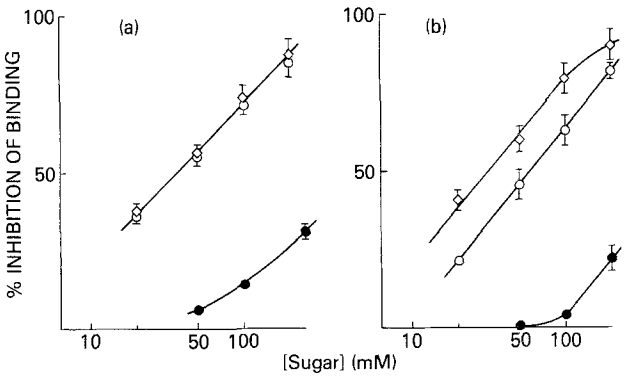

Fig. 5. Inhibition of equilibrium phlorizin binding by α-methyl-D-glucoside (◇), D-glucose (○) and D-xylose (●) in the presence and absence of sodium. (*a*) Vesicles were prepared in Buffer A containing 100 mM NaCl. The incubation medium was the same buffer containing [^{3}H]-phlorizin and various sugars replacing 200 mM L-glucose isoosmotically. The data represent equilibrium phlorizin binding at 0.22 μM concentration. (*b*) As above at 0.37 μM [^{3}H]-phlorizin with KCl replacing NaCl. The stop solution in both (*a*) and (*b*) was 10 mM Tris-HEPES containing 300 mM NaCl and 300 μM unlabeled phlorizin. The data have been corrected for unspecific binding only. The results of three experiments have been combined

glucose transporter in the absence of sodium as well as in its presence we might expect to see a monosaccaride inhibitable component of phlorizin binding under sodium-free conditions as well. That this is the case is illustrated in Fig. 5.

The data, which have been corrected for unspecific binding, illustrate the relative inhibitory potency of the sugars α-methyl-D-glucoside, D-glucose and D-xylose on phlorizin binding in the presence and absence of sodium. The glucose analogue α-methyl-D-glucoside is known from *in vivo* and *in vitro* studies (Silverman, 1976; Turner & Silverman, 1978*a*) to share the renal BBM D-glucose transporter with approximately the same affinity as D-glucose, however, it does not interact at all with the glucose transporter at the antiluminal membrane (Silverman, 1976). D-Xylose, on the other hand, does not share the BBM D-glucose transporter (Silverman, Aganon & Chinard, 1970) but is transported at the antiluminal membrane (Silverman, 1977). The figure indicates that the inhibition of phlorizin binding by these sugars follows a similar pattern in both the presence and absence of sodium; namely, that the concentration of D-xylose required to significantly inhibit binding is at least an order of magnitude higher than for either α-methyl-D-glucoside or D-glucose. Analysis of the D-xylose used in these experiments by thin-layer chromatography indicates the existence of a contaminant consistent with a D-glucose impurity of several percent. Thus the real inhibition of phlorizin binding by D-xylose is probably considerably less than indicated in Fig. 5.

From the data plotted in Fig. 5*a* we calculate that the K_I's for inhibition of phlorizin binding of the competitive inhibitors α-methyl-D-glucoside and D-glucose are $\simeq 12$ mM, in good agreement with our previously reported values from intact brush-border fragments (Turner & Silverman, 1978*a*). The data in Fig. 5*b* are more difficult to interpret quantitatively since in the absence of sodium the low-affinity site characterized by Eq. (2) represents ~20% of the binding found in the absence of inhibitors. This could account for the significant difference between the inhibitory capacity of α-methyl-D-glucoside and D-glucose seen in Fig. 5*b*. In any case the concentrations of α-methyl-D-glucoside and D-glucose required to inhibit 50% of the phlorizin binding observed in the absence of sodium are approximately 32 and 59 mM, respectively.

It is worth mentioning that the results of Fig. 5*b* are inconsistent with the AS Scheme of Fig. 2 which predicts that phlorizin cannot bind to the BBM D-glucose transporter in the absence of Na^+. However, they could be reconciled with this Scheme if K^+ could act in place of Na^+ in the phlorizin-binding process. In fact, we find that phlorizin binding in the absence of sodium is not enhanced by increasing $[K^+]$ as one would expect if this were the case (data not shown). Furthermore, the results of the experiment illustrated in Fig. 3, which argue against the correctness of the AS Scheme, cannot be explained by the possibility that K^+ can replace Na^+ in the binding event.

Sodium Dependence of Phlorizin Binding

Fig. 6*a* shows the results of an experiment in which the kinetics of phlorizin binding were measured as a function of sodium concentration. The data have been corrected for the unspecific and low-affinity components of binding as discussed above. The lines through the points were obtained by the method of least squares and the parameters corresponding to the fits are shown in Table 1 along with their 95% confidence intervals. It is clear from the Table that the effect of increasing the sodium concentration is to decrease the apparent binding constant, K_d^{app}, while producing no significant change in the apparent number of binding sites, C_o. Similar conclusions have been drawn by Frasch et al. (1970), Glossman and Neville (1972) and Chesney et al. (1974).

