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Kinetics of Sodium D-Glucose Cotransport in Bovine Intestinal Brush Border Vesicles

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Summary. Brush border membrane vesicles (BBMV) purified from steer jejunum were used to study the kinetics of sodium D-glucose cotransport under voltage clamped, zero-*trans* conditions. When the initial rate of glucose transport (J_{gluc}) was measured over a wide range of glucose concentrations ([S] = 0.01–20 mM), curvature of the Woolf-Augustinsson-Hofstee plots was seen, compatible with a diffusional and one major, high capacity (maximal transport rate J_{max} = 5.8–8.8 nmol/mg · min) saturable system. Further studies indicated that changes in *cis* [Na] altered the K_t, but not the J_{max}, suggesting the presence of a rapid-equilibrium, ordered bireactant system with sodium adding first. *Trans* sodium inhibited J_{gluc} hyperbolically. KCl-valinomycin diffusion potentials, inner membrane face positive, lowered J_{gluc}, while potentials of the opposite polarity raise J_{gluc}. At low glucose concentrations ([S] < 0.05 mM), a second, minor, high affinity transport system was indicated. Further evidence for this second saturable system was provided by sodium activation curves, which were hyperbolic when [S] = 0.5 mM, but were sigmoidal when [S] = 0.01 mM. Simultaneous fluxes of ^{22}Na and [^{3}H]glucose at 1 mM glucose and 30 mM NaCl yielded a cotransport-dependent flux ratio of 2 : 1 sodium/glucose, suggestive of 1 : 1 (Na/glucose) high capacity, low affinity system and a ~3 : 1 (Na/glucose) high affinity, low capacity system. Kinetic experiments with rabbit jejunal brush borders revealed two major Na-dependent saturable systems. Extravesicular (*cis*) Na changed the K_t, but not the J_{max} of the major system.

Key Words glucose · brush borders · sodium cotransport · kinetics

Introduction

It is now firmly established that uphill glucose transport across the intestinal brush border membrane is coupled to sodium (Crane, Miller & Bihler, 1961; Schultz & Curran, 1970). Nevertheless, some uncertainty exists about the kinetics of the transport process arising from problems associated with unstirred layers, paracellular shunts, changes in membrane potential, and poor control over intracellular sodium and glucose concentrations. Many of these problems have been resolved using BBMV as first introduced by Hopfer and colleagues (Hopfer, Nelson, Perotto & Isselbacher, 1973; Murer & Hopfer, 1974).

There are two preferred experimental approaches to glucose transport kinetics in vesicles: (1) equilibrium isotope exchange (Hopfer, 1977; Hopfer & Groseclose; 1980), where the uptake of radioactively labeled glucose is measured in the absence of sodium, glucose, or electrical potential (E_m) gradients across the membrane; and (2) measurement of the initial rate of labeled glucose in the presence of known *cis* and *trans* [S], [Na], and E_m. Recent methodological advances allow accurate estimates of initial rates of solute uptake and clamping of membrane potential (Kessler, Tannenbaum & Tannenbaum, 1978; Wright, Kippen & Wright, 1982; Wright et al., 1983). Using these methodologies, we decided to reevaluate the kinetics of glucose uptake under zero-*trans* conditions ([Na]$_{trans}$ and [S]$_{trans}$ = 0). We find that glucose uptake occurs by three processes: diffusion and/or a low affinity, sodium-independent carrier, a major sodium-dependent saturable system, and a minor, high affinity sodium-dependent saturable system.

Materials and Methods

Membrane Isolation

Bovine intestine was obtained from freshly killed steer at a local meat packing facility (Federal, Vernon, Calif.). Mucosal scrapings were obtained from a 1-m length of jejunum (approximately 8 m from the rumen), and brush border vesicles were prepared as previously described for rabbit intestinal membranes (Stevens, Ross & Wright, 1982*a*) and stored under liquid nitrogen until use (Stevens et al. (1982*b*). The brush borders were enriched approximately 14-fold with respect to the original homogenate for the enzyme alkaline phosphatase. The membranes were additionally assayed for sodium-D-glucose cotransport, which is known to

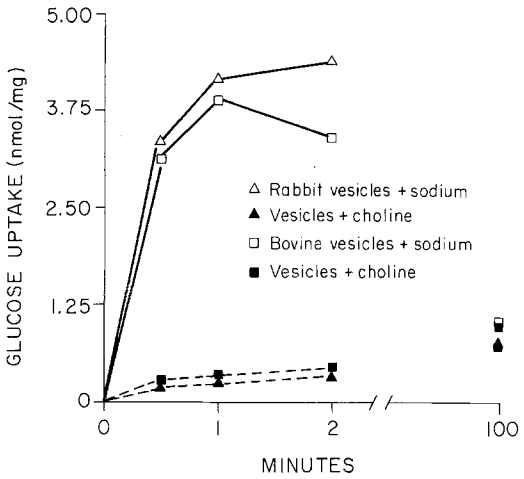

Fig. 1. Time course of D-glucose uptake into brush border membrane vesicles prepared from rabbit or bovine intestine. Vesicles were pre-equilibrated in 300 mM D-mannitol, 2 μM valinomycin, 50 mM HEPES/Tris, pH 7.5, 100 mM KCl. Transport buffers consisted of 0.5 mM D-[^{14}C(U)] or D-[6-^{3}H(N)]glucose, 2 μM valinomycin, 100 mM KCl, and either 100 mM NaCl or choline chloride. Isosmolarity was maintained with mannitol, buffered with 50 mM HEPES/Tris, pH 7.5. The data points are means of two samples. Calculated data: (bovine/rabbit), time to peak overshoot (min) 2/2, uptake at peak overshoot (pmol/mg) 4.2/4.4, mean equilibrium space (μl/mg) 1.8/2.1, peak overshoot/equilibrium uptake 4.1/5.5

exist only in the brush border membrane, by measuring the specific activity of transport in the presence of 100 mM NaCl and 0.5 mM D-glucose under zero-*trans* voltage-clamped conditions.

