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Similarity in Effects of Na^+ Gradients and Membrane Potentials on D-Glucose Transport by, and Phlorizin Binding to, Vesicles Derived from Brush Borders of Rabbit Intestinal Mucosal Cells

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Summary. Both the presence of sodium and of an electrical potential difference across the membrane have been found to be necessary in order to achieve optimal D-glucose-protectable phlorizin binding to brush border membranes from rabbit small intestine. The effect of $\Delta\tilde{\mu}_{Na}$ on phlorizin binding shows a close similarity to that on D-glucose transport, confirming that phlorizin is indeed bound to the D-glucose transporting protein. Possible modulations of binding by a transmembrane potential are discussed on the basis of some models.

Sugar transport across the brush border of intestinal or renal epithelia cells is Na^+-dependent and is generally considered to involve a cotransport of the sugars and the cation [2, 3, 8, 32]. Consequently, the influx of sugar into vesicles derived from brush borders of mucosal cells is considerably enhanced in the presence of an inward gradient of Na^+, leading to a transient increase (overshoot) of sugar concentrations within the vesicles, considerably above those of the medium [17]. The overshoot induced by the Na^+-gradient is larger if Cl^- is replaced by the more permeant anion, SCN^-, suggesting that the sugar influx may be modulated by the membrane potential (which would be determined by the relative rates of influx of Na^+ and anion) [28]. This interpretation is supported by the finding that in vesicles loaded with K^+, the sugar influx (overshoot) is increased by the addition of valinomycin [28]. Kinetic models for Na^+ and sugar cotrans-

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port, based on a mobile carrier hypothesis, predict effects of membrane potentials depending on whether the Na^+-binding site of the carrier is charged either in the unloaded or loaded state [13]. The exact form of the potential dependance, however, depends on the explicit assumptions in the model concerning rate limiting factors.

The sugar binding site of the transport system can also bind the agent, phlorizin, a potent competitive inhibitor; the K_i for the inhibitory action being about 8×10^{-6} M [35, 36]. High affinity binding of phlorizin, comparable to the K_i, has been directly measured in membranes prepared from brush borders of renal epithelial cells [4, 6, 7, 14, 34]. Although there is a similarity in the reported K_d values and our data, the wide range of values reported in the literature (*compare*, e.g., [6] and [34]; *see also* [37]) is an indication of the complex nature of the K_d function and of the importance of the experimental conditions in its determination (*see Discussion*).

Because the initial step in glucose transport involves the binding of this sugar to carrier(s) at the outside of the membrane, and because the same carriers may be directly measured by phlorizin binding, a series of studies were undertaken to determine the effects of Na^+, Na^+ gradients, electrical potentials, and anion substitution on sugar transport and on phlorizin binding. Vesicles prepared from intestinal mucosal cells of rabbit were used as a test system [19]. Some of the results described in the following were presented in a preliminary form [35, 36].

Materials and Methods

Rabbit intestine was collected from freshly killed commercial rabbits at a local slaughterhouse. It was cleaned and frozen before being brought to the laboratory for storage. Vesicles were prepared from the frozen tissue on the day of each experiment according to the procedure of Schmitz *et al.* [30] as modified by Kessler *et al.* [19]. Briefly, 20 g frozen tissue in 1 to 1.5-g pieces was allowed to thaw in 60 ml of 300 mM mannitol, 12 mM Tris-HCl, pH 7.5. The thawed tissue was mixed in solution with a Chemap Vibro-Mixer for 1.5 min to loosen the mucosal cell fragments from the connective tissue. The mixture was filtered through a Büchner funnel and the connective tissue discarded. The cell fragments were diluted sixfold with water, homogenized in a blender for 3 min at full speed, and then $CaCl_2$ was added to a final concentration of 10 mM. After standing for 20 min, the mixture was centrifuged at 3,000 $\times$ g for 15 min and the pellet discarded. The supernatant was then further centrifuged at 27,000 $\times$ g for 30 min.

The pellet was resuspended in 40 ml of buffer, homogenized in a Teflon Potter Elvehjem homogenizer, centrifuged at 27,000 $\times$ g for 30 min, and resuspended in the appropriate medium.

Influx or Binding

Unless stated otherwise, they were both measured at room temperature (ca. 20 °C). 10 μl of vesicles were carefully placed in the bottom of a clear polystyrene test tube fitted into a vibration device controlled by an electric timer. 10 μl of radioactive medium was placed as a separate drop at the bottom of the tube. At the start of the timer, the shaking of the vibrator rapidly mixed the two drops together (less than 80 msec). At the chosen time, 1.5 ml of cold isoosmolar NaCl was automatically injected into the incubation tube slowing down the reaction. (For details of the apparatus, *see* [20]). Then the sample was quickly filtered through a wet Sartorius microfilter (0.6 μm) and washed with a further 5 ml of the cold isoosmolar NaCl. The time required was 10 sec. After dissolution in 10 ml of toluene, Triton X-100, butyl PBD, acetic acid scintillation solvent, it was counted.

Uptake of glucose or phlorizin binding in the presence of an ion gradient was determined in the following way: sodium, phlorizin, and either glucose or fructose were all in the incubation medium. The vesicles were thus exposed to all three ligands simultaneously at the start of the incubation. When binding in the absence of a gradient was determined, the vesicles were allowed to come to equilibrium (1 hr) in appropriate ion solution before the phlorizin and either glucose or fructose incubation was begun.

Protein was determined according to Lowry *et al.* [24].

Choline thiocyanate was prepared as follows: an ion exchange resin, Dowex 50 W- H^+-form was charged with choline hydroxide until the eluate was strongly alkaline. Then KSCN was passed through the resin column and the choline thiocyanate was collected. Choline sulfate was prepared in a similar manner with K_2SO_4 replacing the KSCN. The concentration of the solutions were measured osmotically by freezing point depression. There was no potassium detectable in the final solutions when tested with a flame photometer.

D-[1 – ^{3}H]-glucose was from Amersham, Buks. (5.2 Ci/mmole). [^{3}H(*G*)]-phlorizin, lot No. 929–221, was obtained from New England Nuclear. The radioactive phlorizin yielded with cold phlorizin a single spot by both UV and radioactive detection. It had the same R_f value on Whatman 3 MM, using water as the chromatographic solvent at room temperature (J.S. Cook, 1977, *personal communication*), as authentic recrystallized phlorizin from ICN Pharmaceuticals, Inc., Life Science Group, Plainview, N.Y. The authenticity of the nonradioactive sample was checked by NMR.

Results

Phlorizin Inhibition of Glucose Transport in Rabbit Intestinal Brush Border Vesicles

Determination of the effects of phlorizin on D-glucose transport are illustrated in Fig. 1. After addition of labeled D-glucose plus NaSCN to the vesicles a transient overshoot of labeled glucose uptake occurs, with the internal concentration considerably exceeding that of the medium. The overshoot is not blocked by D-fructose, but it is inhibited by D-glucose or by phlorizin as described by others in preparations from kidney or from rat intestine [17, 21].

