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PHLORIZIN AS A PROBE OF THE SMALL-INTESTINAL Na^+,D-GLUCOSE COTRANSPORTER

A MODEL *

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(1)'Uptake' of phlorizin by intestinal brush border membrane vesicles is stimulated, much as that of D-glucose, by the simultaneous presence of Na^+_{out} and $\Delta\psi \ll 0$. However, phlorizin contrary to D-glucose, fulfills all criteria of a non-translocated ligand (i.e., of a fully competitive inhibitor) of the Na^+,D-glucose cotransporter. (2) The stoicheiometry of Na^+/phlorizin binding is 1, as shown by a Hill coefficient of approx. 1 in the Na^+_{out}-dependence of phlorizin binding. (3) The preferred order of binding at $\Delta\psi \ll 0$ is Na^+ first, phlorizin second. (4) The velocity of association of phlorizin to the cotransporter, but not the velocity of its dissociation therefrom, responds to $\Delta\psi$. These observations, while agreeing with the effect of $\Delta\psi \ll 0$ on the K_d of phlorizin binding in the steady-state time range, also confirm that the mobile part of the cotransporter bears a negative charge of 1. (5) A model is proposed describing the Na^+,$\Delta\psi$-dependent interaction of phlorizin with the cotransporter and agreeing with a more general model of Na^+,D-glucose cotransport. (6) The k_{on}, k_{off} and K_d constants of phlorizin interaction with the Na^+,D-glucose cotransporter are smaller in the kidney than in the small-intestinal brush border membrane, which results in a number of quantitative differences in the overall behaviour of the two systems.

Introduction

Previous work from this laboratory has shown that phlorizin binds to the Na^+,D-glucose cotransporter of the small-intestinal brush border membrane [1,2], optimal binding being obtained in the presence of a $\Delta\psi$, negative inside the membrane vesicles. Similar observations have been reported for the analogous cotransporter of renal cortex brush border [3]. Phlorizin binding to the two transporters, however, shows some differences, the most conspicuous being that the binding to small intestinal vesicles goes through a maximum at about 2 s, whereas binding to renal cortex vesicles has a much smaller association rate.

The ultimate goal of studies of phlorizin binding to the cotransporter is, of course, that of using this probe as a non-penetrating ligand in order to elucidate some of the step(s) in the transport mechanism. Indeed, at the end of this paper we suggest a model for phlorizin binding to the transporter, which is a portion of our more general model for Na^+,D-glucose cotransport [5]. Towards this goal, we have addressed ourselves to the following questions: (i) To what an extent do the

* Dedicated to Professor A.E. Braunstein, Moscow, on the occasion of his 80th birthday.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

conditions yielding optimum phlorizin binding compare to those yielding (in the absence of phlorizin) optimum D-glucose influx? (ii) If these conditions are identical, would it be possible that phlorizin is, rather than a fully-competitive inhibitor (i.e., a non-translocated ligand), a poorly translocated substrate *? As it will be seen, this question could not be answered in a totally unequivocal manner, although conditions were indeed encountered which influenced D-glucose transport in a different manner than phlorizin binding, thus making virtually certain that phlorizin is a non-translocated ligand. Phlorizin binding can thus be used to probe the first steps in Na^+,D-glucose cotransport. (iii) What is the stoicheiometry and the order of binding of Na^+ and phlorizin (at $\Delta\psi \ll 0$)? These questions have been recently answered for the kidney system, which is more amenable to experimentation [4]. For the small-intestinal transporter through a different kinetic approach we come to the similar conclusion, i.e., to a Na^+/phlorizin stoicheiometry of 1:1, and to a preferred order, Na^+ binding before phlorizin. (iv) How large are the k_{on} and k_{off} constants? Does their ratio agree with the K_d value as measured independently? Do they respond to $\Delta\psi$?. We have seen that both k_{on} and k_{off} are small, that they agree with the K_d, that k_{off} is not affected by $\Delta\psi$ ($\ll 0$). The last observation agrees with a similar one from the kidney system [3], and confirms independent conclusions [5] that the mobile part of the small-intestinal carrier (i.e., the 'gate' in a 'gated channel' model [6]) bears, in the substrate-free form, a negative charge of 1, and is made, at least in part, of a COO^--group (Weber, J. and Semenza, G., in preparation). (v) How do the kinetics of phlorizin binding in the small-intestine compare with those in kidney cortex?

Materials and Methods

Preparation of vesicles. Intestinal brush border membrane vesicles were prepared from frozen rabbit small intestine or from scrapings of fresh rat small intestine by the calcium precipitation method ([7], as modified in Ref. 8). In short, non-brush-border membranes in the homogenate were precipitated by 10 mM Ca^{2+} and spun down in a low-speed centrifugation ($3000 \times g$, 15 min). The brush border vesicles were then collected from the supernatant at $27000 \times g$ for 30 min. The pellet was washed once in the buffer used in the experiment. Unless stated otherwise, this was a solution containing 100 mM mannitol, 0.03% KN_3 and either 10 mM Tris-Hepes, pH 7.5, or 10 mM Tris-Mes, pH 6.5. In some experiments where the mannitol concentration was 300 mM, the preparation was modified as follows: (i) after the homogenization in a blender, the vesicles were diluted with 300 mM mannitol instead of water. (ii) After the second centrifugation the vesicles were suspended in 300 mM mannitol, 0.03% KN_3 and 10 mM buffer. The protein concentration was 10–15 mg/ml. Preloading the vesicles with Na_2SO_4 was achieved by a 60 min preincubation at room temperature.

Brush border membrane vesicles from kidney cortex were prepared by essentially the same procedure [9,10] with the exception that Mg^{2+} was used instead of Ca^{2+} to precipitate non-brush-border membranes. One more difference was that the Mg^{2+}-precipitation and the subsequent differential centrifugation was done twice. Aminopeptidase M, a brush border membrane marker, showed a 12.5-fold increase in the specific activity in the final vesicle material as compared to the homogenate; other enzymes known not to be associated with the brush border membrane were not enriched. Therefore, the procedure worked out in Refs. 9 and 10 proved satisfactory also with the minor modifications mentioned above.

Incubation and filtration. Uptake of D-glucose or of phlorizin was measured by the filtration technique (in this report the term 'uptake' will refer to the amount of substrate associated with vesicles, irrespective of whether it is bound to the membranes or trapped in the internal compartment). All incubations were performed at room temperature, according to the following general scheme: 10 μl of the vesicle suspension were rapidly mixed with 10 μl of a solution containing, in addition to mannitol and buffer as described above, the radioactively labelled substrate or ligand and the further additions as indicated in the legends. The

* Here and in the following we indicate as the 'substrate' the sugar and Na^+ as the 'co-substrate'.

incubation was terminated by injecting 2.5 ml of an ice-cold stop solution containing 250 mM NaCl and 1 mM Tris-HCl, pH 6.5. Start and termination of the incubation were completely automated, which permitted incubation times as short as 0.5 s [11]; a short-time-incubation apparatus as used in our experiments is now available from Innovativ Labor AG, 8134 Adliswil, Switzerland. The diluted vesicles were quickly filtered through a prewet Sartorius filter (pore size: 0.6 μm). The filter was washed twice with 5 ml of the stop solution. The time required for the whole stop, filtration and wash procedure was about 10 s and was kept as constant as possible.

In phlorizin binding studies, parallel incubations were performed in the presence of either 25 (or 5) mM D-fructose or D-glucose, which were added to the vesicles at the same time as phlorizin. D-Glucose at these concentrations completely prevented phlorizin from binding to its high-affinity binding site on the D-glucose transporter [2] (binding of phlorizin is inhibited by D-glucose with a K_i of approx. 0.1 to 0.2 mM, a value similar to the K_m of D-glucose transport) without affecting a low-affinity binding, presumably to membrane lipids. In the control incubation, D-fructose was added instead, because this sugar is known to be transported by a separate, Na^+-independent, phlorizin-insensitive agency and because it does not affect D-glucose transport [12,13]. In this report 'phlorizin binding' always refers to the high affinity, D-glucose protectable part of the total phlorizin binding, calculated from the difference between the two parallel incubations. In the 2-s incubations used, it amounted to more than 75% of the total phlorizin bound. Transmembrane potentials were generated as ion diffusion potentials, e.g., by imposing a gradient of the highly permeant anion SCN^- across the membrane. Blanks of phlorizin- and D-glucose uptake were measured by adding 10 μl each of vesicle and of substrate containing solution separately to the stop solution and then proceeding as with the samples.