Figure 1 depicts the time course of 0.5 mM D-glucose uptake across bovine and rabbit brush borders (*see* Kaunitz, Gunther & Wright, 1982). The time-to-peak overshoot (bovine/rabbit, 2:2 min), absolute uptake at peak overshoot (4.2:4.4 pmol/mg), peak overshoot/equilibrium ratios (4.1:5.5) and equilibrium space (1.8:2.1 μl/mg) were comparable. Thus, bovine BBMV appear functionally similar to rabbit BBMV with respect to sodium-coupled D-glucose transport and were chosen for further kinetic studies of the intestinal sodium-D-glucose transporter.

Transport Measurements

A rapid filtration technique described previously (*see* Kaunitz et al., 1982) was used. A 15-μl droplet of the membrane suspension (protein concentration 8–15 mg/ml) was pipetted into the bottom of a 15 × 85 mm polystyrene culture tube. 85 μl of radioactive uptake medium were then rapidly pipetted onto the membrane droplet. The reaction was quenched by pipetting 825 μl of ice-cold stop solution containing 100 mM KCl (osmolarity maintained with $MgSO_4$) after the appropriate time interval. The quenched solution was filtered with 0.45 μm nitrocellulose filters (Sartorius or Schleicher & Scheuer) and quickly rinsed with an additional 4 ml of ice-cold stop solution. Timing for short intervals was done with an electronic metronome. The filters were dissolved, counted, and uptake was calculated and expressed in moles/g protein.

Preloading

Preloading the vesicle interior with KCl was achieved by an incubation in 100 mM KCl on ice in the presence of valinomycin (2 μM) for at least 30 min. Pre-equilibration with NaCl was accomplished by overnight preincubation with NaCl on ice.

Data Analysis

All data analysis was calculated on a NorthStar Horizon microcomputer with standard software. Multiparameter curve fitting programs were furnished by Terry Reedy of the Center for Ulcer Research and Education, Los Angeles, Calif.

Initial Rate Determinations

We chose 3 sec as the earliest time point that we could reliably reproduce under all experimental conditions. To validate the use of a single time point in estimating the rate of glucose uptake, we had to establish that (1) time courses were reasonably linear over the designated period, and (2) the extrapolation of the curve to zero time went through the origin. We thus compared rates calculated from a single 3-sec time point to those estimated from a progress curve such as Fig. 2. The tangent to the curve at zero time was calculated by the Gauss-Newton approximation, using the Taylor series

$$U = A_o + A_1 t + A_2 t^2 + A_3 t^3 \cdots A_n t^n \quad (1)$$

with an iterative least-squares multiparameter curve-fitting program modified from the method of Booman and Neiman (1956) where U is the measured uptake, A_o is the Y intercept, A_1 is the initial rate, $A_{2\ldots n}$ are additional coefficients, and t is time. We found that the use of coefficients of higher order than the quadratic (A_2) were not necessary for analysis of time courses < 10 sec in duration. In our curve-fitting method, no assumption of the value of the y intercept was made. In a series of eight time courses with points between 1 and 6 sec and [S] between 0 and 10 mM and [Na] between 0 and 100 mM, no consistent over- or underestimate of the initial rate occurred with the single 3-sec time point when compared with the Gauss-Newton curve-fitting method, with a 1 ± 10% (SEM) average underestimate overall. Because of uncertainties inherent in any empirical curve-fitting method, we chose not to "correct" our results and use single (3 sec) time points as a reasonable approximation of the initial rate.

Effect of E_m

Since sodium-glucose cotransport can generate changes in electrical potentials in brush border vesicles (Schell, Stevens & Wright, 1983) that can affect glucose transport rates (Murer & Hopfer, 1974; Aronson & Sacktor, 1975), it is important to prevent any generation of (E_m) during glucose uptake. We thus clamped E_m at 0 mV with 100 mM [KCl] in the intra- and extravesicular solutions in the presence of the potassium ionophore valinomycin. When necessary, E_m was varied between −60 and +60 mV by altering the intra- and extracellular KCl concentrations and calculating the Nernst potential for potassium, since experiments have shown that potassium permeability in the pres-

ence of valinomycin greatly exceeds the permeability of all other ions present (Gunther, Schell & Wright, 1984). The ionophore was added to the buffers as an ethanolic stock solution, with a final ethanol concentration of <0.2%. All experiments were repeated at least twice on the same and at least one other membrane preparation. Solutions were buffered to pH 7.5 with 50 mM HEPES[1]/Tris, with the exception of the stop solution, which was buffered with 1 mM HEPES/Tris. Choline chloride was used to maintain constant ionic strength of the uptake buffers, with overall osmolarity of the incubation buffer maintained equal to the intravesicular buffer with mannitol.

Trans Effects

Since *trans* solute may inhibit influx of *cis* solute in cotransport systems (Kessler & Semenza, 1983; Wright et al., 1983), it is also important to know if either the *trans* sodium or glucose concentration accumulated during the incubation period is sufficient to influence the uptake of *cis* glucose. The *trans* [NaCl] and [*S*], calculated using equilibrium vesicular volume and ^{3}H and ^{22}Na uptake data (e.g., Kaunitz et al., 1982, Fig. 3) were 4.5 and 0.7 mM, respectively, after a 3-sec incubation in the presence of *cis* 100 mM NaCl and 5 mM glucose. Other experiments (*see* below, and Kessler & Semenza, 1983) indicate that <30 mM *trans* glucose has negligible effect on D-glucose influx, and 4.5 mM *trans* NaCl inhibits J_{gluc} only 8% (Fig. 7). Furthermore, the combination of *trans* nonelectrolyte and sodium has less inhibitory effect on nonelectrolyte influx than either solute alone (Kessler & Semenza, 1983, Wright et al., 1983). Thus, the *trans* solute concentration after a 3-sec incubation did not appear to greatly affect our estimates of inward D-glucose flux.

Chemicals

D-[6-^{3}H(N)]glucose was obtained from New England Nuclear and Amersham. [^{22}Na]Cl and D-[^{14}C(U)]glucose were obtained from New England Nuclear. All organic compounds were of the highest quality available from Sigma. Reagent grade inorganic salts were obtained from Mallinckrodt.

