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Energy-Dependence of Phlorizin Binding to Isolated Renal Microvillus Membranes

Evidence Concerning the Mechanism of Coupling Between the Electrochemical Na^+ Gradient and Sugar Transport

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Summary. In order to elucidate the mechanism by which the electrochemical Na^+ gradient energizes glucose transport, the energy-dependence of high affinity phlorizin binding to isolated renal microvillus membrane vesicles was examined. Phlorizin is a competitive inhibitor of glucose transport but is not itself transported.

Extravesicular Na^+ accelerated the rate of phlorizin binding and inhibited the rate of dissociation of bound glycoside. Maneuvers to enhance intravesicular electronegativity stimulated phlorizin uptake and those to enhance intravesicular electropositivity inhibited. However, alterations in electrical potential were without effect on the rate of release of bound phlorizin. Intravesicular Na^+ inhibited the phlorizin uptake rate.

The results are consistent with a model of the glucose transporter in which (i) Na^+ increases the binding affinity of the carrier, (ii) the free carrier is negatively charged, and (iii) the translocation of the carrier is inhibited by the binding of Na^+ in the absence of sugar. The electrochemical Na^+ gradient thus energizes both glucose transport and phlorizin binding through its effect on the affinity and appearance of the free carrier at the membrane surface rather than through an effect on sugar translocation per se.

Recent studies indicate that the electrochemical Na^+ gradient energizes uphill D-glucose transport by isolated renal and intestinal microvillus membranes [2, 3, 4, 16, 18, 21]. The unidirectional flux of sugar into membrane vesicles is stimulated by extravesicular Na^+ and inhibited by intravesicular Na^+ [2, 3, 16, 18]. Maneuvers that enhance intravesicular electronegativity augment glucose uptake [4, 21]. However, the mechanism by which glucose transport is coupled to the Na^+ gradient remains unknown.

There are two possible steps at which coupling between sugar transport and an ion gradient might occur. The interaction of sugar with its binding site at the membrane surface could be energy-dependent. Such would be the case if either the appearance or binding affinity

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of a sugar carrier was linked to the electrochemical ion gradient. Alternatively, the translocation of sugar across the membrane might be energy dependent. In studies of the coupling between the electrochemical H^+ gradient and lactose transport in *Escherichia coli* membrane vesicles, it has been observed that membrane binding of nontranslocated transport inhibitors is energy-dependent, thus suggesting that it is the binding step rather than the translocation step which is energized by the H^+ gradient [23, 25]. The present study examines the energetics of phlorizin binding in an attempt to similarly identify the step at which glucose transport is coupled to the electrochemical Na^+ gradient in renal microvillus membrane vesicles.

Phlorizin is a competitive inhibitor of proximal tubular [33] and intestinal [11] glucose transport but is not itself translocated across the brush border membrane by the glucose transporter [27, 30]. Two classes of phlorizin binding sites on isolated renal microvillus membranes have been identified. High affinity phlorizin binding (K_D 0.2–8 μM) is Na^+-dependent and competitively inhibited by D-glucose and those sugar analogs which share the glucose transport system [7, 12, 14, 28]. Low affinity phlorizin binding ($K_D > 100$ μM) is Na^+-independent and less specific [7, 14]. Only the high affinity phlorizin binding site, therefore, has been thought to be identical with the D-glucose transporter [7, 12, 14, 28, 33].

The present study demonstrates that high affinity phlorizin binding to renal microvillus membrane vesicles is indeed influenced by the electrochemical Na^+ gradient. The findings concerning the electrical dependence of phlorizin binding have been presented in abstract form previously [1]. An effect of Na^+ salt gradients on the binding of phlorizin to intestinal brush borders has also recently been reported [31].

Materials and Methods

Membrane Preparation

Rabbit renal microvillus membranes were isolated by the Mg-aggregation method of Booth and Kenny [6] with the following modifications: (i) Kidneys were freshly removed from male New Zealand white rabbits (2–3 kg) sacrificed by intravenous air injection; (ii) the homogenizing solution consisted of 300 mM mannitol, 1 mM Tris-Hepes[1] pH 7.5, rather than hypotonic buffer; (iii) homogenization was performed by completing 10 full

1 The abbreviations used are: Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Mes, 2-(N-morpholino) ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

strokes with a Potter-Elvehjem pestle (clearance 0.15 mm) at 2000 rpm; (iv) $MgSO_4$ was substituted for $MgCl_2$; (v) three aggregation steps were performed rather than two; the slow speed centrifugations to sediment aggregated material were at 2500, 3100 and $3700 \times g$ for 12 min and the higher speed microvillus collections at $27{,}000 \times g$ for 20 min; (vi) the purified membranes were suspended in 300 mM mannitol, 10 mM $MgSO_4$, 1 mM Tris-Hepes pH 7.5 at a concentration of 5–15 mg protein/ml.