Phlorizin is a weak acid with pK_a 7.4 [2] and it has been shown [2] that its affinity for the carrier decreases at pH values above 7.5. D-Glucose transport measurements were conducted at pH 6.5 and 7.5; essentially identical results were obtained at either pH. The phlorizin binding studies reported in the present paper were carried out at pH 6.5.

Protein assay. Protein was measured according to Lowry et al. [14] with bovine serum albumin as standard.

D-[^{3}H]Glucose and [^{3}H]phlorizin were obtained from New England Nuclear, Boston, MA. All chemicals were analytical grade.

Results

A. Similar effects of transmembrane potentials on D-glucose and phlorizin 'uptake'

The amount of phlorizin associated with brush border vesicles is greatly increased when a transmembrane potential (inside negative) is applied to

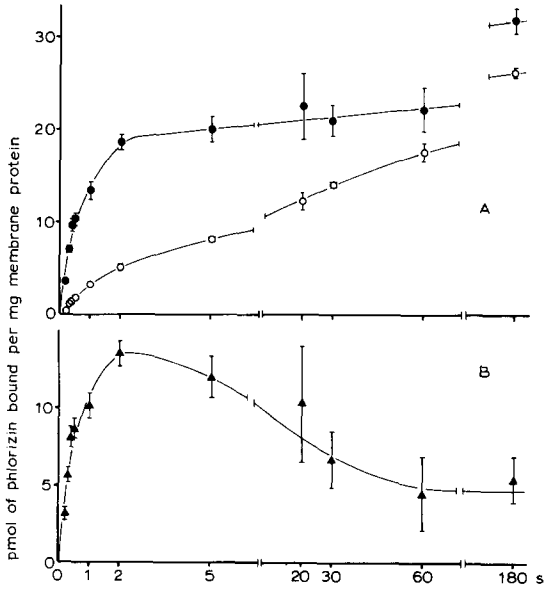

Fig. 1. Time-course of phlorizin binding to brush border vesicles under conditions of a dissipating NaSCN gradient. ●——●, total phlorizin associated with the vesicles; ○——○, phlorizin bound in the presence of 25 mM D-glucose, i.e., phlorizin being displaced from its high-affinity site on the D-glucose transporter; ▲——▲, difference between the two curves of Fig. 1A, representing D-glucose protectable high-affinity phlorizin binding to the D-glucose carrier. The brush border vesicles were suspended in 100 mM mannitol and 10 mM Tris-Mes, pH 6.5. At zero time, 5 μM tritiated phlorizin was added to the vesicles together with 100 mM NaSCN and either 25 mM D-fructose (●) or 25 mM D-glucose (○) (all concentrations are final concentrations in the incubation medium). Values are mean and S.E. of four determinations. Separate blanks were determined for the incubations containing D-fructose and D-glucose, respectively.

the membrane (for further data, see Refs. 1 and 2). This is similar to the uptake of D-glucose, where the initial influx rate is very much larger in the presence of a potential imposed by an SCN^- gradient as compared to conditions with $\Delta\psi \approx 0$ [8]. The apparent analogy between phlorizin binding and D-glucose uptake can be extended further by comparing the time-course of 'uptake' of these two substances in the presence of an NaSCN gradient. Phlorizin associated with the cotransporter shows a sharp initial rise, reaches a short-lived plateau at about 2 s and then steadily falls to a level which corresponds to the level reached in the absence of an initial $\Delta\tilde{\mu}_{Na^+}$ (Fig. 1). A similar, albeit longer-lived, overshoot is commonly observed during the uptake of D-glucose and of other substrates which are transported by an Na^+-coupled mechanism (Fig. 2A). It has been verified in various ways [8,15] that D-glucose is taken up into an intravesicular compartment and that the overshoot of D-glucose uptake is due to accumulation in the osmotic space inside the vesicles beyond the equilibrium concentration. The driving force for the uphill movement of D-glucose resides in the asymmetric distribution of Na^+ and in the presence of a transmembrane potential acting on the electrogenic Na^+-coupled D-glucose transport system [16,17].

Also quantitatively, the changes in $\Delta\psi$ to which phlorizin binding and D-glucose uptake respond, coincide: in Figs. 3A and B the initial anion-diffusion potential (negative inside the vesicles) was reduced or collapsed by increasing progressively the permeability of the membrane to Rb^+. (2 s incubations were used throughout in order to gain very similar, if not identical, $\Delta\psi$ values in phlorizin binding and in corresponding D-glucose transport experiments). This manipulation resulted in closely

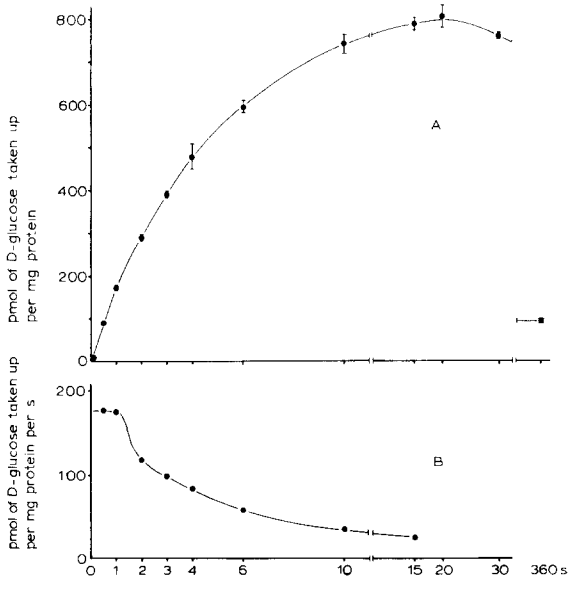

Fig. 2. (A) Time-course of D-glucose uptake under conditions of a dissipating NaSCN gradient. The experimental conditions were equivalent to those in Fig. 1. At zero time, 100 μM D-glucose and 100 mM NaSCN were added to the vesicles. Values are means and S.E. of 2–4 measurements. (B) Approximate time course of the D-glucose uptake rate determined from the values in (A). The increments in each time interval were divided by the length of the respective interval.

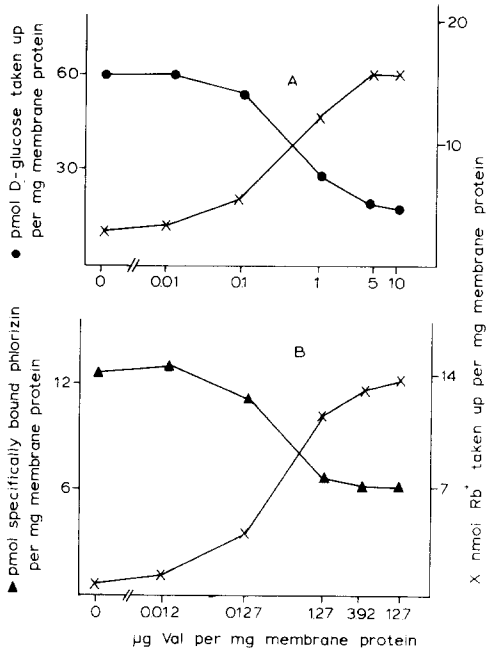

Fig. 3. Effect of reduction or collapse of $\Delta\psi$ across small-intestinal brush border membrane vesicles on D-glucose uptake (●), phlorizin 'uptake' (▲) and Rb^+ uptake (×). Incubation time, 2 s, 20°C, pH 6.5; varying amounts of valinomycin (Val), as indicated, in alcohol (final concentration of alcohol, 2%). At the beginning of the incubation NaSCN (50 mM), $^{86}Rb_2SO_4$ (25 mM) and D-[^{3}H]glucose (0.1 mM) or [^{3}H]phlorizin (5 μM) were added simultaneously (in the case of phlorizin uptake, two incubations were carried out, one in the presence of 25 mM unlabelled D-fructose, the other in the presence of 25 mM unlabelled D-glucose; the 'specific phlorizin binding' indicated is the difference between the two). The uptake values given are the amounts associated with the vesicles after 2 s.

parallel effects on phlorizin binding and D-glucose uptake.