Results

Uptake Kinetics

The kinetics of D-glucose transport in the presence of *cis* NaCl were measured with bovine jejunal BBMV using 3 sec time points and zero-*trans*, voltage-clamped conditions. The kinetic curve was analyzed with a linear transformation of the Henri-Michaelis-Menten equation with initial uptake rate (J_{gluc}) as the ordinate and $J_{gluc}/[S]$ as the abscissa (Woolf-Augustinsson-Hofstee or Hofstee plot). When plotted according to Hofstee, a nonsaturable

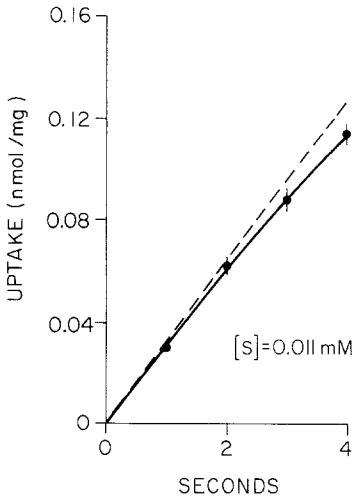

Fig. 2. Initial rate of D-glucose uptake into bovine intestinal vesicles. Vesicles were pre-equilibrated as in Fig. 1. Transport buffers consisted of 100 mM KCl, 2 μM valinomycin, 0.01 mM D-[6-^{3}H(N)]glucose and 100 mM NaCl. Isosmolarity was maintained with mannitol, buffered with 50 mM HEPES/Tris, pH 7.5. In this and succeeding figures, data points are means of at least three samples ± SEM. If error bars are absent, the graphical representation of the mean is smaller than the error bar

(diffusional) process appears as a vertical line with *X* intercept equal to the permeability coefficient *P*, expressed in our case as μl/mg-min, while a saturable transport system conforming to Henri-Michaelis-Menten type kinetics is linear with *Y* intercept = J_{max} and slope = $-K_t$. Curvature of the plot is consistent with multiple and/or non-Michaelis-Menten transport systems.

Vesicles prepared from steer intestine had a first order, diffusional type glucose transport pathway as well as saturable systems, as shown in Fig. 3. After subtraction of the diffusion component, the Hofstee plot was linear for $[S] > 0.25$ mM, consistent with a single Michaelis-Menten type system. The kinetic constants (J_{max} = 8.8 nmol/mg-min; K_t = 0.08 mM; P = 1.1 μl/mg-min) were calculated from the data depicted in Fig. 3 with an iterative nonlinear curve fitting computer using the Michaelis-Menten equation with an added diffusion component.

When $[S] < 0.25$ mM, there is a suggestion of a second saturable uptake system, as shown in Fig. 4 where the uptake rates at $[S]$ = 11 and 26 μM deviate markedly from those expected if one saturable system were present. We estimate that the J_{max} and K_t for the second saturable system are in the range of 0.5 nmol/mg-min and 0.01 mM, respectively, or roughly one order of magnitude lower maximal transport rate and higher affinity than the major system.

[1] HEPES N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

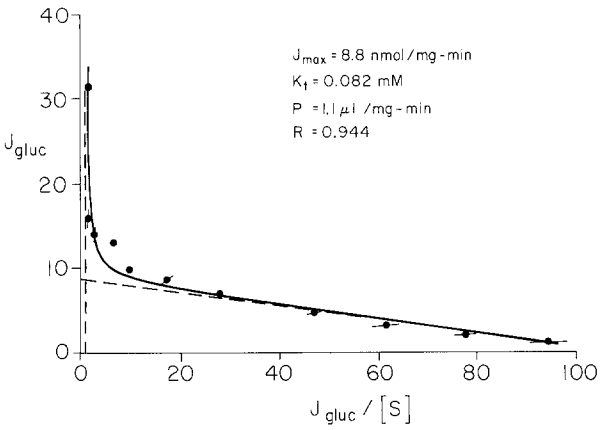

Fig. 3. Kinetic plot of the initial rate of glucose uptake (J_{gluc}) as a function of glucose concentration [S] in bovine intestinal vesicles. Vesicles were pre-equilibrated as in Fig. 1. Transport buffers contained 100 mM KCl, 100 mM NaCl, 2 µM valinomycin, and 0.01–20 mM D-[6-^{3}H(N)]glucose. Isosmolarity was maintained with mannitol, buffered with HEPES/Tris, pH 7.5. Uptake was terminated after 3 sec. The data were plotted using coordinates derived from a linear transformation of the Henri-Michaelis-Menten equation (Woolf-Augustinsson-Hofstee plot). The solid line depicts the best fit of the data points to an equation with one saturable and one diffusive component (*see* text). The slanted and vertical dashed lines depict the resolved saturable and diffusive components, respectively. In this and succeeding Hofstee plots, the abscissa is expressed in µl/mg-min; the unit of the ordinate is nmol/mg-min

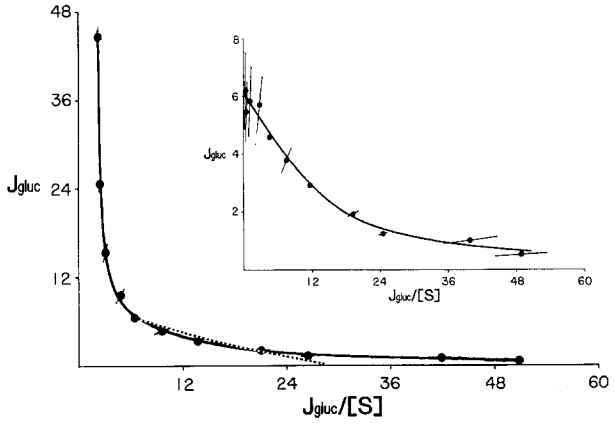

Fig. 4. Hofstee plot J_{gluc} as a function of [S] measured in bovine vesicles from a batch different from those in Fig. 3. Conditions were identical to those described for Fig. 3. The solid line depicts the best fit of the data points to an equation with two saturable and one diffusional component. The dashed line depicts the best fit of the data points to an equation with one saturable and one diffusional component (*see* text). *Inset:* Replot of the same data corrected for diffusion. The diffusional uptake component of the data shown was calculated from the permeability component P for each [S] and subtracted from the total uptake value. Calculated parameters: major saturable system: J_{max} = 5.3 nmol/mg-min; K_t = 0.20 mM; minor saturable system: J_{max} = 0.75 nmol/mg-min; K_t = 0.0083 mM; P = 1.9 µl/mg-min; R = 0.99

Effects of Cis [*Na*]

Varied [*S*]. Figure 5 depicts diffusion corrected Hofstee plots of D-glucose transport kinetics measured in the presence of three *cis* sodium concentrations. For sodium concentrations of 30, 50, and 100 mM, the J_{max} and K_t were 9 ± 0.03 and 0.30 ± 0.02 (nmol/mg-min; mM), 9 ± 0.4 and 0.14 ± 0.01, and 9 ± 0.4 and 0.087 ± 0.007, respectively. The permeability coefficient P varied between 1.1 and 1.5. In the diffusion-corrected Hofstee plots, the increase in apparent standard error of the measurements at high [S] reflects the diminishing contribution of the saturable system(s) to the overall transport rate as [S] increases.

