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Involvement of multiple sodium ions in intestinal D-glucose transport

(membrane vesicles/brush borders/coupling stoichiometry)

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ABSTRACT **Brush border membrane vesicles isolated from rabbit small intestine were used to measure the interactions between sodium and glucose transport with a rapid uptake technique. A plot of glucose uptake rate vs. increasing sodium concentration yielded a sigmoid curve. Hill analysis revealed a coefficient of 1.9 ± 0.02 (±SEM), consistent with at least two sodium ions involved in glucose transport. Transport coupling was then measured directly with double-label experiments in which the uptakes of D-glucose and sodium were determined in the presence and absence of cotransported solute. At the earliest time point, the ratio of cosubstrate-dependent sodium transport to glucose transport was 3.2 ± 0.7 (±SEM). We conclude that two or more sodium ions are coupled to glucose transport across the intestinal brush border membranes.**

It is generally accepted that the mechanism of concentrative glucose transport across the brush border membrane of the intestine involves coupling with an inwardly directed sodium flux down its electrochemical potential gradient (1).

Isolated brush border membrane vesicles have become powerful tools for demonstrating the sodium dependence, effect of membrane potential, and independence from cellular metabolism of sodium-coupled D-glucose transport (2–5). Much work has also been done with vesicles to investigate the kinetics of coupled transport, including calculation of the kinetic constants (6, 7), elucidation of the reaction mechanism (ordered vs. random), and order of reactant binding and unbinding (8–10).

Because an accurate determination of coupling stoichiometry is necessary for the precise modeling of a transport system and because previous estimates of the coupling ratio between sodium and glucose in isolated membrane vesicles have been indirect (5, 10), we have sought to demonstrate coupling stoichiometry directly. A problem with direct determination of stoichiometry has been the high permeability of the vesicles to sodium, which has made it difficult to observe glucose-dependent sodium transport. In intestinal (3) and renal (11) membranes, external D-glucose was capable of stimulating ^{22}Na transport but only at low sodium concentrations (≤5 mM). Hilden and Sacktor (11) concluded that demonstrating coupling stoichiometry directly would require extensive manipulation of the experimental conditions to optimize both glucose and sodium transport.

In view of the recent success with measuring sodium–citrate cotransport in renal brush border membranes (12, 13), we have reinvestigated sodium–glucose coupling in intestinal brush border membrane vesicles.

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EXPERIMENTAL PROCEDURES

Membranes. Rabbit intestinal brush borders were prepared by a Ca^{2+} precipitation technique similar to that of Schmitz *et al.* (14) and described in detail by Stevens *et al.* (15). The specific activities of sucrase and alkaline phosphatase were enriched approximately 15-fold with respect to crude mucosal homogenate. Membranes were rapidly frozen and stored under liquid N_2 (15) so that a series of experiments could be performed on each batch of membranes.

Transport. Transport experiments were performed by using a rapid filtration procedure modified from Kessler *et al.* (16). Fifteen microliters of brush border suspension was pipetted into the bottom of a 12 × 75 mm polystyrene test tube. Transport was initiated by pipetting 85 μl of transport buffer containing labeled substrate and appropriate salt (isosmolarity maintained with mannitol) onto the membrane droplet. Transport was terminated by pipetting 825 μl of ice-cold stop solution (adjusted to isosmolarity with $MgSO_4$) into the reaction mixture, filtering the quenched reaction with a prerinsed cellulose nitrate filter (Sartorious) of 0.45-μm pore size, and rinsing with an additional 4 ml of ice-cold stop solution. The length of incubation was timed by an electronic metronome. The filters were dissolved in 10 ml of scintillation fluid (PCS, Amersham) and the radioactivity was counted in a liquid scintillation spectrometer. Counts were corrected for filter binding, quench, and spillover, and uptakes were expressed as mol per mg of membrane protein. By using this technique, uptakes could be performed at intervals of 3 sec or more, with a standard error of triplicate samples of 5% or less.