Fig. 6*b* illustrates the results of an experiment where we endeavor to measure the K_d^{app} for phlorizin binding in the absence of sodium. Under sodium-free

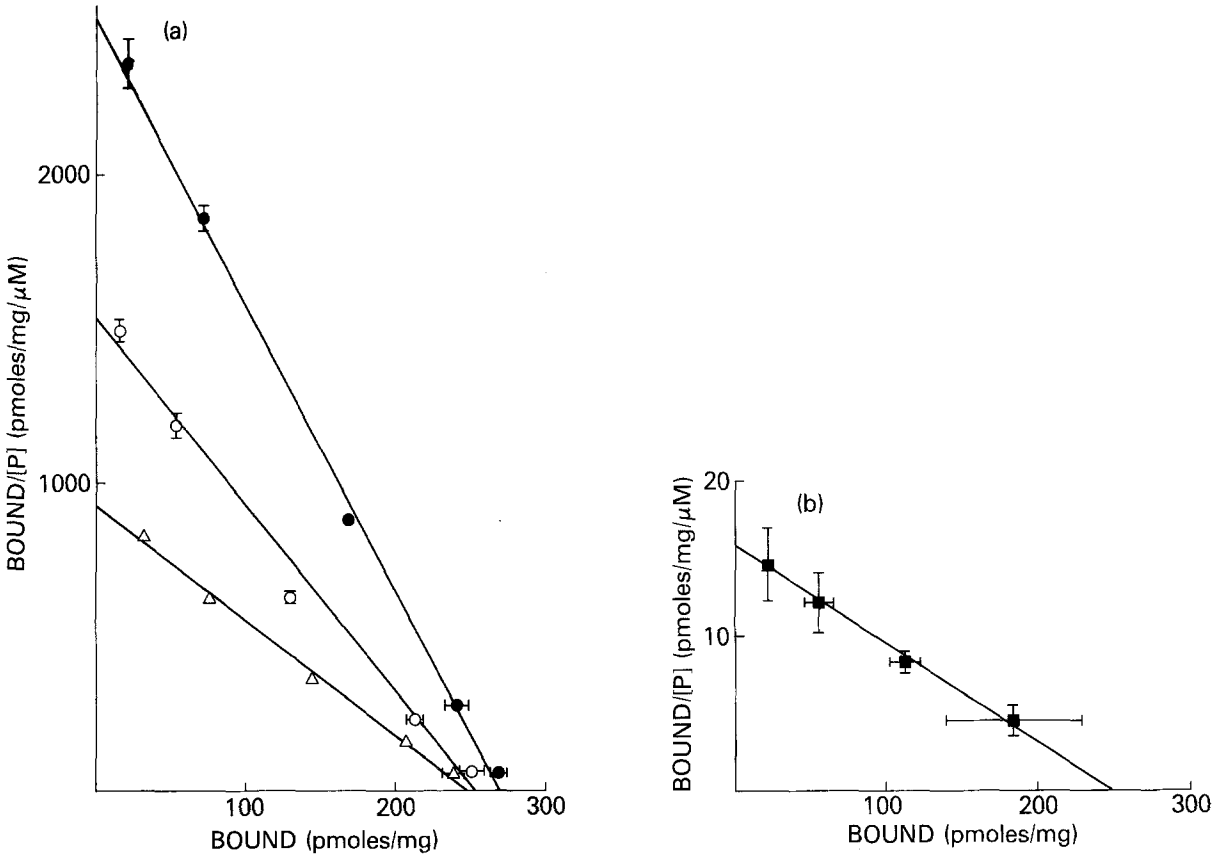

Fig. 6. Kinetics of equilibrium phlorizin binding as a function of sodium concentration. (*a*) [Na]=25, 50 and 100 mM. Vesicles were prepared in Buffer A containing 100 mM NaCl plus 100 mM KCl (●), 50 mM NaCl plus 150 mM KCl (○) or 25 mM NaCl plus 175 mM KCl (△). The incubation media were the same buffers containing appropriate concentrations of [^{3}H]-phlorizin. The stop solution was 10 mM Tris-HEPES with 300 mM NaCl and 300 μM unlabeled phlorizin. The data have been corrected for unspecific and low-affinity binding as described in the text. (*b*) [Na]=0 mM. Vesicles were prepared in Buffer A containing either 100 mM NaCl or 100 mM KCl. The incubation media were the same buffers containing appropriate labeled phlorizin concentrations over the range 0.035 to 40 μM. The stop solution was 10 mM Tris-HEPES with 300 mM NaCl and 300 μM unlabeled phlorizin. The results obtained in the presence of NaCl were analyzed as described for Fig. 1, yielding a high-affinity site with $K_d^{app}=0.104\pm0.040$ μM and $C_o\approx289\pm45$ pmoles/mg protein. The results obtained in the presence of KCl and analyzed as described in the text are shown in the Figure with their accumulated experimental errors. The points representing the two highest phlorizin concentrations were done in sextuplicate

Table 1. Kinetic parameters for phlorizin binding at various sodium concentrations[a]

[Na] (mM)	C_0 (pmoles/mg protein)	K_d^{app} (μM)	Plotted in figure
25	249±31	0.267±0.030	6*a*
50	253±41	0.164±0.031	6*a*
100	270±33	0.107±0.013	6*a*
0	249±37	15.7 ±2.7	6*b*

[a] The errors indicated are the 95% confidence intervals.

conditions the low-affinity component of binding represents a significant contribution to the total binding (~20%). Hence, rather than relying on an approximation such as Eq. (2) to correct for this component, we have obtained our data as follows. An experiment similar to the one shown in Fig. 1 was carried out including a series of points where 100 mM KCl replaced NaCl. The data obtained in the presence of sodium were then analyzed as described for Fig. 1 and the binding due to the low-affinity component corresponding to the inset of Fig. 1 was obtained. These estimates of the low-affinity binding were then used to correct the data obtained in the presence of KCl by a direct point-for-point subtraction. The results, presumably representing only binding to the BBM D-glucose transporter, are shown in Fig. 6*b*. The least-squares fit to these data is included in Table 1.