Constant [*S*]. We previously found in rabbit BBMV (Kaunitz et al., 1982) that measurement of J_{gluc} as a function of extravesicular [Na] can yield indirect information on the number of sodium ions involved in the transport of each glucose molecule. To further test our hypothesis that two saturable transport systems may be present, we constructed sodium activation curves at conditions that would favor either a high or low affinity saturable system.

Figure 6*a* and *b* are plots of J_{gluc} as a function of *cis* sodium concentration at two glucose concentra-

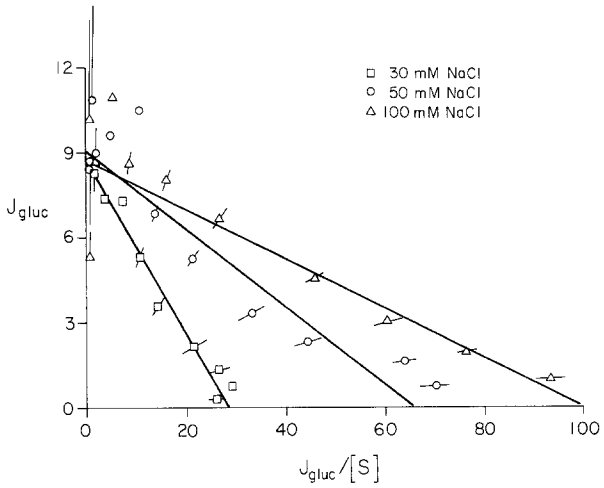

Fig. 5. Kinetic plot of D-glucose uptake in bovine vesicles in the presence of three fixed extravesicular [Na]. Experimental conditions were identical to those described in Fig. 3 with the exception of the [Na] in the transport buffer, which was either 30, 50, or 100 mM NaCl (isotonicity with respect to [Na] maintained with choline chloride). Data analysis was identical for that used in Fig. 4

tions. At low glucose concentrations (0.01 mM) the relationship is clearly sigmoidal, while at higher D-glucose concentrations (0.5 mM), the relationship is essentially hyperbolic. The linear transformation (Hill plot; Fig. 6, insets) yields a slope = n or apparent number of ligand binding sites, and $K_{0.5}$ value ([S] when $J_{gluc} = J_{max}/2$). The Hill plot calculated for [S] = 0.01 mM was linear with a slope of 2. For [S] = 0.5 mM, the Hill plot was curvilinear with slope ~1.3 at low [Na] and reaching a value of 1.1 at high [Na]. The change in sigmoidicity as a function of [S] was observed in all vesicle batches, including those used in the experiment depicted in Figs. 3 and 5.

Effect of Trans [Na]

To determine the effect of intravesicular(*trans*) sodium of D-glucose influx, the vesicles were preloaded with 5-100 mM NaCl. Figure 7 depicts J_{gluc} (0.5 mM) plotted as a function of intravesicular [NaCl]. *Cis* sodium was held constant at 100 mM. A Dixon plot ($[Na]_{intravesicular}$ *vs.* $1/J_{gluc}$) yielded a straight line (r = 0.97), consistent with a Henri-Michaelis-Menten type process. The apparent K_i for *trans* sodium in the presence of 100 mM *cis* NaCl and 0.5 mM *cis* glucose was 54 ± 3 mM. These results suggest that one Na ion interacts with the

