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The Small-Intestinal Na^+, D-Glucose Cotransporter: An Asymmetric Gated Channel (or Pore) Responsive to $\Delta\psi$

Markus Kessler and Giorgio Semenza

Laboratorium für Biochemie der ETH, ETH-Zentrum, CH-8092 Zürich, Switzerland

Summary. At $\Delta\psi \approx 0$, D-glucose influx into, and efflux out of, membrane vesicles from small-intestinal brush borders are affected by trans Na^+ and trans D-glucose to different extents. D-glucose influx and efflux respond to $\Delta\psi$ (negative at the trans side) to different extents. The small-intestinal Na^+,D-glucose cotransporter is thus functionally asymmetric. This is not unexpected, in view of the structural asymmetry previously found. The characteristics of the $\Delta\psi$-dependence of transinhibition by D-glucose are compatible with the mobile part of the cotransporter bearing a negative charge of at least 1 (in the substrate-free form). They are not compatible with its mobile part being electrically neutral. Pertinent equations are given in the Appendix. Partial Cleland's kinetic analysis and other criteria rule out (Iso) Ping Pong mechanisms and makes likely a Preferred Ordered mechanism, with Na^+_{out} binding to the cotransporter prior to the $sugar_{out}$. A likely model is proposed aimed at providing a mechanism of flux coupling and active accumulation.

Key Words small intestine · cotransport · flux coupling · D-glucose transport (Na^+-dependent)

Introduction

Among the few structural features of the small-intestinal Na^+,D-glucose transporter known at present are that, in all likelihood, it spans the brush border membrane asymmetrically with respect to the plane of the membrane. This was deduced from its susceptibility to inactivation by proteases and by mercurials (and reactivation by thiols) from the cytosolic (and/or hydrophobic) side, but not from the luminal, extracellular side (Klip et al., 1979*a–c*; 1980*a, b*). This stable asymmetry of the transporter is indeed not unexpected, in view of the current ideas on the vectorial insertion of intrinsic membrane proteins during (or shortly after the beginning of) their biosynthesis (for reviews, *see* Blobel & Dobberstein, 1975*a, b*; Palade, 1975; Sabatini & Kreibich, 1976; Di-Rienzo, Nakamura & Inouye, 1978; Wickner, 1979, 1980; Inouye & Halegoua, 1980; Engelman & Steitz, 1981; Kreil, 1981; Sabatini et al., 1982), and in view of the improbable flip flop of an intrinsic membrane protein having both hydrophobic and hydrophilic surface areas (Singer, 1974, 1977) (as a transporter is presumed to have).

These considerations (i) rule out an antiparallel orientation of identical subunits (in the case that this transporter is oligomeric, for which or against which no evidence is presently available, however); (ii) rule out "diffusive" or "rotative" modes of operation, making a "gated pore or channel" (Crane & Dorando, 1979, 1980; Semenza, 1982) or a "snip snap" mechanism [as suggested for the adenine nucleotide transporter (Klingenberg, 1980)] most likely; (iii) make a *functional* asymmetry also likely (in addition, of course, to the functional asymmetry which can be further imposed onto the transporter by an asymmetric distribution of substrate(s) and/or by a $\Delta\psi \neq 0$).

In the present paper we have addressed ourselves mainly to point (iii), also because even typical "equilibrating" transporters such as the sugar (e.g., Widdas, 1980) or the anion carrier (e.g., Cabantchik, Knauf & Rothstein, 1978; Rothstein & Ramjeesingh, 1980; Knauf, 1979, 1982) of the erythrocyte membrane are known to be functionally asymmetric.

We have also carried out a partial Cleland kinetic analysis of the initial out→in rates both at $\Delta\psi \simeq 0$ and at $\Delta\psi \ll 0$, to the extent possible in the kinetically complicated and experimentally tricky system. Previous kinetic studies either included corrections for the deviation from linearity in nearly initial uptake rates (e.g., Crane & Dorando, 1979, 1980) (others ignored

this deviation) or considered equilibrium tracer exchange fluxes at $\Delta\psi \simeq 0$ (Hopfer & Groseclose, 1980). In our work we made use of a short-time incubation apparatus (Kessler, Tannenbaum & Tannenbaum, 1978*b*) which allows uptake rates to be measured well within the initial linear range. The preparation of rabbit small intestinal brush border membrane vesicles which we used (Schmitz et al., 1973; Kessler et al., 1978*a*) is the same as that used by Crane's, Hopfer's and Wright's (Kaunitz, Gunther & Wright, 1982) groups. The vesicles are stable, tight and right-side-out better than 95% by various criteria (Tannenbaum et al., 1977; Kessler et al., 1978*a*; Klip et al., 1979*a*).

The results to be presented below show that the Na^+,D-glucose cotransporter is intrinsically asymmetric in its mode of functioning, rule out some kinetic models and make (an) other(s) likely, and allow a plausible model to be put forward.

There is now growing evidence that the small-intestinal brush border membrane is endowed with two, rather than one, Na^+, D-glucose cotransporters (*see* Discussion). Whether or not specifically stated in the following text, our observations and the conclusions drawn therefrom hold true in either case, i.e., whether this membrane is endowed with a single, or with two Na^+, D-glucose cotransporters acting in parallel.

Materials and Methods

Preparation of Brush Border Vesicles

Brush border membrane vesicles were prepared from frozen rabbit small intestine by the calcium precipitation method (Schmitz et al., 1973) as reported elsewhere (Kessler et al., 1978*a*). In short, intestinal mucosa was homogenized in a Waring blender in 300 mM mannitol, 2 mM Tris/HCl, pH 7.1. Nonbrush border membranes were precipitated by 10 mM Ca^{2+} and spun down at $3000 \times g$ for 15 min. Brush border membranes were then collected from the supernatant by a centrifugation at $27{,}000 \times g$ for 30 min. The pellet was resuspended in 300 mM mannitol, 20 mM HEPES/Tris (pH 7.5) and spun down once again at $27{,}000 \times g$ for 30 min. The final pellet, obtained from 25 g of intestine, was resuspended in 1 to 1.5 ml 300 mM mannitol, 20 mM HEPES/Tris (pH 7.5). The protein concentration was about 20 mg/ml. The vesicles obtained are right-side-out better than 95% as judged from the lack of stimulation of sucrase activity upon disruption with Triton X-100 (Kessler et al., 1978*a*), from the quantitative solubilization of sucrase by papain added to the outside of the vesicles under conditions not affecting their permeability properties (Tannenbaum et al., 1977) and from the non-accessibility of a core protein to trypsin added to the outside of the vesicles (Klip et al., 1979*a*).

HEPES is the abbreviation of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

Transport Measurements

Incubations were done at room temperature with an automated procedure which allows working with incubation times as short as 0.3 sec (Kessler et al., 1978*b*). The standard procedure was as follows: The incubation medium containing the radioactively labeled substrate (10 µl or more) was placed on the bottom of a clear polystyrene tube fitted into a vibration device controlled by an electric timer. 5 to 10 µl vesicles were placed approximately 1 mm apart from the incubation medium. At the start of the timer, the shaking of the vibrator rapidly mixed the two drops together (within less than 80 msec). At the chosen incubation time, 3 ml of "stop solution" (250 mM NaCl, 1 mM Tris/HCl (pH 7.5), cooled to 0 °C) were automatically injected into the test tube. The sample was then quickly filtered through a wet cellulose nitrate microfilter (∅ 0.6 µm) and washed twice with 5 ml of the stop solution.

All solutions of preincubations and incubations contained 300 mM mannitol and 20 mM HEPES/Tris (pH 7.5), unless stated otherwise. Further additions as well as incubation procedures different from the one described above are detailed in the legends.

Loading the vesicles with D- and L-glucose and with salts for efflux studies and for transinhibition studies was achieved by preincubating them in the presence of these compounds for 90 to 120 min at room temperature. D-Glucose is not metabolized into nontransportable compounds under these conditions: D-glucose was preincubated with vesicles for 4 hr at room temperature and then separated from the vesicles; this 'treated' D-glucose showed identical Na^+-dependent uptake as fresh D-glucose.

Protein

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Materials

All reagents were of highest purity available. D-[^{3}H] Glucose was from Radiochemical Centre, Amersham, England; L-[^{14}C] glucose was from New England Nuclear, Boston, Mass. Microfilters were purchased from Inchema SA, CH-1249 Avully, Switzerland (Cellpore, CP-060, 0.6 µm ∅) and from Sartorius, Göttingen, Germany. The apparatus for the automated short-time incubations is produced by Innovativ-Labor AG, Feldblumenstrasse 76, CH-8134 Adliswil, Switzerland.

Results

I. Transeffects on D-Glucose Influx

A. Transinhibition of D-Glucose Unidirectional Influx by Na^+_{in} at $\Delta\psi \simeq 0$. The initial uptake velocity of D-glucose, measured at constant outer concentrations of D-glucose and of Na^+, is strongly reduced if Na^+ is present inside the vesicles (Fig. 1, *A–E*). This so-called transinhibition appears at Na^+_{in} concentrations

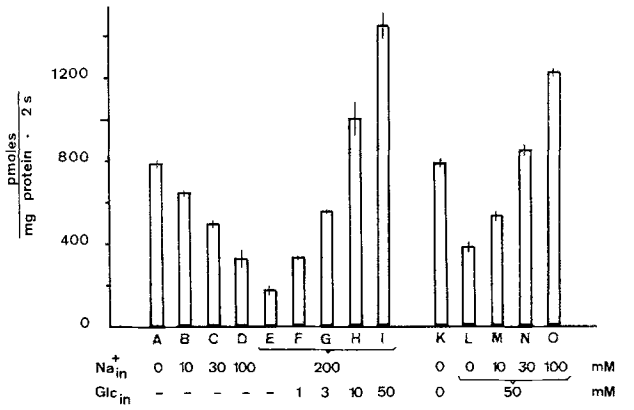


Fig. 1. Transinhibition of D-glucose influx by Na^+_{in} and Glc_{in} at $\Delta\psi \approx 0$. The vesicles were prepared in 300 mM mannitol, 20 mM HEPES/Tris, pH 7.5. In order to load them with the indicated concentrations of Na^+ and D-glucose, they were preincubated at room temperature for 2 hr in 200 mM mannitol, 20 mM HEPES/Tris, pH 7.5, 100 mM choline NO_3, 100 mM (Na_2SO_4 + choline$_2$ SO_4) and D-glucose. At $t=0$, 4 µl of this suspension (containing about 80 µg protein) were rapidly mixed with 196 µl of an incubation solution to give the following final outer concentrations: 200 mM mannitol, 20 mM HEPES/Tris, pH 7.5, 100 mM choline NO_3, 100 mM Na_2SO_4, 1 mM D-glucose (including D-glucose from the preincubation) and 4 µCi ^{3}H-D-glucose. After 2 sec incubation, 2 ml ice-cold stop solution (250 mg NaCl, 1 mM Tris/HCl, pH 7.5) were added. The incubations were done at 20 to 22 °C. Na^+_{in} and Glc_{in} refer to the intravesicular concentrations of Na^+ and of D-glucose, respectively

Table 1. Transinhibition by Na^+_{in} at $\Delta\psi \approx 0$; comparison with other cations[a]

Additive to preincubation solution (≈ intravesicular concentration during the incubation)		D-glucose uptake (pmol/mg protein × sec)
none	–	18.0 ± 0.5
mannitol	150 mM (totally 450 mM)	18.4 ± 0.2
choline$_2$$SO_4$	50 mM	17.6 ± 0.4
Na_2SO_4	50 mM	2.0 ± 0.1
K_2SO_4	50 mM	16.0 ± 0.9
Rb_2SO_4	50 mM	16.0 ± 0.7
Li_2SO_4	50 mM	12.6 ± 0.1
NH_4SO_4	50 mM	13.0 ± 1.0
$MgCl_2$	50 mM	17.7 ± 1.1
$Tris_2SO_4$	50 mM	14.8 ± 0.6

[a] All solutions of the preincubations and the incubations contained 300 mM mannitol, 20 mM HEPES/Tris, pH 7.5, and 100 mM choline NO_3. Only further additions will be mentioned in the Table. The vesicles were preincubated for 90 min at room temperature in solutions containing the components listed in the left column. At the start of the incubation the vesicle suspensions were diluted fivefold in incubation media containing 125 mM Na_2SO_4 and 12.5 µM D-[^{3}H]-glucose. Incubation time was 1 sec.

as low as 10 mM already, and becomes as large as 75 to 90% at Na^+_{in} concentrations in the order of 100 to 200 mM. It is specific for Na^+, no other cation tested having comparable influence on D-glucose uptake, although the small inhibition by internal Li^+, NH_4^+ or Tris should be mentioned (Table 1; as a side observation, the small transinhibition by Li^+, NH_4^+ and $Tris^+$ should be mentioned also). The inhibition is clearly due to a true reduction of the initial uptake rate and not to an early deviation from linearity (Fig. 2). In all three experiments, the membrane potential difference $\Delta\psi$ was kept close to zero by having equal concentrations of the highly permeant anion NO_3^- inside and outside the vesicles, thus ruling out the possibility that Na^+_{in} may exert its inhibitory effect via changing the membrane potential.

Since brush border membranes possess a Na^+/H^+ exchange system (Murer, Hopfer & Kinne, 1976), a Na^+ gradient may be partially converted into a pH gradient. However, at the high buffer concentrations used (10 to 50 mM HEPES/Tris, pH 7.5) an alkalinization of the internal compartment produced via Na^+/H^+

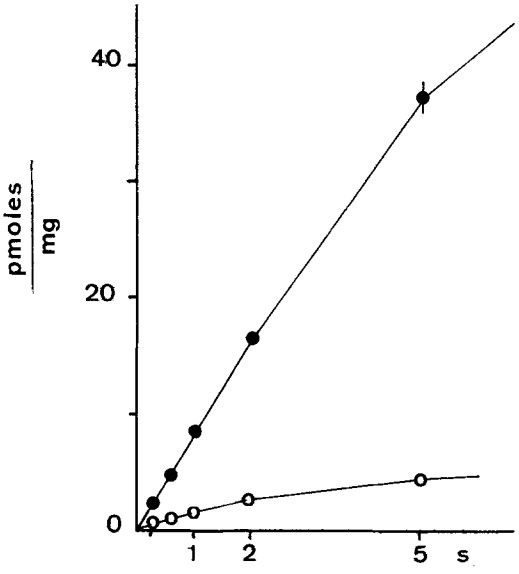


Fig. 2. Initial uptake rates in the presence or absence of internal Na^+ at $\Delta\psi \approx 0$. Vesicles were preincubated (2 hr at 20 to 22 °C) in 300 mM mannitol, 20 mM HEPES/Tris, pH 7.5, 100 mM choline nitrate, and either 100 mM choline sulfate (●) or 50 mM choline sulfate *plus* 50 mM Na_2SO_4 (○). The final concentrations in the incubation (outer) medium were: 300 mM mannitol, 20 mM HEPES/Tris, pH 7.5, 100 mM choline nitrate, 100 mM Na_2SO_4 and 10 µM ^{3}H-D-glucose. Most standard errors were smaller than the size of the symbol in the Figure

Table 2. Effect of internal pH on D-glucose uptake at $\Delta\psi \approx 0$[a]

pH in preincubation (≈pH of internal compartment)	pH in incubation solution (≈pH of external compartment)	Na^+_{in}	pmol/mg protein × sec
7.5	7.5	0	9.26 ± 0.38
7.5	7.5	100	1.47 ± 0.09
8.5	7.5	100	1.59 ± 0.10
8.5	7.5	0	7.14 ± 0.22

[a] For the final centrifugation of the preparation, the vesicles were suspended in 300 mM mannitol without HEPES/Tris buffer. They were then preincubated in solutions containing 300 mM mannitol, 100 mM choline NO_3, 50 mM of either Na_2SO_4 or $choline_2SO_4$ and 50 mM of HEPES/Tris of either pH 7.5 or pH 8.5. Preincubation time was 90 min. To start the incubation, 5 µl vesicles were diluted with 20 µl of an incubation mixture with the following concentrations: 300 mM mannitol, 100 mM choline NO_3, 125 mM Na_2SO_4, 12.5 µM D-glucose and 75 mM HEPES/Tris, pH 7.5. Incubation time was 1 sec.

Table 3. Transinhibition of D-glucose influx by Glc_{in}[a]

D-glucose concentration inside the vesicles (mM)	D-glucose taken up during 2 sec (pmol/mg)
0	591 ± 30
10	546 ± 71
40	406 ± 26
100	270 ± 19

[a] In this experiment the vesicles were diluted to 9/10 of the final incubation volume 2 sec before the addition of NaSCN and the tracer (*see* text). Incubation time was 2 sec. The vesicles were pre-equilibrated for half an hour in 200 mM mannitol, 20 mM HEPES/Tris (pH 7.5) and the D-glucose concentrations listed in the Table (D-glucose concentrations below 100 mM were complemented by additional mannitol). Two sec before starting the incubation, 5 µl were diluted into 450 µl of 300 mM mannitol, 20 mM HEPES/Tris, pH 7.5, and enough D-glucose to a total concentration of 1.11 mM (including the D-glucose originating from the preincubation medium). At $t=0$, 50 µl 1 M NaSCN containing ^{3}H-D-glucose were added. Concentrations of D-glucose and of NaSCN in the outer medium were 1 and 100 mM, respectively.

exchange must be small, certainly less than 1 pH unit. We have thus assured that a gradient of one pH unit cannot mimic the effects discussed here (Table 2).

The transinhibition by Na^+_{in} is of the noncompetitive type, the main effect being on the maximum velocity V_{max}. Eadie-Scatchard plots for Na^+_{in} concentrations of 0 and 100 mM will be shown below (at $\Delta\psi \ll 0$).

B. Transinhibition of D-Glucose Unidirectional Influx by D-Glucose$_{in}$. In vesicles containing a high concentration of (unlabeled) D-glucose (at $Na^+_{in}=0$) the initial influx of externally added D-glucose is inhibited (Fig. 1, *K, L*; Table 3). Thus D-glucose induces a transinhibition similar to that described above for Na^+_{in}. This kind of experiment required a protocol slightly different from the usual one in that the glucose-preincubated vesicles were diluted into a large incubation volume at $t=0$ in order to reduce the external D-glucose concentration down to 1 mM. The following controls made sure that the observed inhibition was not simulated by incomplete mixing (i.e. by a cis effect): (i) When the preincubation for loading the vesicles with glucose (usually at room temperature for at least one hour) was done at 0 °C for 30 sec only, i.e. under conditions not allowing complete equilibration of D-glucose, virtually no inhibition was observed (*not shown*). (ii) In some experiments (e.g. the one of Table 3) the D-glucose preincubated vesicles were first diluted into 9/10 of the final volume 2 sec before the addition of Na^+ and the tracer D[^{3}H]-glucose. Unlabeled D-glucose was added to the dilution media to give a total concentration of 1.11 mM (together with the D-glucose originating from the preincubation medium). Thus, at the moment the tracer was added, the vesicles in all samples were suspended in an identical, well-stirred solution. Therefore, the inhibition is clearly caused by *internal* D-glucose.

The transinhibition by D-glucose can only be observed in incubation lasting less than about 5 sec, while the internal Na^+ concentration is still close to zero; thereafter, the deviation from the initial uptake rate is large enough to obscure the inhibition. Most of the experiments presented here were done at 0.6 to 2 sec incubation time.