From Table 1 it is clear that the affinity of phlorizin binding to BBM vesicles is relatively low in the

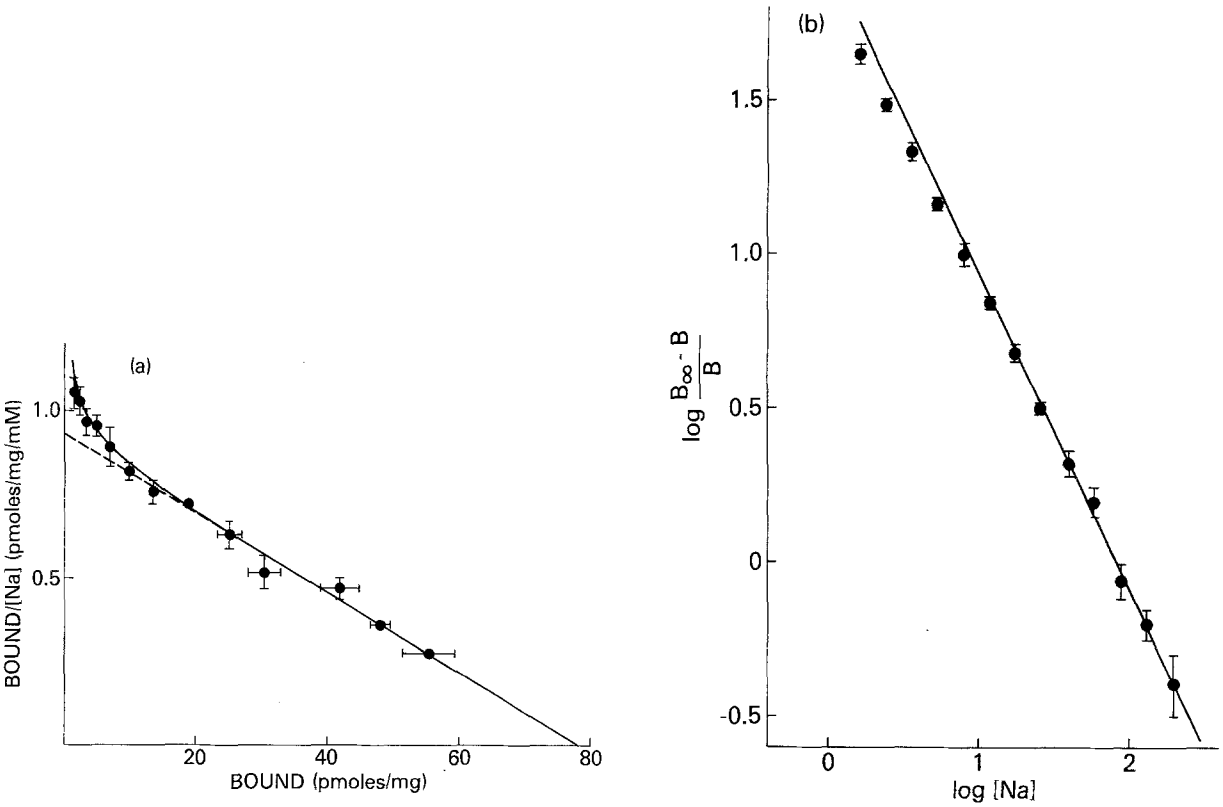

Fig. 7. High-affinity equilibrium phlorizin binding measured as a function of sodium concentration. Vesicles were prepared in Buffer A containing NaCl at various concentrations over the range 1.60 to 200 mM with NaCl replacing 200 mM KCl isoosmotically. The incubation media were the same buffers containing [^{3}H]-phlorizin. The stop solution was 10 mM Tris-HEPES with 300 mM NaCl and 300 μM unlabeled phlorizin. The data represent equilibrium phlorizin binding at 0.02 μM concentration and have been corrected for unspecific and low-affinity binding as described in the text. The points representing the three lowest sodium concentrations were determined in sextuplicate, all other points were done in triplicate. (*a*) Scatchard-type plot. The solid line through the points is a theoretical fit to the data (*see text*). The straight line (dashed plus solid) is a least-squares fit to the seven points representing the highest sodium concentrations (18–200 mM). The slope of the least-squares line is $-0.0119 \pm 0.0016\,\text{mM}^{-1}$ and its intercept on the horizontal axis is 78.5 ± 6.7 pmoles/mg. The significance of this line is discussed in the text. (*b*) Log-log plot of the data. The symbol B in the vertical legend represents bound phlorizin and B_∞ is binding at limitingly large sodium concentrations ($[\text{Na}] \to \infty$). B_∞ has been evaluated from the least-squares fit in Fig. 7*a* to be 78.5 ± 6.7 pmoles/mg

absence of sodium but is markedly enhanced even at sodium concentrations well below physiological levels.