The similarity in the response of phlorizin and D-glucose 'uptakes' towards $\Delta\psi$ and Na^+ led us to the question of whether the potential-induced transient 'binding overshoot' of phlorizin may indicate, rather than binding, a transient accumulation of this glucoside in the inner osmotic space of the vesicles; that is, that the fully-competitive kinetics of phlorizin inhibition of D-glucose transport may be due to the glucoside being a (slowly) transported substrate, and not to it being a non-transported ligand of the Na^+,D-glucose cotransporter. Thus, the following experiments were carried out; their results make the latter alternative the more likely (as is also generally believed).

B. Different responses of phlorizin 'uptake' and D-glucose influx towards various parameters

The obvious experiment would have been that which answers the question: does the amount of phlorizin associated with the vesicles in 2-s incubations depend on the size of the intravesicular osmotic space? The answer to this question was found to be no. Yet the interpretation of this finding was blurred by the fact that the amount of D-glucose found associated with the vesicles in 2-s incubations was not clearly dependent on the size of the osmotic space. Other, indirect, criteria had thus to be resorted to.

B1. Differences in the time courses of phlorizin and D-glucose 'overshoots'. D-Glucose uptake in the presence of an initial NaSCN gradient is linear for at least 2 s, reaches a maximum accumulation after 15–45 s and comes to equilibrium with D-glucose in the incubation medium not before 10- min (Fig. 2, see also Refs. 2, 11). In clear contrast, phlorizin 'uptake' reaches its maximum level already at about 2–5 s, declining thereafter to a final level within 30–60 s (Fig. 1B, see also Fig. 9B).

The time-course of D-glucose uptake indicates that the driving force generated by an initial NaSCN gradient (out $\gg$ in) is at 15–30 s still large enough to sustain a 10-fold accumulation of D-glucose. This driving force presumably decreases with time in a quasi-exponential way. Now, substrates transported by the same agency, with identical flux ratios, etc., but at widely different rates will differ, also, in the height of the maximum accumulation ratio reached: the earlier the overshoot (i.e., the faster the substrate is transported), the higher the overshoot must be, because at early times the driving force is still large. Contrary to this, slowly transported substrates will produce, if at all, overshoots which must be both late and low. In actual fact, the 'overshoot' of phlorizin is instead both early and low as compared with that of D-glucose (Figs. 1B and 2A), indicating that these two substances are not handled identically by the transporter.

The lack of coincidence between D-glucose uptake and phlorizin 'uptake' led us, therefore, to another comparison, namely to that between phlorizin 'uptake' and the D-glucose uptake rate. The latter is shown in Fig. 2B, the uptake rates were calculated from the uptake curve of Fig. 2A. During the first 2 s, the uptake rate is nearly constant, but declines rapidly thereafter. This decline, although partially caused by the onset of efflux, is primarily due to a decline of the influx rate proper (data not shown), presumably as a consequence of the partial dissipation of the $\Delta\tilde{\mu}_{Na^+}$. The time course of phlorizin 'uptake' resembles more the time-course of the D-glucose uptake rate than that of the D-glucose accumulation. The rate of D-glucose uptake primarily reflects alterations in the state of the transport protein as a function of $\Delta\tilde{\mu}_{Na^+}$, and accordingly, the time-dependent changes in phlorizin 'uptake' can be understood to reflect a time-dependent interaction of phlorizin with (a) given form(s) of the D-glucose transporter.

B2. Simultaneous changes in phlorizin binding and its apparent K_i for D-glucose uptake. If the increase in phlorizin 'uptake' in Fig. 1 between 0 and 2 s is due to an increase in binding (rather than in transport into the internal space), a concomitant decrease of the apparent K_i value for inhibition of D-glucose transport may be expected. This is indeed the case, as shown in Fig. 4B. (The apparent K_i values were estimated from the increments of D-glucose uptake in time intervals of 0.3 to 0.5 s, Fig. 4A). The apparent K_i values decreased from 40–50 μM at 0.1 s to a value of approx. 7 μM in the time interval 1.3–1.8 s. This decrease must reflect events related to phlorizin binding proper (and/or changes in the transporter induced by it), and not events related to the dis-

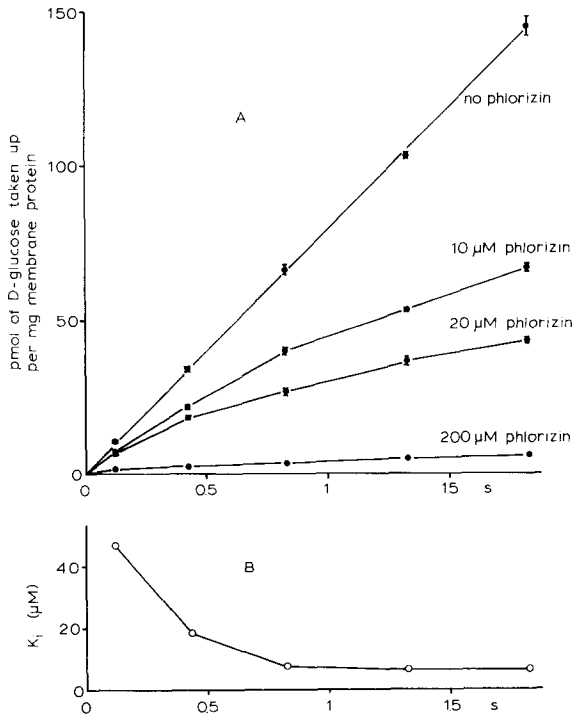

Fig. 4. (A) D-Glucose uptake in the presence of different phlorizin concentrations as a function of time. The pH in this experiment was 7.5. At zero time, 10 μM D-glucose and 100 mM NaSCN were added to the vesicles with 0, 10, 20 or 200 μM phlorizin. Values are mean and S.E. of 2–5 determinations. (B) Apparent K_i values of phlorizin on D-glucose uptake, determined for each time interval separately. For this computation the increments of D-glucose uptake at 0, 10 and 20 μM phlorizin concentration were plotted separately for each of the chosen time intervals according to Dixon. The uninhibited D-glucose uptake was linear over the whole time range, contrasting the clearly bent curves obtained in the presence of phlorizin. Because the increments at 0 μM phlorizin could be determined with much higher accuracy, they were weighted with a factor 5 in the linear regression.

TABLE I

INHIBITION OF D-GLUCOSE UPTAKE AND OF PHLORIZIN 'UPTAKE' BY Zn^{2+}

2 mM $ZnSO_4$ was added to the vesicles together with 100 mM NaSCN and the radioactively labelled substrates. Incubation time was 2 s. D-Glucose-protectable phlorizin binding is the amount of phlorizin bound in the presence of D-fructose minus that bond in the presence of D-glucose.

Compound	pmol/mg protein taken up at 2 s incubation time		% inhibition
	Control	+2 mM $ZnSO_4$	
10 μM D-glucose	75	7	91
5 μM phlorizin (+5 mM D-fructose)	13.6	2.9	
5 μM phlorizin (+5 mM D-glucose)	6.2	3.2	
D-Glucose-protectable phlorizin binding	7.4	0	100

sipation of $\Delta\tilde{\mu}_{Na^+}$: in fact, in the time range considered D-glucose uptake (in the absence of phlorizin) was strictly linear, which ruled out that the initial dissipation of $\Delta\tilde{\mu}_{Na^+}$ may have affected the rate-limiting step.