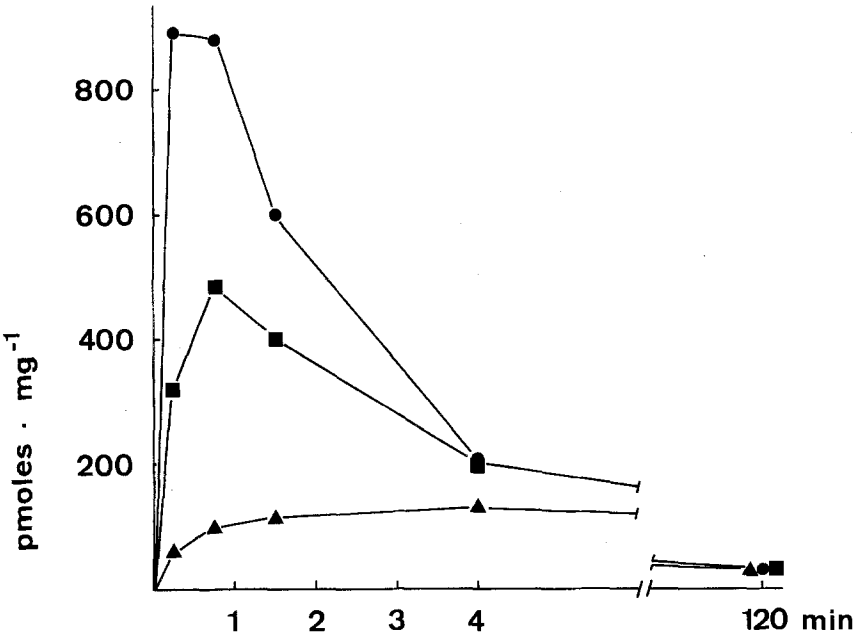

Fig. 1. Phlorizin inhibition of D-Glucose uptake by brush border vesicles. (●) control; (■) 20 and (▲) 100 μM phlorizin. D-Glucose concentration, 30 μM. Initial NaSCN gradient, 100 mM out, 0 in

Phlorizin Binding to Rabbit Intestinal Brush Border Vesicles. Binding under Equilibrium Conditions

High affinity binding of phlorizin, consistent with the K_i for its inhibition of sugar transport, has been reported for vesicles derived from brush borders of renal cells from rats or dogs [4, 34]. The same experimental conditions, i.e., incubation for several minutes with vesicles equilibrated with Na^+, applied to vesicles from brush borders of rabbit intestinal cells was however, unsuccessful. A large amount of low affinity binding was observed with no evidence of a high affinity component that could be correlated with the high inhibitory potency of the phlorizin. This could be due to various factors, including the action of phlorizin hydrolase which occurs in the intestine [10, 26] but not in the kidney [11]. However, the large low affinity binding was not related to sugar transport, for it was not significantly reduced in the presence of D-glucose.

Rapid Onset of Phlorizin Inhibition of Na-Dependent D-*Glucose Transport into Intestinal Brush Border Membrane Vesicles*

Using a newly developed rapid technique for measuring sugar uptake [20] (*see Materials and Methods*), it was found that the phlorizin inhibition was essentially maximal within less than 1 sec at 20 °C [36]. This indicated that an incubation time of 1 sec should be sufficient to measure high affinity phlorizin binding. In order to directly compare the binding with the inhibitory effect, experimental conditions were used that were identical with those for measuring sugar transport. At zero time phlorizin and/or glucose was added together with NaSCN to vesicles which did not contain any Na^+. Thus, phlorizin binding and glucose transport were determined in the presence of a transmembrane NaSCN gradient (*see Materials and Methods*).

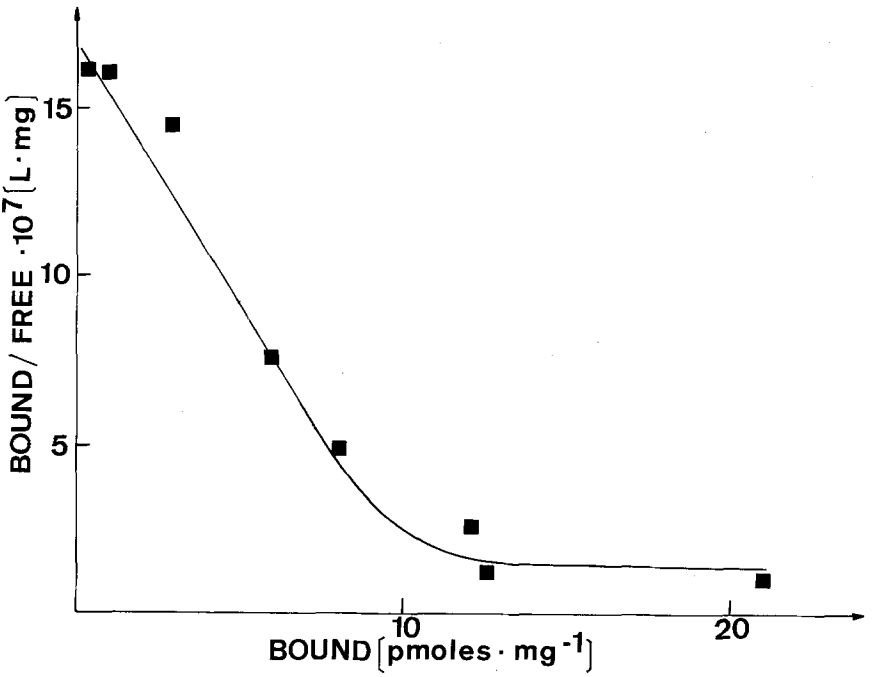

Fig. 2. D-Glucose-protectable phlorizin binding to brush border vesicles in the presence of an initial inward NaSCN gradient (100 mM). The exposure time of the membranes to phlorizin was 2 sec. Each point is the difference between the amount of phlorizin bound in the presence of D-fructose (25 mM) and D-glucose (25 mM)

Rapid High-Affinity Binding of Phlorizin

In preliminary experiments, a high affinity binding was indeed observed within 1 sec; the time of 2 sec was chosen for most experiments. After a longer period of time, it was submerged by a massive amount of low affinity binding. A typical set of data is illustrated in Fig. 2, plotted in the form of the Scatchard equation, so that the slopes represent the "affinities" and the abscissa intercepts the "number of sites". Although the lower portion of the plot, at higher phlorizin concentrations, curves off to the right (also seen in experiments on renal brush borders [34]), a substantial part of the data can be fitted by a straight line. The K'_d (apparent dissociation constant at the pH of the experiment reported) calculated from the slope was 4.62 μM and the number of sites calculated from the intercept was 13.8 pmoles per mg of protein. These values are reasonably close to those found for kidney brush borders [34]. D-fructose had little effect but D-glucose abolished the high affinity component to the extent of about 80% (Fig. 3).