The Stoichiometry of Phlorizin Binding

In the experiment shown in Fig. 7*a* we have measured high-affinity phlorizin binding as a function of sodium concentration. The data have been corrected for unspecific and low-affinity binding as previously described and plotted as a Scatchard-type diagram. It can be shown by the usual methods of enzyme kinetic analysis (Segel, 1975) that if phlorizin and sodium bind to the BBM D-glucose transporter according to the AS Scheme of Fig. 2 one would expect these data to lie on a straight line on this type of plot. Alternatively if either the SA or Random Scheme hold one would expect a curvilinear plot (concave up) which approaches a straight line for large sodium concentrations ($[\text{Na}] \gg \kappa_{-4}/\kappa_4$).[4] Thus the curvilinearity of Fig. 7*a* provides additional evidence against the AS Scheme.

Furthermore at limitingly large sodium concentrations ($[\text{Na}^+] \to \infty$) it can be shown that the SA

[4] In simple terms this difference between the predictions of the three schemes results from the additional phlorizin binding that can occur in the SA and Random Schemes as CP. This additional component only becomes significant at low sodium concentrations since for high $[\text{Na}^+]$ ($\gg \kappa_{-4}/\kappa_4$) virtually all bound phlorizin occurs as the fully occupied complex CNa^+P in all three schemes. In a recent publication (Turner & Silverman, 1980) to be discussed in the next section we have analyzed the binding of a non-transported competitive inhibitor to cotransport models of the carrier type based on the three schemes of Fig. 2. Using these results the interested reader can verify the predictions of these models for Fig. 7*a*.

Scheme predicts total occupancy of binding sites at any phlorizin concentration. Fitting the linear portion of the curve in Fig. 7*a* by the method of least squares (*see caption*) and extrapolating to $[Na] \to \infty$ we find that the SA Scheme predicts 78.5 ± 6.7 pmoles of binding sites/mg protein. From Table 1 it can be seen that this number is a factor of three lower than typically found in our preparation. In fact in a control point carried out simultaneously with the experiment shown in Fig. 7 we found 203 pmoles of bound phlorizin/mg protein at 0.3 μM [^{3}H]-phlorizin and 200 mM NaCl. This measurement represents 2.5 times the maximum number of binding sites predicted by the SA Scheme from Fig. 7*a*. Thus the data shown in Fig. 7 argue strongly against the SA Scheme as well. As we show in detail in the next section, however, the results of this experiment are consistent with the suggestion made earlier in this paper that the binding of phlorizin and sodium to the BBM D-glucose transporter is a random process.

The fact that the points shown in Fig. 7*a* approach a straight line for larger sodium concentrations ($\gtrsim$20 mM) also indicates that the binding stoichiometry of sodium and phlorizin is 1:1. This can be seen more clearly in Fig. 7*b*. Here we have replotted the data on a log-log (Hill-type) plot whose slope gives the binding stoichiometry directly. The line shown in the figure is a least-squares fit to the seven points representing the highest sodium concentrations (18–200 mM) and has a slope of 1.02 $\pm$0.07). We also note that as the sodium concentration decreases the slope of the log-log plot also decreases indicating that less than one sodium is being bound per phlorizin molecule at low [Na] as one would expect from a random binding scheme.

We would like to emphasize that although the correction for low-affinity binding we have applied to the data shown in Fig. 7 is necessarily rather approximate [*see* earlier discussion following Eq. (2)] this does not significantly affect the above conclusions. Even for the lowest sodium concentration shown (1.60 mM) this correction is less than 7% of the total phlorizin binding.

The experimental results presented here will be discussed in more detail in the following section in terms of a simple theoretical model for phlorizin binding to the BBM D-glucose transporter. At the present time, however, we can conclude that the above observations are consistent with the following interpretation:

1. There are three components of phlorizin binding to BBM vesicles, a nonspecific component [*c.f.* Eq. (1)], a specific but low-affinity component characterized approximately by Eq. (2), and a third component consisting of a single high-affinity site representing the binding of phlorizin to the BBM D-glucose transporter.

2. This latter component of binding is sodium dependent, the effect of sodium being to decrease the apparent binding constant without significantly changing the number of binding sites.

3. The binding of phlorizin and sodium to their respective sites on the BBM D-glucose transporter is a random rather than an ordered process.

4. The ratio of phlorizin to sodium binding sites on the transporter is one-to-one.

A Model for Phlorizin Binding to the Brush-Border Membrane D-Glucose Transporter

In a recent publication (Turner & Silverman, 1980) we have analyzed the binding of nontransported competitive inhibitors to several cotransport models of the carrier type. Our model based on the Random Scheme of Fig. 2 is shown in Fig. 8. Here the subscripts 1 and 2 refer to the two sides of the membrane, k_{12}, k_{21}, g_{12}, and g_{21} are the translocation rate constants for the free and loaded carrier and K_{P_1}, K_{PNa_1}, K_{Na_1} and K_{NaP_1} are binding constants defined in the usual way (Turner & Silverman, 1980). We limit our treatment here to binding on one side of the membrane only. The solution to the model can be written as

$$\text{total binding} = [CP_1] + [CNa^+P_1] = \frac{C_0[P_1]}{K_{d_1}^{app} + [P_1]} \tag{3}$$