The D-glucose$_{in}$ concentrations required to elicit transinhibition are rather high, 50 mM D-glucose$_{in}$ inducing only 30 to 50% inhibition. At concentrations below 10 mM virtually no inhibition is observed (Table 3).

C. Transeffects on D-Glucose Unidirectional Initial Influx by the Simultaneous Presence of Na^+ and of D-Glucose Inside the Vesicles at $\Delta\psi \simeq 0$. The transinhibition exerted by either Na^+_{in} or D-glucose$_{in}$ is reverted completely by increasing concentrations of the other cosubstrate (Fig. 1, *E–I, L–O*). Indeed, influx at high transconcentrations of *both* cosubstrates exceeds the influx observed in the controls

Table 4. $\Delta\psi$ dependence of the transinhibition by Glc_{in}[a]

	0 mM trans Glc	20 mM trans Glc	% inhibition
Na_2SO_4 gradient ($\Delta\psi$ small)	65.0 ±1.5	40.1 ±0.7	38%
NaSCN gradient ($\Delta\psi \ll 0$)	338 ±8	261 ±5	23%

[a] The vesicles were preincubated for 60 min with or without 20 mM D-glucose (D-glucose being replaced by mannitol in the control). Five µl of this suspension were diluted at $t=0$ into 95 µl of a medium containing 100 mM mannitol, 20 mM HEPES/Tris (pH 7.5), 0,02% KN_3, labeled D-glucose and either 100 mM NaSCN or 50 mM Na_2SO_4 or 50 mM K_2SO_4. The medium of the control contained further 1.05 mM unlabeled D-glucose to match D-glucose in the incubation medium of the glucose-preincubated samples. Incubation time was 0.6 sec. The values obtained in the presence of a K_2SO_4 gradient were subtracted as background from the respective values determined in the presence of Na^+. Values are means and SE from 4 or 5 determinations, expressed as pmol/mg protein.

(without either Na^+_{in} or D-$glucose_{in}$) by roughly a factor 2. This release from the transinhibition (which will also be referred to in the following as "transstimulation") occurs at moderately low concentrations of the other cosubstrate: the half-maximal concentration $K_{1/2}$ of Glc_{in} capable of reversing the transinhibition by Na^+_{in} appears to be in the order of 5 to 10 mM, i.e., clearly smaller than the Glc_{in} concentration required to elicit transinhibition in the absence of internal Na^+ (*see* Table 3).

D. $\Delta\psi$-Dependence of Transeffects. The transeffects reported above were all measured at a membrane potential close to zero. Table 4 compares those results with the transinhibition measured at $\Delta\psi \ll 0$ (negative inside). The fractional inhibition at a given concentration of transglucose was consistently found to be decreased with increasing membrane potential difference $\Delta\psi$ (inside negative).

E. Counterflow Accumulation. We will indicate as "counterflow accumulation" the accumulation of a substrate α beyond its equilibrium concentration, driven by an imposed gradient of a second substrate β, which shares the same transporter and binding site (Rosenberg & Wilbrandt, 1957; LeFevre, 1975). This phenomenon (though not the actual extent of accumulation) is a consequence of the laws of thermodynamics; it is found with all transport mechanisms whereby binding sites are alternatively exposed to the inner and the outer compartment (mobile carriers, gates, pores, etc.) and it is, therefore, independent of the actual kinetic parameters of an individual transport system. Counterflow accumulation of Na^+-coupled D-glucose translocation is shown in Fig. 3 with unlabeled D-glucose acting as the elicitor (substrate β, inside) for the accumulation of labeled D-glucose (substrate α, outside). Both, in the presence of an inwardly directed Na^+ gradient (Fig. 3*A*) and in vesicles preequilibrated in 200 mM Na^+ (Fig. 3*B*), the *accumulation* at 20 to 180 sec is larger in the presence of outflowing elicitor, which inhibits competitively the backflux of tracer glucose due to its high concentration inside the vesicles.

Transstimulation and counterflow accumulation have sometimes been confused with one another, mainly in disregard of the fact that the former term describes flux rates, i.e. kinetic properties of a transporter, whereas the latter means accumulation due to flux coupling, (and thus is a consequence of conservation of chemical energy, as described by thermodynamic laws). This difference becomes clear by comparing initial uptake rates and accumulation in Fig. 3*A*. At early incubation times (up to 5 sec) the uptake of externally added D-glucose is inhibited by the high concentration of unlabeled D-glucose inside the vesicles, corresponding to the transinhibition in Fig. 1 and Table 3. At later incubation times, however, this initial inhibition is overcompensated by the accumulation due to counterflow, which is much larger in vesicles containing unlabeled D-glucose than in control vesicles. Likewise, in Fig. 3*B* the increased uptake rate of D-glucose during the first seconds is essentially kinetic in nature and can be attributed to the transstimulation by D-$glucose_{in}$, i.e. to the reversal of the transinhibition exerted by Na^+_{in}. Counterflow accumulation alone, however, is responsible for the subsequent accumulation beyond equilibrium. Figure 3 clearly documents the importance of measuring true initial flux rates at appropriately short incubation times in order to determine the kinetics of a transporter.

For a discussion of the two concepts of "transstimulation" and "counterflow," the reader is referred to Heinz's monograph (1978).

II. Transeffects on D-Glucose Efflux; Asymmetry of D-Glucose Transport

A. Measurement of D-Glucose Efflux. In principle, efflux could be measured directly by

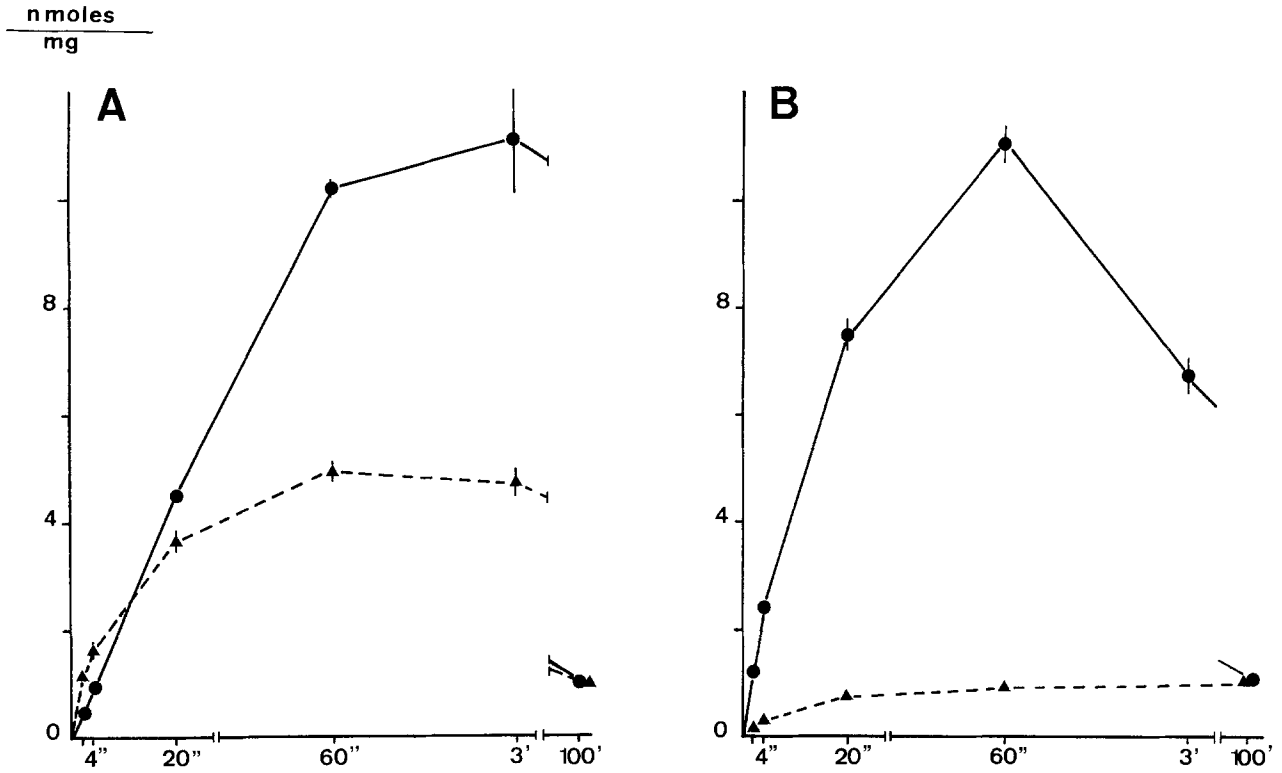


Fig. 3. Counterflow accumulation of labeled D-glucose elicited by internal unlabeled D-glucose at $\Delta\psi \simeq 0$. *A:* In the presence of a Na^+ gradient (out→in). *B:* In Na^+ pre-equilibrated vesicles. Composition of the preincubation solutions: 200 mM mannitol, 20 mM HEPES/Tris, pH 7.5, 200 mM choline$^+$ (*A*) or 200 mM Na^+ (*B*), 100 mM NO_3^-, 50 mM SO_4^{2-} and 30 mM D-glucose (●) or mannitol (▲). At the start of the incubation, the vesicles were diluted 30-fold into a medium containing the same concentrations of mannitol, buffer, NO_3^- or SO_4^{2-} as the preincubation solutions, *plus* further 200 mM Na^+ and ^{3}H-D-glucose + cold glucose to give a final concentration of 1 mM

washing the vesicles free of external D-glucose and then collecting the amount of substrate leaving the vesicles during a given incubation time. Although this technique has proven very useful in studies with erythrocytes, it is less so with the tiny membrane vesicles from intestinal and renal microvilli, because a significant amount of substrate leaks out of the vesicles during the rather long lasting procedure of centrifugation and proper resuspending, even if the vesicles are kept at 0 °C.

Therefore we measured efflux instead by diluting the preincubated vesicles into a 20- to 100-fold volume of efflux medium and determining the amount of D-glucose associated with the vesicles both at 0 sec and at the desired incubation time and calculating the difference between these two values. The determination of exact initial efflux rates would require measurements to be done while less than 10% of the D-glucose initially present had left the vesicles; however, the standard error of such a small difference is similar in size to the difference itself. It thus proved impossible to obtain reliable initial rates, precise kinetic parameters or detailed wash-out curves. (A few approximate determinations of wash-out curves are reported elsewhere: Toggenburger, Kessler & Semenza, 1982. They are not described by a single exponential, nor should they be expected to be: *see* Hopfer, 1977.) In the efflux measurements to be reported below some 20 to 40% of the D-glucose load had left the vesicles. Although the data do not allow kinetic parameters to be calculated, they did lead to valid conclusions as to the possible transeffects by (external) D-glucose or Na^+ and as to the effect of $\Delta\psi$ (negative *outside* the vesicles) on D-glucose efflux.

First of all, the following observations ascertained that the release of D-glucose from the vesicles proceeded through the same Na^+-dependent transport system as influx (Table 5). The release of D-glucose was faster than that of L-glucose, depended on the presence of (internal) Na^+ and was inhibited by externally added phlorizin. Moreover, in vesicles equilibrated with 100 mM Na^+, the efflux rate was comparable in size to the influx rate (*not shown*; strict

Table 5. Efflux of D- and L-glucose; Inhibition by phorizin[a]

Intravesicular concentrations at $t=0$ (=additions in preincubation)		Extravesicular concentrations during efflux (additions in efflux solutions)	Efflux time (sec)	Amount of sugar found to have left the vesicles after 10 or 3 sec, as indicated (pmol mg^{-1} protein)
salt	glucose			
Expt. A				
KCl 100 mM	D-glc* 0.1 mM	KCl 100 mM	10	16±2
NaCl 100 mM	L-glc* 0.1 mM	NaCl 100 mM	3	2±2
Expt. B				
NaCl 100 mM	D-glc* 0.1 mM	NaCl 100 mM	3	38±1
NaCl 100 mM	D-glc* 0.1 mM	NaCl 100 mM + 100 µM phlorizin	3	10±5
NaCl 100 mM	D-glc* 0.1 mM	NaCl 100 mM + 500 µM phlorizin	3	4±3
Expt. C				
NaCl 100 mM	D-glc* 0.1 mM + D-glc 0.4 mM	NaCl 100 mM	3	38±2
NaCl 100 mM	D-glc* 0.1 mM + D-glc 5 mM	NaCl 100 mM	3	12±5
NaCl 100 mM	D-glc* 0.1 mM + D-glc 20 mM	NaCl 100 mM	3	2±2

[a] All solutions contained the standard concentrations of mannitol and buffer, only further additions are listed. Efflux time was 3 sec unless stated otherwise. The amount of D-glucose in the vesicles at $t=0$ was around 140 pmol/mg protein. To start efflux measurements ($t=0$), the preincubated vesicles were diluted 40-fold. The efflux values were calculated for labeled glucose only (marked with an *); unlabeled D-glucose added to the incubations shown in the last three lines was treated like a competitive inhibitor.

equality of influx and efflux rates can only be expected for fluxes in equilibrium tracer exchange experiments, where both Na^+ *and* D-glucose concentrations are equal on both sides of the membrane).[1]

B. Saturation of D-Glucose Efflux. Table 5 shows that the efflux of tracer D-glucose is little inhibited by 0.5 mM (unlabeled) D-glucose, but more than 50% inhibited by 5 mM D-glucose. This allows a rough estimate of the K_m (with 100 mM Na^+ at both sides of the membrane and $\Delta\psi \simeq 0$) of efflux to be in the range of 1 to 5 mM. The corresponding K_m for influx is approximately 0.4 mM (*see below*).

C. Transeffects by D-Glucose and Na^+ on D-Glucose Efflux. The presence of 200 mM Na^+ or 50 mM D-glucose or of both of them in the *efflux medium* had virtually no effect on the efflux rate of D-glucose (Table 6). This experiment was carried out with at least five different vesicle preparations and under a number of different conditions (e.g., different internal and external D-glucose and Na^+ concentrations; with SO_4^{2-}, or Cl^-, or NO_3^- as the major anion). However, the efflux rates were in all cases within 20% of the control.[2]

Table 6. Lack of transeffects on D-glucose efflux[a]

Transsubstrate present	pmol D-glucose released in 2 sec
none	1.09±0.02
200 mM Na^+	1.07±0.05
50 mM D-glc	1.11±0.05
50 mM D-glc + 200 mM Na^+	0.99±0.03

[a] The vesicles were first preincubated for 2 hr (20 to 22 °C) with 300 mM mannitol, 20 mM HEPES/Tris (pH 7.5) and 50 mM Na_2SO_4. Then ^{3}H-D-glucose was added to give a final concentration of 0.1 mM. After 30 sec (by that time, D-glucose was equilibrated) 4 µl of this suspension were diluted into 100 µl efflux solution consisting of 20 mM HEPES/Tris (pH 7.5), 150 mM $choline_2SO_4$ or (100 mM Na_2SO_4 + 50 mM $choline_2SO_4$) and 200 mM mannitol or (150 mM mannitol + 50 mM D-glucose). Efflux time was 2 sec. The values are expressed as pmol per sample, the samples containing about 30 µg protein. The value for the D-glucose content at $t=0$ was 2.57±0.11 pmol.

[1] In a few preparations D-glucose efflux was found to be slow and hardly distinguishable from L-glucose release. These preparations were discarded. Only preparations showing fast, Na^+-dependent D-glucose efflux were used for the experiments shown.

[2] When both D-glucose and Na^+ were present on the outside, efflux appeared to be slightly inhibited; however, this was apparently due to a competitive inhibition of the tracer efflux by unlabeled D-glucose rapidly entering the vesicles via the carrier (when inward transport proceeds at its V_{max}, the internal D-glucose concentration built up within 2 sec may be 1 to 2 mM, i.e. well in the range of the K_m suggested for efflux).

Table 7. Response of D-glucose efflux to $\Delta\psi$ (negative trans)[a]

Major salt present in the (outer) efflux medium	Expected $\Delta\psi$	Amount of D-glucose released from the vesicles in 3 sec (pmol × mg^{-1} protein) $\bar{x} \pm$ SE (3 expts.)
$NaNO_3$	$\Delta\psi \approx 0$	31 ± 3
NaCl	$\Delta\psi \ll 0$ (i.e. negative *outside* the vesicles)	42 ± 3

[a] Vesicles were pre-equilibrated for 1 hr at 20 to 22 °C with 100 mM $NaNO_3$, 0.1 mM ^{3}H-D-glucose, 300 mM mannitol, 20 mM HEPES/Tris buffer, pH 7.5. They were then diluted 40 times into a solution containing 300 mM mannitol, buffer, and either 100 mM $NaNO_3$ or 100 mM NaCl. Efflux time was 3 sec.

D. Effect of $\Delta\psi$ (Negative at the Trans Side) on D-Glucose Efflux. D-glucose *influx* (at low D-glucose concentrations, 100 mM Na^+ at both sides of the membranes) is accelerated 3 to 5 times by the membrane potential difference generated by the following initial bi-anion gradient: 100 mM NO_3^- out, zero in; with zero Cl^- out, 100 mM in (*data not shown*). A corresponding experiment on *efflux* was carried out by preloading the vesicles with NO_3^-[3] and labeled D-glucose and diluting them into a solution containing either NO_3^- at the same concentration as inside (control, $\Delta\psi \simeq 0$), or into a solution without NO_3^- outside ($\Delta\psi \gg 0$). Table 7 shows that efflux is somewhat promoted in the presence of the $\Delta\psi$; however, the acceleration factor is less than 1.5 and thus much smaller than that for influx.

To summarize, efflux is only minimally sensitive (i) to Na^+ and D-glucose present in the external medium and (ii) to changes of $\Delta\psi$ (negative of the trans side). Both findings strongly contrast with the results obtained from influx studies and thus indicate that the D-glucose transporter is asymmetrical.

The high degree of functional asymmetry becomes even more apparent when uptake rates are compared to efflux rates measured at mirrored, but otherwise identical experimental conditions (Fig. 4). When D-glucose influx is measured in the presence of both a Na^+ gradient ($Na^+_{in} = 0$) and a membrane potential negative inside (e.g., when a NaSCN or a $NaNO_3$ gradient is applied) and if the D-glucose concentration is below 0.1 mM, the amount of D-glucose taken up within 2 sec usually reaches an internal concentration 2 to 5 times that in the incubation medium (in Fig. 4 a D-glucose content of about 12 pmol/mg protein corresponds to an internal D-glucose concentration of 10 μM, i.e., to the concentration of D-glucose in the incubation medium). If D-glucose efflux is measured at equivalent, but mirrored conditions, i.e., under a Na^+ gradient directed outward and a potential negative outside, only about 20 to 50% of the D-glucose present initially leave the vesicles within 2 sec. Thus, the efflux velocity is about 10 times smaller than the equivalent influx velocity.

[3] SCN^- could not be used for this purpose, since this anion inhibits the cotransporter from the cytosolic and/or the hydrophobic surface (data not shown).