B3. Different inhibition of D-glucose efflux and phlorizin release from vesicles by Zn^{2+}. An inhibitor of the D-glucose transport system should block efflux of D-glucose from glucose-preincubated vesicles and it should similarly block the release of phlorizin, if the latter were a transported substrate like D-glucose. Zn^{2+} was chosen as inhibitor, because it inhibits D-glucose uptake and phlorizin binding equally well (Table I) and because the inhibition is instantaneous. Zn^{2+} does not affect the integrity of the membrane, as shown by the identical equilibrium values of D-glucose uptake at 2 h incubation time in the presence and absence of Zn^{2+} (data not shown).

Zn^{2+} inhibits the efflux of D-glucose from the vesicles as expected, but it does not affect the release of phlorizin which has been taken up during a 2 s preincubation period (Fig. 5). Clearly, phlorizin and D-glucose are handled differently by the carrier. The lack of inhibition of phlorizin release indicates once more that the phlorizin taken up at 2 s, i.e., at the time of maximum phlorizin 'accumulation', had not been transferred and released into the same compartment as D-glucose.

C. Na^+/phlorizin stoicheiometry of binding

The data reported above make thus very likely that the widely accepted belief is indeed true: i.e., that phlorizin is a fully-competitive inhibitor (a non-transported ligand) and not a substrate of the small-intestinal Na^+,D-glucose cotransporter. Phlorizin binding depends on Na^+_{out} (Fig. 6A) and shows saturation, the $K_{1/2}$ for Na^+_{out} being (under

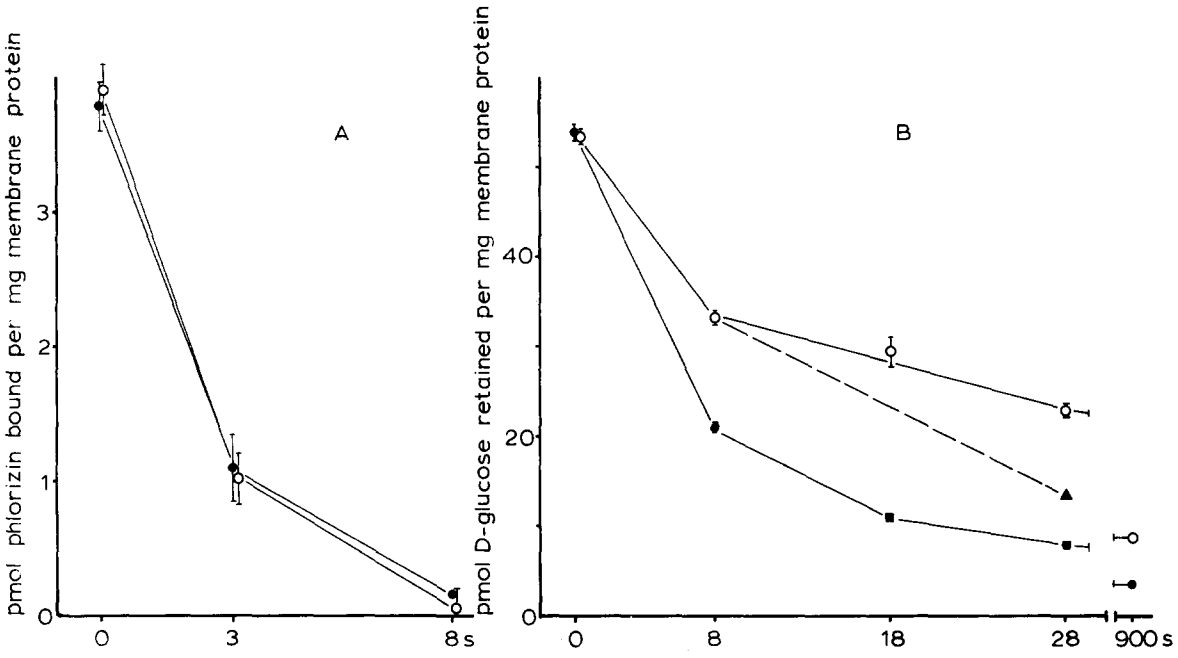

Fig. 5. Release of phlorizin (A) or D-glucose (B) from vesicles diluted into a 25-fold volume of substrate-free solution in the absence (●) and in the presence (○) of 2 mM $ZnSO_4$. The vesicle suspension, the incubation solution and the dilution medium contained 300 mM mannitol, 10 mM Tris-Mes, pH 6.5, and 50 mM Na_2SO_4. Loading the vesicles with substrate (D-glucose or phlorizin) was achieved by a 2 s incubation in a solution containing the following additions: 100 mM choline SCN and 5 μM phlorizin (with either 25 mM D-fructose or 25 mM D-glucose (A)) or 100 mM choline SCN and 115 μM D-glucose (B). Aliquots of 20 μl were then diluted with 500 μl of the dilution medium with or without 2 mM $ZnSO_4$. Incubation and dilution were done at 20°C. After the selected 'efflux' times the samples were mixed with 2.5 ml of ice-cold stop solution containing 250 mM NaCl, 1 mM Tris-Mes, pH 6.5, and 3 mM EDTA and processed as usual. Reversal of Zn inhibition of D-glucose efflux (B) was demonstrated by adding 3 mM EDTA to the dilution medium after 8 s of efflux time (▲).

the conditions of the experiment, see legend to Fig. 6) 30 ± 6 mM (S.E.).

Due to the fairly high nonspecific binding of Na^+ to the membrane vesicles (e.g., Ref. 8), it is not possible to measure the phlorizin-dependent Na^+ binding to any acceptable accuracy. However, the slope of the Hill plot in Fig. 6B is close to 1, which is strongly indicative of an Na^+/phlorizin binding ratio of 1 *.

D. Estimation of the k_{on} and k_{off} constants of Na^+-dependent phlorizin binding to small-intestinal brush border membrane vesicles; the effect of $\Delta\psi$

From experiments like those in Figs. 1 and 7, in the presence of an initial NaSCN gradient (100 mM out, 0 in), the k_{on} constant was estimated to be approx. $1.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$ and the k_{off} constant to be approx. $0.4\ s^{-1}$. The k_{off}/k_{on} ratio (approx. 2.7 μM) compared favorably, therefore, with the K_d, as determined from the quasi-steady-state (2 s) time range, i.e., 4.6 ± 0.9 μM (Ref. 2).

Essentially identical k_{off} constants were found in the presence of an initial SCN gradient (out ≫ in)

* The Na^+/phlorizin binding ratio of 1 agrees with the Na^+/D-glucose flux ratio of 1 derived from similar kinetic arguments in the same transport system (e.g., Refs. 5, 39; see also Ref. 18) and is at variance with the Na^+/3-methylglucose flux ratio of 2 reported by others [40] in isolated chicken enterocytes, in the presence of inhibitors blocking the sugar transport system(s) of the basolateral membrane. The reason(s) for this discrepancy have not yet been identified, but some may be envisaged (different species; unidentified side-effects of the inhibitors on the brush border sugar translocator; the Ca^{2+} treatment [7] of the brush border membrane vesicles [8,39], etc.). It should be noted, however, that a stoicheiometric ratio of 2, while complicating somewhat the treatment of our own data, would not change the substance of our conclusions.