where C_0 is the total number of carriers and $K_{d_1}^{app}$ is the apparent binding constant to be discussed in detail below. Notice that the model predicts a Michaelis-Menten type dependence on $[P_1]$ with a constant number of binding sites, C_0. Under sodium equilibrium conditions we find that (*see* Table II and Eq. (5*b*) of Turner and Silverman, 1980)

$$K_{d_1}^{app} = \frac{[Na]}{K_{PNa_1} + [Na]} \left[K_{\infty_1} + \frac{K_{0_1} K_{PNa_1}}{[Na]} \right] \tag{4}$$

where

$$K_{\infty_1} = K_{NaP_1} \left(\frac{g_{12}}{g_{21}} + 1 \right) \tag{5a}$$

$$= K_{NaP_1} \left(\frac{K_{Na_1} k_{12}}{K_{Na_2} k_{21}} + 1 \right) \tag{5b}$$

and

$$K_{0_1} = K_{P_1} \left(\frac{k_{12}}{k_{21}} + 1 \right). \tag{6}$$

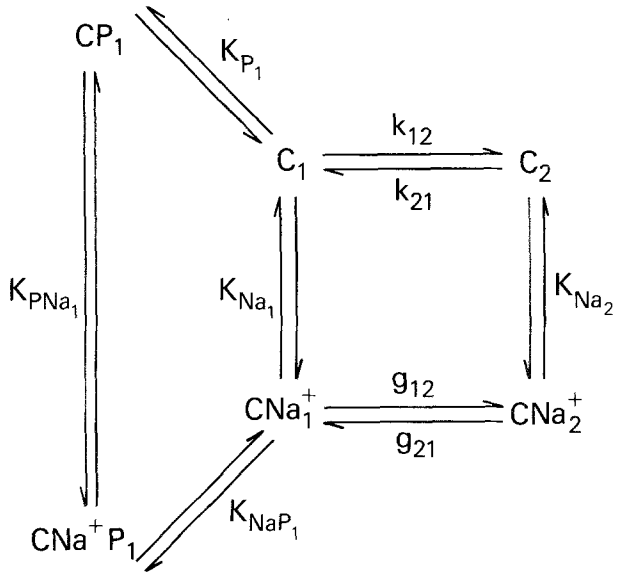

Fig. 8. Random Model for phlorizin binding to a carrier-type representation of the BBM D-glucose transporter. (*Note:* the model is referred to as the "General Model" in Turner and Silverman, 1980)

The quantities K_{∞_1} and K_{0_1} have a simple kinetic interpretation; they are the apparent binding constants when $[Na] \to \infty$ and $[Na] = 0$, respectively. Eq. (5b) is the more general expression for K_{∞_1}; Eq. (5a) holds only when the complex CNa^+ is mobile, i.e. when $g_{12}, g_{21} \neq 0$.

As previously mentioned it can be shown in a straightforward way from Eqs. (3) and (4) that for $[Na] \gg K_{PNa_1}$ a plot of the type shown in Fig. 7*a* should be linear. The predicted slope of this line is given by $-(K_{\infty_1} + [P_1])/K_{0_1} K_{PNa_1}$ and its intercept on the horizontal axis by $C_o[P_1]/(K_{\infty_1} + [P_1])$. Taking the values of the slope and intercept from the least-squares fit given in the caption of Fig. 7*a* and requiring that $K_{d_1}^{app} = 0.107\,\mu M$ when $[Na] = 100\,mM$ (*cf.* Table 1) we find $C_o = 272 \pm 29$ pmoles/mg protein, $K_{\infty_1} = 0.049 \pm 0.009\,\mu M$ and $K_{0_1} K_{PNa_1} = 5.8 \pm 1.1\,\mu M\,mM$. Finally, taking the value $K_{0_1} = 15.7 \pm 2.7\,\mu M$ from Table 1 we calculate that $K_{PNa_1} = 0.37 \pm 0.09\,mM$. The above constants represent a complete parameterization of the equilibrium binding predictions of the model in Fig. 8. The corresponding theoretical curve is shown as a solid line in Fig. 7*a*. It is not surprising that this curve fits the experimental points well in the linear ($[Na] \gg K_{PNa_1}$) region, since the relevant parameters of the model (K_{∞_1} and the product $K_{0_1} K_{PNa_1}$) were calculated from these data. However, the fit to the nonlinear region is also quite good and the parameter K_{PNa_1} which characterizes the deviation from linearity [*cf.* Eq. (4)] was calculated using the value of K_{0_1} taken from an independent experiment (Fig. 6*b*).

In Turner and Silverman (1980) we also discuss two simplified versions of the binding model shown in Fig. 8. These are based on the ordered binding sequences of the AS and SA Schemes (Fig. 2) and are referred to as the AS and SA Models, respectively. In the above paper we show that a plot of K_d^{app} *vs.* $1/[Na]$ is expected to be linear for the AS Model while a plot of $1/K_d^{app}$ *vs.* $[Na]$ should be linear for the SA Model. These plots for the data given in Table 1 are shown in Fig. 9. The curvilinearity of