The purpose of these modifications was to isolate membrane vesicles that were fully equilibrated with an isosmotic mannitol solution in the absence of permeant anions. Purity of the membranes was equivalent to that produced by the original method. The enrichment in specific activity (final pellet/homogenate) of the luminal membrane marker γ-glutamyl transpeptidase [13] was 11–16 ×, while that of the basolateral marker Na, K-ATPase [15] was consistently < 1.0. Electronmicroscopy revealed that the preparation consisted predominantly of individual microvilli with intact core structures, as reported by Booth and Kenny [6], suggesting that the membranes were isolated in a "right-side-out" configuration. Preliminary experiments using these membranes demonstrated no significant differences in the characteristics of the Na^+-dependent D-glucose transport system from those described previously using rabbit renal brush borders isolated by a different method [3]. Glucose was found to equilibrate with an intravesicular volume of approximately 1.5 µl/mg membrane protein in the present preparation. Isolated renal plasma membranes contain no phlorizin hydrolase activity [7, 14].

Binding Measurements

Uptake of [^{3}H] phlorizin was assayed by modification of the Millipore filtration technique previously utilized to study glucose transport [3]. In general, 10 µl of membrane suspension were preincubated for 1 min at 20 °C and then incubation at 20 °C initiated by the addition of 40 µl of buffered mannitol medium containing [^{3}H] phlorizin (2×10^5 cpm) and other constituents as required. Replacement of mannitol in the incubation medium by salts was always performed isosmotically. All solutions contained 10 mM $MgSO_4$ and 1 mM Tris-Hepes pH 7.5 unless otherwise indicated. Incubations were terminated after the desired interval by rapid addition of 3.5 ml of an iced "stop" solution consisting of 150 mM NaCl, 10 mM $MgSO_4$ and 1 mM Tris-Hepes pH 7.5. The mixture was immediately poured on a 0.65 µm Millipore filter (DAWP) prewetted with distilled water. The incubation tube was washed once with 3.5 ml of iced "stop" solution, which was poured on the filter, and then the filter itself washed with an additional 7 ml of "stop" solution. The entire termination and washing process was completed in 13–15 sec. The rate of release of bound phlorizin into the iced "stop" solution was found to be approximately 10% per 60 sec. Filters were dissolved in Aquasol (New England Nuclear Corp.) and the radioactivity measured in a scintillation counter. Values for the nonspecific retention of radioactivity by the filters were subtracted from the values of the incubated samples [3]. Any deviations from the procedures are noted.

All incubations were performed in triplicate using membranes prepared the same day. An initial phlorizin concentration of 0.2 µM was employed in all experiments. Protein was assayed by the method of Lowry *et al.* [20]. While absolute phlorizin binding expressed per mg membrane protein varied over a twofold range from day to day, relative changes resulting from experimental manipulations were extremely reproducible. Data from representative experiments are illustrated.

Materials

[^{3}H] phlorizin (5.2 Ci/mmol) was obtained from New England Nuclear Corp. with radiochemical purity > 98% by thin layer chromatography. Unlabeled phlorizin and valino-

mycin were purchased from Sigma Chemical Co. and FCCP from Boehringer-Mannheim. Other chemicals were of the highest purity available from commercial sources. All water used for preparing media was distilled, deionized, and filtered through 0.45 μm Millipore filters. As valinomycin and FCCP were stored in 95% ethanol, equivalent volumes of ethanol were added to control incubations.

Results

Verification of High Affinity Binding Site

A Scatchard plot [24] of equilibrium phlorizin binding in the presence of 120 mM Na^+ is illustrated in Fig. 1. In the range of phlorizin concentrations 0.09–1.3 μM, only a single high affinity binding site was identified. The K_D (1.0 μM) was within the reported range [7, 12, 14, 28], although the concentration of receptor sites (265 pmoles/mg membrane protein) was 3–20 × higher than that previously described. The uptake of 0.2 μM phlorizin was inhibited >90% by removal of Na^+ from the incubation medium (Fig. 2), consistent with the known Na^+-dependence of the high affinity phlorizin binding site [7, 12, 14, 28].

The data in Fig. 2 also clearly indicate that accumulation of free glycoside in the intravesicular space can account, at most, for a trivial component of measured phlorizin uptake. The intravesicular volume in these membranes, as determined by steady state glucose uptake, is ap-

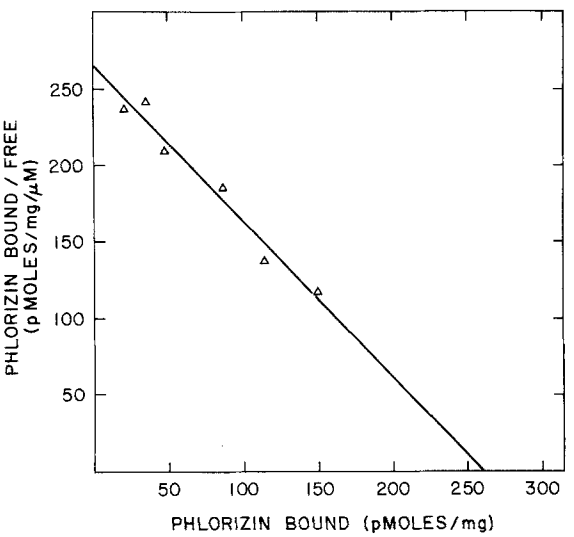

Fig. 1. Scatchard plot of phlorizin binding. Incubations were performed for 36 min at 20 °C in a medium containing 60 mM Na_2SO_4