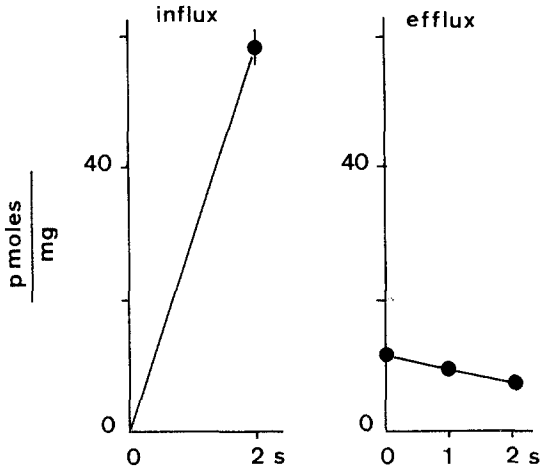


Fig. 4. Comparison of influx (left) and efflux (right) velocities determined in the presence of a Na^+ gradient ($Na^+_{trans} = 0$) and a potential (negative on the trans side). The cis concentrations were: 100 mM Na^+, 10 μM D-glucose. The potential was established by a gradient of 100 mM NO_3^-. A D-glucose content of 12 pmol/mg protein corresponds to an intravesicular concentration of about 10 μM

III. Effect of Varying Initial Concentrations of External Na^+ on the Apparent V_{max} and K_m for Initial D-Glucose Uptake

Since Na^+-dependent D-glucose transport is electrogenic and $\Delta\psi$ affects the transport rate (Murer & Hopfer, 1974), the partial Cleland analysis to be reported below was carried out both in voltage-clamped (near zero) vesicles and in vesicles with $\Delta\psi \ll 0$ (negative inside).

A. In Vesicles with $\Delta\psi \simeq 0$. In the experiment of Fig. 5, *A – C*, the vesicles were both pre-incubated (for 2 hr) and incubated in the presence of the highly permeant (Liedtke & Hopfer, 1977) NO_3^- (100 mM in the pre-incu-

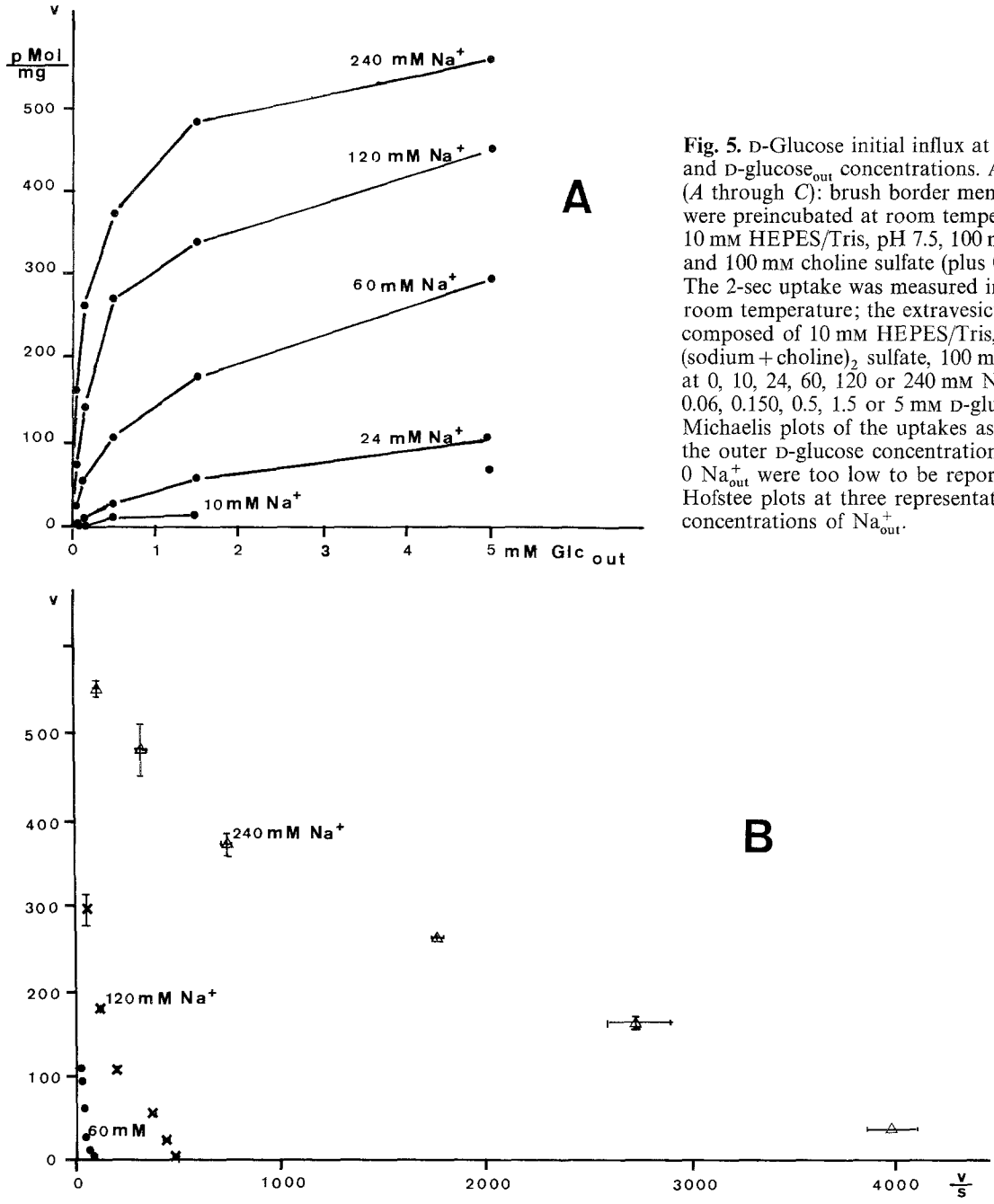
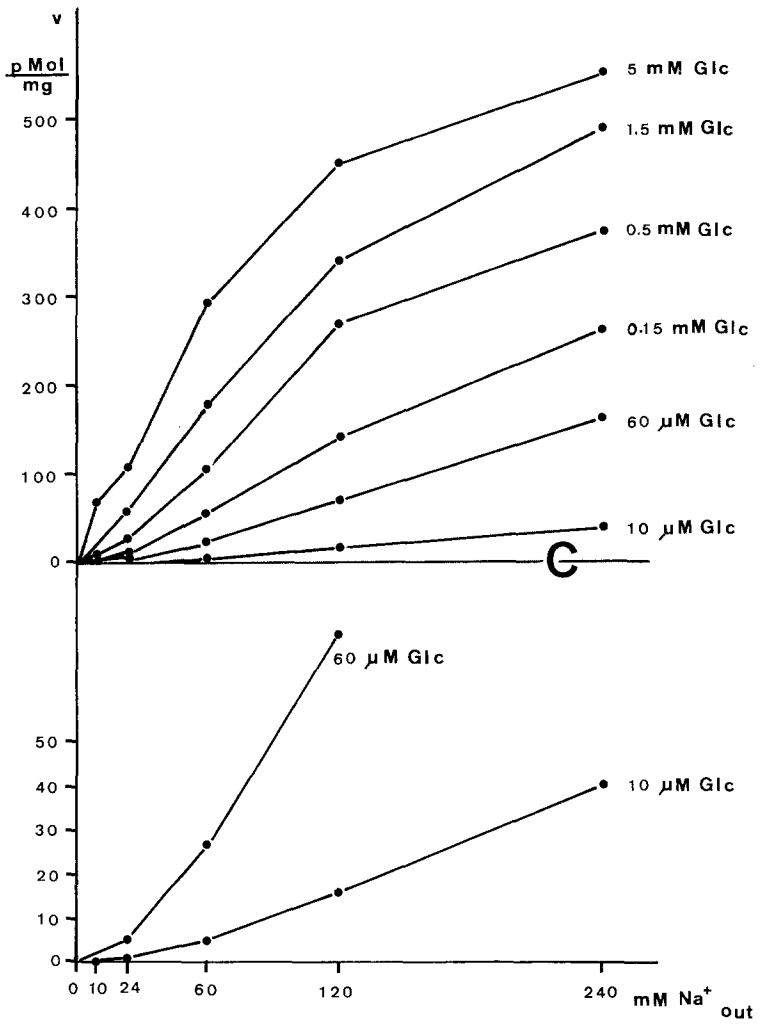


Fig. 5. D-Glucose initial influx at various Na^+_{out} and D-glucose$_{out}$ concentrations. At $\Delta\psi \simeq 0$ (*A* through *C*): brush border membrane vesicles were preincubated at room temperature for 2 hr in 10 mM HEPES/Tris, pH 7.5, 100 mM choline nitrate and 100 mM choline sulfate (plus 0.01% KN_3). The 2-sec uptake was measured in triplicate at room temperature; the extravesicular medium was composed of 10 mM HEPES/Tris, pH 7.5, 120 mM (sodium + choline)$_2$ sulfate, 100 mM choline nitrate at 0, 10, 24, 60, 120 or 240 mM Na^+_{out} and at 0.01, 0.06, 0.150, 0.5, 1.5 or 5 mM D-glucose$_{out}$. *A:* Michaelis plots of the uptakes as a function of the outer D-glucose concentrations (the values at 0 Na^+_{out} were too low to be reported). *B:* Eadie-Hofstee plots at three representative concentrations of Na^+_{out}.

bation and in the incubation). The initial velocities of D-glucose uptake were measured within the linear range, i.e., at 2 sec incubation, at six Na^+_{out} (0 to 240 mM) and seven D-glucose concentrations (0.01 to 50 mM). Representative data only are reported in Fig. 5 (*A*, as Michaelis plot and *B*, as Eadie-Hofstee plot of D-glucose$_{out}$; *C* as a Michaelis plot of Na^+_{out}). It is clear that plot *C* is sigmoidal, and that *A* and *B* also deviate from a Michaelian behavior, but in the opposite direction. Whatever the interpretation of this twofold (and possibly complementary) deviation (*see* Discussion), it is clear that high Na^+_{out} lead to small K_m for D-glucose$_{out}$ and that high D-glucose$_{out}$ lead to small $K_{1/2}$ of Na^+_{out} activation. These observations confirm and expand those of Kaunitz et al. (1982) and of Kaunitz and Wright[4], who clamped the vesicles with K^+/valinomycin, rather than with nitrate, as in Fig. 5, *A* – *C*.

[4] Kaunitz, J.D., and Wright, E.M. Kinetics of sodium D-glucose cotransport in rabbit and bovine intestinal brush border vesicles *(unpublished)*. (*See also* Kaunitz & Wright, 1983.)



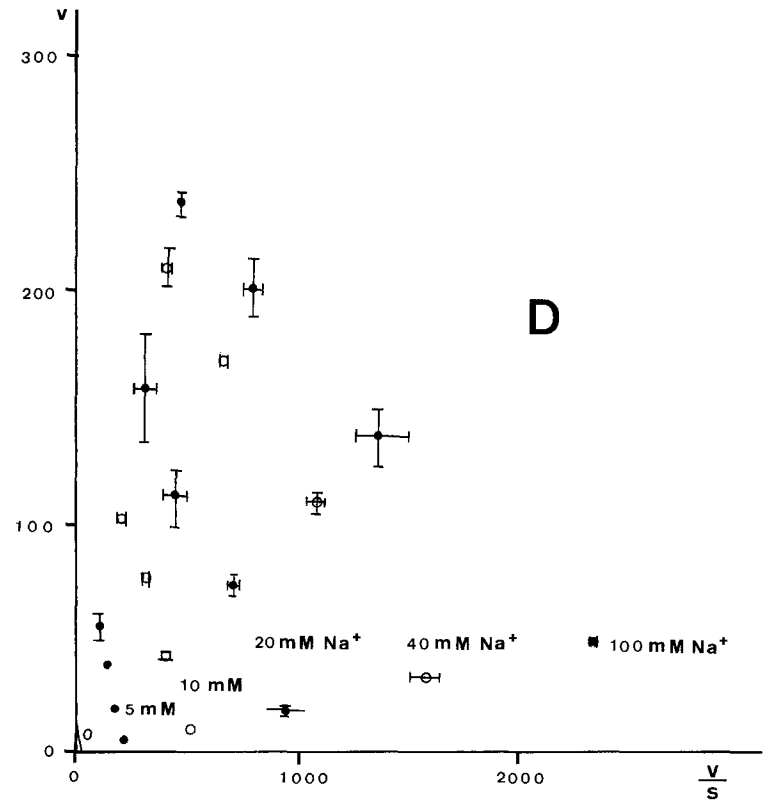


Fig. 5. *C*: Michaelis plot of the uptakes as a function of the outer Na^+ concentrations (in the lower panel the scale at the *y*-axis was expanded). Each value is the average of three determinations.

At $\Delta\psi \ll 0$ (*D* and *E*): brush border membrane vesicles were prepared in 10 mM HEPES/Tris, pH 7.5, 300 mM mannitol. The 2-sec uptake was measured in triplicate at room temperature; the extravesicular medium was composed of 10 mM HEPES/Tris, pH 7.5, 300 mM mannitol, 100 mM (sodium + choline) SCN, at 0, 5, 10, 20, 40 and 100 mM Na^+_{out} and 0.02, 0.10, 0.25 and 0.50 mM D-glucose$_{out}$. *D*: Eadie-Hofstee plots (*s* being the D-glucose$_{out}$ concentration).

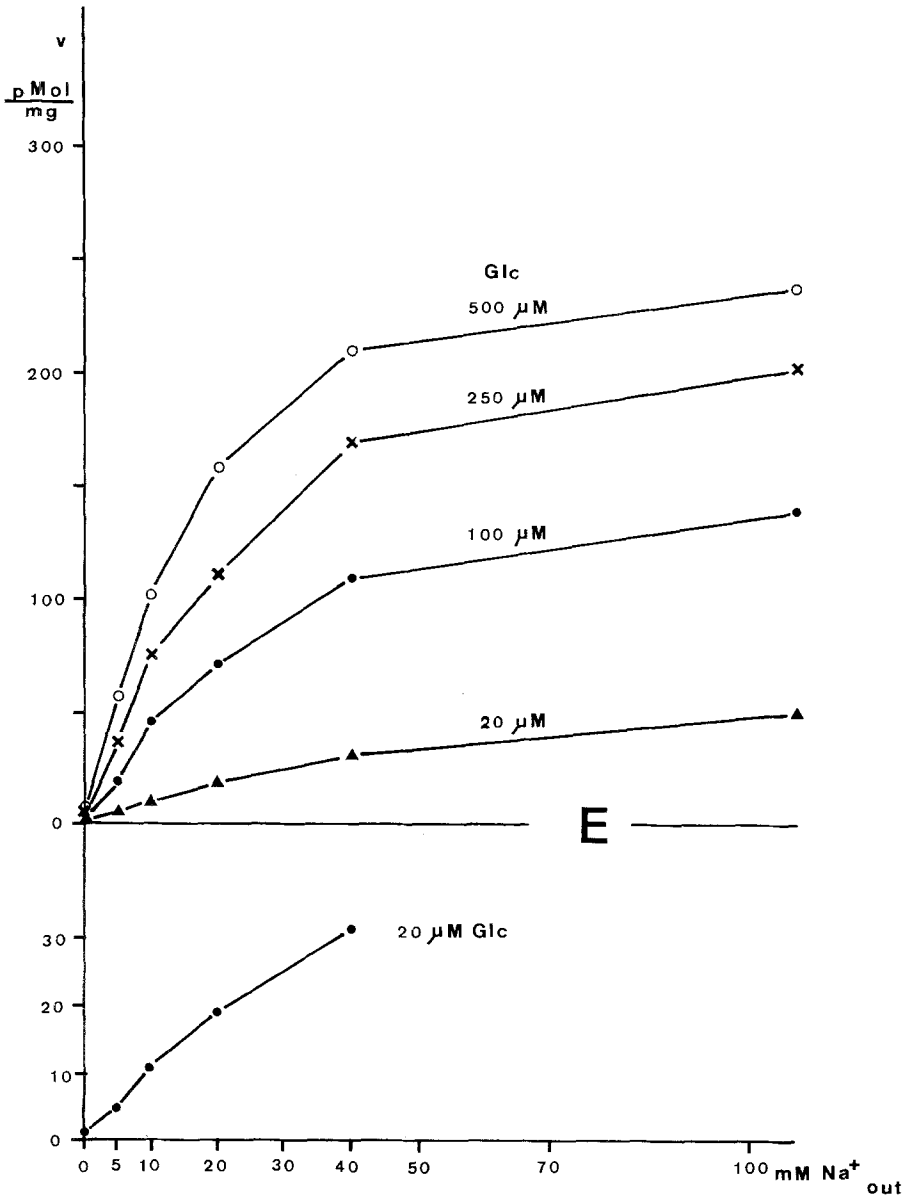


Fig. 5. *E:* Michaelis plot of the uptakes as a function of the outer Na^+ concentrations (in the lower panel the scale at the *y*-axis is expanded). Each value is the average of three determinations; the bars indicate the SEM (when not given, it was smaller than the symbol used). The velocity of uptake is expressed as pmol of D-glucose taken up $\times mg^{-1}$ protein taken up by the sample in the 2-sec incubation

B. In the Presence of an Initial $\Delta\psi \ll 0$. Clearly, it is not possible to keep constant the $\Delta\psi$ or $\Delta\tilde{\mu}_{Na^+}$ across the vesicle membrane for any extended length of time or to produce exactly identical $\Delta\psi$'s by the use of ion gradients of different compositions. For example, in the experiment of Fig. 5*D* the initial $\Delta\psi$'s certainly began to dissipate at time zero; moreover, the initial $\Delta\psi$'s would have been identical in the various determinations of apparent K_m at different Na^+_{out} concentrations, *only* if the electrogenic permeabilities of the membrane for Na^+ and for $choline^+$ were exactly identical, which was certainly not the case.