Because intestinal sugar transport is electrogenic (17) and membrane potential can affect sodium-coupled D-glucose transport (5), steps were taken to minimize effects of membrane potential on transport. The membrane potential was "clamped" at 0 mV by using equimolar (100 mM) KCl solutions in both intra- and extra-vesicular solutions in the presence of 2 μM valinomycin. In addition, 7 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added to the transport medium to minimize the formation of H^+ gradients, and 100 μM amiloride was added to reduce Na^+:H^+ exchange. Previous experiments (unpublished observations) indicated that amiloride inhibits Na^+:H^+ exchange in intestinal brush borders, as is the case with renal brush border membrane vesicles (18). Control experiments (data not shown) showed no effect of amiloride on sodium stimulated D-glucose uptake. Solutions were buffered to pH 7.5 with 50 mM Hepes/Tris, with the exception of the stop solution, which was buffered with 1 mM Hepes/Tris. Experiments were performed with a single membrane preparation; results were verified by repeating experiments with a minimum of two other membrane preparations.

Chemicals. D-[^{3}H]- and [^{14}C]glucose and ^{22}Na were obtained from New England Nuclear. Amiloride was a gift from Merck, Sharpe & Dohme. The remaining chemicals were of the highest quality available from Sigma.

RESULTS

Preliminary Studies. To ensure that our membrane preparation exhibited Na^+-dependent transport ability, the uptake of D-glucose (0.5 mM) was studied in the presence and absence of 100 mM NaCl in the uptake medium. Fig. 1 demonstrates the expected overshoot phenomenon with eventual equilibration into an intravesicular space of 1.7 μl/mg. The peak uptake value of six times the equilibrium level was achieved at 2 min. Three-second uptake values, which represent an approximation of an initial rate, were enhanced 10-fold in the presence of sodium.

Because direct measurements of glucose–sodium stoichiometry require measurement of ^{22}Na uptakes, similar preliminary experiments were performed with 100 mM NaCl in the absence of ionophores and amiloride. Uptakes were approximately linear up to 30 sec. The initial rate (J_{Na}) of sodium uptake was then determined as a function of external sodium concentration. A plot of J_{Na} vs. $J_{Na}/[Na^+]$ (data not shown) exhibited diffusional and carrier-mediated components with a maximal transport rate of J_{max} = 8 nmol/mg per min, transport Michaelis constant of K_t = 4 mM, and the permeability coefficient of 6 nl/mg of protein per sec.

The initial (3 sec) rate of D-[^{3}H]glucose (0.25 μM–10 mM) was then measured under voltage-clamped conditions in the presence of 30 mM NaCl in the external medium. The results are listed in Table 1. Based on the experiments above, 5 mM was chosen as the optimal D-glucose concentration for direct measurement of sodium–glucose coupling.

Determination of Stoichiometry. *Indirect experiments.* If the initial rate of glucose uptake (J_g), at tracer glucose concentrations, is plotted as a function of external [Na^+], valuable indirect information regarding coupling ratios can be obtained. If one sodium ion is involved in glucose transport, a hyperbolic relationship is expected; if more than one sodium ion are involved, a sigmoid relationship is predicted, as with enzyme systems

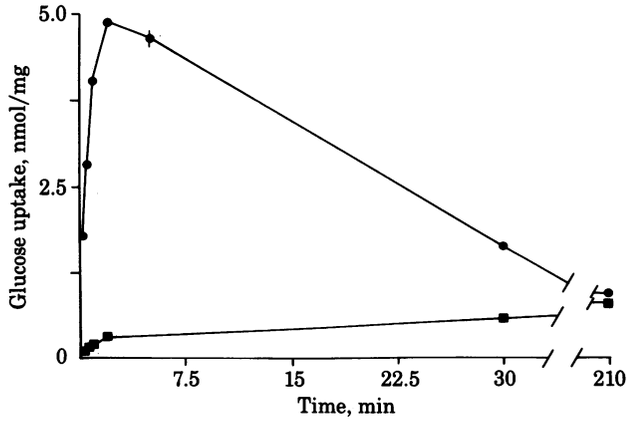

FIG. 1. Time course of D-glucose uptake into intestinal brush border membrane vesicles. Vesicles were preequilibrated in 300 mM D-mannitol/50 mM Hepes/Tris, pH 7.5/ 100 mM KCl. Transport buffers consisted of 0.5 mM D-[^{14}C(U)]glucose, 2 μM valinomycin, 7 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 100 mM amiloride, and either 100 mM NaCl (●) or D-mannitol (no NaCl) (■). Isosmolarity was maintained with D-mannitol buffered with 50 mM Hepes/Tris. In this and succeeding figures, data points are means of at least three samples ± 1 SEM. If vertical bars are absent, the graphical representation of the mean is larger than the error bar.