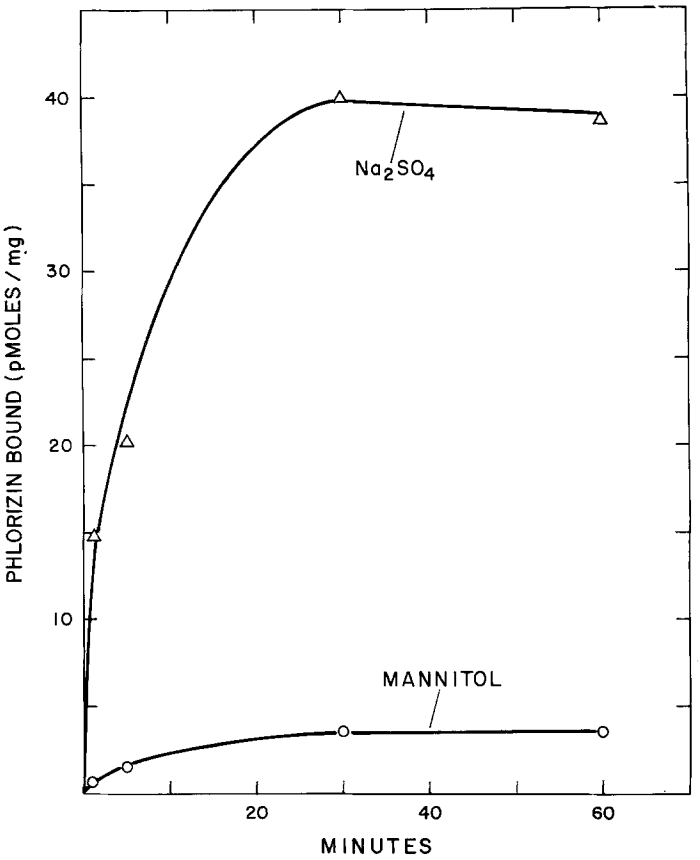

Fig. 2. Effect of Na^+ on equilibrium phlorizin binding. The uptake of 0.2 μM phlorizin at 20 °C was assayed in the presence (△) or absence (○) of 60 mM Na_2SO_4

proximately 1.5 μl/mg membrane protein. Equilibration of 0.2 μM phlorizin into this volume would account for a measured phlorizin uptake of only 0.3 pmole/mg membrane protein. Thus $<1\%$ of the phlorizin uptake in the presence of Na^+ and $<10\%$ of the uptake in the absence of Na^+ can be attributed to accumulation of free glycoside in the intravesicular space. Indeed, as will be addressed in the *Discussion*, it is extremely unlikely that any phlorizin is transported across the microvillus membrane.

Effect of Extravesicular Na^+ on Unidirectional Rates of Phlorizin Association and Dissociation

The effect of extravesicular Na^+ on the initial rate of phlorizin association was estimated from incubations performed for 5 sec after the simul-

Table 1. Effect of extravesicular Na^+ salts on initial rate of phlorizin binding

Incubation medium (mM)	Phlorizin bound (pmoles/mg)
300 Mannitol	0.71±0.02
60 Na_2SO_4 + 120 mannitol	7.87±0.06
120 NaCl + 60 mannitol	10.92±0.36
120 NaSCN + 60 mannitol	14.38±0.42

The uptake of 0.2 μM phlorizin for 5 sec at 20 °C was measured ± the addition of Na^+ salts at the initiation of the incubation. Results are expressed as the mean pmoles bound per mg membrane protein ± the SE for three determinations.

taneous addition of [^{3}H] phlorizin and replacement of mannitol by each of the Na^+ salts indicated in Table 1. The rate of glycoside binding was stimulated >10-fold by the addition of 120 mM Na^+, even with an impermeant accompanying anion such as $SO_4^=$.

Figure 3 illustrates the effect of extravesicular Na^+ on the rate of release of phlorizin that had previously been bound to the high affinity site. In this experiment, membranes were equilibrated with [^{3}H] phlorizin in the presence of 120 mM Na^+ and then diluted 1:20 into either the standard Na^+-free mannitol medium, or a medium containing 150 mM Na^+. The rate of dissociation of bound phlorizin, measured after dilution, was inhibited 75% by high extravesicular Na^+.

These findings concerning the effects of extravesicular Na^+ are consistent with the results of equilibrium binding studies which indicate that Na^+ increases the affinity (reduces the K_D) of the phlorizin receptor with no significant change in the total number of high affinity binding sites [7, 12, 14]. The increased affinity induced by Na^+ may thus be attributed to stimulation of the association rate constant and inhibition of the dissociation rate constant.