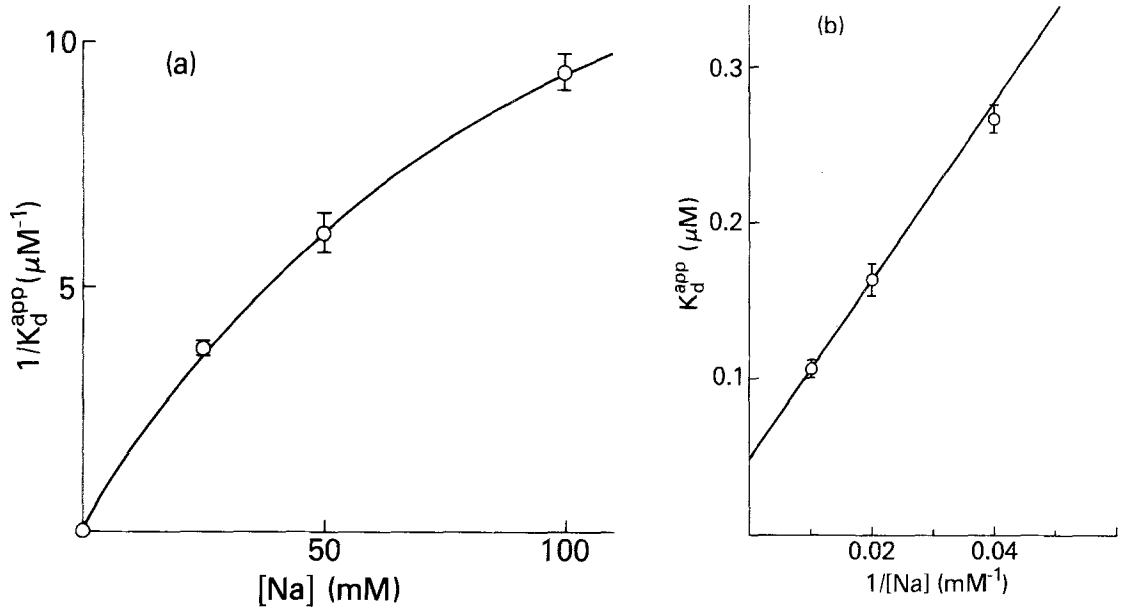

Fig. 9. Plots of K_d^{app} from Table 1 versus [Na]. The error bars represent the standard deviations calculated from least-squares fits. The lines through the points correspond to the theoretical fit to the Random Model derived in the text

Fig. 9*a* argues again against the correctness of the SA Model as does our earlier conclusion that $K_{\infty_1} = 0.049 \pm 0.009$ μM since in the SA Model $K_d^{app} \to 0$ as $[Na] \to \infty$ (Turner & Silverman, 1980, Table 2). The apparent linearity of Fig. 9*b* indicates that the AS Model is a good representation of the binding process for $[Na] \geqq 25$ mM; however, deviations from linearity in this plot are expected at lower sodium concentrations. The lines drawn through the data points in Fig. 9*a* and *b* represent the theoretical predictions of the Random Model as determined above.

We conclude this section with some interesting calculations which provide an internal consistency check on our parameterization of the model shown in Fig. 8. From Fig. 3 we can estimate the rate constants κ_{-2} and κ_{-3} (*cf.* Fig. 2) for phlorizin dissociation from the carrier in the presence and absence of sodium, respectively. Using Eqs. (9a) and (9b) of Turner and Silverman (1980) we have that ratio of the amount of phlorizin bound as $[CP_1]$ to that bound as $[CNaP_1]$ is equal to $K_{PNa_1}/[Na_1]$ where here $K_{PNa_1} = 0.37$ mM (*see above*). Thus at 100 mM NaCl virtually all phlorizin is bound as $[CNaP_1]$ and κ_{-2} can be taken directly from Fig. 3 to be $\simeq 6.0 \times 10^{-3}\,\text{sec}^{-1}$. At 1 mM NaCl the rate of phlorizin dissociation is given by $\kappa_{-2}[CNaP_1] + \kappa_{-3}[CP_1]$ which from Fig. 3 is equal to $3.1 \times 10^{-2}\,\text{sec}^{-1}$. Again applying Eqs. (9a) and (9b) of Turner and Silverman (1980) we find that $\kappa_{-3} \simeq 9.9 \times 10^{-2}\,\text{sec}^{-1}$.

The rate of association of phlorizin to the carrier in the absence of sodium is given by $\kappa_{+3}[P_1][C_1] = \kappa_{+3}[P_1]C_0/(k_{12}/k_{21}+1)$. Thus from Fig. 4 we can calculate that $\kappa_{+3}/(k_{12}/k_{21}+1) \simeq 1.02 \times 10^{-2}\,\mu\text{M}^{-1}\,\text{sec}^{-1}$. (Here we have assumed that $C_o = 258$ pmoles/mg protein. This value is taken from the experiment shown in Fig. 1 which was done on the same crude membrane fraction.) Owing to the existence of the low-affinity component of phlorizin binding [*cf.* Eq. (2)] this value is probably an overestimate of this quantity. Combining this result with the value for κ_{-3} obtained above, we can now calculate that $K_{0_1} = (\kappa_{-3}/\kappa_3)(k_{12}/k_{21}+1) \simeq 9.7$ μM which, considering its approximate nature, is in good agreement with the value of 15.7 ± 2.7 μM given in Table 1.