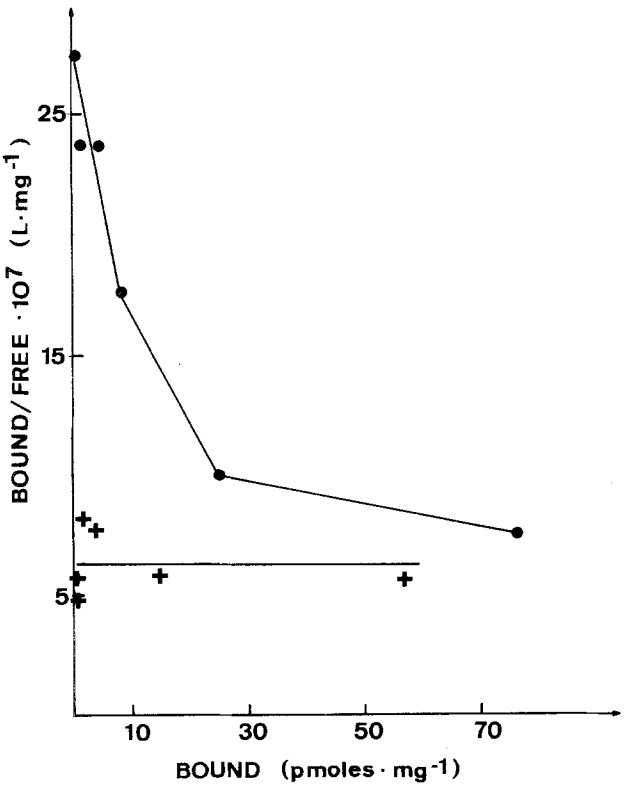

Fig. 3. D-Glucose inhibition of phlorizin binding to brush border vesicles. (●) 25 mM D-fructose; (+) 25 mM D-glucose. Initial conditions as in Fig. 2

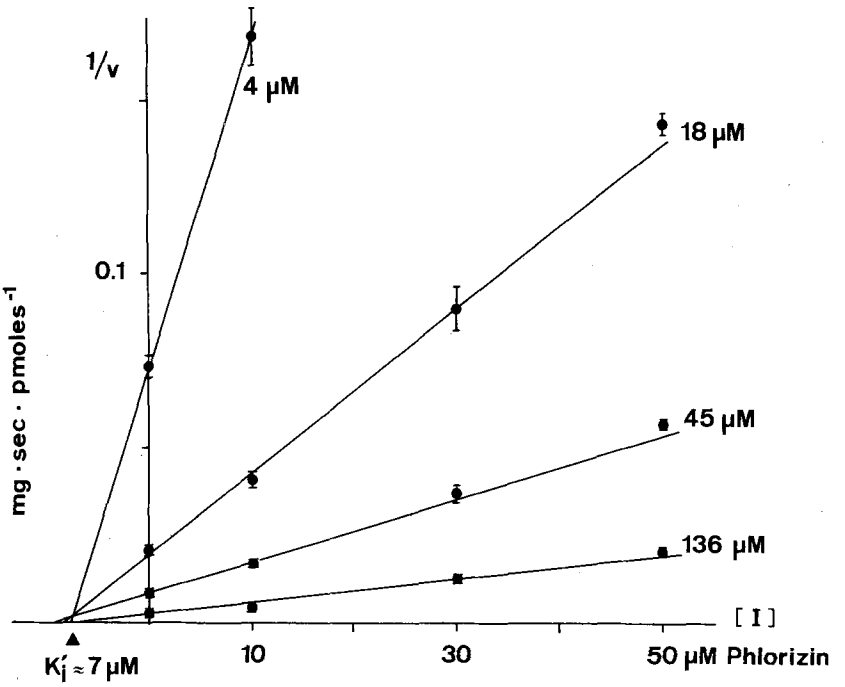

Fig. 4. Dixon plot of the inhibition of D-glucose uptake into brush border vesicles by phlorizin in the presence of a 100 mM inward NaSCN gradient. Incubation time, 2 sec. D-Glucose concentrations: 4, 18, 45, and 136 μM

Comparison of Phlorizin Binding and Phlorizin Inhibition of Glucose Transport

Using the rapid technique [20], it was determined that the rate of sugar uptake was linear for at least 2 sec and fell off thereafter. The kinetics of phlorizin inhibition were measured after 2 sec, so that the values represent the initial linear rate of sugar uptake. The conditions were also identical with those used for measuring phlorizin binding (Fig. 2). The resulting data are given in Fig. 4 in the form of the Dixon plot. The data fall along a straight line and the calculated K_i' is 7 μM, almost the same as the measured K_d' for the high affinity component of phlorizin binding from Fig. 2.

Rate of Dissipation of Sodium Gradient

The overshoot in D-glucose uptake into these vesicles in the presence of an initial NaSCN gradient (100 mM out, 0 in) peaks at 30 to 60 sec [19].

During this time the NaSCN gradient is dissipating and presumably has almost completely disappeared by 10 min. The rate of dissipation of the Na^+ gradient was determined directly by measuring the uptake of Na^+ under conditions identical to those used in measuring D-glucose uptake. At the external Na^+ concentration used (100 mM) the half time for Na^+ equilibration was 1–1.5 min, and the initial rate of uptake was independent of D-glucose concentration in the range 25–325 μM. (For a 1:1 Na^+, glucose flux ratio, any glucose-dependent change in Na^+ uptake would have been below the detection range.) During the 2-sec interval chosen for measurements of binding and of initial rate of D-glucose uptake, the uptake of Na^+ was thus less than 8%.