Effect of Transmembrane Electrical Potential on the Unidirectional Rate of Association

In the experiment shown in Table 1, imposition of out > in anion gradients was observed to modify the rate of phlorizin binding measured in the presence of 120 mM Na^+. Uptake was stimulated 39% by Cl^- and 83% by SCN^- when these anions were substituted for $SO_4^=$. Similar sensitivity to anion gradients has been described for the Na^+-dependent glucose transport system in renal [4, 18] and intestinal [21] brush border

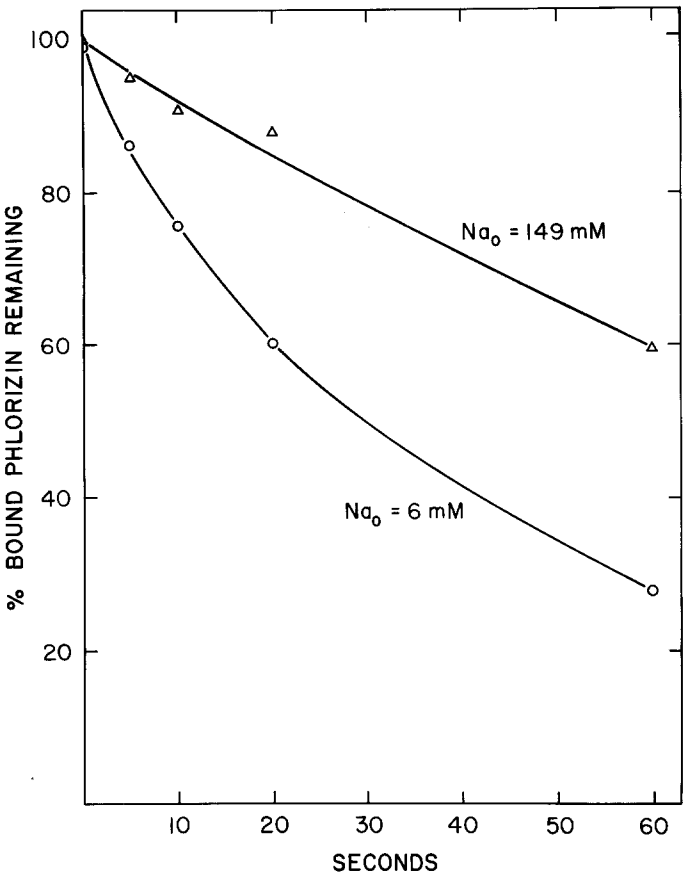

Fig. 3. Effect of extravesicular Na^+ on the rate of release of bound phlorizin. Membranes were equilibrated with 0.2 μM phlorizin for 60 min at 20 °C in the presence of 60 mM Na_2SO_4, then diluted 1:20 with either phlorizin-free mannitol medium (○) or 75 mM Na_2SO_4 (△), and the release of phlorizin assayed during reincubation at 20 °C. The zero time (100%) phlorizin binding was 27.2 pmoles per mg protein

membranes and has been thought to represent an effect of the transmembrane electrical potential difference on sugar transport. Presumably, imposition of an out > in gradient of a permeant anion renders the intravesicular space relatively more electronegative than does a similar gradient of a poorly permeant anion such as $SO_4^=$. The greater effectiveness of SCN^- than Cl^- in this regard is explained by the fact that the SCN^- conductance exceeds that of Cl^-, as recently found to be the case in isolated intestinal microvillus membranes [19]. These results concerning anion sensitivity therefore suggest that the rate of high affinity phlorizin binding is stimulated when intravesicular electronegativity is enhanced.

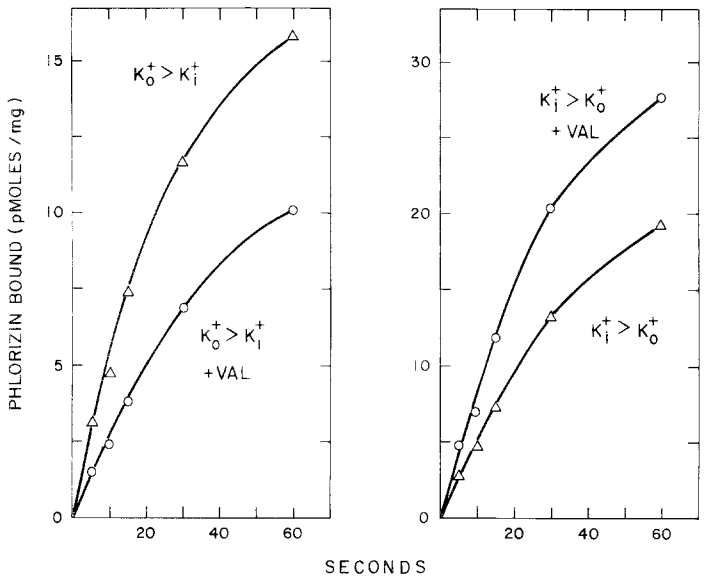

Fig. 4. Effect of valinomycin on the rate of phlorizin binding. $K_o^+ > K_i^+$: Uptake of 0.2 μM phlorizin at 20 °C in the presence of 30 mM Na_2SO_4 and 30 mM K_2SO_4 was determined using membranes preincubated for 60 min in mannitol medium with (○) or without (△) valinomycin (9.8 μg/mg membrane protein). $K_i^+ > K_o^+$: Uptake in the presence of 60 mM Na_2SO_4 and 7.5 mM K_2SO_4 was determined using membranes preincubated in 37.5 mM K_2SO_4 with (○) or without (△) valinomycin