However, short of imposing a constant $\Delta\psi$ across the membrane, one can impose an *initial* $\Delta\psi$ producing a maximal constant stimulation of transport. Since this approach is not quite obvious it needs qualification. Transport agencies in which the substrate binding site(s) is (are) exposed alternatively (but not simultaneously) to the opposite sides of the membrane are defined as, and show the kinetic characteristics of, "mobile carriers" (Läuger, 1980). That is, by whatever mechanism the actual transport takes place, in mobile carrier the transport of substrate(s) is accompanied by a simultaneous and related movement of a portion of the transporting protein itself. Clearly, the velocity of this transport-related movement cannot increase indefinitely in response to a given parameter: in particular, higher and high-

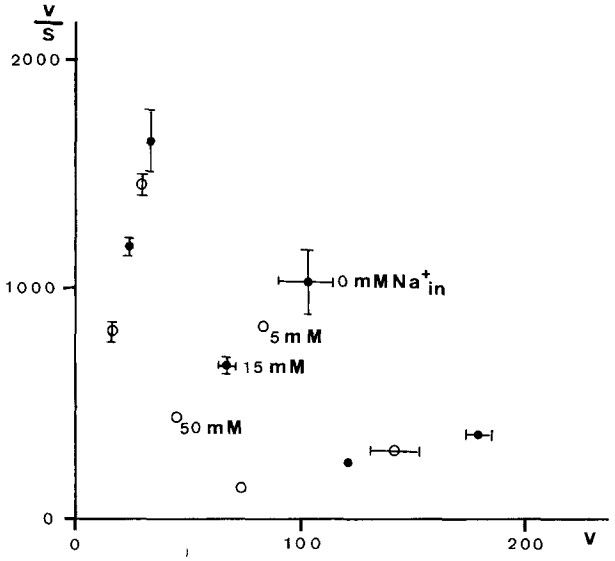


Fig. 6. Transinhibition by Na^+_{in}/D-glucose influx at $\Delta\psi \ll 0$, 100 mM Na^+_{out} and various Na^+_{in} concentrations (Eadie-Hofstee plot). The vesicles were preincubated (2 hr at 20 °C) in 300 mM mannitol, 10 mM HEPES/Tris (pH 7.5), 0.03% KN_3, and 25 mM (Na_2SO_4 + choline$_2SO_4$) to give the Na^+_{in} concentrations indicated in the Figure (0, 5, 15, 50 mM). To start the incubation, D-glucose and NaSCN were added to give the following final concentrations in the incubation medium: 300 mM mannitol, 10 mM HEPES/Tris (pH 7.5), 20, 100 or 500 μM D-glucose, 100 mM NaSCN and Na_2SO_4/choline$_2SO_4$ at half the concentration of the preincubation. *V* and [Glc] are expressed as in Fig. 5. Incubation time 2 sec

er $\Delta\psi$ (or $\Delta\tilde{\mu}_{Na^+}$) values will yield (in electrophoretic systems such as the one studied here) faster and faster transport velocities, till the fastest possible movement of the related protein portion will be attained. Further increase in $\Delta\psi$ (or $\Delta\tilde{\mu}_{Na^+}$) will fail to stimulate further transport, i.e., a kind of "saturation" by $\Delta\psi$ or ($\Delta\tilde{\mu}_{Na^+}$) is to be expected (*see also* Fig. 10 of the Appendix, as well as Geck & Heinz, 1976; Heinz & Geck, 1977; Geck, 1978; Turner, 1981; Van den Broek & Van Steveninck, 1982). Conversely, starting from very high $\Delta\psi$ (or $\Delta\tilde{\mu}_{Na^+}$) a *limited* decrease of either of these parameters will fail to lead to a detectable decrease of transport velocity, as long as $\Delta\psi$ (or $\Delta\tilde{\mu}_{Na^+}$) is still in the range of producing the maximum stimulation of transport. In other words, it is essential that the initiating collapse of $\Delta\psi$ (or $\Delta\tilde{\mu}_{Na^+}$) does not lead to a change in the rate-limiting step of the transport which is being measured. In practice this means that it is essential to make sure that the initiating collapse of $\Delta\psi$ (or $\Delta\tilde{\mu}_{Na^+}$) does not lead to a decrease of the transport velocity during the incubation – or, to put it differently, that uptake measurements are carried out during the very initial time range, while the uptake velocities are still linear. In the presence of an initial NaSCN gradient (100 mM out, zero in) the uptake of D-glucose into the vesicles studied here is linear until at least 3 to 4 sec (*see also* Tannenbaum et al., 1977; Toggenburger et al., 1978, 1982). As a rule, therefore, the uptakes were measured at incubations lasting as short as 0.6 to 2 sec. (For more arguments, *see* Toggenburger et al., 1978, 1982.) Furthermore, if $\Delta\psi$ is described by Goldman's equation, the $\Delta\psi$ produced by an initial NaSCN gradient, 100 mM out, zero in, is equal to $\frac{RT}{F}\ln\frac{P_{Na^+}}{P_{SCN^-}}$ as long as $SCN^-_{in}\cdot P_{SCN^-} \ll Na^+_{out}\cdot P_{Na^+}$. Since P_{SCN^-}/P_{Na^+} is approximately 5 to 10 (Kessler & Semenza, 1979), the initial $\Delta\psi$ remains fairly constant as long as $SCN^-_{in} \ll Na^+_{out}\cdot 0.1$.

As to the experiments in which the concentrations of Na^+_{out} were varied, the initial SCN^- gradient was kept constant, while a cation with a smaller electrogenic permeability than Na^+ was chosen as its substitute, i.e., choline. (Most if not all of the movement of this cation across this membrane is electrically silent, since different anion diffusion potentials yielded essentially identical initial velocities of choline uptake: Kessler et al., 1978*a*.) Thus, if an initial NaSCN gradient 100 mM out, zero in, already produced a "supramaximal" or "saturating" stimulation of D-glucose uptake (as documented by the linear uptake velocity), the more so must have done a combined (Na^+ + choline$^+$) SCN^- gradient, the initial SCN^- gradient being the same in all incubations. Naturally, these considerations refer only to the use of "saturating" $\Delta\psi$'s or $\Delta\tilde{\mu}_{Na^+}$'s. They do NOT apply to incubations carried out in the presence of low (but not nil) $\Delta\psi$'s or $\Delta\tilde{\mu}_{Na^+}$'s yielding intermediate degrees of stimulation: their initiating collapse at the beginning of the incubations undoubtedly lead to an *immediate* decrease of the uptake velocities and to a change in the rate-limiting step being measured.

The experiments in Figs. 5*D–E* and 6 were thus carried out in the presence of initial SCN^- gradient (100 mM out, zero in) and the measurements were confined to the linear time range. Here also, the uptake values at high D-glucose concentrations (1 and 3 mM) showed a deviation from linearity upwards (*not shown*) and the Michaelian plot *vs.* Na^+_{out} (Fig. 5*E*) indicated some sigmoidicity, nowhere comparable, however, to that observed at $\Delta\psi \simeq 0$ (Fig. 5*C*).

Discussion

Any attempt at working out a realistic model, even if partial, of the possible mode of functioning of a fairly complicated transport system, such as the small-intestinal Na^+,D-glucose cotransporter, must combine a variety of kinetic and chemical approaches. In the following we will discuss one feature at a time, as we deduce it and as we progressively restrict the number of alternatives left.

I. The Asymmetry: A "Channel" (or "Pore")

Current ideas on the vectorial insertion of intrinsic membrane proteins during or after their biosynthesis and obvious thermodynamic considerations justify the expectation that intrinsic membrane proteins having on their surface hydrophilic and hydrophobic areas be inserted (during or immediately after their biosynthesis) in the membrane asymmetrically with respect to the plane of the membrane, and that they remain in this positioning thereafter. Transport agencies should belong to this class of proteins, and indeed it has been conclusively shown even in so-called "equilibrating" transport agencies that they are asymmetrically inserted and that they have different overall kinetic parameters at both sides of the membrane. (For reviews on the sugar transporter of the erythrocyte, *see* Widdas, 1980; on that of the anions in the same membrane, *see* Cabantchik et al., 1978; Knauf et al., 1978; Rothstein et al., 1978; Rothstein & Ramjeesingh, 1980; and on that of adenine nucleotides of the mitochondrial inner membrane, *see* Klingenberg, 1980.) Naturally, thermodynamics sets the frame of the kinetic asymmetries possible (*see more below*).

As to the Na^+,D-glucose cotransporter of the small intestine, we have shown previously (Klip et al., 1979*a*, *b*; 1980*a*, *b*) that this transport agency also is inserted asymmetrically with respect to the plane of the membrane: the structures which it exposes to the two sides of the membrane react differently with SH-reagents or proteases. It would be odd if an asymmetric structure were to have totally symmetric kinetic parameters, and indeed we have shown in the present paper that the Na^+,D-glucose does have asymmetric functional properties. This functional asymmetry was demonstrated here in four different aspects: (i) at $\Delta\psi \simeq 0$, transinhibitions and their release ("transstimulations") were observed for influx only, not for efflux (Fig. 1, Tables 1, 3, 6). (ii) Influx is more accelerated by a membrane potential difference (negative on the trans side) than by efflux (Tables 4 and 7). (iii) The estimated K_m for efflux appears to be larger than the K_m's determined for influx. (iv) Uptake and efflux rates may differ by a factor of 10 measured at equivalent, but mirrored conditions. Some of these aspects of asymmetry are interrelated. The scheme of Fig. 7 summarizes and relates to one another the individual features of asymmetry in transinhibitions and in membrane potential effects, as follows. We begin with equilibrium tracer exchange experiments, where Na^+ and D-glucose concentrations are equal on both sides of the membrane and where influx and efflux rates must be equal for thermodynamic reasons (Fig. 7, columns 4 and 5). Omitting D-glucose on the trans side has little influence on the flux rates from the cis side at the low D-glucose concentration assumed in this scheme (10 μM; columns 3 and 6). Reducing further trans-Na^+ from 100 to 0 mM does not affect efflux, but allows the influx velocity to be roughly 4 times larger (columns 2 and 7). Finally, the additional presence of a membrane potential difference, negative on the trans side, further stimulates *influx* by a factor of 3 to 4, but *efflux* by a

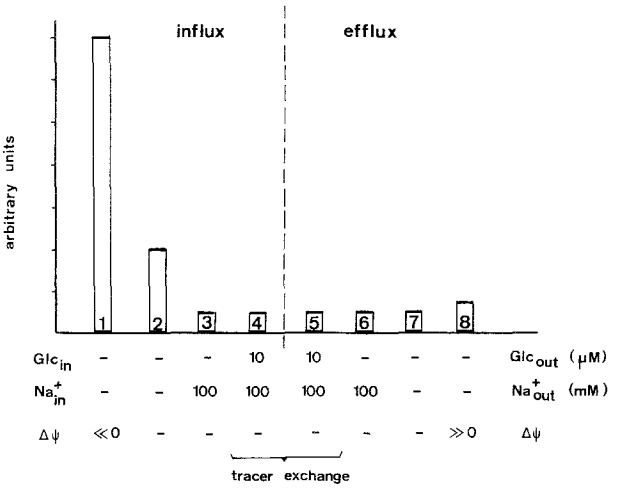


Fig. 7. Schematic representation of the size of influxes and effluxes under various experimental conditions aiming at correlating the very different values of influx and efflux in Fig. 4. The columns are drawn to an arbitrary scale. Experimental conditions are arranged symmetrically with respect to the dashed line. Trans concentrations of the substrates are indicated under the columns, cis concentrations are 100 mM Na^+ and 10 mM D-glucose. Columns 4+5: tracer exchange. Columns 3+6: zero trans D-glucose, equilibrated cis and trans Na^+ concentrations. Columns 2+7: Na^+ gradient (cis→trans). Columns 1+8: Na^+ gradient, plus a membrane potential difference, negative at the trans side, as in Fig. 4. *See text*

factor of 1.5 or less (columns 1 and 8). The large (*ca.* 10-fold) difference in influx and efflux rates in the experiment of Fig. 4 results, therefore, from the combined asymmetries in transinhibitions by substrates and in stimulation by $\Delta\psi$.

If the concentration of D-glucose in the experiment of Fig. 7 is increased above 0.1 mM, a smaller asymmetry between uptake and equivalent efflux rate is observed. This is mainly due to the different values in apparent K_m for D-glucose at the two sides of the membrane: the apparent K_m values for D-glucose influx are in the range 80 to 450 μM (for 100 to 200 mM Na^+_{out}), depending on the magnitude of the $\Delta\psi$ and on the concentration of Na^+_{in} (*see* Figs. 5, 6, 8, and also Kessler, Tannenbaum & Tannenbaum, 1978*b*). In comparison, the apparent K_m values in equivalent efflux experiments were estimated to be in the range 1 to 5 mM (Table 5). In other words, the influx rates saturate at smaller glucose concentrations than the corresponding efflux rates; the result is that high glucose concentrations stimulate efflux more than influx, and thus progressively reduce the difference between the two.

Transinhibition by Na^+_{in} has been described earlier by various authors (e.g., by Aronson and Sacktor, 1975, in renal brush border membrane vesicles). However, it has been occasionally misinterpreted as being just the trivial and obvious consequence of the reduction in the Na^+ gradient ($\Delta\mu_{Na^+}$ or $\Delta\tilde{\mu}_{Na^+}$) across the membrane, i.e., of the driving force for D-glucose accumulation. This thermodynamic interpretation is undoubtedly correct for relatively long incubations; also, thermodynamic and kinetic aspects are mutually related. There is little doubt, however, that the transinhibition of truly *initial* rates (Fig. 2) by Na^+_{in} demands a purely kinetic interpretation, the more so as this transinhibition is relieved by the simultaneous presence of D-$glucose_{in}$ (Fig. 1), and no comparable transinhibitions are observed in efflux experiments (Table 6). Pertinent kinetic models will be discussed in a later section.

The strong transinhibition of influx rates by Na^+_{in} is a most prominent and reproducible feature in other Na^+-coupled transport systems also (for L-ascorbate; Toggenburger et al., 1981; for L-leucine; Förster, 1979) and may well be present in all Na^+-coupled transport systems. Johnstone (1978) has likewise observed an intrinsic kinetic asymmetry of the Na^+, glycine cotransporter in Ehrlich cells.

II. A Transport Agency with the Kinetic Properties of a "Mobile Carrier": A "Gated Channel (or Pore)"

A stable structural and functional asymmetry rules out a "diffusive" or "rotative" mode of functioning and makes a "pore" or a "channel" the most probable mechanism(s). But this does not mean that transport is not accompanied, actually made possible, by the movement of a portion of the transport agency, "carrier" being defined (Läuger, 1980) as a transport agency whose substrate binding site(s) can be exposed to both sides of the membrane, but not simultaneously. Indeed, the Na^+,D-glucose cotransporter studied here does have the kinetic properties of a "mobile carrier," the most classical one being that of counterflow accumulation (*see* for example, Hopfer et al., 1973 and also Fig. 3*A* and *B* of the present paper). Also, the $\Delta\psi$ dependence of Na^+-dependent phlorizin binding to this cotransporter (Toggenburger et al., 1978, 1982) strongly indicates the motion of a portion of the cotransporter to be associated with its functioning. Finally, the fairly low turnover number (estimated to be approximately 20 sec^{-1}, Toggenburger et al., 1978) is more compatible with the classical ideas on "mobile carriers" than of freely open "channels" or "pores." A "gated channel (or pore)" is thus the most likely model for this Na^+,D-glucose cotransporter. For the purposes of the present paper we define as "gate" that portion of the channel or pore whose movement is associated with or related to the translocation of the substrate(s).

III. The "Gate" Bears a Negative Charge of 1 or 2 ($z=-1$ or $z=-2$)

This conclusion is based on, or is compatible with, the following experimental observations:

(i) $\Delta\psi$-dependence of transinhibition. Geck and Heinz (1976), Heinz and Geck (1977) and Turner (1981) have shown that the $\Delta\psi$-dependence of V_{max}/K_m or (what is equivalent) of fluxes (at very small substrate concentrations) can be used to determine whether the gate is electrically neutral ($z=0$) or whether it carries a negative charge ($z=-1$, thus becoming neutral after binding of Na^+). However, the reader may easily overlook that most of the equations and all the Figures presented by the authors were calculated for symmetrical transport systems only. In Appendix I we show that strongly asymmet-

ric transport systems with $z=-1$ may become virtually indistinguishable from $z=0$ systems; in other words, the test criteria given by these authors are not applicable to transport systems such as the one for Na^+-dependent glucose transport studied in the present paper. In the following (and in Appendices II and III) we present another test to determine the size of z which can be applied to transport systems with any degree of asymmetry, namely the $\Delta\psi$-dependence of transinhibitions.

The transinhibition studies presented above were mostly done at a membrane potential difference close to zero, which was established by having equal concentrations of the highly permeant anion NO_3^- (or of other highly permeant anions or cations) on both sides of the membrane. Static surface potential can be expected to be small, due to a rather high ionic strength of the media. The experimental set-up of the transinhibition studies presented above made sure that $\Delta\psi$ be minimally affected, if at all, by variations of the internal Na^+ concentrations or by any increase of the overall membrane permeability for Na^+ due, e.g., to the D-glucose-associated Na^+ influx.

A $\Delta\psi \ll 0$ (negative inside the vesicles) reduces the transinhibitions of the influx rates, which are brought about by either Na^+_{in} or D-glucose$_{in}$ (a representative experiment using D-glucose$_{in}$ is shown in Table 4). In principle, the decreased transinhibition might be brought about by $\Delta\psi$ via an effect on the static distribution of cations at the membrane surface, i.e., via a decreased effective Na^+ concentration near the Na^+ binding site(s) of the cotransporter. This mechanism can be ruled out, however, because our experiments were carried out at fairly high ionic strength, and it is even more unlikely in the example reported (Table 4), in which the uncharged co-substrate (D-glucose$_{in}$) was used to elicit transinhibition.

It seems more logical, therefore, to attribute the effect of $\Delta\psi$ on the transinhibition by Na^+_{in} and by Glc_{in} to an effect of $\Delta\psi$ on the orientation of the mobile portion of the "gated channel" or "mobile carrier" (i.e., of the "gate"). As it will be mentioned in the next section, it is quite possible that the Na^+,D-glucose stoichiometric ratio is equal to two. In discussing the effect of $\Delta\psi$ on the orientation of the "gate" we thus have to consider both possible stoichiometric ratios 1 and 2.

If the stoichiometry ratio is equal to 1, two models can be envisaged (*see* Appendices): (a) The gate bears no electric charge ($z=0$), when in the substrate-free form. Thus only the translocation (or reorientation) probabilities of the translocator-Na^+ (if mobile at all) and of the translocator-Na^+-Glc complexes will respond to $\Delta\psi$. Increasing $\Delta\psi$ (negative inside) will accelerate the inward translocation of these complexes and induce a new steady-state distribution of the translocator-substrate complexes, whereby an increased amount of translocator faces inward. Accordingly a *larger* fraction of translocator is sequestered by a transsubstrate inside in the form of little mobile binary complex, leading to an *increased* fractional inhibition. (b) If the gate bears a negative charge ($z=-1$), only the translocation probabilities of the substrate-free translocator and the translocator-Glc complex (if mobile at all) will respond to the membrane potential. A $\Delta\psi$ (negative inside) will accelerate the *outward* translocation of these complexes and induce a new steady-state distribution with less translocator facing inward. Accordingly the transinhibition by any transsubstrate will be *reduced*. Thus, as shown more rigorously in Appendix II, the $\Delta\psi$-dependence of transinhibition provides the possibility to distinguish between these two models.

If the stoichiometric ratio is $2\,Na^+$ transported per one D-glucose, the situation is obviously more complex, and three models have to be considered (*see* Appendix III): a) the gate bears no electric charge ($z=0$) in the substrate-free form: as in the case discussed in the previous paragraph, $\Delta\psi$ (negative inside) will produce an *increase* in transinhibition. (b) The gate bears two negative charges ($z=-2$): as in case *b* of the previous paragraph $\Delta\psi$ (negative inside) will produce a *decrease* in transinhibition. (c) The gate bears one negative charge ($z=-1$): in this case $\Delta\psi$ will recruit the form(s) of the cotransporter occupied by $2\,Na^+$ towards the "in" side, while "pushing" the Na^+-free form(s) towards the "out" side.

As shown in Appendix III this model also predicts a *decrease* of the extent of transinhibition by $\Delta\psi \ll 0$ (negative on the trans side).

In actual fact (Table 4) the transinhibition by D-glucose$_{in}$ follows the prediction of the models with $z=-1$ or $z=-2$ and is not compatible with model with $z=0$. We conclude that the "gate" of the translocator bears, in the substrate-free form, at least one negative charge.

Independently of the actual mechanism, the partial relief by $\Delta\psi$ of the transinhibition of in-

flux by Glc_{in}, is likely to have physiological significance: it reduces or prevents the "braking" of D-glucose entry into the cell by the substrate already present at the cytosolic side of the membrane.

(ii) Lack of effect of $\Delta\psi \ll 0$ on the rate of dissociation of phlorizin from the cotransporter. If the gate carries a negative charge of 1 and the Na^+/phlorizin stoichiometry of binding is one (which is made likely by the $\bar{n}$ coefficient being equal to one: Toggenburger et al. (1982), for the intestinal and Turner and Silverman (1981), for the renal cotransporter), the ternary complex (phlorizin-Na^+-cotransporter$^-$) is neutral and the rate of phlorizin dissociation should not be affected by $\Delta\psi \ll 0$. This is indeed the case for both the intestinal (Toggenburger et al., 1982) and the renal (Aronson, 1978) cotransporter.