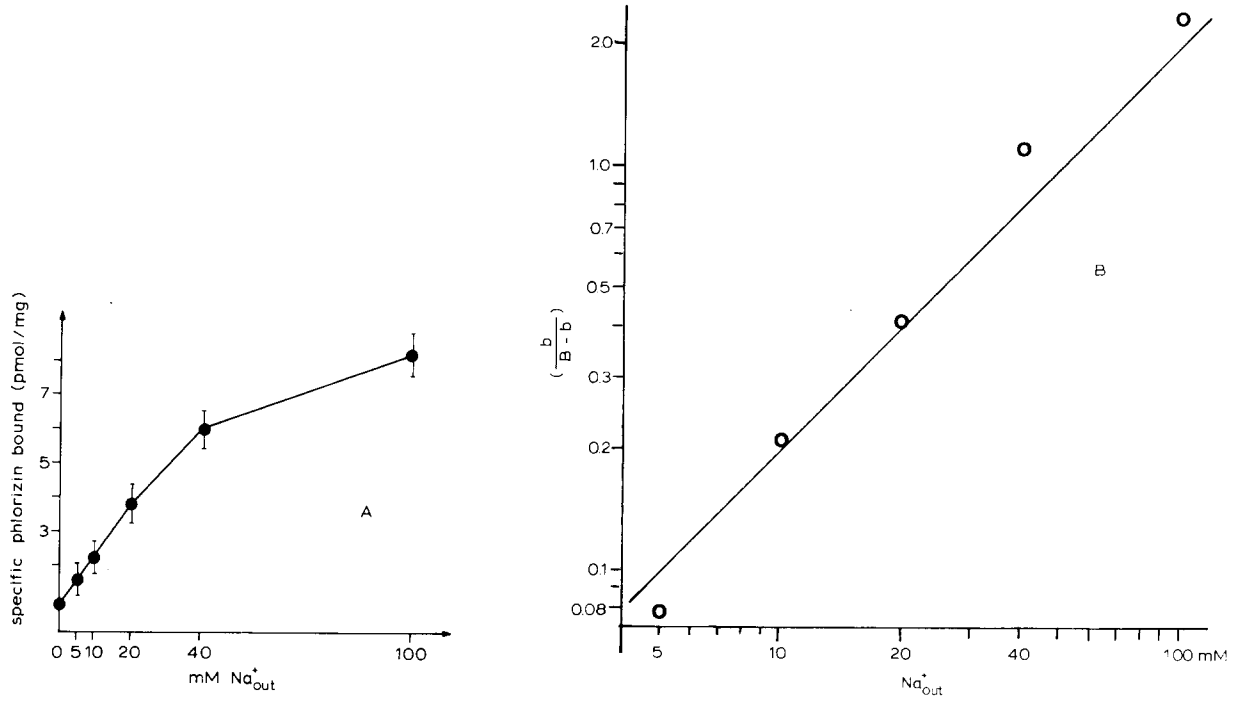

Fig. 6. Na^+_{out} dependence of specific phlorizin binding. Incubation time, 2 s; 20°C; phlorizin, 5 μM (+either 25 mM D-fructose or 25 mM D-glucose); pH 6.5. At time zero, 100 mM (Na+choline) SCN was added. (A) Direct plot. (B) Hill plot of the same data, after substraction of the amount of phlorizin bound at zero $[Na^+_{out}]$ (i.e., approx. 0.8 pmols·mg^{-1}). *b*, phlorizin bound at a given $[Na^+_{out}]$; *B*, phlorizin bound at infinitely large Na^+_{out}, extrapolated from a Scatchard plot (not shown). The line was drawn for a calculated Hill $\bar{n}$ of 1.

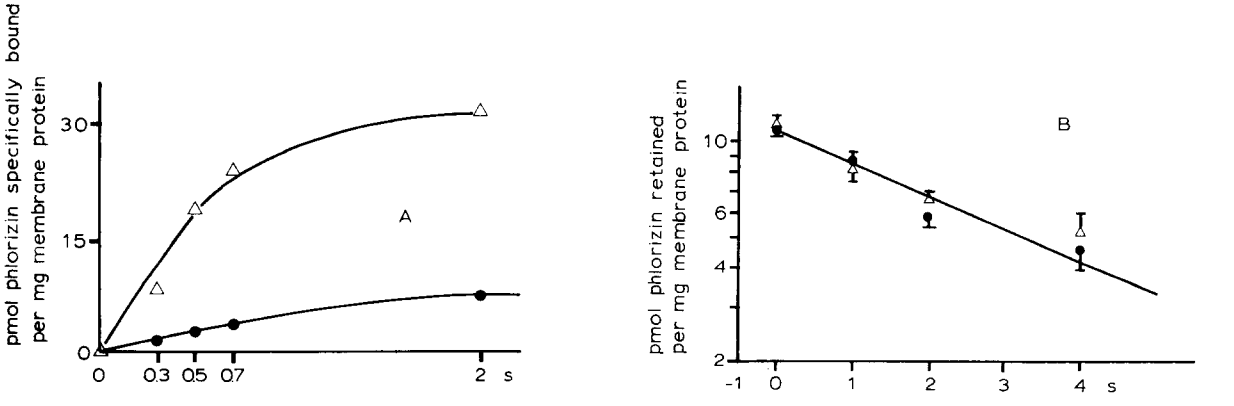

Fig. 7. Time-course of phlorizin binding (A) and release (B) in the presence of an initial (out>in) gradient of NaSCN (△) or sodium cyclamate (●). In A, specific phlorizin binding (phlorizin 10 μM, pH 6.5; a 100 mM out, 0 in gradient of either NaSCN or sodium cyclamate) was obtained as the difference in binding in the presence of 25 mM D-fructose minus that in the presence of 25 mM D-glucose. In B, the vesicles were first pre-equilibrated in 100 mM sodium cyclamate, pH 6.5. At time −1 s phlorizin (10 μM) was added and a choline SCN gradient (100 out, 0 in) was established. At time zero the vesicle suspension was diluted 50-times in either 100 mM NaSCN (△) or 100 mM sodium cyclamate (●).

or at very small $\Delta\psi$ (Fig. 7B), which showed that this constant was not influenced by $\Delta\psi$. As opposed to this observation, k_{on} becomes very small in the absence of a strongly negative $\Delta\psi$ inside the vesicles: in the experiment of Fig. 7A it was estimated to be approx. $0.18 \cdot 10^5\,M^{-1} \cdot s^{-1}$. The k_{off}/k_{on} ratio (approx. 22 μM) was in excellent agreement with the K_d value determined from the quasi-steady-state range under similar (albeit not identical) conditions ($10 - 30\,\mu M$ in the presence of an initial NaCl gradient, 100 mM out, 0 in (Ref. 2 and Schmidt, U. (1982) unpublished data)).

E. Order of binding

The usual approach, i.e., studying the dependence of apparent K_d of phlorizin binding on $[Na^+_{out}]$ (which has been used successfully on the kidney system [4]), does not lead to reliable conclusions in the case of the small intestine, due to the lower capacity of phlorizin binding (12–30 pmol/mg protein [2], as compared to 270 pmol/mg protein [4]) and to its time course (compare, for example, Fig. 1 with Fig. 8 below). However, the preferred (not necessarily compulsory) order of binding could be identified as Na^+_{out} first, phlorizin second. The argument goes as follows.

From experiments of the kind shown in Fig. 1 it could be calculated that small-intestinal brush border vesicles bind approx. 50 pmol of phlorizin per s per mg protein (extrapolated to saturating phlorizin concentrations; at initial NaSCN gradient, 100 mM out, 0 in). Under the same conditions (but of course in the absence of phlorizin) the transport of D-glucose is at least 10-times faster at saturating glucose concentrations. Now, since the interactions of phlorizin and of D-glucose with the substrate binding site of the cotransporter are affected by a number of parameters in identical fashion (e.g., Na^+, $\Delta\psi$), it seems safe to assume that some events (steps) are common to D-glucose transport and to phlorizin binding. The events in common (including the binding of Na^+_{out} to the free form of the cotransporter) must be as fast as, or faster than, the overall D-glucose transport. Thus, the free form of the cotransporter, at the outer side of the membrane, must interact with Na^+_{out} at least 10-times faster than with phlorizin$_{out}$; this is paramount to saying that it has a better than 10-fold chance of reacting with Na^+_{out} than with phlorizin$_{out}$. Turner and Silverman [4] have likewise found a random sequence of binding with much larger binding velocity for Na^+ than for phlorizin (i.e., a preferred order) in the case of the Na^+,D-glucose cotransporter of kidney cortex.