To test this further, the effect of ionophores on phlorizin uptake was evaluated. As illustrated in Fig. 4, the K^+ ionophore valinomycin inhibited the rate of phlorizin binding (by 51% at 5 sec) in the presence of an out > in K^+ gradient. Under these conditions, the effect of valinomycin on transmembrane potential should be to enhance intravesicular electropositivity. Supporting the interpretation that the inhibition by valinomycin was due to an electrical effect is the observation, also indicated in Fig. 4, that valinomycin stimulated the initial phlorizin association rate (by 69% at 5 sec) when the direction of the K^+ gradient was reversed. Under these conditions, valinomycin should enhance intravesicular electronegativity. Valinomycin causes comparable stimulation of Na^+-dependent glucose transport into renal [4] and intestinal [21] microvillus membrane vesicles in the presence of in > out K^+ gradients.

Analogous experiments using pH gradients and the H^+ ionophore FCCP are illustrated in Fig. 5. FCCP inhibited the initial rate of phlorizin binding (by 36% at 5 sec) in the presence of an out > in H^+ gradient,

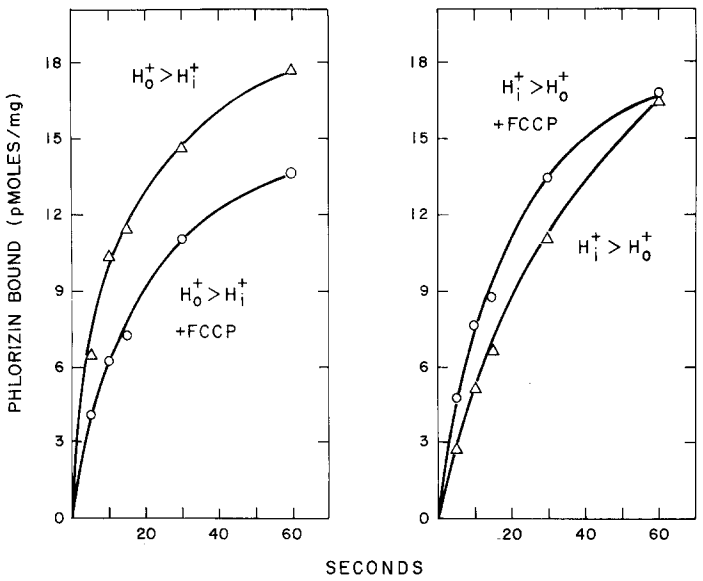

Fig. 5. Effect of FCCP on the rate of phlorizin binding. $H_o^+ > H_i^+$: Uptake of 0.2 μM phlorizin at 20 °C in the presence of 60 mM Na_2SO_4, 50 mM Tris-Mes, 4 mM Tris-Hepes, pH 5.8 with (○) or without (△) 15 μM FCCP was determined using membranes preincubated for 60 min in mannitol medium containing 21 mM Tris-Hepes pH 7.4. $H_i^+ > H_o^+$: Uptake in the presence of 60 mM Na_2SO_4, 4 mM Tris-Mes, 50 mM Tris-Hepes, pH 7.4 with (○) or without (△) FCCP was determined using membranes preincubated in mannitol medium containing 20 mM Tris-Mes, 1 mM Tris-Hepes, pH 5.6

but stimulated uptake (by 73% at 5 sec) when the H^+ gradient was reversed. However, under the latter circumstance, the effect of FCCP was short-lived, being no longer evident at 60 sec. Presumably the Na^+-H^+ exchange system which exists in the microvillus membrane [22] caused a rapid dissipation of the in > out H^+ gradient since an out > in Na^+ gradient was also imposed at the initiation of the incubation. Stimulation of Na^+-dependent glucose transport by proton conductors in the presence of in > out H^+ gradients has been previously reported [4, 21].

The effects of FCCP on phlorizin uptake are consistent with the results concerning anion gradients and valinomycin noted earlier. In every case, maneuvers to enhance intravesicular electropositivity caused inhibition, and those to enhance intravesicular electronegativity caused stimulation, of the initial rate of phlorizin association.