One might object that an extremely asymmetrical (inwardly directed) cotransporter with $z=0$ (and thus with a net charge of $+1$ when bound to 1 Na^+ plus phlorizin) would likewise not respond to $\Delta\psi$ in its rate of dissociation of phlorizin, because a positively charged, phlorizin occupied "gate" cannot change its positioning in response to $\Delta\psi$ (negative inside) if it is already *totally* oriented towards the inner phase. However, such a possibility can be ruled out, because it implies also that the cotransporter in the presence of Na^+ (i.e., when carrying a positive charge of at least $+1$) should not respond to $\Delta\psi$ (negative inside) in *any* respect. But we do know that the cotransporter in the presence of Na^+ does respond to $\Delta\psi$ (negative inside) both as far as D-glucose transport (Murer and Hopfer, 1976) and as far as phlorizin binding (Toggenburger et al., 1978) are concerned.

(iii) The apparent K_m values for D-glucose uptake are independent of the pH in the 6.5–9.5 range; the apparent K_m at pH 5.5 is some fivefold larger (Toggenburger et al., 1978). It is quite possible, therefore, that the negative charge in the "gate" (or elsewhere in one of the sites binding either substrate) is a carboxylate group.[5]

(iv) This conclusion would also agree with recent observations by Weber and Semenza (1983, *in preparation*) that D-glucose transport is inhibited irreversibly by dicyclohexenylcarbodiimide.

(v) Finally, $\Delta\psi$ (negative inside) accelerates D-glucose influx into and increases phlorizin binding onto the (renal) brush border cotransporter even in the *absence of* Na^+ (Hilden & Sacktor, 1982). Phlorizin binds to the (intestinal) cotransporter in the neutral form (Toggenburger et al., 1978) and, of course, so does D-glucose. Thus, the mobile portion of the cotransporter must carry a net charge when unoccupied and when bound to an electrically neutral ligand.

IV. The Na^+, D-Glucose Flux Ratio

Hopfer and Groseclose (1980, from equilibrium tracer exchange in brush border vesicles) and Goldner, Schultz and Curran (1969, from initial uptake velocities across the brush border membrane of surviving small intestine) have suggested a Na^+/D-glucose flux ratio of one: the transport velocities were Michaelian functions of the external Na^+ concentrations. Both approaches have been recently criticized, however: the former authors have corrected for the large "leak" rates by *assuming* a Michaelian behavior (as a function of $\text{D-glucose}_{\text{in and out}}$) at each Na^+ concentration (Semenza, 1982; Toggenburger et al., 1982); the latter have not corrected the uptakes for the effect of decreased $\Delta\psi$ produced by the electrogenic Na^+,D-glucose flux itself (Kimmich & Randles, 1980).

Kaunitz et al. (1982) have recently reported that the Na^+_{out}-dependence of D-glucose uptake into small-intestinal brush border membrane vesicles is highly sigmoidal (the $\Delta\psi$ was clamped near zero with K^+/valinomycin; Na^+/H^+ exchange was minimized with amiloride and H^+ gradients with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). The $\bar{n}$ coefficient was approximately 2. We confirm this sigmoidicity in vesicles clamped with NO_3^- (Fig. 5*C*) and find little or no sigmoidicity in the presence of $\Delta\psi \ll 0$ (Fig. 5*E*). The sigmoidicity by itself does not prove a 2:1 Na^+/D-glucose stoichiometry ratio, particularly if accompanied by an opposite deviation from a Michaelian behavior in the D-glucose dependence (*see more below*). However, flux ratios of 2 have been found by measuring directly the sugar-dependent Na^+ flux under various conditions, particularly in the presence of inhibitors depressing

[5] Barnett, Ralph and Munday (1970) have suggested an electrically silent transesterification of position 2 of D-glucose with a carboxyl ester group occurring within the sugar binding site of the cotransporter. This should not be confused with the "gate"-COO^- which we are discussing as a part of the Na^+ binding site. We have no data pertinent to Barnett's mechanism.

the D-glucose independent Na^+ flux (Kimmich & Randles, 1980; Kaunitz et al., 1982; Kimmich et al., *personal communication*, 1982). Our own data on the D-glucose-dependent Na^+ uptake in vesicles with $\Delta\psi \ll 0$ and in the absence of inhibitors are too imprecise to allow a definite conclusion (Kessler et al., 1978*a*). Thus, at the moment of writing a Na^+/D-glucose flux ratio of 2 must be considered as a real possibility – although an effect of the experimental conditions (e.g., of $\Delta\psi$) on the flux ratio cannot be ruled out.

As mentioned above, the $\bar{n}$ coefficient of $\Delta\psi$-dependent phlorizin binding to the cotransporter is equal to one (Toggenburger et al., 1982). If this indicates a Na^+/phlorizin binding stoichiometry of one, it is conceivable that the Na^+-phlorizin-cotransporter complex may be nonfunctional for the very reason of not binding a second Na^+, if indeed only the complexes of the type Na_2^+-sugar-cotransporter have large translocation probabilities (*see* below). This mechanism, which is represented here as a mere suggestion only, will have to be investigated experimentally in the future.

V. The Translocation Probabilities of Partially Occupied Cotransporter Complexes

In view of the considerations in the preceding section, we will indicate as "fully occupied" cotransporter that form of it in which all binding sites for the substrates and cosubstrates are occupied by the respective ligands. The "fully occupied" cotransporter has, therefore, the composition D-glucose-Na_n^+-cotransporter, where n is equal to 1 or 2, depending on how large the stoichiometric flux ratio is (*see above*). Likewise, we will indicate as "partially occupied" cotransporter that form of it in which one or more (but not all) substrate and cosubstrate binding sites is (are) occupied by the respective ligand(s). In the "empty" cotransporter all binding sites for substrates and cosubstrates are unoccupied.

Heinz (1978) has pointed out that, to provide for an efficient energy conversion, the translocation probabilities of the partially occupied cotransporters must be low as compared with those of the fully occupied and of the empty forms. In actual fact, whatever evidence is available does indicate that the translocation probabilities of many, if not all, of the partially occupied cotransporters are indeed small, as follows.

At $\Delta\psi \simeq 0$, the transinhibitions by either Na_{in}^+ or D-glucose$_{in}$ (Fig. 1, Table 3) indicate that the in→out translocation probabilities of the respective partially occupied cotransporter forms are small.[6] As to the out→in probabilities, that of the D-glucose-cotransport complex must be very small (as deduced from the data of Fig. 5*A*, for example). This last conclusion was reached by Hopfer and Groseclose, too, under conditions of tracer equilibrium exchange (1980). The out→in probability of the Na_n^+-cotransporter form(s) are also likely to be very small (at least in the presence of amiloride: Kaunitz et al., 1982).

At $\Delta\psi \ll 0$ (negative inside) the transinhibitions by Na_{in}^+ (Fig. 6) and by D-glucose$_{in}$ (Table 4) again indicate small in→out translocation probabilities for the respective partially occupied cotransporter forms. As to the out→in translocation probability of the D-glucose-cotransporter complex it must be small at $\Delta\psi \ll 0$ also (Fig. 5*D* and *E*).

We will thus proceed under the likely assumption that all translocation probabilities of the partially occupied cotransporters are very small as compared to those of the empty and of the fully occupied forms.

[6] The transinhibition by either substrate is, in all likelihood, due to the sequestration of a part of the translocators in a binary complex with slow translocation probability. However, two further mechanism, which also might lead to transinhibition, shall be briefly discussed in the following: (i) Influx might be inhibited due to the interaction of an internal substrate with a regulatory site located at the cytoplasmic surface of the translocator. Such a mechanism seems, however, unlikely because of the complete reversal of the transinhibition by the cosubstrate. (ii) Na_{in}^+ might retard the release of D-glucose into the intravesicular space. Two groups have proposed models, which are based on steady-state rather than rapid-equilibrium kinetics, i.e., binding and dissociation rates are assumed *not* to be fast as compared with the translocation probabilities (Crane & Dorando, 1979, 1980; Hopfer & Groseclose, 1980). In a steady-state model, the release of inwardly translocated D-glucose from the carrier might be retarded by Na_{in}^+ to such an extent that the overall dissociation rate of D-glucose becomes much smaller than the outward translocation rate of the translocator-Na^+-Glc complex. As a consequence, D-glucose is carried back to the outside before being released into the intravesicular space. This might occur both in models with a compulsory binding sequence, in which D-glucose cannot be released prior to Na^+ (at the inside) or in affinity type models with random substrate binding. Although transinhibition by Na_{in}^+ might be readily interpreted in this way this mechanism seems not to provide an explanation for the complete reversion of the transinhibition by Glc_{in}. If Na_{in}^+ would solely retard the release of (labeled) D-glucose, the additional presence of unlabeled D-glucose in the internal compartment should not interfere with this transinhibition.

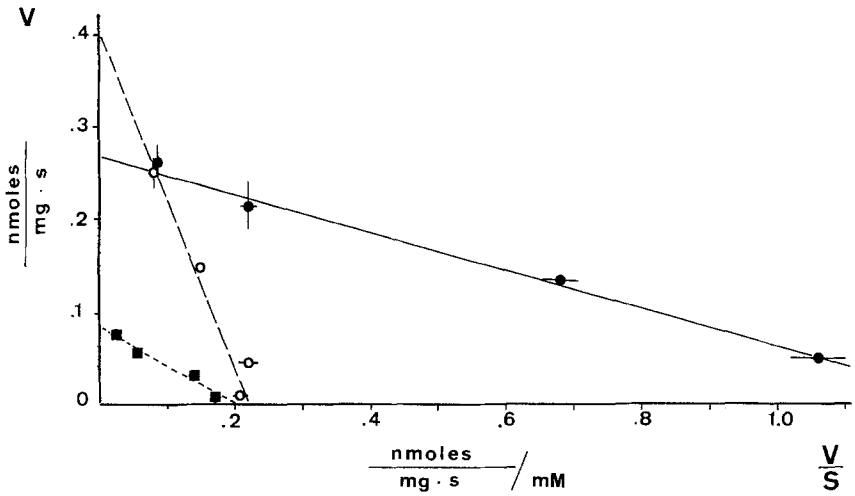


Fig. 8. Hofstee plots of D-glucose influx at $\Delta\psi \approx 0$, measured in Na^+-equilibrated vesicles (■----■), in the presence of a Na^+ gradient (●——●) or in vesicles equilibrated with both Na^+ and D-glucose (tracer-equilibrium exchange, o – – o). The membrane potential was short-circuited by the presence of 100 mM SCN^- on both sides of the membrane. The vesicles were pre-equilibrated in 200 mM mannitol, 10 mM HEPES/Tris, pH 7.5, 100 mM NaSCN or 100 mM KSCN, and 0 mM (●, ■) or 0.05, 0.2, 1 or 3 mM (o) D-glucose. To start the incubation, the vesicles were mixed with a solution to give the following final concentrations: Na^+ 100 mM, mannitol 200 mM, HEPES/Tris (pH 7.5) 10 mM, SCN^- 100 mM and D-glucose 0.05, 0.2, 1 or 3 mM. Incubation time 1 sec. The K_m values calculated by linear regression were: 200 ± 14 μM (Na^+ gradient), 450 ± 48 μM (Na^+ equilibrated), 1.62 ± 0.35 mM (tracer exchange). These "*apparent K_m-values*" were calculated only for the purpose of gross comparison. (*See* text)

This is not to say that the translocation probabilities of the parially occupied complexes always need be equal to zero. Indeed, Hilden and Sacktor (1982) have recently shown that $\Delta\Psi$ (negative inside the vesicles) accelerates the out → in transport of D-glucose by the renal cotransporter even in the absence of Na^+. Thus, at least the out → in translocation probability of the D-glucose loaded (renal) cotransporter(s) must be of detectable (albeit small) magnitude.

VI. The K_m values for D-Glucose Influx from Zero-Trans Glucose and from Equilibrium Tracer Exchange Experiments

The *apparent* K_m values for D-glucose in vesicles equilibrated in 100 mM Na^+ or in the presence of a NaSCN gradient (100 mM out, zero in) are, in the Michaelian concentration range (i.e., up to approximately 1 to 3 mM D-glucose), close to 0.1 mM (Toggenburger et al., 1978; Kessler et al., 1978*b*; *see also* Figs. 5, 6 and 8). Contrary to this, the apparent K_m values reported for equilibrium tracer exchange are 14.2 mM (Hopfer, 1977) or 2.4 mM (Hopfer & Groseclose, 1980). The difference does not disappear even if one corrects the values of $\Delta\psi \ll 0$ for the deviation from a Michaelian behavior.

Indeed, it would be surprising if these three different experimental conditions were to yield identical apparent K_m values, since these K_m's are different kinetic quantities: the equilibrium tracer exchange rates, for example, are described by the full equation of one of the Bi Bi (or Ter Ter) mechanisms, for the special case in which $[Glc_{out}]$ (substrate A or B) is the same as $[Glc_{in}]$ (product P or Q), and $[Na^+_{out}]$ (substrate B or A) is the same as $[Na^+_{in}]$ (product Q or P). That is, the K_m^{app} for the velocity of glucose tracer exchange is dictated both by the varying $[Glc_{out}]$ and by the transeffect of the identically varying $[Glc_{in}]$. These transeffects, as shown above, depend also on the Na^+ inside the vesicles.

Figure 8 tries to illustrate this point. Since at the glucose concentrations used (up to 3 mM) little or no deviation was observed from a Michaelian behavior, for the comparative purposes of this experiment we will neglect the corrections which should otherwise have to be introduced in the *apparent* K_m values, in order to account either for the parallel operation of a second low-affinity cotransporter or for the kinetic mechanism being perhaps a Random Non Equilibrium (*see more below*). Thus, the K_m values in this paragraph should be regarded as operational parameters only.

In all the experiments of Fig. 8 the membrane potential difference was clamped near

zero by high concentrations of a permeant anion (SCN) at both sides of the membrane. The K_m for D-glucose influx in the presence of an initial Na^+ gradient (100 mM out, 0 in) was 0.2 mM (●——●). In the absence of a Na^+ gradient (100 mM both in and out) the K_m was slightly larger (0.45 mM, ■- - -■), with influx rates strongly reduced at all $glucose_{out}$ concentrations. Let us now consider the curve for tracer exchange equilibration (○——○), again at 100 mM Na^+, both in and out: very low internal D-glucose concentrations (far below saturation) do not relieve the transinhibition by Na^+_{in}; thus, tracer exchange fluxes at low D-glucose concentrations should approach the rates of influx observed at high $Na^+_{in} = Na^+_{out}$. In actual fact, the two regression lines (○——○ and ■- - -■) do intersect close to the x-axis. As the internal and external D-glucose concentrations are increased simultaneously, two effects add to one another: (i) the influx tends to saturate as $[Glc]_{out}$ exceeds 0.2 to 0.4 mM, and at the same time (ii) the increasing $[Glc]_{in}$ releases the transinhibition exerted by Na^+_{in} (as in Fig. 1, columns *F* through *I*). The net result of this transstimulation is that high $Glc_{in} = Glc_{out}$ concentrations yield much higher rates than in vesicles equilibrated in Na^+ alone (■), larger even than those observed in the presence of a Na^+ (out > in) gradient (●). (This latter finding is noteworthy in itself, because it indicates that the outward translocation, or reorientation probability of the fully occupied translocator is larger than that of the empty form.)

Similar observations were made previously back in the 1950's on other systems by Heinz and Durbin (1957) and by Heinz and Walsh (1958). As a result, the apparent K_m for D-glucose in the system investigated in the present paper is shifted from about 200 μM in net uptake conditions (●——●) to 1.6 mM in tracer equilibrium exchange conditions (○——○).

Hopfer (1977) maintains that equilibrium tracer exchange alone yields K_m values reflecting "true" characteristics of the transporter: in all other experimental set-ups the D-glucose-associated Na^+ influx would lead to a collapse (or even inversion) of $\Delta\psi$, which results in the "braking" of D-glucose influx and thus simulate saturation at $[Glc_{out}]$ as low as 0.1 to 0.4 mM. However, this explanation can be safely ruled out from the evidence presented in Fig. 8, where again a roughly 10-fold difference in apparent K_m values was found, although the membrane potential was clamped near zero. Additional evidence that our determinations of apparent K_m values in the presence of a dissipating $\Delta\tilde{\mu}_{Na^+}$ are not grossly distorted by the D-glucose-associated Na^+ influx or by the initiating collapse of $\Delta\psi$ has been presented elsewhere (Kessler et al., 1978*b*) (*See also* under Results Section III.B).

VII. A Gated Channel (or Pore) with Preferred Inward Orientation

In a recent paper (Toggenburger et al., 1982) we indicated that at $\Delta\psi \simeq 0$ the substrate binding site of the Na^+, D-glucose cotransporter is not freely accessible to the substrate from the outer, luminal, side of the membrane. The effect of $\Delta\psi$ (negative inside the vesicles) merely consists in reorienting the mobile part of the cotransporter (i.e., the negatively charged "gate," *see* Section III above) towards the outside, thereby allowing — after the binding of Na^+ — the binding of phlorizin to the substrate site. Deoxycholate-disrupted membranes (naturally, at $\Delta\psi = 0$) bind phlorizin with the same K_d value as vesicles in the presence of $\Delta\psi \ll 0$.

It is remarkable that this same model — of a negatively charged "gated channel" having preferred inward orientation at $\Delta\psi \simeq 0$ — can satisfactorily explain the transinhibition by substrates, their asymmetry, and the very small response of *efflux* rates to $\Delta\psi \ll 0$ (negative at the trans side). The only addition to the model which we make here is (as discussed in Section V): that the translocation probabilities of the partially occupied cotransporter forms from the inside ($''$) to the outside ($'$) are negligibly small in comparison with the corresponding translocation probabilities of the empty and of the fully occupied forms.

In fact, if the substrate-free cotransporter at $\Delta\psi \simeq 0$ has a predominantly inward orientation (i.e., if $p'_o > p''_o$), sequestering of much cotransporter into slowly or nontranslocating partial complexes at the $''$ side will result in a strong transinhibition of influx; (conversion of the partial complexes into the more mobile, fully occupied form would relieve the transinhibition). On the other hand, sequestering much cotransporter at the outer ($'$) side may be difficult (due to the predominant orientation toward the inside); the transinhibition (from the outside) of efflux, if any, may go undetected.

Furthermore, if the empty form of the cotransporter (or, rather, its mobile, negatively charged "gate") already at $\Delta\psi \simeq 0$ has a predo-

minantly inward orientation, a $\Delta\psi \gg 0$ (positive inside the vesicles) can affect its orientation only little. (The same can be said for the binary complex with glucose, if formed at all.) Thus, the model of an asymmetric cotransporter with a predominantly inward orientation predicts also little or no response of the efflux rates to $\Delta\psi$ (negative at the trans side, i.e., at the outside of the vesicles). This is actually observed experimentally (Table 7).