The Effects of Na^+, Na^+-Gradient, and Membrane Potential on High-Affinity Phlorizin Binding

(1) *Initial NaSCN gradient:* The high-affinity component of phlorizin binding in Fig. 2 was measured in the presence of an initial NaSCN

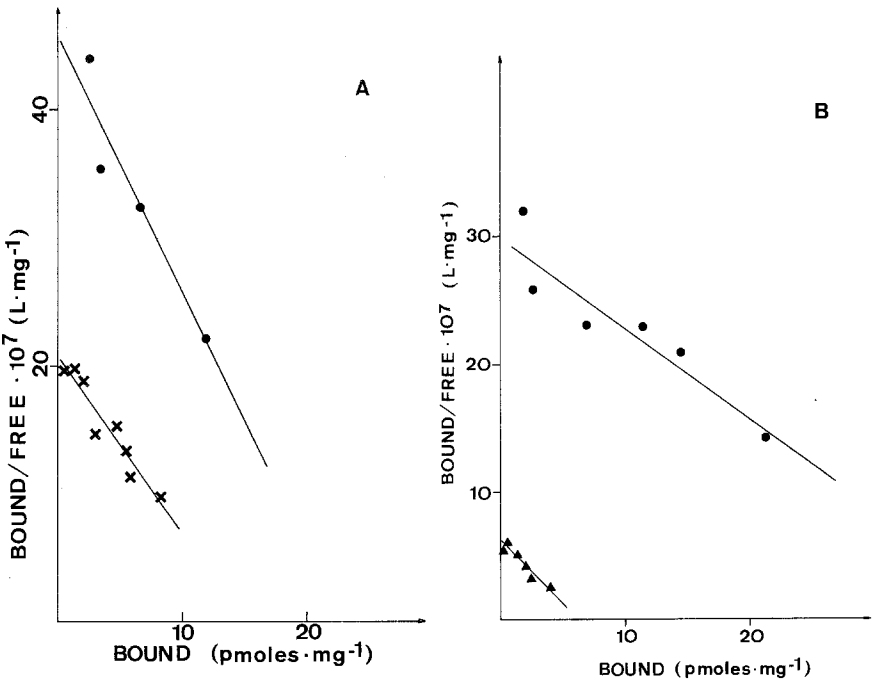

Fig. 5. D-Glucose protectable phlorizin binding to brush border vesicles in the presence (●) or absence (×, ▲) of a 100 mM inward NaSCN gradient. To eliminate the gradient, vesicles were incubated for 1 hr in 100 mM NaSCN prior to addition of phlorizin. Phlorizin incubation time, 2 sec. (*A*): Binding at pH 7.5. (*B*): Binding at pH 6.5

gradient. If the gradient was allowed to dissipate by allowing the vesicles to equilibrate in NaSCN for 1 hr, before the addition of phlorizin (but still measuring the binding after 2 sec), phlorizin binding was markedly reduced (Fig. 5*A* and *B*).

(2) *The influence of membrane potential on high-affinity phlorizin binding* was determined by comparing the effects of NaSCN, NaCl and Na_2SO_4. The "overshoot" in D-glucose uptake was considerably smaller with NaCl than with NaSCN gradients, a behavior attributed to the lower rheogenic permeability of the membrane to Cl^- as compared to SCN^- [23] which leads to the formation of membrane potentials of different sizes.

In parallel, phlorizin binding was significantly lower in a NaCl than in a NaSCN gradient. The extent of reduction in phlorizin binding was approximately the same as the extent of reduction in the initial rate of D-glucose uptake and in the apparent affinity of phlorizin when measured as a fully competitive inhibitor of D-glucose transport (Fig. 4); i.e., about 4-times (Table 1).

(3) *Thiocyanate-induced potential in the absence of Na^+ gradient:* A D-glucose overshoot can be produced by an initial SCN^- gradient (by addition of choline thiocyanate) in the absence of an inward Na^+ gradient (Fig. 6) with a parallel increase in phlorizin binding (Fig. 7). Addition of

Table 1. Interaction of phlorizin with membrane vesicles from rabbit small intestinal brush borders: K_i'-values from phlorizin inhibition of initial D-glucose uptake and K_d' values for the high-affinity, D-glucose protectable phlorizin binding sites[a], all determined at pH 7.5

	Phlorizin inhibition of D-glucose uptake	Phlorizin binding[b]	
	K_i' (μM) $\bar{x} \pm$ SE (n)	K_d' (μM) $\bar{x} \pm$ SE (n)	Approximate concentration of high-affinity, glucose-protectable binding sites (pmole per mg membrane protein): $\bar{x} \pm$ SE (n)
With NaSCN gradient	7.1 ± 1.1 (4)	4.62 ± 0.85 (5)	13.80 ± 3.5 (5)
With NaCl gradient	31 ± 3 (2)	28 ± 5 (2)	> 30[b]

[a] (Primed K's indicate K_i and K_d values referring to the total concentration, charged + uncharged, of phlorizin.)

[b] Due to the curvature of some Scatchard plots, quantitative comparison can only be made among experiments carried out under strictly identical conditions.

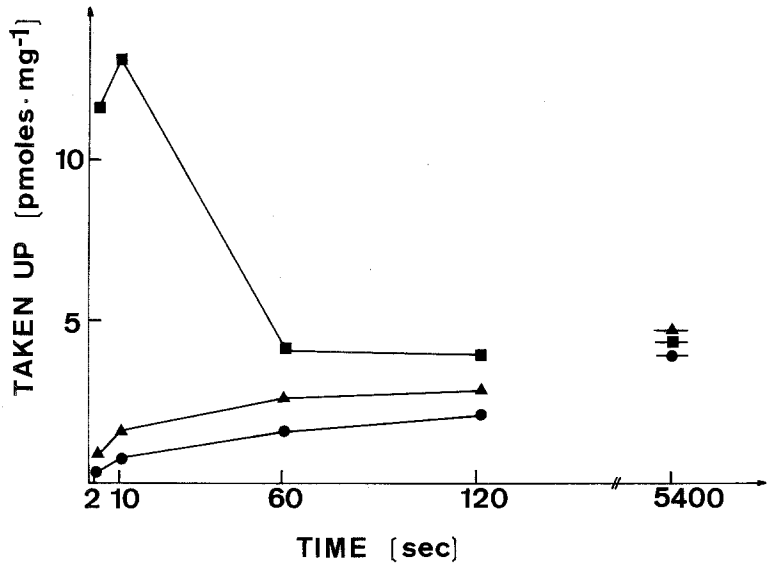

Fig. 6. Resolution of the effects of Na^+ and of SCN^--induced potential gradient on D-glucose transport by brush border vesicles. (■) Vesicles pre-equilibrated in 50 mM Na_2SO_4; transport measured in the presence of a 100 mM choline thiocyanate gradient. (▲) 50 mM Na_2SO_4 pre-equilibration; 50 mM choline sulfate gradient. (●) 50 mM choline sulfate preequilibration; 100 mM choline thiocyanate gradient. All incubations were at pH 7.5. D-Glucose concentration, 10 μM

choline sulfate (Figs. 6 and 7), rather than choline thiocyanate, on the other hand, had no effect on either D-glucose transport or phlorizin binding. The extent of stimulation of D-glucose transport and of phlorizin binding was influenced in parallel (in Na^+-preequilibrated vesicles) by different sizes of the choline thiocyanate imposed (Fig. 8). The observation that phlorizin binding is increased twofold by a 90 mM choline thiocyanate gradient and D-glucose transport is increased fivefold by the same gradient (Fig. 8) could be a result of the transport system recycling many times during the 2-sec incubation. The binding of phlorizin to the maximum number of sites would be complete after only one cycle. Experiments with a potassium gradient (in > out) and valinomycin yielded ambiguous results due to the high permeability of these membranes to potassium even in the absence of valinomycin and perhaps also to inhibition by K^+.