Table 1. Kinetics of D-glucose uptake

Type of plot	Medium Na^+, 30 mM J_{max}, nmol/mg/min	K_t, mM
J vs. $J/[S]$	7.5	1.7
$[S]/v$ vs. $[S]$	8.2	2.1
Nonlinear regression (19)	8.4	2.3
Average (±SEM)	8.0 ± 0.3	2.0 ± 0.2

Vesicles were preequilibrated as in Fig. 2 but in the presence of 2 mM valinomycin for at least 30 min. Transport buffers consisted of D-[6-^{3}H(*N*)]glucose (0.25–10 mM), 2 μM valinomycin, 100 mM amiloride, 100 mM KCl, and 30 mM NaCl. Isosmolarity was maintained with mannitol buffered with 50 mM Hepes/Tris at pH 7.5. [S], substrate concentration.

(20). Fig. 2 illustrates such an experiment. In the absence of sodium, a small sodium-independent uptake of glucose was observed (1.1 nmol/mg per min). J_g increased with sodium concentrations in a sigmoid fashion. Measurable enhancement of J_g occurred at 20–30 mM and approached a maximal value at 100 mM NaCl. Uptake was analyzed by expressing J_g as the sum of two components

$$J_g^{tot} = J_g^{dep} + J_g^{ind}, \quad [1]$$

in which J_g^{tot} is the measured total uptake rate, J_g^{dep} is the uptake rate attributed to sodium coupling, and J_g^{ind} is the uptake rate in the absence of sodium.

The relationship between J_g^{dep} and the medium [Na^+] can be described by the Hill equation

$$J_g^{dep} = \frac{J_{g(max)}^{dep} \times [Na^+]^n}{K + [Na^+]^n}, \quad [2]$$

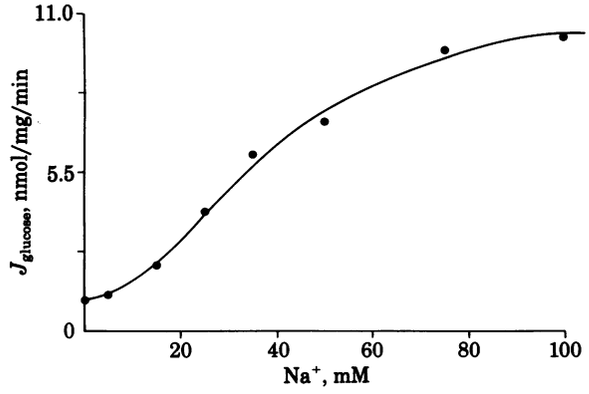

FIG. 2. Effect of increasing the medium [Na^+] on the initial rate of D-glucose uptake by brush border vesicles. Uptake was terminated at 3 sec. Vesicles were preequilibrated in 300 mM D-mannitol/50 mM Hepes/Tris, pH 7.5/100 mM KCl. Transport buffers consisted of 0.4 mM D-[^{14}C(U)]glucose, 2 mM valinomycin, 7 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 100 μM amiloride, 100 mM KCl, and increasing concentrations of NaCl. Isosmolarity was maintained with D-mannitol. The line drawn was obtained by calculating the regression line (R = 0.998) of the data plotted on the coordinates $\log[(J_g^{dep}/J_{g(max)}^{dep}) - J_g^{dep}]$ vs. $\log[Na^+]$, adding J_g^{ind}, and replotting the calculated line and data points on the coordinates J_g^{tot} vs. [Na^+]. Error bars have been omitted for clarity. The standard error of all points was <5% with the exception of those at 5 and 100 mM NaCl, which were 8%. J_{max} was estimated by using the value that best linearized the data on a logarithmic Hill plot. See text for explanation of symbols.

in which $J^{dep}_{g(max)}$ is the sodium-dependent glucose uptake at infinite external sodium concentrations, $[Na^+]$ is the external sodium concentration, K is a constant, and n is the Hill coefficient, which in this case is the apparent number of sodium ions involved in the transport of one glucose molecule. By using 11.2 nmol/mg per min as the value for $J^{dep}_{g(max)}$—which was the value that best linearized the data on a logarithmic Hill plot—n was calculated to be 1.9 ± 0.02 (±SEM). This suggests that a minimum of two sodium binding sites exist on the glucose transport protein.