F. Differences in phlorizin binding to intestinal and renal brush border vesicles

Most of the phlorizin binding studies reported in literature were done with brush borders or brush border vesicles from kidney [3,4,19]. With the latter vesicles, incubation times of 30 s or longer were required to reach binding equilibrium. A dependence on the membrane potential has been described by Aronson [3]; however, no 'overshoot' of phlorizin binding was observed. This raised the question of whether the much faster binding we had found with intestinal vesicles was due to methodological differences or whether there is a true difference in the way the D-glucose transporter(s) handle(s) phlorizin in kidney and intestine.

Fig. 8 shows that phlorizin binding to renal vesicles from the rat requires indeed much longer incubation times to attain full binding as compared to intestinal vesicles. In both cases, binding was similarly enhanced in the presence of a membrane potential; however, in renal vesicles the membrane potential probably has dissipated by the time binding equilibrium is reached, whereas in the intestine phlorizin binding is fast enough to reach binding equilibrium while the state of the D-glucose transporter is still modulated by the membrane potential, thereby producing the observed 'overshoot'.

An even larger difference in the time course of phlorizin binding was observed in renal vs. intestinal vesicles from rabbit (not shown). In any case, the binding velocity appears to correlate with the dissociation constant K_d and the inhibitor constant K_i. In the rat, the K_i on D-glucose transport in renal vesicles (0.8 μM after 60 s preincubation with phlorizin) was smaller than the K_i values obtained in intestinal vesicles (smallest value determined was 2 μM). Similarly, the K_d of phlorizin binding to rabbit renal vesicles (0.5 μM at 10 min incubation time) was about 10-times smaller than K_d values we have reported for phlorizin binding to rabbit intestinal vesicles (smallest value was 4 μM, see Ref. 2).

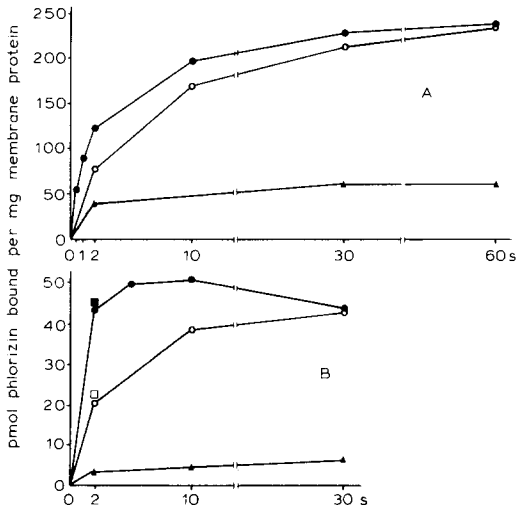

Fig. 8. Time-course of phlorizin binding to renal (A) and intestinal (B) brush border vesicles from rat, measured under conditions of dissipating Na^+ salt gradients. The vesicles were prepared in 500 mM mannitol, 20 mM Tris-Hepes, pH 7.5. [^{3}H]Phlorizin and salts were added at the start of the incubation to give the following final concentrations: 3 μM [^{3}H]phlorizin; 100 mM NaSCN (●) or 100 mM sodium isethionate (○) or 100 mM KSCN (▲) or (100 mM NaSCN + 100 mM sodium isethionate) (■) or 200 mM sodium isethionate (□); 350 mM (●, ○, △) or 250 mM (■, □) mannitol; 20 mM Tris-Hepes, pH 7.5, 20°C. The values shown represent total phlorizin binding. Specific phlorizin binding to the D-glucose transporter is obtained after substracting the value determined in the absence of Na^+ (▲). The S.E. values were smaller than 5% of the mean values shown in the figure.

One might object that phlorizin binding proper to the D-glucose translocator is the same in renal and intestinal vesicles and that the observed differences are caused by the presence of phlorizin hydrolase which is present in intestinal, but not in renal, vesicles. Indeed, if a major fraction of the total phlorizin present in the incubation media (of intestinal vesicles) were hydrolysed within 30 s, a binding 'overshoot' similar to that in Fig. 1B (but of phloretin taken up into the lipid phase) might be obtained. This objection can be refuted because phlorizin hydrolysis was found to be negligibly small during an incubation time of 1 min [20]. Furthermore, if the D-glucose transporters in renal and intestinal vesicles were identical and had the same K_d for phlorizin, the dissociation rate of bound phlorizin should be the same in both types of vesicle, and it should be independent of the incubation time of the vesicles with phlorizin. This is clearly not the case (Table II). The dissociation rate of phlorizin bound to intestinal vesicles is 40-times faster than the equivalent dissociation rate from renal vesicles, indicating that the D-glucose translocators in kidney and intestine indeed have different affinities towards phlorizin.

From Aronson's work [3] it is possible to estimate the k_{on} and k_{off} constants in his renal vesicles prepared from the same species we have used in the preparation of the small intestinal ones (the rabbit). In the presence of a $\Delta\psi < 0$, at pH 7.5, k_{on} is approx. $1.65 \cdot 10^4\,M^{-1} \cdot s^{-1}$; k_{off} is approx. $0.031\,s^{-1}$ (in fair agreement with our own estimate, Table II). The ratio between the two, 1.8

TABLE II

DISSOCIATION RATES OF PHLORIZIN FROM RABBIT INTESTINAL AND RENAL VESICLES

Data for intestinal vesicles are from Fig. 5A (phlorizin release in Fig. 5A was measured at pH 6.5; however, essentially identical dissociation rates were obtained at pH 7.5). Kidney vesicles were prepared in 300 mM mannitol/20 mM Tris-Hepes (pH 7.5) and preincubated for 1 h in an identical solution containing in addition 100 mM NaCl. 10 min after the addition of 0.2 μM [^{3}H]phlorizin, aliquots were diluted 40-times in solutions containing 100 mM NaCl or 100 mM KCl, 300 mM mannitol and Tris-Hepes (pH 7.5), at 20°C.

	Cation in dilution medium	Time after dilution	% phlorizin released	Dissociation rate (s^{-1})
Intestinal vesicles (from Fig. 5A)	100 mM Na^+	3 s	70	0.40
		8 s	>90	
Kidney vesicles	100 mM Na^+	10 s	9	0.011
	100 mM K^+	10 s	40	0.05

μM, agrees well with Aronson's K_d (1.0 μM) from the steady-state range.

In spite of the scatter in the K_d for phlorizin in kidney vesicles [3,4,19] and in spite of the uncertainties in the estimation of the k_{on} constant in the small intestine, it seems safe to say that the k_{on}, k_{off} and K_d constants are smaller in the kidney than in the small intestine, and that this difference is related to the tissue and not to methodological differences although some constants may show species differences for the same organ (Gibbs, E.M. and Diedrich, D.F., unpublished data).

Discussion

A. Binding vs. transport

In 1962 the inhibition of sugar transport by phlorizin in the small intestine was identified kinetically as being fully competitive in nature [21]. Subsequent observations showed that phlorizin also: (i) inhibits sugar-evoked increases in transmural potentials and currents in the small intestine [22,23]; (ii) releases the Na^+-dependent heterologous inhibition of amino acid transport systems by glucose and its analogues in the small intestine [24,25]; (iii) inhibits D-glucose efflux from vesicles isolated from intestinal brush border membranes [15]. Although the simplest explanation for these observations is that phlorizin is a fully competitive inhibitor (i.e., a a non-transported ligand) of Na^+-dependent monosaccharide transport (and indeed this is the view generally held), none of these studies allowed any discrimination to be made between a fully-competitive inhibitor and a slowly transported substrate. As a matter of fact, there is at least one report in the literature favouring the latter possibility: phlorizin produces a change in the transmural potential across rat ileum of the same sign as, but of smaller amplitude than, that produced by monosaccharides [26].

Deciding between these two possibilities (or at least providing more data favouring one or other alternative) became necessary also because of our observations showing a close analogy between phlorizin binding to small-intestinal membrane vesicles and D-glucose transport: both phenomena respond to the presence of Na^+_{out} and of $\Delta\psi$ (negative inside) ([1,2], see also present paper, Fig. 3) in very similar fashion. Furthermore, the dependence of phlorizin binding (Fig. 6) and of D-glucose (out $\rightarrow$ in) transport on Na^+_{out} (Refs. 5, 18) shows a stoicheiometric ratio of 1 in both systems.