(4) *Na^+ requirement:* Although the effects of the thiocyanate gradient on the rate of D-glucose influx or on phlorizin binding could be produced without an inward Na^+ *gradient*, no such effects were seen in the *absence* of Na^+ (Fig. 6, 7). Thus, a Na^+-requiring process is involved. If Na^+ was

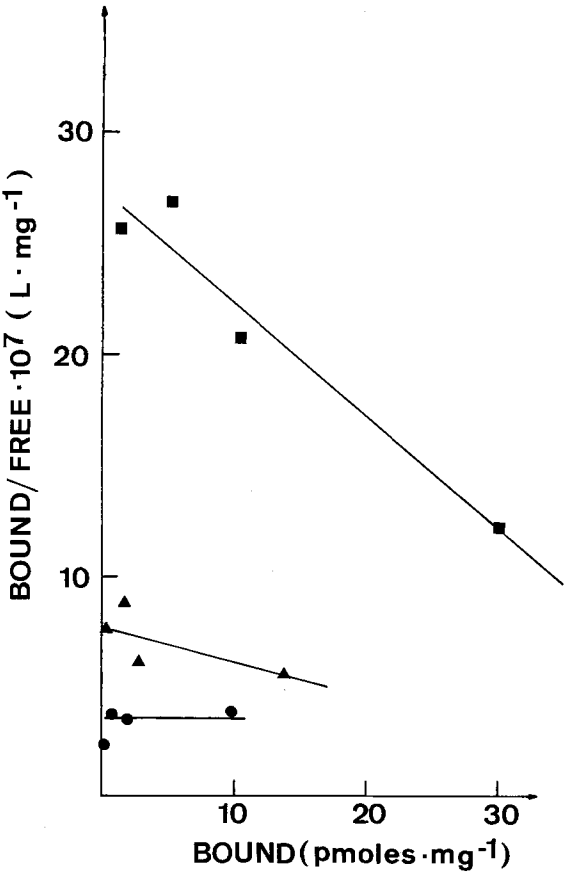

Fig. 7. Resolution of the effects of Na^+ and SCN^- induced potential gradient on phlorizin binding to brush border vesicles. (■) Vesicles pre-equilibrated in 50 mM Na_2SO_4; binding determined in the presence of a 100 mM choline thiocyanate gradient. (▲) 50 mM Na_2SO_4 pre-equilibration; 50 mM choline sulfate gradient. (●) 50 mM choline sulfate pre-equilibration; 100 mM choline thiocyanate gradient. All incubations were at pH 7.5 for 2 sec

replaced by choline then, even in the presence of an electrical membrane potential (choline thiocyanate gradient), there was a reduction in phlorizin binding (Fig. 7). Thus, the binding is somewhat increased by the presence of Na^+, and is increased to a much greater extent in the presence of a NaSCN gradient. This behavior is analogous to that observed for the effects of sodium and membrane potential on D-glucose transport; the addition of sodium increased the rate of glucose uptake and the further imposition of a membrane potential yielded a further increase in uptake rate (Fig. 6).

(5) *Ruling out osmotic effects, ionic strength and chaotropic action as major factors in high-affinity phlorizin binding:* Sets of data which were

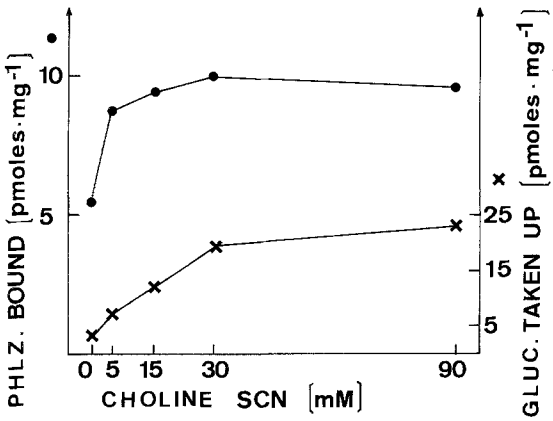

Fig. 8. Effect of increasing choline thiocyanate gradients on D-glucose uptake by, and phlorizin binding to, brush border vesicles. (●) D-Glucose (25 mM) protectable phlorizin binding. Phlorizin concentration, 2 μM. (×) D-Glucose uptake (10 μM). All vesicles were pre-equilibrated for 1 hr in 50 mM Na_2SO_4. Incubations were at pH 6.5 for 2 sec

conceived as controls in experiments discussed above also provided evidence that high-affinity phlorizin binding is not primarily due to either osmotic effects or high ionic strength; e.g., choline SCN gradient in the absence of Na^+, or preincubation in NaSCN without $\Delta\psi$ imposed, etc., all failed to produce high-affinity phlorizin binding. Finally, high affinity phlorizin binding is probably not produced, at least directly, by the chaotropic action of SCN^-, because it was absent or strongly reduced under either of the following conditions: (i) in the presence of a *choline* SCN^- gradient (Fig. 7, ●) and (ii) in the presence of 100 mM NaSCN at *both* sides of the membrane (Fig. 5, × and ▲).

Phlorizin binding and pH: Titrations of phlorizin under the conditions similar to those of the experiments on D-glucose uptake and phlorizin binding gave a pK'_a of 7.4, i.e., similar to that reported for the aglycone phloretin, 7.3 [18]. Thus, at the pH of the above experiments, about one half of the phlorizin in the bulk phase is dissociated, carrying one negative charge.

In the case of phloretin, the inhibitory action on sugar transport in red blood cells has been attributed to the uncharged form, based on increased potency at reduced pH [22]. The observation that phlorizin binding critically depends on the *electro*chemical gradient of Na^+ made it imperative to investigate the effect of pH on phlorizin inhibition of D-glucose uptake and on high-affinity phlorizin binding.