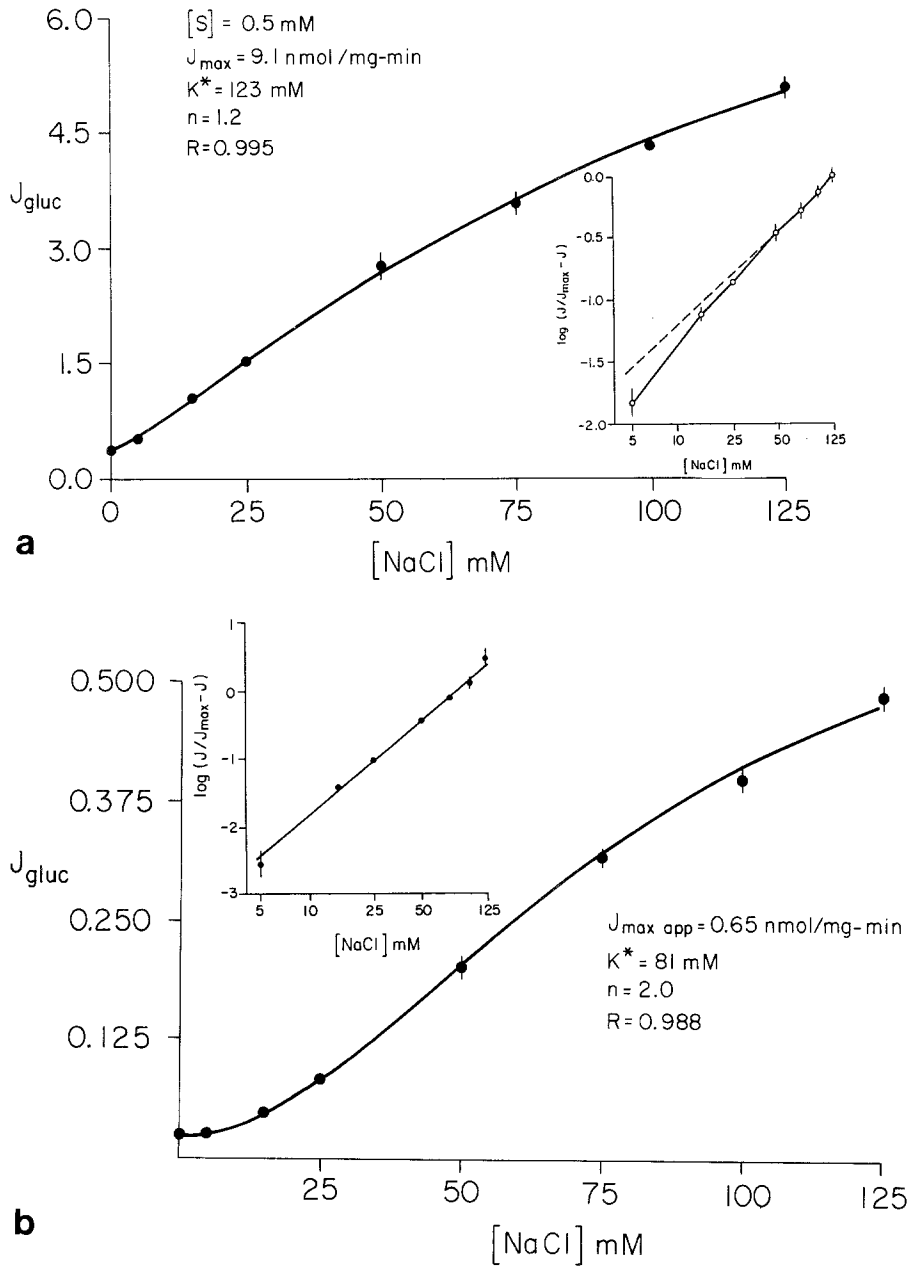

Fig. 6. Effect of varying [Na] on total glucose uptake rate at two fixed glucose concentrations. Vesicles were pre-equilibrated as in Fig. 1. Transport buffers were identical to those described in Fig. 1 with the exceptions that [S] = 0.5 mM in *a* and 0.01 mM in *b*. The lines drawn were obtained by subtracting sodium-independent from sodium-dependent glucose uptake and fitting the data to the Hill equation (*see* text). (*a*): [S] = 0.5 mM; J_{max} = 9.1 nmol/mg-min; $K_{0.5}$ = 123 mM; n = 1.2; R = 1.0. (*b*): J_{max} = 0.65 nmol/mg-min; $K_{0.5}$ = 81 mM; n = 2.0, R = 0.99. *Insets:* Logarithmic Hill plots (ln [Na] *vs.* ln ($J/J_{max} - J$)) of the data in *a* and *b*, using the values of J_{max} listed above. The dotted line in *a* is for reference purposes with slope 1.1

carrier at the inner membrane face under the experimental conditions favoring uptake via the major system.

Effects of E_m

Figure 8 is a plot of J_{gluc} as a function of E_m, assuming $E_m = E_K$. When the E_m is negative with respect to the vesicle interior, J_{gluc} decreases, while the opposite polarity increases J_{gluc}. The relationship between E_m and J_{gluc} for a symmetrical carrier is given by

$$J_{gluc} = J'_{gluc} 10^{(-0.43u\eta)} \quad (2)$$

where J'_{gluc} is the transport rate when $E_m = 0$, $u = zFE_m/RT$, z is the net charge of the transported species, and η characterizes the point where the transition from *cis* to *trans* occurs in the membrane within the limits $1 \geq \eta \geq 0$ (Turner, 1981; Wright et al., 1983). Since sodium-independent glucose transport is electroneutral (Schell et al., 1983), variations in E_m affect only the sodium-dependent glucose transport rate (J^{dep}_{gluc}). Assuming $z = +1$ and an uncharged unloaded carrier (which is implicit in Eq. 3), $\eta = 0.09$, which may be compared with $\eta = 0.24$ for sodium-succinate cotransport with renal vesicles (Wright et al., 1983). It is clear that a potential difference, vesicle interior positive, inhibits sodium-coupled D-glucose transport, while the opposite polarity promotes transport, in agreement with previous qualitative observations made with glucose transport in intestinal and renal vesicles (Murer & Hopfer, 1974; Aronson & Sacktor, 1975).

Direct Measurement of Coupling Ratio

Simultaneous fluxes of ^{3}H glucose and ^{22}Na were measured in bovine vesicles using a method published previously (Kaunitz et al., 1982; Wright et al., 1982). D-[^{3}H]glucose (1 mM) uptake was measured in the presence and absence of 30 mM NaCl; the uptake of 30 mM [^{22}Na]Cl was measured in the presence and absence of 1 mM D-glucose. Amiloride (0.1 mM) was present in all uptake buffers to reduce nonglucose-dependent sodium flux. The initial rate of sodium uptake was obtained by linear regression of the 1, 3 and 5 sec time points, as sodium flux has been shown to be linear under comparable conditions for as long as 30 sec (Gunther et al., 1984). Subtracting nonactivated from cosubstrate-activated rates yielded cosubstrate-dependent rates for both sodium and glucose (Table). The ratio of glucose-dependent sodium transport/sodium-dependent glucose transport under these experimental conditions was 2.0–2.3. Attempts to directly measure the coupling ratio under near saturating conditions for the major system ([Na] = 100 mM, [S] = 10 mM) were unsuccessful due to the high rates of non-

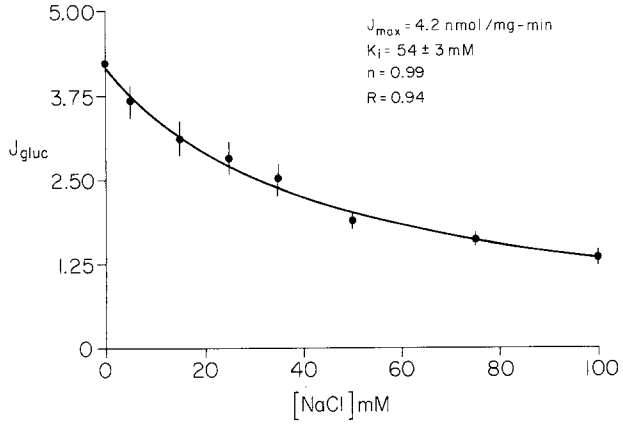

Fig. 7. Effect of *trans* sodium on glucose influx in bovine intestinal vesicles. Vesicles were pre-equilibrated in 100 mM KCl, 2 μM valinomycin, 300 mM mannitol, 50 mM HEPES/Tris, and 0–100 mM NaCl (isotonicity maintained with choline chloride) overnight on ice. Transport buffers consisted of 100 mM KCl, 2 μM valinomycin, 0.5 mM D-[6-^{3}H(N)]glucose, and 100 mM NaCl. Isotonicity with respect to ions was maintained with choline chloride, with overall osmolarity maintained with mannitol, buffered with HEPES/Tris, pH 7.5