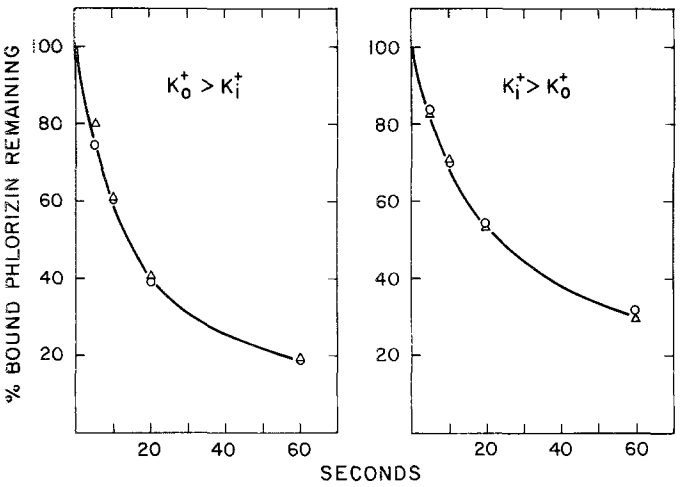

Fig. 6. Effect of valinomycin on the rate of release of bound phlorizin. $K_o^+ > K_i^+$: Membranes were equilibrated with 0.2 μM phlorizin for 60 min at 20 °C in the presence of 60 mM Na_2SO_4 with (○) or without (△) valinomycin (8.9 μg/mg membrane protein), then diluted 1:20 with phlorizin-free medium containing 75 mM K_2SO_4 and release assayed during reincubation. Zero time (100%) phlorizin binding was 31.4 and 33.8 pmoles per mg protein, with and without valinomycin, respectively. $K_i^+ > K_o^+$: Membranes were equilibrated with phlorizin in the presence of 30 mM Na_2SO_4 and 30 mM K_2SO_4 with (○) or without (△) valinomycin, then diluted 1:20 with phlorizin-free mannitol medium and reincubated. Zero time (100%) phlorizin binding was 22.4 and 24.7 pmoles per mg protein, with and without valinomycin, respectively

Effect of Transmembrane Electrical Potential on the Unidirectional Rate of Dissociation

In the experiment illustrated in Fig. 6, valinomycin and K^+ gradients were employed to examine the effect of the transmembrane electrical potential difference on the rate of dissociation of previously bound phlorizin. To evaluate the effect of enhanced intravesicular electropositivity, the possible influence of valinomycin on phlorizin release was determined in the presence of 143 mM extravesicular K^+ and in the absence of intravesicular K^+. No effect of valinomycin was noted. It should be recalled (Fig. 4) that approximately the same valinomycin concentration markedly inhibited the rate of phlorizin uptake in the presence of only 60 mM extravesicular K^+.

To evaluate the effect of enhanced intravesicular electronegativity, the influence of valinomycin on release was determined in the presence of 3 mM extravesicular K^+, using membranes that had been K^+ preloaded by incubation in 60 mM K^+. Again, no effect of valinomycin was noted

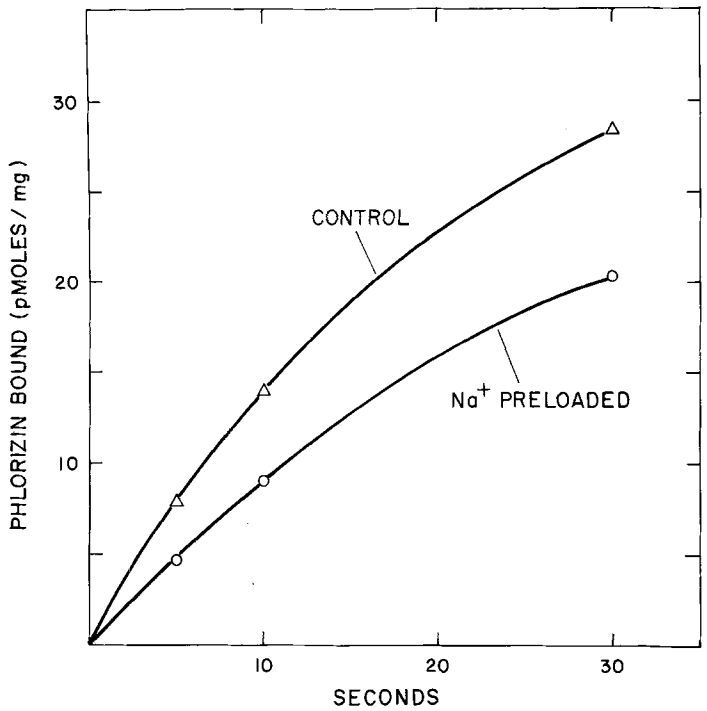

Fig. 7. Effect of intravesicular Na^+ on the rate of phlorizin binding. Uptake of 0.2 μM phlorizin at 20 °C in the presence of 75 mM NaCl was determined using membranes preincubated for 5 min in either mannitol medium (△) or 75 mM NaCl (○)

despite the previous observation (Fig. 4) that valinomycin markedly stimulated phlorizin uptake in the presence of 15 mM extravesicular K^+, using membranes that had been K^+ preloaded in 75 mM K^+.

Not illustrated are other phlorizin release experiments in which 10-fold greater valinomycin concentrations still failed to affect the glycoside dissociation rate. Similar experiments were also performed at high extravesicular Na^+ and, although the rate of phlorizin release was slower than that observed in Fig. 6, valinomycin was again without effect.