This model of "preferred inward orientation" (at $\Delta\psi \simeq 0$) is essentially based on the assumption of an asymmetry in the translocation probabilities (i.e., $p'_o > p''_o$). For thermodynamic reasons, this asymmetry must be compensated for by other asymmetries in other translocations probabilities and/or in the dissociation constants of the binary, ternary (and quaternary, if the case) complexes at both sides of the membrane. For example, in the model with a Na^+:D-glucose stoichiometry of *one* the following equations must be satisfied at $\Delta\psi \simeq 0$ (Geck & Heinz, 1976):

$$K'_G \frac{p'_o}{p'_G} = K''_G \frac{p''_o}{p''_G} \quad \text{(I)}$$

$$K'_N \frac{p'_o}{p'_N} = K''_N \frac{p''_o}{p''_N} \quad \text{(II)}$$

$$r' \frac{p'_o p'_{GN}}{p'_G p'_N} = r'' \frac{p''_o p''_{GN}}{p''_G p''_N} \quad \text{(III)}$$

G: glucose; N; Na^+; p: translocation probabilities: p_o: of the unloaded translocator, p_G: of the translocator-glucose complex, p_N: of the translocator-Na^+ complex, p_{GN}: of the tertiary complex; ′: 'outside' or 'inward translocation'; ″: "inside" or "outward translocation"; K: dissociation constants: $K_N = (Na^+)\cdot(C)/(C-Na^+)$; $K_G = (G)\cdot(C)/(C-G)$; $K_G^N = (C-Na^+)\cdot(G)/(CGNa^+)$; $K_N^G = (C-G)\cdot(Na^+)/(CGNa^+)$; C: cotransporter; r: factor by which the affinity is increased by the cosubstrate, i.e.: $r = \frac{K_G}{K_G^N} = \frac{K_N}{K_N^G}$.

In addition, it can be shown easily that, if the four translocation probabilities of the *binary* complexes are negligibly small as compared with those of the substrate-unloaded and of the Na^+,D-glucose-loaded translocator the following relations must hold true at $\Delta\psi \simeq 0$:

$$\frac{p''_o}{p'_o} \cdot \frac{p'_{GN}}{p''_{GN}} = \frac{K_G^{N'} K'_N}{K_G^{N''} K''_N} = \frac{K_N^{G'} K'_G}{K_N^{G''} K''_G}.$$

Similar equations can be derived for the model with a 2:1 Na^+, D-glucose stoichiometry.

The transeffect experiments allow one more conclusion to be drawn. The fact that both substrates, Na^+_{in} and Glc_{in} can elicit transinhibition indicates that each substrate can bind to the translocator in the absence of the other substrate. This virtually rules out any mechanism with a strictly compulsory binding sequence in the inward-facing state.

Summing up the considerations above, the small-intestinal Na^+,D-glucose cotransporter, in addition to being structurally asymmetric (with respect to the plane of the membrane) – which rules out diffusive and rotating modes of operation and makes a gated channel or pore, or a snip snap mechanism likely – is *functionally* asymmetric also. At $\Delta\psi \simeq 0$ and in the absence of substrates its substrates' binding sites have a predominantly inward orientation (or accessibility); it has very small outward translocation probabilities of partially occupied forms; it has a negative charge of at least 1 (presumably, a carboxylate group) in the mobile part (the "gate") responding to the membrane potential difference. The translocation probability of the fully occupied cotransporter is probably larger in the out→in than in the in→out direction. In order to restrict further the number of possible kinetic models, a partial Cleland kinetic analysis was carried out.

VIII. Ruling Out (Iso) Ping Pong Mechanisms and Making One of the (Iso) Random Mechanisms Likely

If the translocation probabilities of the binary complexes are negligibly small and the flux ratio is one, the possible kinetic mechanisms left for consideration restrict to those belonging to the two groups called in enzymology (Iso) Ping Pong Bi Bi and (Iso) Random Bi Bi, (the (Iso) Ordered Bi Bi mechanisms being special cases for the latter), A and P (or B and Q) being Na^+_{out} and Na^+_{in}, respectively, and B and Q (or A and P) being D-$glucose_{out}$ and D-$glucose_{in}$, respectively. Likewise, if the transition probabilities of the binary and tertiary com-

plexes are negligibly small and the Na^+/D-glucose flux ratio is two, the kinetic mechanisms to be considered are the (Iso) Ping Pong Ter Ter and the (Iso) Random Ter Ter.

Since the kinetic properties are those of a "mobile carrier," a portion of the "channel" or "pore" must move in conjunction with substrate transport. That is, the kinetic mechanism must include isomerization steps and belong to the "Iso" groups.

In principle, a complete Cleland's kinetic analysis[7] could discriminate among many of the Bi Bi (or of the Ter Ter) mechanisms. In practice, however, such an analysis is impossible in this system, since measurements of efflux are subjected to considerable experimental error and those of Na^+ influx are likewise inaccurate, due to the very large D-glucose-independent Na^+ influx in the absence of inhibitors. Finally, the uncertainties still present as to the flux ratio, as to the transition probabilities of the partially occupied forms of the cotransporter – and even as to whether one or two types of cotransporters act in parallel in this membrane – further increase the difficulties of sorting out the proper kinetic mechanisms. However, some discrimination among groups of mechanisms can be achieved.

We will first consider the following hypothetical mode of operation. It is conceivable (although to our knowledge never been proposed) that a cotransporter binds one substrate only at the time, and never both substrates simultaneously, be they at the same or at opposite sides of the membrane. Flux coupling would take place as follows: the one substrate binds at its own binding site, which is exposed at side ′ of the membrane; be transferred to or otherwise released into side ″; in this "flipped over" form the cotransporter would now expose to side ′ the binding site of the other substrate, which would now, in its turn, be transferred to side ″, etc. This mechanism corresponds in enzymology to an Iso Ping Pong Bi Bi mechanism, in which EA and FB isomerize to FP and EQ, respectively. (Similar mechanisms can be formulated if three or more substrates are involved.)

These Iso Ping Pong mechanisms can be ruled out on the basis of various observations. First of all, in all (Iso) Ping Pong Bi Bi mechanisms varying the concentrations of the one substrate affect the apparent V_{max} and K_m of the other substrate by the *same factor*; in particular, increasing concentrations of the one substrate make the K_m for the other increase. Now, whether one dissects the data of Fig. 5 into more than one Michaelian function or forces them into a single one, it is clear that increasing Na^+_{out} never leads to an increase of the K_m for D-glucose$_{out}$.

Secondly, the apparently noncompetitive nature of the inhibition by Na^+_{in} (Fig. 6) is also hardly compatible with (Iso) Ping Pong Bi Bi mechanisms (*cf.* Segel, 1975).

Thirdly, experiments of tracer equilibrium exchange make all (Iso) Ping Pong mechanisms – whether Bi Bi or Ter Ter or Quater Quater – most unlikely. In fact, as mentioned above, the key feature of Ping Pong mechanisms is that the "fully occupied" form of the cotransporter is never generated – or, to put it another way, that the binding of the one or more substrates hampers the binding of the remaining ones, or at least of the last substrate still left to bind. As a consequence, the transport velocity at high, saturating $(Na^+)_{in,out}$ and $(Glc)_{in,out}$ should be smaller, or at least not larger than that observed under other conditions. In actual fact (Fig. 8) it seems to be larger.

It seems, therefore, that Ping Pong mechanisms can be ruled out safely. We are thus left with the Iso Random Bi Bi (or Ter Ter) family of mechanisms with or without Rapid Equilibrium. The double deviation from Michaelian behavior observed at $\Delta\psi \simeq 0$ (i.e., the sigmoidicity with Na^+_{out}, Fig. 5, *A–C*) reminds one of

[7] Hopfer (1977) and Hopfer and Groseclose (1980) have attempted to draw conclusions on the kinetic mechanism of the Na^+,D-glucose cotransporter from equilibrium exchange rates alone. This experimental set-up, in addition to being subjected to larger experimental errors and requiring large corrections for the "leak" rates (which may explain the different K_m values for D-glucose$_{out}$ reported in their two papers), does not allow to distinguish (as claimed by Hopfer and Groseclose, 1980) between Random Bi Bi and Ordered Bi Bi: in fact, the only phenomenon used by the former authors to this purpose (inhibition of exchange rates by high concentrations of the cosubstrate) can be found in both mechanisms (*see* Hopfer & Liedtke, 1981, Figs. 2*a* and 3). For additional discussion, *see* Semenza, 1982, *and Toggenburger et al.*, 1982). *Naturally, this* is not to say that equilibrium exchange experiments cannot produce valuable information in other respects. Finally, the growing evidence for the existence of two Na^+, D-glucose cotransporters acting in parallel, and for a stoichiometry larger than 1 (*see* text) advise one not to use Hopfer's equations (Hopfer, 1977; Hopfer & Liedtke, 1981), which were derived assuming the existence of a single Bi Bi system.

the kinetics which can be observed in Non-Rapid Equilibrium Random Bi Bi (Ferdinand, 1966) and indeed such a kinetic mechanism has been proposed for the Na^+,D-glucose cotransporter studied here (Crane & Dorando, 1979, 1980, 1982). At low Glc_{out} the prevailing route is that of Na^+_{out} first, D-glucose$_{out}$ second; as Glc_{out} increases, the outer route, i.e., D-glucose$_{out}$ first, Na^+_{out} second ceases from being negligibly small. Also, it is possible that within the family of Iso Random (including Iso Ordered) mechanisms some substrates may follow preferentially one kinetic route, and others another; and that some experimental conditions may favor one kinetic route over the others. One major factor is undoubtedly $\Delta\psi$, which clearly must accelerate some steps and brake others: this may result in a change of the prevailing kinetic route or type within the family of related mechanisms (e.g., produce an Iso Ordered from an Iso Random, or vice versa, and/or change the mechanism from a Rapid Equilibrium to a Non-Rapid Equilibrium, or vice versa).

On the basis of Cleland-type kinetic analysis, at least three major mechanisms have been put forward: a Non-Rapid Equilibrium Random Bi Bi (Crane & Dorando, 1979, 1980, 1982), a Non-Rapid Equilibrium Ordered Bi Bi (having "some degree of randomness," however) with glide symmetry (Hopfer & Groseclose, 1980) and a Rapid Equilibrium Ordered with Na^+ first, glucose second, for two carriers working in parallel having Na^+/glucose flux ratios of 3 and 1, respectively (Kaunitz & Wright, 1983[4]). The experimental basis of the model "Ordered Bi Bi with glide symmetry" is weak[7], although the model itself is physically plausible (*see also* Semenza, 1982; Toggenburger et al., 1982). As mentioned in the previous paragraph it is a special case of the more general models of Crane and Dorando, and of Kaunitz and Wright.

In a very recent review Crane[8] has pointed out that a careful perusal of all the published data in the field strongly indicates that the discrepancies among the various reports can all be reconciled with one another if *two* Na^+-D-glucose cotransporters occur in the small-intestinal brush border membrane: some experimental conditions would lead to results stemming essentially from the one cotransporter, other conditions to results stemming from the other. Turner and Moran (1982*a–c*) have produced conclusive evidence, based on physical separation, of the existence in the kidney of *two* Na^+-D-glucose cotransporters: (i) the one, prevailing in the *outer cortex* (and corresponding to the "classical" renal Na^+, D-glucose cotransporter), having low affinity for D-glucose (K_m approx. 6 mM); high sensitivity to phlorizin (K_d approx. 0.18 μM); a 1:1 Na^+/glucose stoichiometry; and being little inhibited by D-galactose; (ii) the other cotransporter, prevailing in the *outer medulla*, has high affinity for D-glucose (K_m approx. 0.35 mM), low sensitivity to phlorizin ($K_i \gg 1$ μM), a 2:1 Na^+/glucose stoichiometry; and being inhibited by D-galactose. Indeed, the existence of two cotransporters in the small intestinal brush border membrane has been suggested by Honegger and Semenza (1973) and by Kaunitz and Wright[4] on the basis of kinetic arguments and by Honegger and Gershon (1974) on the basis of a partial physical separation. Their properties correspond quite closely to those reported by Turner and Moran for the kidney, the major difference being that cotransporter (ii) seems to be more represented in the small intestine than in the kidney (minor discrepancies still remaining may be related to species differences). We thus share Crane's view[8]. (For older observations, also related to human pathophysiology, *see* the references quoted by Honegger and Semenza (1973) and by Crane[8]).

The very likely existence of two cotransporters operating in parallel and the experimental difficulties of the system severely limit the amount of information which a Cleland type of analysis can provide. Since, however, each of the models mentioned above – indeed each of the models in the Iso Random families – is compatible with the mechanistic model to be proposed below, we will limit ourselves to the following considerations. They are based on the preferred order of binding of phlorizin and may hold true for "poor substrates" (if phlorizin is to be regarded as an "infinetely slowly" transported substrate). The argument goes as follows: optimal binding of this fully-competitive inhibitor to the cotransporters of the small-intestinal brush border requires the simultaneous presence of Na^+_{out} and $\Delta\psi$ (negative inside) (Tannenbaum et al., 1977; Toggenburger et al., 1978. This holds true for the renal cotransporter also: Aronson, 1978).

Let us compare with one another the velocities of phlorizin *binding* and of D-glucose *trans-*

[8] Crane, R.K. Diversity in Na^+ gradient-coupled transport. (*submitted*)

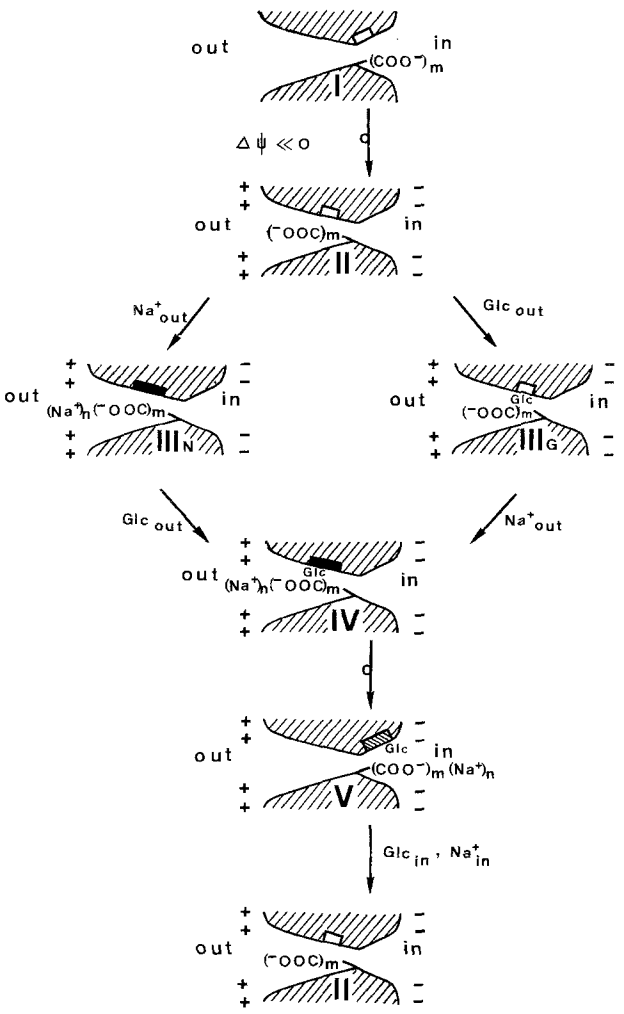


Fig. 9. A likely mode of operation of the small-intestinal Na^+, D-glucose cotransporter: An asymmetric gated channel (or pore) responsive to $\Delta\psi$. The D-glucose (and phlorizin) binding site is indicated by: ▭ (low affinity), or ▬ (high affinity), or ▨ (indefinite affinity). For a detailed discussion, *see* text. *m*: it is not known yet whether *m* is 1 or 2. *n*: 2 (if the Na^+/D-glucose flux ratio is 2) or 1 (if the flux ratio is 1)

port (out→in) under identical conditions (in particular, in the presence of an initial $\Delta\tilde{\mu}_{Na^+}$; Toggenburger et al., 1982): one mg of vesicle protein binds approximately 50 pmol of phlorizin · sec^{-1} (extrapolated at saturating phlorizin concentrations) or transports 520–580 pmol of D-glucose · sec^{-1} (at high D-glucose concentrations). Thus, the velocity of D-glucose transport is much larger than that of phlorizin binding.

Now, in the specific $\Delta\tilde{\mu}_{Na}$-dependent binding of phlorizin to the carrier one can distinguish between two (groups of) events: (i) the changes (positioning and/or reactivity) triggered in the carrier by $\Delta\psi$ and Na^+_{out}; and (ii) phlorizin binding proper (+possible alterations induced in the carrier by this very binding.) Since the requirements for optimal phlorizin binding are identical with those for optimal D-glucose transport, the events of group (i) must be identical with a part of the series of events leading to Na^+, D-glucose cotransport; each of these events must be as fast as, or faster than, the overall Na^+,D-glucose cotransport. That is, form II (Fig. 9), when exposed simultaneously to Na^+_{out} and $phlorizin_{out}$ must bind the former at least 10 times faster. Form III N, then, is the form of the cotransporter which binds phlorizin optimally – a conclusion for which other evidence also is available (Toggenburger et al., 1982). This is tantamount to say that $\Delta\psi$, Na^+_{out}-dependent phlorizin binding follows a Preferred (although not necessarily compulsory) Ordered Bi Bi sequence, Na^+_{out} binding first. (Similar conclusions were reached by Turner and Silverman (1981) for phlorizin binding to kidney cortex membranes on the basis of different kinetic arguments; they called this mechanism "Random Bi Bi" with large differences in the K values for phlorizin and for external Na^+).

The Preferred Ordered sequence of phlorizin binding (and possibly of slowly transported substrates) is, of course, a special case of other, more general (e.g., of Random) mechanisms. It may also mimick – if two Na^+ are transported per D-glucose – the first two steps in the transport of 2 Na^+ along with 1 D-glucose. If the $\bar{n}$ coefficient for Na^+-dependence of phlorizin binding (which is equal to 1; Toggenburger et al., 1982) means that only one Na^+ is bound per phlorizin to the cotransporter, it is conceivable that the Na^+-phlorizin cotransporter cannot bind (for whatever still unknown reason) the second Na^+. That is, the "partially occupied" cotransporter would be, in the case of phlorizin, incapable of reacting further (save for dissociating) and would have (like other partially occupied cotransporter forms) very small translocation probabilities. Whether this is the reason for phlorizin not being transported, only future work will decide.

IX. A Plausible Mechanistic Model

In an important paper Läuger (1980) has pointed out that the kinetic and thermodynamic properties of "mobile carriers" are shown by those transport agencies which can expose the substrate binding site at both sides of the membrane, but *not simultaneously* (*see also* Patlak,

1957). In the case of the Na^+-D-glucose cotransporter it is clear that it can exist in two major families of forms, the one prevailing at $\Delta\psi \simeq 0$ (e.g. Form I, Fig. 9) and the other one (the "energized" forms) prevailing at $\Delta\psi \ll 0$ (negative at the in-side) (e.g., Forms II through V). Each of the two (families of) forms operate as a "mobile carrier" in Läuger's sense, but with different energetic profiles (and thus with at least some of the individual rate constants different) in the "translocation" events. Thermodynamically, the difference between "nonenergized" and "energized" forms is apparent in the capability of the latter (but absent in the former) of producing accumulation of substrates starting from initial conditions $[Na^+]_{in} = [Na^+]_{out}$ and $[Glc]_{in} = [Glc]_{out}$.

Even at the cost of stating the obvious we want to emphasize that the displacement of the substrate binding sites depicted in Fig. 9 need not imply their actual movement. Indeed a change in their accessibility from either side of the membrane (which could be related with protein fluctuation) is a perfectly logical and likely possibility (Läuger, 1980). Accordingly, "translocation" and "change in accessibility" are used for the purposes of this paper, as synonymes.

In the following we will discuss a plausible mode of operation mainly of the "energized" Na^+,D-glucose-cotransporter (i.e., in the presence of a $\Delta\psi \ll 0$, negative inside) (*see* Fig. 9). In so doing, we will combine all the features which have been enlisted and discussed in the previous sections.