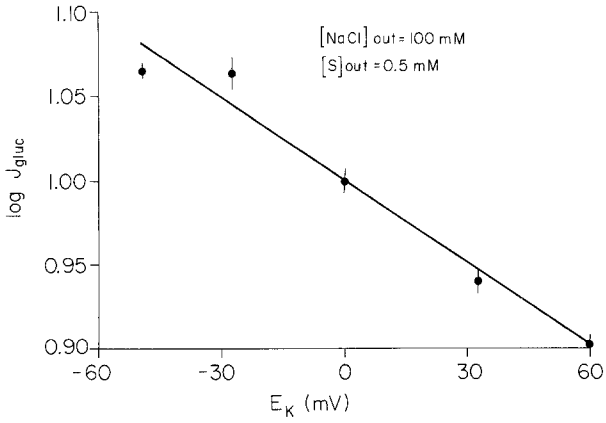

Fig. 8. The effect of transmembrane potential difference on the uptake of D-glucose. Log J_{gluc} (normalized to 1 when $E_m = 0$) is depicted on the ordinate. Actual J_{gluc} when $E_m = 0$ was 4.9 nmol/mg-min. Vesicles were pre-equilibrated as in Fig. 1 with the exception that the [KCl] was 100 or 10 mM. Transport buffers consisted of 0.5 mM D-[6-^{3}H(N)]glucose, 100 mM NaCl, 2 μM valinomycin, and 10–100 mM KCl (isotonicity with respect to KCl adjusted with choline chloride). Overall isosmolarity was maintained with mannitol, buffered with 50 mM HEPES/Tris, pH 7.5. Voltages are with respect to the vesicle interior, calculated with the use of the Nernst equation (*see* text), assuming that $E_m = E_K$

glucose-dependent sodium flux at high sodium concentrations.

Rabbit Vesicles

In addition to the sodium activation analysis and direct measurement of the coupling ratio described previously (Kaunitz et al., 1982), parallel kinetic studies were done with rabbit jejunal vesicles (Fig. 9). The diffusion corrected Hofstee plot was distinctly curved when J_{gluc} was measured in the presence of extravesicular sodium and could be mathematically separated into two Michaelis-Menten type saturable systems, with the J_{max} of the minor system 20–55% of that for the major system. In the absence of extravesicular sodium, the J_{gluc} *vs.* $[S]$ plot was linear, with slope equal to the vertical asymptote of the Hofstee plot of data measured in the presence of extravesicular sodium. Further studies were then performed to examine the effect changes in extravesicular sodium ion had on glucose kinetics. In order to minimize the contribution of the minor saturable system, the kinetic constants were calculated by fitting of all data points for $[S] \geq 0.25$ mM to the Michaelis-Menten equation with an added diffusion component. The results are in agreement with data obtained with bovine vesicles, with extravesicular sodium changing the K_t, with no change of the J_{max}.

Table. Na/glucose coupling coefficient

Experiment	Flux (nmol/(mg · min)	
Sodium (30 mM)		
+ glucose (1 mM)	29 ± 1.1	26 ± 0.5
− glucose	17 ± 0.5	18 ± 0.5
Δ	12 ± 1	8 ± 0.5
Glucose (1 mM)		
+ Na (30 mM)	7.9 ± 0.2	5.3 ± 0.1
− Na	2.6 ± 0.1	1.3 ± 0.1
Δ	5.3 ± 0.2	4.0 ± 0.1
Ratio	2.3 ± 0.2	2.0 ± 0.2

Initial rates of D-glucose and sodium uptakes were measured as described previously (Kaunitz et al., 1982; Wright et al., 1982). The initial rate of (^{3}H)-glucose uptake was obtained at 3 sec, while the initial rate of ^{22}Na uptake was obtained by linear regression of the 1, 3 and 5 sec uptakes as described by Gunther et al. (1984). In these two separate experiments the rates were obtained from triplicate uptakes at each time point and are given with the standard deviations.

Discussion

In this study of intestinal brush border vesicles, we have used initial rate measurements under voltage-clamped conditions to explore the pathways and kinetics of glucose uptake. When J_{gluc} is measured over a wide concentration range (10 μM − 20 mM), the kinetics can be resolved into two components: a diffusional[2] system with an apparent permeability constant comparable to those for neutral amino acids (Stevens et al., 1982*a*); and two sodium-dependent saturable systems, one a high capacity, low

[2] Ling, Im and Faust (1981) have given evidence for a very low affinity (K_t > 50 mM) sodium-independent facilitated diffusion system which appears first order at $[S] < 20$ mM.