The observation of Fig. 7, i.e., the reduction of high affinity phlorizin

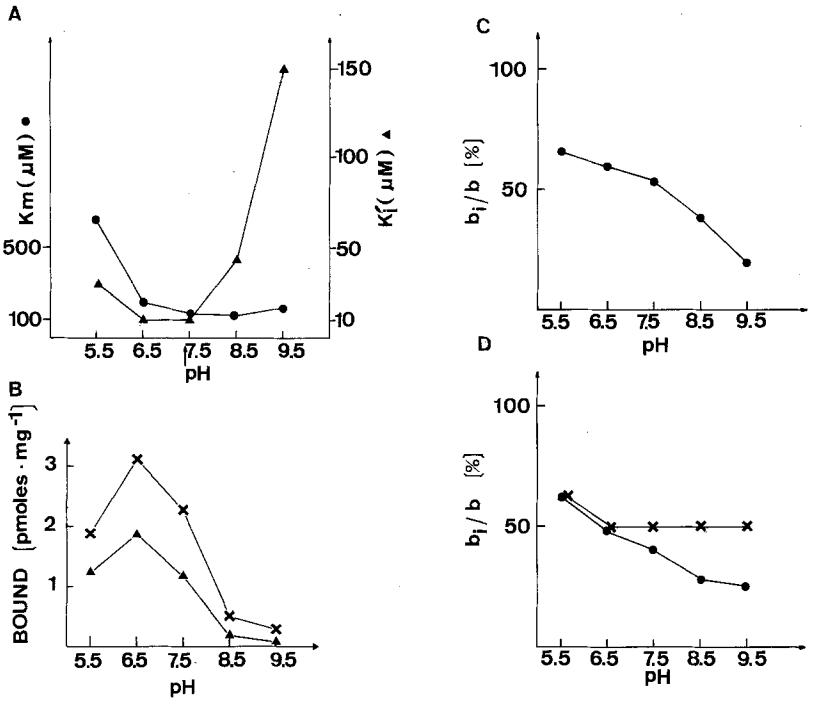

Fig. 9. Effect of pH on D-glucose transport (K_m), on phlorizin inhibition of this transport (K_i), and on D-glucose inhibitable phlorizin binding to brush border vesicles. (*A*): Variation in K_m and K_i with pH. K_m was determined from Eadie plots; D-glucose concentrations: 20, 60, 150, and 400 μM. K_i was determined from Dixon plots; D-glucose concentration, 20 μM; phlorizin concentrations, 0, 10, 25, and 50 μM. (*B*): Variation in D-glucose protectable phlorizin binding with pH. D-Glucose concentration, 50 mM; phlorizin concentration, 10 μM. (×) 100 mM NaSCN gradient. (▲) 100 mM NaSCN gradient plus 400 μM D-glucose. (*C*): Ratio of 400 mM inhibited phlorizin binding (b_i) to uninhibited binding (b) as a function of pH. Calculated from data of (*B*). (*D*): Theoretical plots of b_i/b *vs.* pH. (×) pH independent binding; i.e., phlorizin binds in both forms. (●) pH dependent binding; only the protonated form binds. (Constants used for the calculation: $K_d = 5$ μM; $pK'_a = 7.4$; $K_m = 130$ μM; at pH 5.5, $K_m = 230$ μM.) The arrow in *A* indicates the pK'_a-value of phlorizin, as determined titrimetrically under conditions similar to those under which D-glucose uptake was measured

binding in the presence of a membrane potential, but in the absence of Na^+, already indicated that this phlorizin binding was more than a simple electrostatic phenomenon. This conclusion was further substantiated by the experiment of Fig. 9 where the K_m-value for glucose transport did not significantly change in the pH-range 6.5 to 9.5 (Fig. 9 *A*), but both phlorizin inhibition of glucose transport and the degree of glucose-protectable, high-affinity phlorizin binding did decrease at pH values higher than approximately 8. The observed degree of inhibition of (high-affinity) glucose-protectable phlorizin binding by 0.4 mM D-glucose (Fig. 9 *C* and *D*) agreed

fairly well with the degree of inhibition calculated from the K_m- and K_i'-values of D-glucose transport and phlorizin inhibition (Fig. 9*A*). These observations, while again pointing to the identity of high-affinity phlorizin binding sites with (a part of) the Na^+-dependent D-glucose carrier(s), and to the fact that high-affinity phlorizin binding is not merely an electrostatic phenomenon, indicate either one or both of the following mechanisms: (i) phlorizin binds to the Na^+-dependent D-glucose carrier only in the protonated (uncharged) form; and/or (ii) the aglycone-binding region preferably binds phlorizin when the site is in a protonated form.

Discussion

Kinetic Parameters of D-*Glucose Uptake*

Many of the experiments described in this paper were done under conditions of ion gradients that were dissipating during the course of the experiment. Most of the comparisons of phlorizin binding and rate of sugar uptake were made two seconds after the gradients were established. Considerable evidence suggests that during this short time only a small fraction of the gradient is dissipated and that the system can be considered to be in a *quasi steady state*. Evidence supporting this contention can be listed: (i) the rate of uptake of D-glucose is linear for at least 2 sec, indicating that the driving force for the Na^+, glucose cotransport is not substantially changed in 2 sec; (ii) the kinetics of sugar transport determined from the 2-sec rates gives a straight line on a kinetic (Dixon) plot (Fig. 4) supporting the same conclusion; (iii) the values of K_m (for D-glucose) and K_i' (for phlorizin) were unchanged when incubation times were varied among 1, 2 and 7 sec [20, 36]; (iv) the peak of the overshoot in various experiments occurs in 30 to 60 sec. In two seconds only a fraction of the gradient could be dissipated; (v) direct measurement of the Na^+ fluxes indicated that less than 8% of the cation gradient is dissipated in 2 sec and that Na^+ fluxes were independent of the concentration of D-glucose at least in the range used by us, i.e., 25–325 μM. It should be noted, furthermore, that this rate of dissipation is maximal for it occurs with the maximum Na^+-gradient used, and with an electrical gradient produced by the permeant anion, SCN^-. In other experiments with either no Na-gradient or a reduced electrical gradient (through use of a less permeant anion), the rate of dissipation of ion gradients should be even less.

In addition to these experimental observations, there are other reasons why experimentally determined 2-sec transport parameters should reflect

true *initial* kinetic parameters. It can be deduced from the Goldman equation [15] that if the permeability of the membrane to the cation (sodium and perhaps hydrogen and buffer cations) is not insignificant compared to the permeability to thiocyanate[1] the transmembrane potential initially generated is a function of the ratio between the permeation rates of cations and anions across the vesicle membrane (out → in). The transmembrane potential should, therefore, remain essentially constant until the thiocyanate concentration inside the vesicles reaches a level where the thiocyanate distribution ratio becomes the overriding factor in determining the $\Delta\psi$ across the membrane. These considerations indicate that the collapse of the transmembrane potential should be minimal during the first seconds after establishing a NaSCN gradient across the membrane.

A second theoretical reason for accepting the kinetic parameters deduced from the 2 sec measurements as reliable is apparent from the equations derived by Geck and Heinz [13]; in cation-driven transport systems, at very high values of membrane potential difference (as are likely to be created by the initial NaSCN gradients which we have used) substrate fluxes and K_m-values are independent of moderate decreases in $\Delta\psi$.