In addition, we will add two more features which, although plausible, do not have a direct experimental support: (i) The "gate" is (a part of) the Na^+ binding site (the alkali cations vastly prefer 0 over *N* or *S* as the ligand) and, (ii), the "orientation" (or "accessibility"), as well as the affinity state of the substrate binding sites must change in a concerted and identical fashion. (That is, no significant amount of the cotransporter ever has the binding site of one substrate at low and the other at high affinity; or one binding site facing one side of the membrane and the other facing the other: these conformational states would be a part of an Iso Ping Pong mechanism and/or be conductive to the transport of one substrate alone, both of which possibilities are unlikely; *see* previous sections.) The "gated channel (or pore)" of the Na^+,D-glucose cotransporter is asymmetric with respect to the plane of the membrane. In particular, at $\Delta\psi \simeq 0$ and at low substrate concentrations (Fig. 9, *I*) all binding sites have a spontaneously inwardly directed orientation. In Form *I*, then Na^+_{in} and D-glucose$_{in}$ each have access to the respective binding site and trap the cotransporter as a slowly (or non-) translocating binary complex. This is the basis of the transinhibitions by Na^+ or by D-glucose from the "in" side (Fig. 1, Tables 1 and 3) and for their lack from the "out"-side (Table 6), as discussed in a previous section.

In the presence of a $\Delta\psi \ll 0$ (negative inside) the "gate" (and with it, by an ill-understood mechanism, the D-glucose binding site also) "moves" towards the outside (or is made otherwise accessible), from the "out"-side (Form *II*). Na^+_{out} can now bind to the gate (Form *IIIN*), which increases the affinity of the sugar binding site. The mechanism whereby the binding of Na^+ to its site affects the affinity (or, rather, the K_m; *see* Fig. 5) of the sugar binding site for its own substrate is still a matter of speculation. However, the drastic change in the coulombic field near the COO^--gate upon Na^+ binding and the (even if minute) reorientation of the "gate" under $\Delta\psi$ provide ample possibilities.

Alternatively (but less likely with poor sugar substrates, *see* above), D-glucose as the first may bind to its outwardly exposed binding site (Form *II*), leading to Form *IIIG*. Neither Form *IIIN* nor Form *IIIG* crosses past the membrane (or, rather, expose and liberate the lone bound substrate to the in-side). We do not know why the binary complexes *IIIN* and *IIIG* have low or nil translocation probabilities. However, this is a prerequisite for efficient flux coupling (Heinz, 1978). Also in the case of cotransporter(s) binding $2\,Na^+$ and 1 D-glucose, our model is compatible with any order of substrate binding.

From either Form *IIIN* or *IIIG* the "fully occupied" Form *IV* is generated, in which the gate charge is neutralized by Na^+, or even made positive (if the gate carries a charge of -1 and can bind $2\,Na^+$). This makes the gate snap back (Form *V*) in the "spontaneous" inwardly directed positioning (as in Form *I*), the more so if it is now positively charged. In Form *V* the binding sites (and thus the substrates) are exposed towards the inside, where they are liberated (we do not know in which order). Reappearance of the negative charge in the gate makes it again respond to $\Delta\psi$ and snap towards the outside (Form *II*). The combined effect of Na^+ on/off and of $\Delta\psi$ would

thus be that of modulating the function of the sugar binding site and of changing the energy profile of the channel so as to favor the exposure of the sugar binding site towards one or the other side of the membrane. An important condition for this "shoveling" mechanism to operate is that, as suggested above, the translocations (or changes in accessibility) of *all* substrate binding sites must take place in a concerted, identical fashion.

The model provides a unifying and realistic basis to at least three of the major characteristics of the small-intestinal Na^+, D-glucose cotransporter: the Na^+,($\Delta\psi$)-dependent phlorizin binding (Form *IIIN* only binds phlorizin optimally, as discussed in detail by Toggenburger et al., 1982); the flux coupling between Na^+ and D-glucose (Form *IV* mainly, the fully occupied complex, liberates the substrates at the trans side), and perhaps most important of all, the accumulation of an unloaded compound (D-glucose) being driven by $\Delta\psi$, with the proviso that Na^+ is also present. It is an attempt to explain in molecular terms the phenomenon of flux coupling in secondary active transport which was put forward for the first time by Crane, Miller and Bihler in 1961 (*see* Crane, 1977, for a comprehensive review).

It seems very likely that the mechanism depicted in Fig. 9 be common to the kindred kidney cortex Na^+, D-glucose cotransporters, the mobile part of which also probably bears, in the free form, a negative charge of 1 (Aronson, 1978). (For a discussion of the quantitative differences between these two transport systems, *see* Toggenburger et al., 1982.) Also a recent, independent observation of Hilden and Sacktor (1982) on the kidney cotransporter(s) fits beautifully in the mechanistic model of Fig. 9. In cortex membrane vesicles in the total absence of Na^+, $\Delta\Psi$ (negative inside) promotes phlorizin binding and a (slow) uptake of D-glucose, the K_d- and K_m-values being rather large. This is exactly what the transition between Form I and Form II (Fig. 9) predicts: a $\Delta\Psi$ (negative inside) leads to exposure of the substrates' binding sites towards the outside, leaving them, however, in the low-affinity form. In addition, Form II (when combined with D-glucose) must have a small but detectable out $\rightarrow$ in translocation probability. In addition, many of the known properties of Na^+- or H^+-coupled symport systems indicate that the "shoveling mechanism" of Fig. 9 may have a more general validity.

Needless to say, the "shoveling" model of Fig. 9 is a minimum mechanism. In addition to completion, it may well need revision in some steps, but it is remarkable that it can explain most, if not all, the known characteristics of the cotransporter. We still ignore the role, if any, of the thiol(s) which have been found to occur in the cytosolic and/or hydrophobic surface of the cotransporter (Klip et al., 1979*b*, 1980*a*). Equally unknown is the role of other thiols or amino groups which are essential for its functioning (or for keeping the correct conformation) (Biber, Weber & Semenza, 1983). Whether some of these thiols play the same role in this system, which Robillard and Konings (1982) have suggested in electrogenic H^+-symport systems, cannot be ruled out.

Finally, in the model of Fig. 9 we do not state whether the Na^+ binding site(s) are located in the same or in another subunit as the sugar binding site. The latter possibility is suggested by the recent work of Guffanti et al. (1981) and of Zilberstein et al. (1982) on the genetics of Na^+-dependent transport systems in procaryontes.

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References

Aronson, P.S. 1978. Energy-dependence of phlorizin binding to isolated renal microvillus membranes. *J. Membrane Biol.* **42**:81–98

Aronson, P.S., Sacktor, B. 1975. The Na^+ gradient-dependent transport of D-glucose in renal brush border membranes. *J. Biol. Chem.* **250**:6032–6039

Barnett, J.E.G., Ralph, A., Munday, K.A. 1970. Structural requirements for active intestinal transport. The nature of the carrier-sugar bonding at C-2 and the ring oxygens of the sugar. *Biochem. J.* **118**:843–850

Biber, J., Weber, J., Semenza, G. 1983. Chemical modification of the small intestinal Na^+, D-glucose cotransporter: Sugar transport and phlorizin binding inactivation after blocking sulfhydryl or amino groups. *Biochim. Biophys. Acta* **728**:429–327

Blobel, G., Dobberstein, B. 1975*a*. Transfer of protein across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell. Biol.* **67**:835–851

Blobel, G., Dobberstein, B. 1975*b*. Transfer of proteins. II. Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* **67**:852–862

Cabantchik, Z.I., Knauf, P.A., Rothstein, A. 1978. The anion transport system of the red blood cell: The role of membrane protein evaluated by the use of "probes." *Biochim. Biophys. Acta* **515**:239–302

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

Crane, R.K., Dorando, F.C. 1979. On the mechanism of Na^+-dependent glucose transport. *In:* Function and Molecular Aspects of Biomembrane Transport. E. Quagliariello et al., editors. pp. 271–278. Elsevier, Amsterdam

Crane, R.K., Dorando, F.C. 1980. On the mechanism of Na^+-dependent glucose transport. *Ann. N.Y. Acad. Sci.* **339**:46–52

Crane, R.K., Dorando, F.C. 1982. The kinetics and mechanism of Na^+ gradient-coupled glucose transport. *In:* Membranes and Transport. A. Martonosi, editor. Vol. 2, pp. 153–160. Plenum, New York

Crane, R.K., Miller, D., Bihler, I. 1961. The restrictions on possible mechanism of intestinal active transport of sugars. *In:* Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors., pp. 439–449. *Czech. Acad. Sci. Press (Prague)*

DiRienzo, J.M., Nakamura, K., Inouye, M. 1978. The outer membrane proteins of gram-negative bacteria: Biosynthesis, assembly and functions. *Annu. Rev. Biochem.* **47**:481–532

Engelman, D.M., Steitz, T.A. 1981. The spontaneous insertion of proteins into and across membranes: The helical hairpin hypothesis. *Cell* **23**:411–422

Ferdinand, W. 1966. The interpretation of non-hyperbolic rate curves for two-substrate enzymes. A possible mechanism for phosphofructokinase. *Biochem. J.* **98**:278–283

Förster, R. 1979. Kinetik des Natrium-abhängigen L-Leucin Transportes an Bürstensaummembran-Vesikeln des Kaninchendünndarms. Diplomarbeit ETH Zürich

Geck, P. 1978. Die Bedeutung des Membranpotentials für die Kinetik des Carrier-vermittelten Cotransportes. Habilitationsschrift an der Johann Wolfgang Goethe-Universität, Frankfurt am Main, GFR

Geck, P., Heinz, E. 1976. Coupling in secondary transport. Effect of electrical potentials on the kinetics of ion linked co-transport. *Biochim. Biophys. Acta* **443**:49–63

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

Guffanti, A.A., Cohn, D.E., Kaback, H.R., Krulwich, T.A. 1981. Relationship between the Na^+/H^+ antiporter and Na^+/substrate symport in *Bacillus alcaliphilus*. *Proc. Natl. Acad. Sci. USA* **78**:1481–1484

Heinz, E. 1978. Mechanism and energetics of biological transport. *In:* Molecular Biology, Biochemistry and Biophysics. A. Kleinzeller, G.F. Springer and H.G. Wittmann, editors. Vol. 29, pp. 1–157. Springer Verlag, Berlin, Heidelberg, New York

Heinz, E., Durbin, R.P. 1957. Studies of the chloride transport in the gastric mucosa of the frog. *J. Gen. Physiol.* **41**:101–117

Heinz, H., Geck, P. 1977. The electrical potential difference as a driving force in Na^+-linked cotransport of organic solutes. *In:* Membrane Transport Processes. J. Hoffmann, editor. Vol. 1, pp. 13–30. Raven Press, New York

Heinz, E., Walsh, P.M. 1958. Exchange diffusion, transport and intracellular level of glycine and related compounds. *J. Biol. Chem.* **233**:1488–1492

Hilden, S., Sacktor, B. 1982. Potential-dependent D-glucose uptake by renal brush border membrane vesicles in the absence of sodium. *Am. J. Physiol.* **242**:*F*340–*F*345

Honegger, P., Gershon, E. 1974. Further evidence for the multiplicity of carriers for free glucalogues in hamster small intestine. *Biochim. Biophys. Acta* **352**:127–134

Honegger, P., Semenza, G. 1973. Multiplicity of carriers for free glucalogues in hamster small intestine. *Biochim. Biophys. Acta* **318**:390–410

Hopfer, U. 1977. Kinetics of Na^+-dependent D-glucose transport. *J. Supramol. Struct.* **7**:1–13

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

Hopfer, U., Liedtke, C.M. 1981. Kinetic features of cotransport mechanism under isotope exchange conditions. *Membr. Biochem.* **4**:11–29

Hopfer, U., Nelson, K., Perrotto, J., Isselbacher, K.J. 1973. Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* **248**:25–32

Inouye, M., Halegoua, S. 1980. Secretion and membrane localization of proteins in *Escherichia coli*. *CRC Crit. Rev. Biochem.* **10**:339–371

Johnstone, R.M. 1978. The basic asymmetry of Na^+-dependent glycine transport in Ehrlich cells. *Biochim. Biophys. Acta* **512**:199–213

Kaunitz, J.D., Gunther, R., Wright, E.M. 1982. Involvement of multiple sodium ions in intestinal D-glucose transport. *Proc. Natl. Acad. Sci. USA* **79**:2315–2318

Kaunitz, J.D., Wright, E.M. 1983. Heterogeneity of the intestinal sodium-D-glucose carrier. *Fed. Proc.* **42**:5381 *(Abstr.)*

Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M., Semenza, G. 1978*a*. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. *Biochim. Biophys. Acta* **506**: 136–154

Kessler, M., Semenza, G. 1979. On the efficiency of energy conversion in sodium-driven D-glucose transport across small intestinal brush border membrane vesicles: An estimation *FEBS Lett.* **108**:205–208

Kessler, M., Tannenbaum, V., Tannenbaum, C. 1978*b*. A simple apparatus for performing short-time (1–2 seconds) uptake measurements in small volumes; Its application to D-glucose transport studies in brush border vesicles from rabbit jejunum and ileum. *Biochim. Biophys. Acta* **509**:348–359

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

Klingenberg, M. 1980. The ADP-ATP translocation in mitochondria, a membrane potential controlled transport. *J. Membrane Biol.* **56**:97–105

Klip, A., Grinstein, S., Biber, J., Semenza, G. 1980*a*. Interaction of the sugar carrier of intestinal brush border membranes with $HgCl_2$. *Biochim. Biophys. Acta* **598**:100–114

Klip, A., Grinstein, S., Semenza, G. 1979*a*. Transmembrane disposition of the phlorizin binding protein of intestinal brush borders. *FEBS Lett.* **99**:91–96

Klip, A., Grinstein, S., Semenza, G. 1979*b*. Distribution of the sulfhydryl groups in intestinal brush border membranes. Localization of side-chains essential for glucose transport and phlorizin binding. *Biochim. Biophys. Acta* **558**:233–245

Klip, A., Grinstein, S., Semenza, G. 1979*c*. Partial purification of the sugar carrier of intestinal brush border membranes. Enrichment of the phlorizin-binding com-

ponent by selective extractions. *J. Membrane Biol.* **51**:47–73

Klip, A., Grinstein, S., Semenza, G. 1980*b*. The small-intestinal sodium D-glucose cotransporter is inserted in the brush border membrane asymmetrically. *Ann. N.Y. Acad. Sci.* **358**:374–377

Knauf, P.A. 1979. Erythrocyte anion exchange and the band 3 protein: Transport kinetics and molecular structure. *Curr. Topics Membr. Transp.* **12**:249–363

Knauf, P.A. 1982. Kinetic asymmetry of the red cell anion exchange system. *In:* Membranes and Transport. A. Martonosi, editor. Vol. 2, pp. 441–449. Plenum, New York

Knauf, P.-A., Ship, S., Breuer, W., McCulloch, L., Rothstein, A. 1978. Asymmetry of the red cell anion exchange system: Different mechanisms of reversible inhibition by N-(4-azido-4-nitrophenyl)-aminoethylsulfonate (NAP-taurine) at the inside and outside of the membrane. *J. Gen. Physiol.* **72**:607–630

Kreil, G. 1981. Transfer of proteins across membranes. *Annu. Rev. Biochem.* **50**:317–348

Läuger, P. 1980. Kinetic properties of ion carriers and channels. *J. Membrane Biol.* **57**:163–178

LeFevre, P.G. 1975. The present state of the carrier hypothesis. *Curr. Top. Membr. Transp.* **7**:109–215

Liedtke, C.M., Hopfer, U. 1977. Anion transport in brush border membranes isolated from rat small intestine. *Biochem. Biophys. Res. Commun.* **76**:579–585

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275

Murer, H., Hopfer, U. 1974. Demonstration of electrogenic Na^+-dependent D-glucose transport in intestinal brush border membranes. *Proc. Natl. Acad. Sci. USA* **71**:484–488

Murer, H., Hopfer, U., Kinne, R. 1976. Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *Biochem. J.* **154**:597–604

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. *Science* **189**:347–358

Patlak, C.S. 1957. Contributions to the theory of active transport. II. The gate type non-carrier mechanism and generalizations concerning tracer flow, efficiency, and measurement of energy expenditure. *Bull. Math. Biophys.* **19**:209–235

Robillard, G.T., Konings, W.N. 1982. A hypothesis for the role of dithiol-disulfide interchange in solute transport and energy-transducing processes. *Eur. J. Biochem.* **127**:597–604

Rosenberg, Th., Wilbrandt, W. 1957. Uphill transport induced by counterflow. *J. Gen. Physiol.* **41**:289–296

Rothstein, A., Grinstein, S., Ship, S., Knauf, P.A. 1978. Asymmetry of functional sites of the erythrocyte anion transport protein. *Trends Biochem. Sci.* **3**:126–128

Rothstein, A., Ramjeesingh, M. 1980. The functional arrangement of the anion channel of red blood cells. *Ann. N.Y. Acad. Sci.* **358**:1–12

Sabatini, D.D. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* **92**:1–22

Sabatini, D.D., Kreibich, G. 1976. Functional specialization of membrane-bound ribosomes in eukaryotic cells. *In:* The Enzymes of Biological Membranes. A. Martonosi, editor. Vol. 2, pp. 531–579. John Wiley & Sons, New York

Sabatini, D.D., Kreibich, G., Takashi, M., Adesnik, M. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* **92**:1–22

Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J., Crane, R.K. 1973. Purification of the human intestinal brush border membrane. *Biochim. Biophys. Acta* **323**:98–112

Segel, I.H. 1975. Enzyme Kinetics. Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. pp. 1–945. John Wiley & Sons, New York

Semenza, G. 1982. The small-intestinal Na^+/D-glucose carrier is inserted asymmetrically with respect to the plane of the brush border membrane. A model. *In:* Membranes and Transport: A Critical Review. A. Martonosi, editor. Vol. 2, pp. 175–182. Plenum Press, New York

Singer, S.J. 1974. The molecular organization of membranes. *Annu. Rev. Biochem.* **43**:805–833

Singer, S.J. 1977. Thermodynamics, the structure of integral membrane proteins, and transport. *J. Supramol. Struct.* **6**:313–323

Tannenbaum, C., Toggenburger, G., Kessler, M., Rothstein, A., Semenza, G. 1977. High-affinity phlorizin binding to brush border membranes from small intestine: Identity with (a part of) the glucose transport system, dependence on the Na^+-gradient, partial purification. *J. Supramol. Struct.* **6**:519–533

Toggenburger, G., Häusermann, M., Mütsch, B., Genoni, G., Kessler, M., Weber, F., Hornig, D., O'Neill, B., Semenza, G. 1981. Na^+-dependent, potential sensitive L-ascorbate transport across brush border membrane vesicles from kidney cortex. *Biochim. Biophys. Acta* **646**:433–443

Toggenburger, G., Kessler, M., Rothstein, A., Semenza, G., Tannenbaum, C. 1978. 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. *J. Membrane Biol.* **40**:269–290

Toggenburger, G., Kessler, M., Semenza, G. 1982. Phlorizin as a probe of the small-intestinal Na^+,D-glucose cotransporter. A model. *Biochim. Biophys. Acta* **688**:557–571

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

Turner, R.J., Moran, A. 1982*a*. Heterogeneity of sodium dependent D-glucose transport sites along the proximal tubule: Evidence from vesicle studies. *Am. J. Physiol.* **242**:*F*406–*F*414

Turner, R.J., Moran, A. 1982*b*. Further studies of proximal tubular brush border membrane D-glucose transport heterogeneity. *J. Membrane Biol.* **70**:37–45

Turner, R.J., Moran, A. 1982*c*. Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. *J. Membrane Biol.* **67**:73–80

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

Van den Broek, P.J.A., Van Steveninck, J. 1982. Kinetic analysis of H^+/methyl β-D-thiogalactoside symport in saccharomyces fragilis. *Biochim. Biophys. Acta* **693**:213–220

Wickner, W. 1979. The assembly of proteins into biological membranes: The membrane trigger hypothesis. *Annu. Rev. Biochem.* **48**:23–45

Wickner, W. 1980. Assembly of proteins into membranes. *Science* **210**:861–868

Widdas, W.F. 1980. The asymmetry of the hexose transfer system in the human red cell membrane. *Curr. Top. Membr. Transp.* **14**:166–223

Zilberstein, D., Ophir, I.J., Padan, E., Schuldiner, S. 1982. Na^+ gradient-coupled porters of *Escherichia coli* share a common subunit. *J. Biol. Chem.* **257**:3692-3696

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Appendices

M. Kessler

Determination of the Net Charge z of the Mobile Part of a Cotransporter

I. In Strongly Asymmetric Transport Systems with Preferred Inward Orientation, the $\Delta\psi$ Dependence of V_{max}/K_m Cannot be used to Determine the Net Charge of the Mobile Binding Site. Geck and Heinz (1976), Heinz and Geck (1977), Geck (1978) and Turner (1981) have provided comprehensive treatments of cotransport kinetics, which also take into account the effect of the transmembrane potential difference $\Delta\psi$ on the overall kinetic parameters of transport. They have shown that electrogenic H^+- or Na^+-symport systems exhibit clearly distinct kinetic behavior, depending on whether the binding and mobile portion of the "carrier," i.e., the region presumed to undergo reorientation (or change in accessibility) during, and in conjunction with, the translocation process, has a net negative charge of one ($z=-1$) or is electrically neutral ($z=0$). For $z=0$, the apparent K_m for the electrically neutral substrate approaches zero asymptotically with increasing $\Delta\psi$. For $z=-1$, the K_m is, at large $\Delta\psi$, independent of $\Delta\psi$, and only at fairly small $\Delta\psi$ does show a moderate $\Delta\psi$ dependence. Graphic representations of these types of $\Delta\psi$ dependence have been presented (Heinz & Geck, 1977; Geck, 1978; Turner, 1981); as the authors themselves have stated, these graphs were computed for symmetrical (or nearly symmetrical) cotransport systems. As we have seen in the present paper, the small-intestinal Na^+,D-glucose cotransporter is functionally asymmetrical.