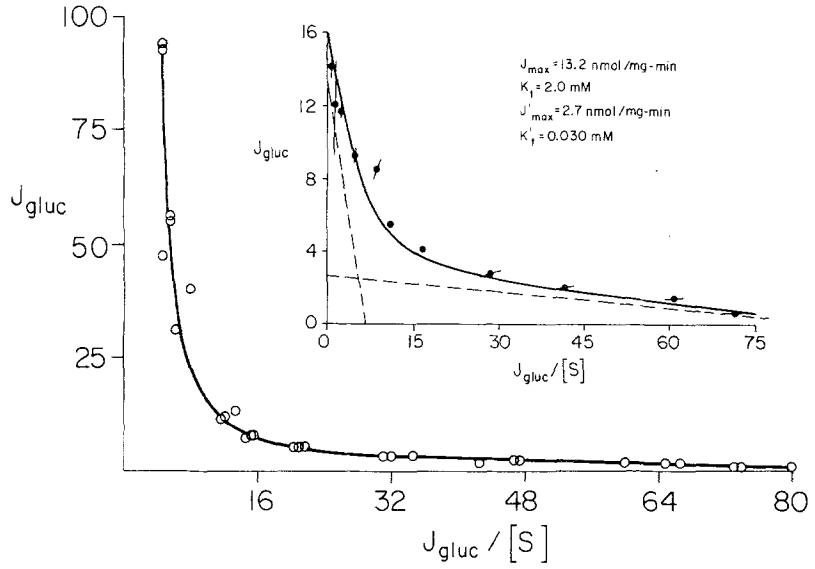

Fig. 9. Hofstee plot of D-glucose uptake in rabbit vesicles in the presence of fixed extravesicular [Na]. Conditions and calculations were identical to those described for Fig. 3 with the exception of [Na] which was 85 mM. *Inset:* Replot of data corrected for diffusion as described in Fig. 4. The dashed lines depict the resolved major and minor saturable systems. Calculated parameters: major saturable system: J_{max} = 13 nmol/mg-min, K_t = 2.0 mM; minor saturable system: J_{max} = 2.7 nmol/mg-min, K_t = 0.030 mM. P = 3.8 μl/mg-min, R = 0.996

affinity system with a Na/glucose coupling ratio of one, and another, of minor importance in the cow, with a lower capacity and higher affinity, and a coupling ratio of greater then one. Optical probe experiments in rabbit brush borders (Schell et al., 1983) can only discern the presence of the low affinity system, which raises the possibility that "uptake" attributed to the high affinity system reflects binding rather than transport. In renal cortex, however, two anatomically distinct sodium-glucose transport systems exist (Turner & Moran, 1982).

To evaluate the kinetic parameters of Na-glucose uptake into brush border vesicles, it is necessary to determine initial rates of uptake under well-defined conditions. For example, in experiments requiring zero *trans* conditions, increases in *trans* glucose and/or Na concentrations during the incubation period could lead to underestimates in the initial rates and result in incorrect estimates of the kinetic parameters. Furthermore, since Na/glucose cotransport depolarizes E_m in vesicles (Schell et al., 1983), and since E_m may alter the rate and kinetics of transport (Murer & Hopfer, 1974; Kimmich & Carter-Su, 1978; Wright et al., 1983), it is necessary to clamp E_m during uptake measurements. We have established that under zero-*trans* conditions, a 3-sec uptake provides a reasonable (±10%) estimate of initial rate of glucose uptake into cow and rabbit brush border vesicles. Under the Na and glucose concentrations we employed, the vesicles accumulate adequate amounts of radioisotope over 3 sec, though the actual amount is <5% of their respective extravesicular concentrations. Optical measurements (Wright et al., 1983; Gunther et al., 1984) have validated the use of potassium with valinomycin to clamp the membrane potential in rabbit vesicles.

Kinetics

It has proved cumbersome to resolve the kinetic parameters of glucose uptake across rabbit brush borders owing to the presence of two relatively high capacity saturable systems and a diffusion component (Fig. 9). In three membrane preparations, the J_{max} of the low affinity system (K_t 0.7–2 mM) ranged between 5 and 13 nmol/mg-min, and for the high affinity system (K_t = 3–20 μM) the J_{max} was between 3 and 6 nmol/mg-min. Fortunately, in the cow one major saturable uptake system prevailed: a single saturable system was resolved in one preparation, while an additional minor high affinity (K_t ~ 0.03 mM), low capacity (J_{max} ~ 0.5 nmol/mg-min) was resolved in other vesicle preparations. The kinetic parameters of the major system in the cow under short circuited, zero-*trans* conditions at 22°C in the presence of 100 mM *cis* NaCl (J_{max} = 5–9 nmol/mg-min and K_t = 0.09 − 0.2 mM) are comparable to those obtained under identical conditions in the rabbit (this study) and by Kessler and colleagues (Kessler et al., 1978; Kessler & Semenza, 1983) for a narrow range of glucose concentrations under open-circuit conditions.

Cis Effects

Under our experimental conditions, glucose uptake via the major saturable component behaves as a rapid equilibrium iso ordered bireactant system with sodium adding first to increase the affinity of the sodium/carrier complex for glucose. This was deduced by observing the effect of *cis* Na on the kinetics of glucose influx. As the Na concentration increased, the measured K_t decreased, with no apparent change in J_{max}, consistent with an affinity effect or competitive activation (Segel, 1975, p. 322). Kessler and Semenza (1983) concluded from rabbit experiments under comparable zero-*trans* conditions that the kinetics were of the "semi-random preferred" type with Na binding first. Crane and Dorando (1982) have given preliminary evidence for a steady-state random system for rabbit intestinal vesicles, while Hopfer and Groseclose (1980) concluded that glucose uptake across rabbit brush borders under equilibrium exchange conditions was consistent with an iso-ordered bi bi system, but were unable to determine whether glucose or Na added first.

In the absence of *trans* solute (or product in the terminology of enzyme kinetics) the isomerization of the carrier does not affect the form of the velocity equation (Segel, 1975, pp. 634–638), allowing sodium-glucose cotransport to be treated as a simple bireactant system. Furthermore, the competitive activation of glucose uptake by sodium implies that the most probable system in the context of rapid equilibrium kinetics (Turner, 1981) is a rapid equilibrium-ordered system in which Na binds first (Cuppoletti & Segel, 1975; Segel, 1975, p. 320)[3].

[3] Steady-state models under certain experimental conditions may also exhibit competitive activation, e.g., steady-state ordered bi bi models in the absence of products when either (i) dissociation of the tertiary complex at the *trans* side is rate limiting (Segel, 1975, p. 564) or (ii) the dissociation of the binary Na/carrier complex at the *cis* side is much greater than other rate constants, i.e., the partial rapid equilibrium ordered bi bi model (Segel, 1975, p. 593). Steady-state random models are complex and they generally do not exhibit Michaelis-Menten kinetics. Nevertheless, product inhibition patterns (Segel, 1975, p. 653) may be helpful in distinguishing between models.