Effect of Intravesicular Na^+ on the Unidirectional Rate of Association

To determine the effect of intravesicular Na^+ on phlorizin uptake, membranes were preincubated for 5 min in the presence or absence of 75 mM NaCl and then binding assayed in a medium with a final NaCl concentration of 75 mM. As indicated in Fig. 7, the initial rate of phlorizin

Table 2. Effect of intravesicular Na^+ on phlorizin binding in valinomycin-treated membranes

Preincubation medium (mM)	Incubation medium (mM)	% Inhibition of binding
15 K_2SO_4	60 NaCl 15 K_2SO_4	0
15 K_2SO_4	30 Na_2SO_4 15 K_2SO_4	13.7±3.2
60 NaCl 15 K_2SO_4	60 NaCl 15 K_2SO_4	33.3±2.7

Membranes containing valinomycin (8.5 μg/mg protein) were K^+ loaded in 15 mM K_2SO_4 for 60 min at 20 °C. The 5 sec uptake of 0.2 μm phlorizin at 20 °C was then determined in the presence of either 60 mM NaCl or 30 mM Na_2SO_4 using membranes preincubated for 5 min in either mannitol medium or 60 mM NaCl. All media contained 15 mM K_2SO_4 and mannitol to maintain isotonicity. The control uptake (0% inhibition) was 1.83 pmoles per mg protein. Each datum is the mean ± the SE for three determinations.

binding was inhibited (by 39% at 5 sec) in the Na^+ preloaded membranes. *Trans*inhibition by Na^+ is also a characteristic of glucose transport in isolated microvillus membrane vesicles [2, 3, 16, 18].

In view of the electrical dependence of phlorizin binding, however, an alternate explanation for the inhibition resulting from NaCl preloading is that the effect is primarily electrical and arises from obliteration of the out > in Cl^- gradient. Such an explanation would be untenable if *trans*inhibition by NaCl was still observed under conditions where alterations in membrane potential were minimized. In theory, valinomycin should serve to fix the membrane potential close to the K^+ diffusion potential and thereby minimize alterations that could arise from variations in the Cl^- gradient.

For the experiments described in Table 2, valinomycin-treated membrane vesicles were K^+ loaded in 15 mM K_2SO_4, the same concentration of which was maintained in all experimental media. Under these conditions, NaCl preincubation caused twice the inhibition (33% *vs.* 14%) as did elimination of the Cl^- gradient by $SO_4^=$ for Cl^- substitution. This finding clearly indicates that phlorizin uptake is inhibited by intravesicular Na^+ *per se*.

Discussion

The significance of these findings rests on the assumption that measured phlorizin uptake represents binding to the microvillus membrane

itself rather than to an intravesicular structure. A possible trivial explanation for the observed energy-dependence of phlorizin uptake is that binding only occurs subsequent to translocation of the glycoside via the Na^+ gradient-dependent D-glucose transport system. This possibility is highly unlikely for several reasons. First, saturable binding of [^{3}H] phlorizin to intestinal brush borders, without intracellular accumulation, has been demonstrated by radioautography [30]. Second, [^{3}H]phlorizin bound to the renal microvillus membrane *in vivo* can be quantitatively recovered in the urine after flushing doses of cold phlorizin, suggesting that phlorizin is bound at the membrane surface [27]. Third, while some accumulation of phlorizin by renal cortical slices may occur after prolonged incubation, such uptake is Na^+-independent, nonsaturable, not inhibited by sugars, and thus not mediated by the Na^+-dependent glucose transport system [7]. Fourth, cold phlorizin displaces bound [^{3}H]-phlorizin from isolated renal microvillus membrane vesicles [7] but inhibits the efflux of [^{14}C]glucose [2, 3, 16, 18]. This indicates that the site of phlorizin binding must be external to the site of glucose sequestration and thus external to the intravesicular compartment. Fifth, if phlorizin gains access to its binding site only after electrogenic transport, then electrical dependence of phlorizin release should have been observed, and it was not.

Another important assumption is that the high affinity phlorizin receptor is identical with the Na^+-dependent glucose transporter. This is primarily based on the fact that high affinity phlorizin binding is Na^+-dependent, inhibited by D-glucose, and inhibited by only those sugar analogs which inhibit glucose transport [7, 12, 14, 28]. Moreover, phlorizin analogs demonstrate the same specificity for high affinity binding in isolated membranes [5] as for inhibition of glucose transport in the intact tubule [33]. Finally, phlorizin, at concentrations $< 10\ \mu M$, is a competitive inhibitor of glucose transport in both isolated membranes [3] and the intact tubule [33].

Membrane Model for Phlorizin Binding

The general model of carrier-mediated cotransport described by Schultz and Curran [26] can be adapted to the problem of phlorizin binding, as illustrated in Fig. 8. The details of the model will be discussed in relation to the experimental findings.

1) Effect of extravesicular Na^+. The stimulation of phlorizin uptake by extravesicular Na^+ implies that k_2, the rate constant for association