An incentive towards trying to distinguish between binding and transport in the case of phlorizin came also from observations [27] on the β-galactoside transport system in *Escherichia coli* membranes. Two supposed 'ligands' were unequivocally identified as poor substrates, rather than fully competitive inhibitors, with the result of seriously questioning many of the conclusions which others had previously drawn [28–31].

In the present paper we show that, although similar in a number of respects, the interaction of phlorizin and that of D-glucose with the (substrate binding site of the) small-intestinal cotransporter are different in a number of others. In short: (i) phlorizin 'uptake' parallels more the uptake rate, rather than the uptake of D-glucose; (ii) the timing of appearance of phlorizin 'overshoot' indicates that this glucoside is not handled in the same way as the typical substrate glucose; (iii) Zn^{2+} inhibits (by whatever mechanism) D-glucose efflux, but affects phlorizin release little, if at all. It seems safe to conclude that phlorizin is either not transported at all, or, if it is, it is so to a minimal extent. One can use, therefore, phlorizin binding (as we and others have done [1–4,32]) with the purpose of dissecting some step(s) in the complex chain of events in Na^+,D-glucose cotransport. The model of Fig. 9 (see below) is a part of a more complex model for flux coupling in Na^+,D-glucose cotransport [5].

B. Working in the presence of a high $\Delta\psi$ (negative inside the vesicles)

In small-intestinal brush border membrane vesicles an initial $\Delta\psi \ll 0$ is clearly necessary for specific phlorizin binding to be detected (small binding capacity, transient binding). If phlorizin binding is to be used as a tool in probing D-glucose transporter, it becomes imperative that transport also should be measured under identical conditions, i.e., during the initial dissipation of $\Delta\psi$, indeed of $\Delta\tilde{\mu}_{Na^+}$. The question can be raised, therefore, of whether one obtains meaningful results from measurements of (transport or binding) velocities during dissipation of their very driving

force. We have discussed this important point elsewhere [5,6,33]. The major condition to be fulfilled for these measurements to be reliable is that the position of the rate-limiting step should not change from one particular individual step to another during the incubation. That is, it is essential to make sure that the measurements are confined to the very initial, still linear, glucose uptake rate (usually lasting 3–4 s or less), during which the initial, partial dissipation of the driving force does not yet affect the velocity of the rate-limiting step.

C. Stoicheiometry and order of binding at $\Delta\psi \ll 0$

The experiment in Fig. 6 provides a strong kinetic indication that one sodium ion interacts with the cotransporter per phlorizin bound *. The order of binding, for the reasons mentioned under Results, section E, is that of preferred (not necessarily compulsory) binding of Na^+_{out} first, phlorizin second.

D. The mobile part of the cotransporter bears one negative charge ($z = -1$*) in the substrate-free form; the* k_{on} *and* k_{off} *constants of phlorizin binding*

Elsewhere we have presented a study on *trans*-inhibition by D-glucose$_{in}$ and by Na^+_{in} [5]. The particular pattern of trans effects observed in the presence of $\Delta\psi \ll 0$ could be explained only if the mobile part of the translocator (the 'gate' in a 'gated channel' model) bears, in the substrate-free form, one negative charge. If so, the mobile part of the translocator, when bound to one sodium ion ($\pm$ one phlorizin) should be electrically neutral and not respond to $\Delta\psi$. This was also found to be the case, (Fig. 7B), the velocity of dissociation of phlorizin being unaffected by $\Delta\psi$. A similar observation has been made by Aronson [3] on the dissociation of phlorizin from kidney cortex membrane vesicles.

As to the velocity of association of phlorizin to the cotransporter, it was found that it is strongly accelerated by $\Delta\psi$, negative inside the vesicles (Fig. 7A), the k_{on} constant being increased by a factor 8–9 in the experiment reported. Since k_{off} is not affected by $\Delta\psi$ (see above), it is to be expected that K_d should be affected by $\Delta\psi$; more specifically, it should be smaller in the presence of highly negative $\Delta\psi$. This is, in fact, the case. The K_d from the steady-state range being 4.6 μM with an initial (out → in) NaSCN gradient, and 28 μM with an NaCl gradient (Table I in Ref. 2).

As mentioned above, the mobile part of the cotransporter bears a negative charge of 1 in the substrate-free form. It is thus this form (plus, if possible at all, the Na^+-free phlorizin-bound form) which responds to $\Delta\psi$. If (see above) the preferred order of binding is (at $\Delta\psi \ll 0$) Na^+_{out} first, phlorizin$_{out}$ second, and if the substrate-free form of the carrier has a preferred inward orientation at low $\Delta\psi$ [5], then all observations on the chartacteristics of phlorizin binding to the small-intestinal Na^+,D-glucose cotransporter can be rationalized in the following sequence of events: $\Delta\psi \ll 0$ 'pushes' the mobile 'gate' (in a 'gated channel model, e.g. Refs. 5, 6; this 'gate' probably bears a COO^- group (Weber, J. and Semenza, G., in preparation)) of the cotransporter towards the outer surface of the membrane (or 'orients' the cotransporter towards the outside, in a 'snip-snap' model); Na^+_{out} binds to the Na^+-binding site; finally, phlorizin binds to the substrate-binding site. At $Na^+_{in} = 0$, the effect of $\Delta\psi \ll 0$ would thus be confined to a so-called 'carrier recruiting', the binding of both Na^+ and phlorizin being per se independent of $\Delta\psi$.

E. A minimum model of the interaction of phlorizin with the small-intestinal Na^+,*D-glucose cotransporter (Fig. 9)*

The model is based on, and aims at explaining in a single framework, the following observations: (i) phlorizin is a non-transported ligand (Results, Section B) and binds at the substrate-binding site [21,1,2]; (ii) its interaction with the cotransporter requires Na^+ and, (iii) in vesicles, is optimal at $\Delta\psi \ll 0$ (negative inside) [1,2]; (iv) the Na^+/phlorizin stoicheiometric ratio is 1 (Fig. 6); (v) the cotransporter is asymmetric with respect to the plane of the membrane and has, at $\Delta\psi \simeq 0$, a predominantly inward orientation [5] (form I in Fig. 9); (vi) events I → II → III (i.e., the effects of $\Delta\psi \ll 0$ and of Na^+ on the cotransporter) are in common with a part of the chain of events which leads (in the absence of phlorizin) to Na^+,D-

* See footnote on p. 563.

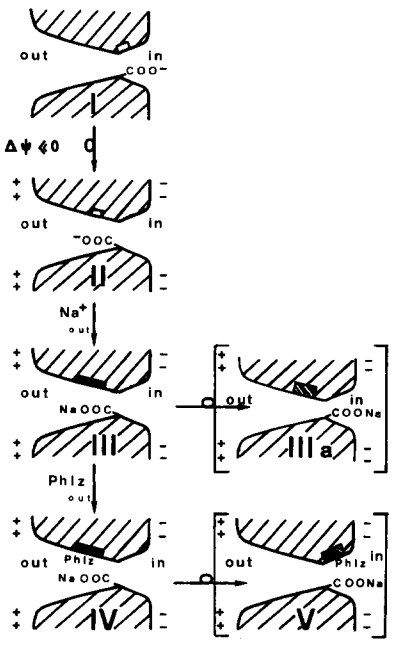

Fig. 9. Proposed minimum model for phlorizin binding to the small-intestinal Na^+,D-glucose cotransporter. □, sugar (phlorizin) binding site at low affinity; ■, same at high affinity; ▨, same at undefined affinity. —o→ indicates transitions in which the orientation of the gate-carboxylate (and of the sugar-binding site) change. In brackets are given improbable forms of the Na^+,D-glucose cotransporter. Note that the drawing is not to scale and is meant to convey the major feature only; in particular, no statement can be offered as to whether the sugar-binding site and the gate-carboxylate are located near to one another or face one another in the proposed gated channel. For details, see text.

glucose cotransport and are fast when compared to phlorizin binding proper; they must, therefore, take place prior to phlorizin binding (Results, Section E); (vii) the Na^+,D-glucose cotransporter, which shows a stable structural asymmetry with respect to the plane of the membrane [38,41], does not operate by 'diffusion' or 'flipping over', but is a 'pore' or 'channel' [6,42,43] endowed with (viii) a mobile portion (a 'gate') which is made of, or carries, a COO^- group ([5] and Results, Section D).