The rate of association of phlorizin to the carrier in the presence of sodium is given by $\kappa_{+2}[P_1][CNa_1] + \kappa_{+3}[P_1][C_1]$ where the second term is necessarily small relative to the first (*see* Fig. 4) and can be neglected for our purposes here. From the results of Turner and Silverman (1980) it can be shown that $[CNa_1] = C_0[Na_1]\ (g_{12}/g_{21}+1)^{-1}(K_{Na_1}^{app} + [Na_1])^{-1}$ where $K_{Na_1}^{app}$, the apparent binding constant for Na^+ on side 1 of the membrane, is given by $K_{0_1}K_{PNa_1}/K_{\infty_1} = 118$ mM. Thus $\kappa_{+2}/(g_{12}/g_{21}+1)$, can be calculated from Fig. 4 to be $\simeq 0.157\,\mu\text{M}^{-1}\,\text{sec}^{-1}$. Combining this result with the value of κ_{-2} obtained from Fig. 3 we have that $K_{\infty_1} = (\kappa_{-2}/\kappa_{+2})(g_{12}/g_{21}+1) \simeq 0.038$ μM. This value also corresponds well with the prediction $K_{\infty_1} = 0.049 \pm 0.009$ μM, obtained from the equilibrium binding data. This agreement between kinetic parameters derived from both equilibrium and initial phlorizin-binding data gives further credence to validity of a random-binding scheme.

Discussion

In this paper we present a number of experiments concerning the phlorizin-binding properties of the BBM D-glucose transporter. Taken together our experimental results provide strong evidence that phlorizin and sodium bind to their respective sites on the transporter in a random fashion and that these sites exist in a one-to-one stoichiometry. These conclusions are further supported by the fact that our data are consistent with a carrier type model of the D-glucose transporter based on the assumption of a random-binding scheme. Given that phlorizin and sodium bind to the transporter in a random fashion it seems highly likely that sodium and glucose do the same.

In a recent publication Hopfer and Groseclose (1980) have analyzed the glucose transport properties of intestinal BBM vesicles in terms of a number of binding schemes for sodium and glucose. In particular, these authors find that the equilibrium exchange flux of both D-glucose and sodium are first stimulated and then inhibited by increasing concentrations of the cotransported species. They demonstrate that these results are consistent with an ordered binding (iso-ordered-bi-bi) model in which the first substrate (sodium or glucose) which binds to the transporter at one side of the membrane is necessarily the first released at the other side. They suggest that the biphasic activation of glucose equilibrium exchange by sodium and sodium equilibrium exchange by glucose argues against a random-binding scheme. However, this type of behavior only excludes a certain class of random models, namely, those in which neither of the binding pathways is kinetically less favorable (Segel, 1975, page 853). In general, biphasic activation curves *are consistent with a random model.* (Boyer & Silverstein (1963) give a numerical example of this phenomenon which can be simply generalized to demonstrate simultaneous biphasic activation for equilibrium exchange of either substrate by the other.) A random-binding scheme can only be excluded in favor of the ordered model suggested by Hopfer and Groseclose (1980) if it can be demonstrated that the equilibrium exchange flux of *both* sub-

strates can be driven to zero by large concentrations of the other (Segel, 1975, page 851). Since Hopfer and Groseclose (1980) have not demonstrated this we must conclude that their results on glucose transport are consistent with a random-binding scheme and thus with the phlorizin-binding data presented here. Owing to the complexity of the mathematics of steady-state random transport models the detailed significance of the biphasic activation curves observed by these authors is not obvious to us at the present time.

In attempting to characterize the BBM D-glucose transporter through its phlorizin-binding properties in this paper we have made several assumptions concerning the nature of the interaction of phlorizin with the carrier which should be mentioned. We have assumed that phlorizin is not transported or so poorly transported by the BBM D-glucose carrier that its interaction may be regarded solely as a binding event. The experimental evidence arguing against phlorizin transport by the BBM D-glucose transporter has been discussed in detail by Aronson (1978) and we will not repeat it here. As also noted by Aronson (1978) phlorizin accumulation in the intravescular space can only account for a small fraction of the total binding found associated with BBM vesicles.

In our analysis of phlorizin binding in terms of the model shown in Fig. 8 we assumed that binding occurs only at one side of the membrane. This assumption is supported by the observation that we do in fact only find one high affinity binding site (*cf.* Fig. 1). There is now good evidence that renal BBM vesicles from the rabbit (Booth & Kenny, 1976), rat (Haase, Schafer, Murer & Kinne, 1978) and dog (K. Amsler, M. Sc. Thesis, University of Toronto, 1977; Grinstein, Turner, Silverman & Rothstein, 1980) are predominantely oriented right-side-out. Furthermore, the rapid onset and competitive nature of phlorizin inhibition of initial D-glucose uptake into BBM vesicles (Turner & Silverman, 1978*b*) indicate that phlorizin is acting from the same side of membrane as the sugar (i.e., the outside) where they compete for a common binding site [e.g., in the case of carrier-type models it can be seen from Eq. (8) of Turner and Silverman (1980) that an inhibitor acting from the *trans* side of the membrane results in *mixed* rather than *competitive* inhibition of substrate flux]. Thus the high-affinity component of binding we observe is almost certainly associated with the outside (extracellular side) of the BBM. *In vivo* studies of phlorizin binding using the multiple indicator dilution technique (Silverman, 1974) also suggest that phlorizin binding occurs at the urine face of the BBM.

There is no compelling reason to associate the low-affinity site we observe in Fig. 1 with the D-glucose transporter; in fact, some residual binding of this type to contaminants in our vesicle preparation (e.g., antiluminal membranes) is expected. However, it is possible that a portion of this component represents binding to the cytoplasmic side of the membrane, i.e., to inside-out vesicles.