The rate of uptake is given by

$$J_{gluc}^{dep} = J_{max}[S]/(K_s(1 + K_{Na}/[Na]) + [S]) \quad (3)$$

where K_{Na} and K_s are the apparent dissociation constants for Na and glucose respectively. Equation (3) reduces to

$$J_{gluc}^{dep} = J_{max}[S]/(K_t + [S]) \quad (4)$$

where $K_t = K_s(1 + K_{Na}/[Na])$ (*see* Segel, 1975; Turner, 1981). Accordingly, at infinitely high [Na], $K_t = K_s$. Estimates of the apparent affinity constants can be obtained from nonlinear regression analysis of the variation of K_t as a function of [Na], or alternately by analysis of sodium activation curves using a form of Eq. (3). In the cow we estimate the $K_s \sim 0.01$ mM and $K_{Na} \sim 1000$ mM. By comparison, K_s and K_{Na} for the sodium-succinate cotransport system in renal brush borders are 0.3 and 33 mM, respectively. As discussed by Turner (1981), these apparent affinity constants are related to the true affinity constants K_s' and K_{Na}' by terms relating to the rates of translocation (isomerization) of the loaded (k_p) and unloaded (k_p') carriers, e.g.

$$K_s = K_s'(k_p'/(k_p' + k_p)). \quad (5)$$

Note that the foregoing arguments are based on a coupling ratio of one for the major saturable system, based on the hyperbolic kinetics observed when [Na] is varied and *cis* glucose is saturating for the high affinity system (Fig. 6*a*).

Trans Na

As illustrated in Fig. 7, *trans* Na inhibits glucose uptake hyperbolically. In contrast to the sigmoidal inhibition of succinate transport by Wright et al. (1983), our data is indicative of a single sodium binding site on the inner membrane face. It is unlikely that this inhibition simply represents efflux from the vesicles, since initial rates were measured. More probably, inhibition of J_{gluc} by *trans* Na is related to the turnover of the carrier, probably as a reduction in J_{max} as reported by Kessler and Semenza (1983) and Wright et al. (1983) for renal succinate transport. Both groups observed a relief of *trans* inhibition by *trans* substrate (glucose or succinate), suggestive that the translocation rates for binary Na/carrier complexes are relatively slow, also in agreement with the conclusions Hopfer and Groseclose (1980) made from the equilibrium exchange studies. Thus, it appears that *trans* sodium "recruits" carriers to the inner membrane face, decreasing the number of carriers that can interact with solute on the *cis* side. The reader should note that the kinetic parameters measured under equilibrium exchange conditions are considerably greater than under zero-*trans* conditions (*see* Kessler & Semenza, 1983).

E_m

The inhibition of glucose uptake by positive E_m (with respect to the *cis* side) and the acceleration by negative E_m (Fig. 8) is consonant with the observation that glucose depolarizes E_m in rabbit vesicles (Schell et al., 1983). The relationship between glucose uptake and membrane potential can be explained for a symmetrical carrier, as discussed by Turner (1981) if the glucose/Na/carrier complex bears a net positive charge and the unloaded carrier is electroneutral. On the other hand, Kessler and Semenza (1983) have concluded that the unloaded carrier bears a net negative charge, based largely on earlier observations on the effect of voltage changes on phlorizin binding. The discrepancy is probably related to assumptions about the symmetry of the carrier (*see* Kessler & Semenza, 1983).

Semenza's group (Toggenburger, Kessler & Semenza, 1982; Kessler & Semenza, 1983) have presented a gated channel model to account for their observations on phlorizin binding and glucose fluxes. The essence of this model is that the mobile part of the cotransporter, which resides on the inner surface of the membrane in the absence of external solute, bears a negative charge. A negative E_m "pushes" the mobile gate of the pore towards the outer surface, where external Na binds to the negative charge, increasing the affinity of the transporter for glucose. The binding of glucose induces a conformational change, which exposes the active site to the vesicle interior, where Na and glucose dissociate to complete the cycle. This model has a certain intellectual appeal, and it offers a viable physical framework for future discussion of Na-glucose cotransport.

Minor Saturable System

There are two kinetic arguments for the presence of a second cotransport system in cow brush borders. First, in two out of three membrane preparations where J_{gluc} was measured over a wide range of [S], the Hofstee plot could not be fitted to an equation with one saturable system with a diffusion component (Fig. 4, cow; Fig. 9, rabbit). The addition of a second saturable system with a J_{max} of 0.5 nmol · mg-min and K_t of 0.03 resulted in a considerably

better fit. Second, sodium activation curves at tracer ([S] = 0.01 mM) were sigmoidal (mean Hill coefficient $n = 2.1 \pm 0.3$ SEM), whereas activation curves when [S] = 0.5 mM were hyperbolic (mean $n = 1.2 \pm 0.09$) in experiments performed with three different vesicle batches. Crane and Dorando (1982) also have cited evidence for the existence of a second, low capacity, high affinity system in rabbit intestine brush borders. Interestingly, sigmoidal activation kinetics were observed at tracer [S] with the vesicles used in Figs. 3 and 5, despite the apparent lack of a second saturable system seen on the Hofstee plot, indicative of a very low capacity minor system.

Our interpretation of these results is that the major transport system (K_t = 0.2 mM) has a 1:1 sodium/glucose coupling ratio, while the minor system (K_t = 10 μM) has a valency for multiple sodium ions. This premise is strengthened by direct measurement of cotransport, where we found that ~2 Na ions are transported with each glucose molecule, under subsaturating conditions for the major system. In rabbit, we previously obtained a slightly higher coupling ratio under similar conditions (Kaunitz et al., 1982). This is consistent with a greater relative contribution of the high affinity system in the rabbit in the presence of 30 mM NaCl and 1 mM glucose.

Conclusions

Sodium-dependent glucose transport in BBMV from bovine intestine is resolvable into a first-order diffusional and at least one saturable system conforming to Henri-Michaelis-Menten type kinetics. The major saturable system behaves as a rapid equilibrium, ordered system with sodium adding first, and is characterized by an apparent 1:1 (sodium/glucose) coupling stoichiometry, inhibition of E_m (vesicle interior negative) and *trans* sodium. In bovine (and rabbit) vesicles, a minor, high affinity system is present, which appears multivalent for sodium.

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