In this Appendix I we show that Geck's test – the $\Delta\psi$ dependence of K_m or of V_{max}/K_m – cannot be used in highly asymmetric transport systems with preferred inward orientation. In fact, as we will see, in these systems the curves for *both* $z=0$ and $z=-1$ are strongly $\Delta\psi$ dependent at small $\Delta\psi$ values; a clear-cut discrimination between the two models (which in principle would be possible) appears at very high $\Delta\psi$ values only, which in practice cannot be attained experimentally.

For a Na^+/D-glucose stoichiometric ratio of one, V_{max}/K_m is given by Eq. (1) (Geck & Heinz, 1976; Geck, 1978):

$$\frac{V_{max}}{K_m}=\frac{\frac{x_{tot}}{K_a}\left[p'_a+\frac{r'b'}{K'_b}\cdot p'_{ab}\cdot\xi^{\frac{m+1}{2}}\right]\cdot\xi^{z-\left(\frac{m+1}{2}\right)}}{B+A\cdot\xi^z\frac{p'_o+\frac{b'}{K'_b}\cdot p'_b\cdot\xi^{\frac{m+1}{2}}}{p''_o+\frac{b''}{K''_b}\cdot p''_b\cdot\xi^{\frac{m-1}{2}}}} \tag{1}$$

with

$$A=1+\frac{b''}{K''_b}\quad\text{and}\quad B=1+\frac{b'}{K'_b}\quad\text{and}\quad\xi=e^{-\frac{F}{RT}\Delta\psi}.$$

a: D-glucose; b: Na^+; ': 'out' or 'translocation out→in'; '': 'in' or 'translocation in→out'; K_a, K_b: dissociation constants; r: factor by which a dissociation constant is changed after binding of the cosubstrate; p_o, p_a, p_b, p_{ab}: translocation rates of the unloaded carrier or the respective binary or ternary complexes; x_{tot}: total number of carriers; z: net charge of the binding site(s); m: a parameter characterizing the $\Delta\psi$ dependence of the carrier; it can assume any value between -1 and $+1$; with $m=+1$, $\Delta\psi$ affects only influx, with $m=-1$ affecting only efflux. Since influx is more accelerated by $\Delta\psi$ than efflux, m supposedly is between 0 and $+1$.

We can further make the following assumptions:

The contribution of the binary complex carrier/Glc to the total translocation of D-glucose must be small compared with that of the ternary complex, i.e.

$$p_a\ll\frac{r'b'}{K'_b}\cdot p'_{ab}.$$

The strong transinhibition observed for $b\neq0$ in influx suggests that p''_b is very small as compared to p''_o. The term

$$\frac{b''}{K''_b}\cdot p''_b\cdot\xi^{\frac{m-1}{2}}$$

can therefore be neglected at first approximation.

Under these assumptions, Eq. (1) reduces to:

$$\frac{V_{max}}{K_m}=\frac{\frac{x_{tot}}{K_a}\cdot\frac{r'b'}{K'_b}\cdot p'_{ab}\cdot\xi^{\left(\frac{m+1}{2}\right)(z+1)}}{B+A\cdot\xi^z\cdot\frac{p'_o}{p''_o}\left[1+\frac{b'}{K'_b}\cdot\frac{p'_b}{p'_o}\cdot\xi^{\frac{m+1}{2}}\right]}. \tag{2}$$

For $z=0$, Eq. (2) reduces to

$$\frac{V_{max}}{K_m}=\frac{\frac{x_{tot}}{K_a}\cdot\frac{r'b'}{K'_b}\cdot p'_{ab}}{B+A\cdot\frac{p'_o}{p''_o}\left[1+\frac{b'}{K'_b}\cdot\frac{p'_b}{p'_o}\cdot\xi^{\frac{m+1}{2}}\right]}\cdot\xi^{\frac{m+1}{2}}. \tag{3}$$

Clearly, for $p'_b\ll p'_o$, the ratio V_{max}/K_m will increase continuously with increasing $\xi=e^{-\frac{F\Delta\psi}{RT}}$. Indeed, for $p'_b\simeq0$, the ratio V_{max}/K_m tends to approach infinitely large values; as V_{max} is finite for all values of ξ, K_m tends to approach zero.

For $z=-1$, Eq. (2) reduces to

$$\frac{V_{max}}{K_m}=\frac{\frac{x_{tot}}{K_a}\cdot\frac{r'b'}{K'_b}\cdot p'_{ab}}{B+A\cdot\xi^{-1}\cdot\frac{p'_o}{p''_o}\left[1+\frac{b'}{K'_b}\cdot\frac{p'_b}{p'_o}\cdot\xi^{\frac{m+1}{2}}\right]}. \tag{4}$$

Here, the numerator is independent of $\Delta\psi$. Since both A and B are usually between 1 and 10, the $\Delta\psi$ will only produce relatively small changes in the ratio V_{max}/K_m, in the case where the carrier operates in a nearly symmetrical fashion (i.e., if $p'_o/p''_o\simeq1$) and if the translocation probability of the binary complex carrier-Na^+ (i.e., p'_b) is negligibly small.

If, however, p'_o/p''_o is much larger than 1, the second term in the denominator may become much larger than B.

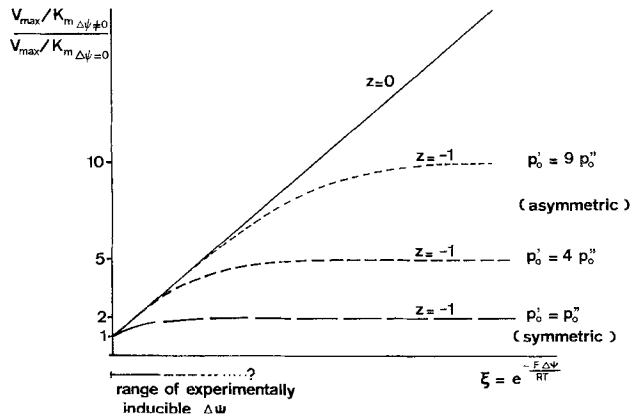


Fig. 10. Scheme representing the $\Delta\psi$ dependence of V_{max}/K_m for symmetric and asymmetric $z=0$ and $z=-1$ systems. Since $v \cong (V_{max}/K_m)\cdot s$ for $s \ll K_m$, the number on the y-axis can be interpreted as the increase of glucose flux by $\Delta\psi$ at very small glucose concentrations, p_o' and p_o'' are the inward and outward translocation rates of the unloaded carrier at $\Delta\psi=0$. The larger the ratio p_o'/p_o'' in $z=-1$ systems, the larger will be the $\Delta\psi$-induced increase of V_{max}/K_m. This scheme is based on the figures previously published by Heinz and Geck (1977) and by Turner (1981), which, however, were computed for *symmetric* sytems. Position and shape of actual experimental curves depend also on other kinetic parameters of the cotransporter

In this case, V_{max}/K_m may exhibit a $\Delta\psi$ dependence similar to that found for $z=0$ at small to intermediate values of $\Delta\psi$. The condition $p_o'/p_o'' \gg 1$ would indicate a carrier having a much larger inward than outward translocation probability in the free form. This is an important feature in the model suggested in the "Discussion" to rationalize our results.

Figure 10 shows in a schematic way how the graphs originally reported by Geck and Heinz (1976), Heinz and Geck (1977) and Turner (1981) have to be modified for asymmetric cotransport systems: the fundamental difference between $z=0$ and $z=-1$ systems still holds true for asymmetric systems. However, the more asymmetric the system (i.e., the larger the p_o'/p_o'' ratio), the more extended becomes the range of ξ in which it is *not* possible to discriminate between $z=0$ and $z=-1$. Since in practice the *degree* of asymmetry of a given transport system is unknown, it is generally not possible to be sure of operating within the range of ξ where the discrimination between $z=0$ and $z=-1$ can be made.

For Na^+-coupled D-glucose transport, the $\Delta\psi$ dependence of V_{max}/K_m is, therefore, of little use to discriminate between $z=0$ and $z=-1$.

II. $\Delta\psi$ Dependence of the Transinhibition. We have thus resorted to another criterion in order to discriminate between $z=0$ and $z=-1$, namely, to the $\Delta\psi$ dependence of the transinhibitions by $glucose_{in}$ and Na^+_{in}. Based on Geck's equations, the following expression $H_a = J_{a''\neq0}/J_{a''=0} = f(\theta)$ was derived, where H_a represents the ratio (influx in the presence of $glucose_{in}$)/(influx in the absence of $glucose_{in}$):

$$H_a = \frac{K+(B+\theta\cdot C)\cdot L\cdot\theta^{2z}}{K+(B+\theta\cdot C)\cdot M\cdot\theta^{2z}} \tag{5}$$

where:

$$K = \left(1+\frac{a''p_a''}{K_a''p_o''}\right)\cdot\frac{1}{p_o'}\cdot\left[1+\frac{a'}{K_a'}+\frac{b'}{K_b'}+\frac{r'a'b'}{K_a'K_b'}\right]$$

$$B = 1+\frac{a'p_a'}{K_a'p_o'}$$

$$C = \frac{1}{p_o'}\left[\frac{b'\cdot p_b'}{K_b'}+\frac{r'a'b'}{K_a'K_b'}p_{ab}'\right]$$

$$\theta = \xi^{\frac{1}{2}}$$

$$L = \frac{1}{p_o''}\left(1+\frac{a''p_a''}{K_a''p_o''}\right)$$

$$M = \frac{1}{p_o''}\left(1+\frac{a''}{K_a''}\right).$$

Note that all the terms in the equations above are >0.

If $z=0$, Eq. (5) reduces to:

$$H_a = \frac{K+B\cdot L+C\cdot L\cdot\theta}{K+B\cdot M+C\cdot M\cdot\theta}. \tag{6}$$

Its first derivative is:

$$\frac{dH_a}{d\theta} = \frac{K\cdot C\cdot(L-M)}{[K+B\cdot M+C\cdot M\cdot\theta]^2}. \tag{7}$$

As all the individual terms are positive, the sign of the equation depends on the relative magnitude of L and M. Since transinhibition is only possible if the translocation rates of the binary complexes are small as compared to those of the unloaded carrier and of the ternary complex, we can assume that $p_o'' \gg p_a''$ and therefore $M>L$. Therefore:

$$\frac{dH_a}{d\theta} < 0 \quad \text{for all values of } \theta;$$

i.e., for $z=0$, the ratio H_a decreases monotonously with increasing $\Delta\psi$ (inside negative) or, in other words, the *transinhibition increases* monotonously with *increasing* $\Delta\psi$.

$z=-1$: In this case, the respective equations are:

$$H_a = \frac{K+B\cdot L\cdot\theta^{-2}+C\cdot L\cdot\theta^{-1}}{K+B\cdot M\cdot\theta^{-2}+C\cdot M\cdot\theta^{-1}} \tag{8}$$

and

$$\frac{dH_a}{d\theta} = \frac{K\cdot\theta^{-2}\cdot(2B\theta^{-1}+C)(M-L)}{[K+B\cdot M\cdot\theta^{-2}+C\cdot M\cdot\theta^{-1}]^2} \tag{9}$$

since $M>L$

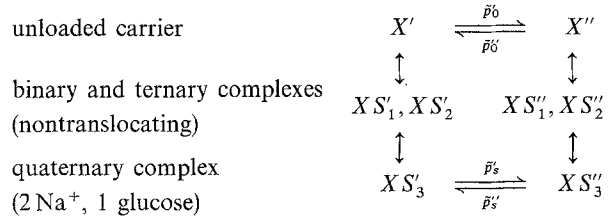
$$\frac{dH_a}{d\theta} > 0 \quad \text{for all values of } \theta;$$

i.e., the ratio H_a increases with increasing $\Delta\psi$ (negative inside) or, in other words, the *transinhibition decreases* monotonously with *increasing* $\Delta\psi$. Systems with $z=0$ and $z=-1$ thus show an *opposite $\Delta\psi$ dependence* of transinhibition.

We have been able to provide an analogous proof for Na^+ as transinhibitor for the case $z=0$ (*not shown*), i.e. transinhibition by Na^+_{in} should always increase with increasing $\Delta\psi$ if $z=0$. However, because of the possible overlap of transinhibition and Na^+-well effect, we have re-

stricted these theoretical considerations on the transinhibition by D-glucose.

III. $\Delta\psi$ Dependence of Transinhibition: 2 Na^+ Transported per Glucose Molecule. As in Appendix II, we assume (i) that the translocation rates are small compared to the association and dissociation rates and (ii) that $\Delta\psi$ only acts on translocation rates, not on binding constants. Since a complete mathematical description of a 3-ligand system is very complex, we introduce the additional simplifying, but well-justified assumption (iii) that the translocation rates of binary and ternary complexes are negligibly small. We can then describe the transport process by the following kinetic scheme:



S: Glc or Na^+; $\tilde{p}_s$: translocation rate of quaternary complex; $\tilde{p}_0$: translocation rate of unloaded carrier; $\tilde{p}'_0 = p'_0 \cdot \xi^{\frac{z}{2}}$; $\tilde{p}''_o = p''_o \cdot \xi^{-\frac{z}{2}}$; $\tilde{p}'_s = p'_s \cdot \xi^{\frac{z}{2}+1}$; $\xi = e^{-\frac{F\Delta\psi}{RT}}$; p'_o, p''_o and p'_s are the respective translocation rates at $\Delta\psi = 0$.

In transinhibition experiments, only one substrate is present in the $''$ compartment, therefore, $XS''_3 = 0$ and $J''_s = XS''_3 \cdot \tilde{p}''_s = 0$.

At given substrate concentrations, the various carrier/ligand associations facing the same side of the membrane are present in fixed, $\Delta\psi$-independent proportions, which are entirely determined by the dissociation constants (assumptions i and ii). Therefore, we can introduce the following parameters:

$$R' = \frac{\text{sum of all binary and ternary complexes on } ' \text{ side}}{X'}$$

$$Q' = \frac{XS'_3}{X'}$$

and similarly R'' for the $''$ side.

Influx J' is described by:

$$J' = \frac{Q' \cdot \tilde{p}'_s \cdot x_{tot}}{(1+R'+Q') + (1+R'')\frac{\tilde{p}'_o + Q' \cdot \tilde{p}'_s}{\tilde{p}''_o}}.$$

We define H_s as the flux in the presence of trans-substrate, divided by the flux in the absence of any trans-substrate:

$$H_s = \frac{J_{s''\neq 0}}{J_{s''=0}}$$

$$= \frac{(1+R'+Q')\,p''_o \cdot \xi^{-\frac{z}{2}} + p'_o \cdot \xi^{\frac{z}{2}} + Q' \cdot p'_s \cdot \xi^{\frac{z}{2}+1}}{(1+R'+Q')\,p''_o \cdot \xi^{-\frac{z}{2}} + (1+R'')\,p'_o \cdot \xi^{\frac{z}{2}} + Q'(1+R')\,p'_s \cdot \xi^{\frac{z}{2}+1}}.$$

$z = 0$:

$$H_s = \frac{(1+R'+Q')\,p''_o + p'_o + Q' \cdot p'_s \cdot \xi}{(1+R'+Q')\,p''_o + (1+R'')\,p'_o + Q'(1+R'')\,p'_s \cdot \xi}$$

$$\frac{dH_s}{d\xi} = \frac{(1+R'+Q')\,p''_o \cdot Q' \cdot p'_s \cdot (-R'')}{(\text{Denominator})^2} < 0.$$

H_s decreases monotonously with increasing ξ, i.e. with increasing $\Delta\psi$, negative on the trans side; transinhibition accordingly increases with increasing $\Delta\psi$, negative on the trans side.

$z = -2$:

$$H_s = \frac{(1+R'+Q')\,p''_o \cdot \xi + p'_o \cdot \xi^{-1} + Q' \cdot p'_s}{(1+R'+Q')\,p''_o \cdot \xi + (1+R'')\,p'_o \cdot \xi^{-1} + Q'(1+R'')\,p'_s}$$

$$\frac{dH_s}{d\xi} = \frac{(1+R'+Q')\,p''_o \cdot R''[2p'_o \cdot \xi^{-1} + Q' \cdot p'_s]\,\xi}{(\text{Denominator})^2} > 0;$$

i.e., H_s increases monotonously with increasing ξ.

$z = -1$:

$$H_s = \frac{(1+R'+Q')\,p''_o + p'_o \cdot \xi^{-1} + Q' \cdot p'_s}{(1+R'+Q')\,p''_o + (1+R')\,p'_o \cdot \xi^{-1} + Q'(1+R'')\,p'_s}$$

$$\frac{dH_s}{d\xi} = \frac{(1+R'+Q')\,p''_o \cdot p'_o \cdot \xi^{-2} \cdot R''}{(\text{Denominator})^2} > 0;$$

i.e., H_s increases monotonously with increasing ξ. For both $z = -1$ and $z = -2$, transinhibition decreases with increasing $\Delta\psi$ (negative on the trans side).