It seems reasonable to assume, furthermore, that this gate-COO^- is (a part of) the Na^+-binding site (Na^+ much prefers oxygen over other ligands). We suggest, also, that the two binding sites for Na^+ and for the substrate, respectively, be they free or occupied, can be exposed to either side of the membrane, but not simultaneously to both sides [44]; and that the simultaneous exposure of the Na^+-binding site at one side of the membrane and of the substrate-binding site at the other side (or vice versa) is unlikely (as discussed elsewhere [5]).

We suggest that the form of the cotransporter binding phlorizin optimally is form III, in which the outwardly oriented carboxylate (of form II) has bound Na^+_{out} and has produced conformational change making the substrate (phlorizin) binding site more affine for and/or more accessible to the substrates from the outside. It is not illogical to assume that the neutralization of a negative charge (by Na^+) in the immediate neighbourhood of the substrate (phlorizin) binding site may change drastically its affinity and/or its 'orientation'. Phlorizin would then bind to form III, without triggering in it a drastic conformational change; i.e., form IV would be similar to or identical with form III (save for the phlorizin bound). In particular, transition IV → V, which is the key step in cotransport, would be possible with transportable sugars, but not with phlorizin, a non-transportable ligand. Transition IV → V could be favoured by the 'spontaneous' orientation of the 'gate' being towards the inside (i.e., of the gate when not subjected to a negative $\Delta\psi$, as in form I, or when neutralized, as in form V); note, also, that we suggest that this 'snapping back' of the gate COONa does not take place in the absence of transportable substrates (or to a minimal extent): the translocation probabilities of the binary complexes are likely to be very small [5]).

The rationale for suggesting form III as the carrier form binding phlorizin optimally is the following: (i) that, in membrane fragments the presence of Na^+ is mandatory for phlorizin binding [38], which makes form I an unlikely candidate for a good phlorizin binder; (ii) that the presence of $\Delta\psi$ (negative inside) alone (i.e., in the absence of Na^+) does not lead to optimal phlorizin binding, which makes form II an unlikely candidate for a good phlorizin binder; (iii) that external Na^+ alone with small or no $\Delta\psi$ is not conducive to optimal phlorizin binding, which indicates again that form I, even in the presence of an out → in Na^+ gradient, hardly binds phlorizin. In actual fact, the K_d values for phlorizin binding at only moderately negative $\Delta\psi$ are fairly large (e.g., up to

30 μM in an initial NaCl gradient, 100 mM out, 0 in, Ref. 2; 45 μM in an initial sodium cyclamate gradient, 100 mM out, 0 in, if equated to the K_i for D-glucose transport inhibition, Ref. 20, p. 33) and cannot be determined reliably at very low $\Delta\psi$-values. (iv) That the hypothetical transition III $\rightarrow$ IIIa (gate-COONa snapping towards the inside) is very unlikely. In fact, if form IIIa existed, internal Na^+, even in the absence of external Na^+, should favour phlorizin binding from the outside. This is not the case, however. Internal Na^+ inhibits, rather than stimulates, phlorizin binding (Table III). Na^+ (100 mM), when present at both sides of the membrane, leads to a decrease of the phlorizin binding sites by about 50% and to an increase of the K_d value (as compared to the data obtained with an initial NaSCN gradient, 100 mM out, 0 in; Schmidt, U. (1982) unpublished data). Also, form IIIa would be part of an iso ping-pong bi-bi mechanism (with the substrate and Na^+ binding sites facing opposite sides of the membrane), and/or of a mechanism transporting Na^+ in the absence of sugars; but each of these mechanisms has been shown to be unlikely [5].

If the accessibility (or translocation) of the binding sites for Na^+ and for sugar is such that both these sites have to 'look' to the same side of the membrane it is reasonable to expect, also, that the inwardly oriented substrate binding site may exhibit high-affinity phlorizin-binding if the (equally inwardly oriented and presumably neighbouring) Na^+-binding site is occupied. That is, form I (Fig. 9) could exhibit Na^+-dependent (and probably $\Delta\psi$-independent) high-affinity phlorizin binding if both phlorizin and Na^+ reach it from the 'in'-side. Unfortunately, it has been impossible to date to obtain inside-out brush border vesicles and thus to carry out the pertinent experiment. However, deoxycholate-disrupted membranes, in which Na^+ and phlorizin have access to both sides and the $\Delta\psi$ is, of course, equal to zero (and in which, by analogy form I, both Na^+ and substrate binding sites are likely to have an inwardly orientation) do exhibit Na^+-dependent phlorizin binding, the K_d being 9.5 μM (Fig. 2B in Ref. 38). In comparison, brush border membrane vesicles pre-equilibrated in Na^+ and with $\Delta\psi \approx 0$ show very poor binding for phlorizin added to the 'out'-side only (Ref. 2 and Schmidt, U. (1982) unpublished data).

Thus, the model of Fig. 9 accomodates satisfactorily a number of observations, both old and new; to the best of our knowledge, it does not disagree with any. It seems very likely to us, therefore, that it has a fair chance of being essentially correct.

We attribute the inhibition of phlorizin binding (from the outside) by Na^+_{in} (Table III) to the trapping of a part of the cotransporter in a form with the gate-COONa snapped towards the inside (i.e., in a form similar to form I, but with Na^+ neutralizing the COO^- group), and thus with the substrate-binding site also oriented towards the inside.

F. A limited comparison with the renal Na^+,D-glucose cotransporter

It was not our purpose to carry out an extensive comparison of the Na^+,D-glucose cotransporters in the kidney and in the small-intestine. However, some comments may be worthwhile.

Renal and intestinal brush border membranes are endowed with very similar enzyme and transport systems, which are subjected in many cases to the same genetic control. This may well hold true for the Na^+,D-glucose transporter(s) also [34,35].

TABLE III

EFFECT OF INTRAVESICULAR Na^+, $CHOLINE^+$ OR K^+ ON SPECIFIC PHLORIZIN BINDING IN THE PRESENCE OF $\Delta\psi \ll 0$ (NEGATIVE INSIDE THE VESICLES: 200 mM SCN^-_{out}, ZERO IN) AND 50 mM Na^+_{out}

Small-intestinal brush border membrane vesicles were preloaded with the given cations by preincubating them for 60 min with either Na_2SO_4, or choline sulphate or K_2SO_4 at 25°C. At time zero the vesicles were mixed with a solution giving the final concentrations: 10 μM [^{3}H]phlorizin, 200 mM choline SCN and 25 mM Na_2SO_4. 'Specific' phlorizin binding is defined as the difference in binding between samples in the presence of D-fructose and in the presence of D-glucose.

Major cation in the intravesicular space	Specific phlorizin binding at 2 s incubation (pmol/mg protein) $\bar{x} \pm$ S.E. (4)
100 mM Na^+_{in}	12.4 ± 1.2
100 mM $choline^+_{in}$	16.8 ± 0.8
100 mM K^+_{in}	18.1 ± 0.8

What, if anything, do the smaller k_{on}, k_{off} and K_d constants of the renal Na^+,D-glucose cotransporter indicate? At least three possibilities, which are not mutually exclusive, come to mind: (i) the cotransporters are similar but not identical proteins in small intestine and in kidney; (ii) the two organs are endowed with two cotransporters each, but in different ratios to one another [34,36,37]; (iii) the cotransporters are identical proteins in kidney and small intestine, but different microenvironments (lipids?, other proteins?, glycosylation?) confer slightly different properties to them (see also Gibbs, E.M. and Diedrich, D.F., unpublished data). At the present stage of knowledge, or rather, of lack of knowledge, it would be futile to speculate further.

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