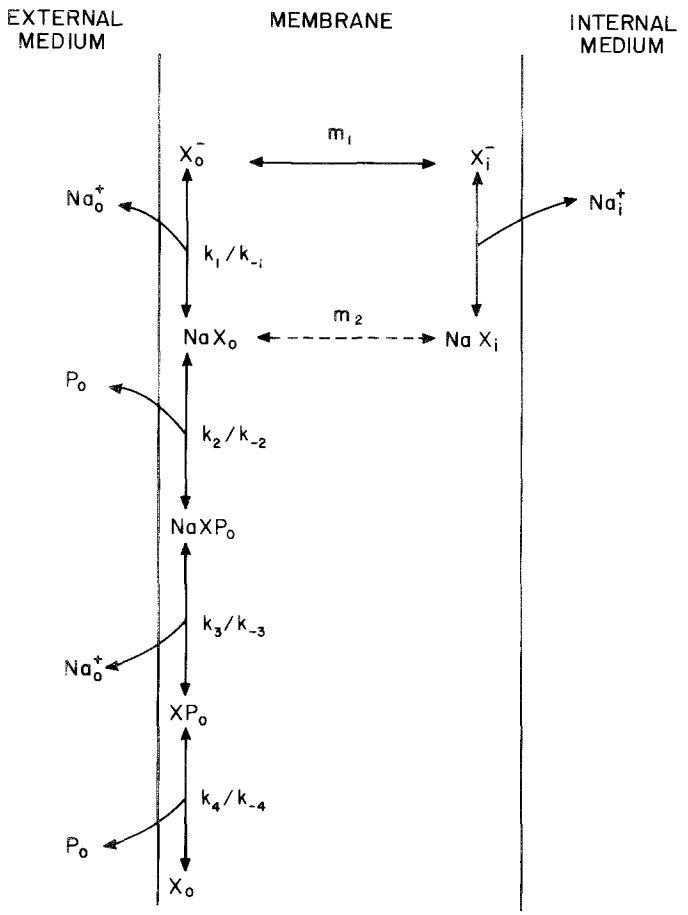

Fig. 8. Membrane model for phlorizin binding. *See text* for details

of phlorizin with the Na^+-carrier complex, is greater than k_4, the constant for phlorizin association with the free carrier. Similarly, the inhibition of release of bound phlorizin by extravesicular Na^+ implies that $k_{-4} > k_{-2}$. Taken together, $k_2/k_{-2} \gg k_4/k_{-4}$, which is to say that binding of Na^+ increases the affinity of the carrier for phlorizin. This is consistent with the known effect of Na^+ to reduce the K_D for equilibrium phlorizin binding [7, 12, 14]. An effect of Na^+ on the binding affinity of the carrier is also consistent with the observation that extravesicular Na^+ reduces the apparent K_m, without an effect on V_{max}, for glucose transport in renal microvillus vesicles [3].

2) Effect of transmembrane electrical potential. Phlorizin uptake was stimulated by increasing intravesicular electronegativity and inhibited by increasing intravesicular electropositivity. One explanation for these

findings is that the free carrier is negatively charged and thus that the appearance of free carrier at the external membrane surface is potential-dependent. A possible alternate explanation is that the free carrier is electroneutral and that there is potential-dependent translocation of the positively charged ternary complex, Na*XP*, to an internal membrane site. However, such an explanation would necessarily predict that release of bound phlorizin also be potential-dependent and this was not observed. Finally, it is possible that alterations in membrane potential induce a conformational change in the phlorizin binding site such that the association rate constant, k_2, is affected. Clearly, the dissociation rate constant, k_{-2}, must be potential-independent.

The observation that intravesicular glucose stimulates glucose uptake by isolated brush border vesicles [2, 16] implies that translocation of the free carrier is rate limiting for sugar transport [26]. Therefore, potential-dependent translocation of a negatively charged carrier provides a reasonable explanation for the known electrical dependence of glucose transport [4, 21]. Potential-dependent translocation of a positively charged ternary complex, as in the case of an electroneutral carrier, could not explain the electrical dependence of sugar flux.

3) Effect of intravesicular Na^+. The inhibition of phlorizin uptake by intravesicular Na^+ may be explained on the basis that m_2, the mobility of the binary complex Na*X*, is smaller than m_1, the mobility of the free carrier. Binding of Na^+ to the carrier at the internal membrane surface would therefore inhibit the appearance of free carrier at the external membrane surface. This mechanism will also account for the *trans*inhibition by Na^+ of glucose transport [2, 3, 16, 18] since, as already discussed, translocation of the free carrier appears to be rate limiting for sugar flux. A very low m_2 has the additional virtue of minimizing a possible pathway, via Na*X* translocation, for dissipating the Na^+ gradient without performing the work of energizing uphill glucose transport.

4) General comments. The present model can account, at least qualitatively, for the energization of phlorizin binding and glucose transport by the electrochemical Na^+ gradient. The key features of this scheme are that Na^+ increases the binding affinity of the carrier, that the free carrier is negatively charged, and that binding of Na^+ to the carrier in the absence of sugar inhibits carrier translocation. In essence, it is the appearance of free carrier at the membrane surface and affinity of the carrier to bind glucose, rather than the step of sugar translocation, which is affected by the Na^+ gradient. A similar model, also involving a negatively charged carrier, has been proposed by Kaback to explain

the coupling of lactose transport to the electrochemical H^+ gradient in *E. coli* membrane vesicles [17].

It should be stressed that this model makes no definitive statement as to the underlying molecular structure of the transport system. The concept of carrier-mediated transport has been used here, as recently discussed by Crane [8], to indicate a transport mechanism in which the substrate binding site functionally alternates between the two surfaces of the membrane. Thus, functional translocation of the carrier could well be achieved by conformational change in a transmembrane protein rather than by movement of a truly mobile carrier [29]. More precise determination of molecular structure will undoubtedly emerge from studies involving isolation [33] and reconstitution [9, 10] of transport proteins from the microvillus membrane.

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