Dependence of High-Affinity, Glucose-Protectable Phlorizin Binding on $\Delta\psi$ and Na^+

Many studies have demonstrated a dependence of sugar transport in epithelial systems on Na^+ [2, 3, 8, 32]. In vesicles derived from brush borders of renal or intestinal epithelial cells from rats or dogs the influx of glucose is enhanced by Na^+ gradients so that the internal concentration transiently exceeds that of the medium by several fold ("overshoot" phenomenon) until the gradient has dissipated [7, 17, 19]. The size of the overshoot is also dependent on the permeability of the membrane to the anion, suggesting some dependence on the electrical potential. This interpretation is supported by the finding that in K^+-rich vesicles, valinomycin will also produce an "overshoot". In this respect, the behavior

1 Unpublished observations of M. Kessler of this laboratory indicated that the permeability of the membrane to SCN^- is some 5–10 times that to Na^+. This is well within the range of our assumptions regarding the application of the Goldman equation. At the beginning of the incubation, when $[SCN^-]_i$, $[Na^+]_i \simeq 0$ and $[Na^+]_0 = [SCN^-]_0$, the Goldman equation

$$E_m = \frac{RT}{F} \ln \frac{P_{Na^+} \cdot [Na^+]_0 + P_{SCN^-} \cdot [SCN^-]_i}{P_{Na^+} \cdot [Na^+]_i + P_{SCN^-} \cdot [SCN^-]_0} \quad \text{reduces to}$$

$$E_m = \frac{RT}{F} \ln \frac{P_{Na^+}}{P_{SCN^-}}.$$

of the brush border membranes with respect to sugar uptake is somewhat similar to that of bacterial vesicles [29, 38, 39] or metabolically depleted *E. coli*, except that H^+ replaces Na^+ in the latter system.

Vesicles derived from the brush border of rabbit intestinal cells used in the present experiments also behave in a similar fashion with respect to D-glucose transport. Without Na^+, no overshoot is produced. In the presence of a NaSCN gradient (at zero time), a large D-glucose overshoot is produced. The importance of the anion is indicated by the reduced overshoot with Cl^-, a less permeant anion [23]. This experiment suggests that the potential (due to the relative rates of influx of Na^+ and the anion) must play a role, but the relative contributions of Na^+ gradient *per se* (due to effects of Na^+ concentrations on the two sides of the membrane) and of $\Delta\psi$ cannot be quantitatively differentiated in the experiments in which both a Na^+ and an anion gradient are present. The effects of $\Delta\psi$, independent of an inward Na^+ *gradient* were, therefore, separately examined as follows. In the experiment of Fig. 7, a choline SCN gradient was imposed on the vesicles in the absence (●) or in the presence (■) of Na^+. High-affinity phlorizin binding was observed in the latter set of data only. (Choline can cross these membranes [19], but its rate of movement is considerably smaller than that of Na^+ [19]. Thus, the $\Delta\psi$ due to a choline SCN gradient must be at least equal to, if not larger than, that produced by a similar NaSCN gradient.) It should be noted that in the experiment with Na^+ (Fig. 7), this cation was present at zero time at both sides of the membrane at concentrations which saturated the glucose transport system (i.e., above 40–50 mM; M. Kessler, *unpublished observations*), so that changes in the concentration of Na^+ due either to $\Delta\psi$ or to osmotic shrinkage of the vesicles should not affect the degree of Na^+ occupancy of the carrier. Also, experiments with equal Na^+ concentrations but different SCN^- gradients (Fig. 8) showed that high-affinity phlorizin binding varies with the size of the SCN^- gradient. Therefore, the effect observed cannot be attributed to osmotic shrinkage which must have been approximately the same in all experiments of Fig. 7.

Finally, the experiments of Figs. 7, 8 also rule out the ionic strength of the medium as the decisive factor in high-affinity phlorizin binding. The conclusion seems inescapable, therefore, that $\Delta\psi$ is a necessary (but, if alone, not sufficient) factor in determining high-affinity, glucose-protectable phlorizin binding; Na^+ also has to be present (at least at the outer surface of the membranes).

On the other hand, and complementary to this conclusion, Na^+ alone without $\Delta\psi$ does not produce optimal high-affinity, glucose-protectable phlorizin binding. Fig. 5 shows a much reduced binding in vesicles

preincubated in NaSCN; $\Delta\psi$ must also be applied. We conclude, therefore, that the simultaneous presence of both Na^+ and $\Delta\psi$, i.e., the presence of a $\Delta\tilde{\mu}_{Na^+}$ across the membrane with *both* the electric and the concentration terms >0, are necessary for optimal high-affinity, glucose-protectable phlorizin binding.

High-affinity phlorizin binding has been determined (by various procedures reported by others) to be present in renal tubuli preparations [4, 6, 14, 21, 34]. To the best of our knowledge, no dependence of high-affinity glucose-protectable phlorizin binding on the $\Delta\tilde{\mu}_{Na^+}$ was observed in these preparations, although the K_d-value was reported to depend on the *presence* of Na^+ [6, 14, 21, 34]. It is not clear whether the conditions employed by previous authors may have generated a $\Delta\tilde{\mu}_{Na^+}$ during binding measurements, or whether the $\Delta\tilde{\mu}_{Na^+}$ dependence may be specific for (rabbit) small intestine, or even for the kind of vesicle preparation which we have used. For example, it is quite possible that two agencies with overlapping substrate specificities, but both requiring Na^+ are operative in the small intestine for the transport of glucose and its analogues across the brush border membrane [16]. It is not known if and how phlorizen binding to each of them depends on $\Delta\psi$ and on $[Na^+]_0$. Now, functional and other differences have been reported between the characteristics of Na^+-dependent monosaccharide transport in renal tubuli and in the small intestine, which may perhaps be attributed to the two transport agencies being present in renal tubuli in a different ratio than in the intestine [27]. Whether this explains the possible lack of $\Delta\tilde{\mu}_{Na^+}$ dependence of phlorizin binding in renal tubuli is a matter for further analysis.

Others have reported the binding of competitive inhibitors and other ligands to membranes to depend on $\Delta\psi$ (e.g., [5, 31]).

Identity of the High-Affinity Phlorizin Binding Sites with (a part of) the Na-Dependent Monosaccharide Carrier

The involvement of the small-intestinal Na^+-dependent monosaccharide transporting system in the high-affinity phlorizin binding is shown by the following observations:

1) D-glucose inhibits high-affinity binding of phlorizin (Figs. 3 and 9).

2) In the pH range tested, i.e., between 5.5 and 9.5, the extent of inhibition of high-affinity binding by 0.4 mM D-glucose corresponds closely to that predicted from the K_m for glucose transport and the K_i' for phlorizin inhibition of glucose transport (Fig. 9 *C*, *D*).

3) The K_d' for high-affinity, glucose-protectable phlorizin binding

corresponds closely to the K_i' for phlorizin inhibition of D-glucose transport; both constants vary with experimental conditions in a similar manner (Table 1).