It has been shown that phlorizin (phloretin-2′-β-glucoside) has several points of attachment to the glucose transporter (Diedrich, 1966) (*i*) via the glucoside moiety and (*ii*) via hydroxyl groups on each of the aromatic rings on the phloretin moiety. It is interesting to note that, although the binding affinity of phlorizin to the D-glucose transporter increases by a factor ~ 160 as the sodium concentration increases from 0 to 100 mM (*see* Table 1), the data shown in Fig. 5 suggest that the K_I for D-glucose inhibition only changes by a factor ~ 6. These results seem to indicate that occupancy of the sodium-binding site not only increases the binding affinity of the glucose site but also increases the binding affinity of the phloretin site(s).

A portion of this work was supported by grant MT4590 from the Medical Research Council of Canada and was carried out while R.J. Turner was an M.R.C. (Canada) Centennial Fellow. The authors wish to acknowledge the technical assistance of Ms. Jennifer Thomas, Ruth Rutherford, and Doris Jones and the patient secretarial assistance of Mrs. Lorraine Hirz. We would also like to thank Drs. Maurice Burg, Joseph Handler, and Clifford Patlak for critical readings of the manuscript, and Drs. Ulrich Hopfer and Rhea Groseclose for sending us their results prior to publication.

References

Aronson, P.S. 1978. Energy-dependence of phlorizin binding to isolated renal microvillus membranes. *J. Membrane Biol.* **42**:81

Baginski, E.S., Foa, P.P., Zak, B. 1974. Glucose-6-phosphatase. *In:* Methods of Enzymatic Analysis. H.U. Bergmeyer, editor. p. 876. Academic Press, New York

Bode, F., Baumann, K., Diedrich, D.F. 1972. Inhibition of [^{3}H] phlorizin binding to isolated kidney brush border membranes by phlorizin-like compounds. *Biochim. Biophys. Acta* **290**:134

Booth, A.G., Kenny, A.J. 1976. A morphometric and biochemical investigation of the vesculation of kidney microvilli. *J. Cell Sci.* **21**:449

Boyer, P.D., Silverman, E. 1963. Equilibrium reaction rates and enzyme mechanisms. *Acta Chem. Scand.* **17**:S195

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248

Chesney, R., Sacktor, B., Kleinzeller, A. 1974. The binding of phlorizin to the isolated luminal membrane of the renal proximal tubule. *Biochim. Biophys. Acta* **332**:263

Diedrich, D.G. 1966. Competitive inhibition of intestinal glucose transport by phlorizin analogs. *Arch. Biochem. Biophys.* **117**:248

Frasch, W., Frohnert, P.P., Bode, F., Baumann, K., Kinne, R. 1970. Competitive inhibition of phlorizin binding by D-glucose and the influence of sodium: A study on isolated brush border membrane of rat kidney. *Pfluegers Arch.* **320**:265

Glossmann, H., Neville, D.M., Jr. 1972. Phlorizin receptors in isolated kidney brush border membranes. *J. Biol. Chem.* **247**:7779

Grinstein, S., Turner, R.J., Silverman, M., Rothstein, A. 1980. Inorganic anion transport in kidney and intestinal brush border and basolateral membranes. *Am. J. Physiol.* **238**:*F*452

Haase, W., Schafer, A., Murer, H., Kinne, R. 1978. Studies on the orientation of brush-border membrane vesicles. *Biochem. J.* **172**:57

Hopfer, U., Groseclose, R. 1980. The mechanism of Na^+-dependent D-glucose transport. *J. Biol. Chem.* **255**:4453

Malathi, P., Preiser, H., Fairclough, P., Mallett, P., Crane, R.K. 1979. A rapid method for the isolation of kidney brush border membranes. *Biochim. Biophys. Acta* **554**:259

Pennington, R.J. 1961. Biochemistry of dystrophic muscle. *Biochem. J.* **80**:649

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

Silverman, M. 1974. The *in vivo* localization of high-affinity phlorizin receptors to the brush border surface of the proximal tubule in dog kidney. *Biochim. Biophys. Acta* **339**:92

Silverman, M., Black, J. 1975. High affinity phlorizin receptor sites and their relation to the glucose transport mechanism in the proximal tubule of dog kidney. *Biochim. Biophys. Acta* **394**:10

Silverman, M. 1976. Glucose transport in the kidney. *Biochim. Biophys. Acta* **457**:303

Silverman, M. 1977. Sugar interaction with the antiluminal surface of the proximal tubule in dog kidney. *Am. J. Physiol.* **232**:*F*455

Silverman, M., Aganon, M.A., Chinard, F.P. 1970. Specificity of monosaccharide transport in dog kidney. *Am. J. Physiol.* **218**:743

Silverman, M., Turner, R.J. 1979. The renal proximal tubule. *In:* Biomembranes, Vol. 10. L.A. Manson, editor, p. 1. Plenum Publishing Corp., New York

Stirling, C.E. 1967. High-resolution radioautography of phlorizin-^3H in rings of hamster intestine. *J. Cell Biol.* **35**:605

Turner, R.J., Silverman, M. 1978*a*. Sugar uptake into brush border vesicles from dog kidney. I. Specificity. *Biochim. Biophys. Acta* **507**:305

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

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

Received 23 April 1980; revised 17 July 1980