4) In particular, for these vesicles, both high-affinity, glucose-protectable phlorizin binding (as discussed above) and D-glucose uptake (Figs. 6, 7 and 8) depend on $\Delta\tilde{\mu}_{Na^+}$.

Thus, the high-affinity, glucose-protectable phlorizin binding site is involved in D-glucose binding to and/or transport across these membrane vesicles. This does not mean that phlorizin binding is confined to the *monosaccharide binding site* of the carrier; it has been suggested by others [1, 9, 10], and not without reason, that the high affinity of phlorizin binding (and inhibition) is due to its simultaneous binding to the monosaccharide binding site and to an adjacent aglycone binding site.

The high-affinity glucose-protectable phlorizin-binding membrane protein may or may not be the whole of the Na-dependent monosaccharide carrier(s). Other components (lipids, other membrane proteins?) may also be involved.

The Scatchard plots of Figs. 2 and 3 allow a calculation of the number of high-affinity, glucose-protectable phlorizin binding sites in the membrane (approximately, 4 pmoles $\cdot$ mg^{-1} membrane protein). This figure, and similar figures published by others for phlorizin binding to renal tubuli [4, 6, 14, 21, 34] should be taken with caution, due to the probably complex significance of this $\Delta\tilde{\mu}_{Na^+}$-dependent "maximum binding". In addition, some Scatchard plots, particularly those in the presence of a NaCl, rather than a NaSCN gradient, were somewhat curved. However, this "maximum number of binding sites" was increased twofold by negative purification by papain treatment, which removed other membrane proteins, apparently not involved in phlorizin binding [36]. At the same time, D-glucose transport was also negatively purified by a factor of two [33, 36].

The close parallelism between high-affinity phlorizin binding and D-glucose transport in these vesicles (*see above*) raises the question of whether phlorizin is a fully competitive inhibitor, as generally but not universally believed [25], or a very poor substrate. This question was discussed elsewhere and it was shown that phlorizin is not taken up by these vesicles in an osmotically active space.[2] Phlorizin is, therefore, a fully competitive inhibitor of Na-dependent monosaccharide transport across small-intestinal brush border membranes.

2 Kessler, M., Toggenburger, G., Rothstein, A., Semenza, G., 1978. Phlorizin as an inhibitor of D-glucose transport by intestinal brush border vesicles. Differentiation between simple binding and transmembrane translocation of phlorizin (*in preparation*).

An Attempt to Integrate $\Delta\psi$ and Na-Dependence of High-Affinity Phlorizin Binding with the Mechanism of Na-Dependent Monosaccharide Transport

Whatever the model assumed, only a movement of the carrier[3] (or of a part thereof) can explain the $\Delta\psi$-dependence of high-affinity phlorizin binding, in the presence of Na^+, to the Na-dependent D-glucose carrier. This dependence can, therefore, be taken as an independent additional criterion for the transport agency being mobile, at least in some part.

More than one mechanism could, in principle, lead to an integrated picture of the dependence of both D-glucose transport into and phlorizin binding by brush border membranes. Since only the *uncharged* form of this glucoside is likely to bind, this dependence indicates that phlorizin binding involves a movement, either "gate opening" or carrier "dislocation", of the transport agency involved.

D-Glucose transport is known to be electrogenic [32]. The direction of the current flow in intact tissue and the increase in uptake by vesicles in response to increasing transmembrane potential (in the presence of Na^+) can be accounted for if either (i) the unloaded carrier is negatively charged and the rate-limiting step in Na^+ D-glucose transport is the reorientation of the empty carrier or (ii) the fully loaded carrier is positively charged and the rate-limiting step is the inward reorientation of this form of the carrier.

If alternative (*i*) (negatively charged unloaded carrier) holds true, then the effect of the transmembrane potential (inside negative) would be to accelerate the rate of conformational change of unloaded carrier from the internal to the external orientation, thereby increasing the number of externally oriented phlorizin (or substrate) binding sites. In addition, Na^+ would also have to be required for phlorizin to bind to the exposed sites. If D-glucose is the ligand, the electrically neutral D-glucose-Na^+-carrier would then, independently of the transmembrane potential, reorient itself towards the inside of the membrane. That the rate limiting step of Na^+-glucose transport may indeed be the reorientation of the empty carrier is indicated, but not necessarily proved, by the observations of Kessler *et al.* [20] that the unidirectional influx of D-glucose under tracer exchange conditions (with $[\text{D-glucose}]_{in} = [\text{D-glucose}]_{out} \gg K_m^{Glc}$, and $[Na^+]_{in} = [Na^+]_{out} \gg K_m^{Na}$) is much larger than when the internal concentrations of both D-glucose and Na^+ are zero.

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3 Hereafter, unless specified otherwise, "carrier" refers to the mobile part of the transport agency, and not to the whole (protein) carrier module.

4 *See* footnote 2, p. 286.

If, in contrast, we assume that alternative (*ii*) above is the correct one, since phlorizin is believed not to be a substrate,[4] its binding should not be affected by the transmembrane potential, unless binding of this glucoside to the carrier ($+Na^+$) results in a reversible, nontransporting reorientation of the carrier with "trapping" of phlorizin and this event requires the presence of a transmembrane potential.

A third, fundamentally different, model can be envisioned in which the sodium and D-glucose binding sites are on opposite sides of the membrane. If in this model the carrier is neutral in the unloaded form, then, in the absence of $\Delta\tilde{\mu}_{Na^+}$, the Na^+ binding site is assumed to occur predominantly on the *external* surface and the phlorizin (or substrate) binding site on the *internal* surface of the carrier. The binding of Na^+ in the presence of a $\Delta\psi$ (negative inside) would induce a flipping over of the carrier and thus the moving of the Na^+ binding site to the internal surface and of the phlorizin (or substrate) binding site to the external surface of the membrane. It would then be possible for phlorizin to bind (or for the substrate to bind and be transported, *after* Na^+ has dissociated from the now internally oriented Na^+ binding site). The equations describing this model, which to the best of our knowledge was never considered before, have not been worked out yet. However, preliminary considerations indicate that this model would function properly only if D-glucose and sodium were alternatively bound and could not bind simultaneously from the same side of the membrane. A possible consequence of this model, which appears to conflict with experimental results, is that the exchange rate of D-glucose under substrate equilibrium conditions would be lower in the presence of internal sodium than in its absence.

As described above, several important factors must be taken into consideration when attempting to formulate a mechanism to explain our experimental results. Is the unloaded carrier neutral or negatively charged? Does phlorizin simply block the D-glucose binding site or does its binding to the carrier result in a nonproductive transformation of the carrier-ligand complex? Do sodium and D-glucose bind to the carrier in an obligate alternating mechanism or can both ligands be bound simultaneously? These factors and many other points already noted can be combined in many ways to give a variety of models. Some of these combinations can be readily discarded, some not. At present, the available experimental evidence does not allow for the selection of one true model.

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