Direct experiments. To determine the coupling coefficient directly, it was necessary to observe the uptake of [^{3}H]glucose or ^{22}Na or both under the following conditions: D-[^{3}H]glucose uptake in the presence of glucose only, ^{22}Na uptake in the presence of sodium only, and simultaneous uptakes of D-[^{3}H]glucose and ^{22}Na in the presence of both glucose and sodium. As discussed above, 5 mM D-glucose and 20–30 mM NaCl were chosen as minimal concentrations that would produce a measurable increase in transport in the presence of cotransported solute.

The results of one experiment are shown in Fig. 3. J^{dep}_{Na} and J^{dep}_{g} were measurable at each time point. The increase resulting from the addition of cotransported solute was assumed to represent the fraction of transport attributable to sodium–glucose coupling. For example, in the experiment shown [^{3}H]glucose uptake in the absence of sodium was 0.66 ± 0.02 nmol/mg (±SEM) and 0.89 ± 0.007 nmol/mg in the presence of 20 mM NaCl at 3 sec, yielding a sodium-dependent uptake value of 0.23 ± 0.02 nmol/mg at 3 sec. In the absence of glucose, ^{22}Na uptake was 2.0 ± 0.07 nmol/mg, which rose to 2.9 ± 0.06 in the presence of 5 mM D-glucose, yielding a glucose-dependent uptake of 0.9 ± 0.09 nmol/mg at 3 sec. The ratio of the calculated glucose-dependent sodium uptake value to the sodium-dependent glucose uptake value (±SEM) is (0.90 ± 0.09)/(0.23 ± 0.02) or 3.9 ± 0.5. The same process was repeated at each time point, and the average ratio of J^{dep}_{Na} to J^{dep}_{g} for four experiments was then calculated. At 3 sec the ratio was 3.2 ± 0.7 (±SEM), whereas at 6 sec it was 2.5 ± 0.5. The apparent decrease in coupling ratio between 3 and 6 sec is probably due to the fact that solute uptake at 6 sec is no longer linear. The small size of the vesicles (0.2 μm) and the rapid uptake rates account for the short duration of linear uptake (16).

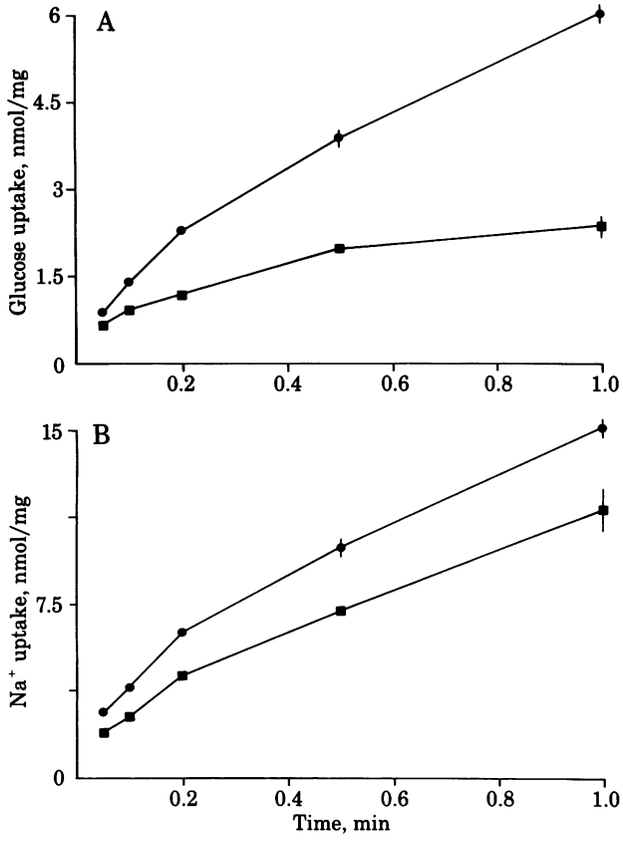

FIG. 3. (*A*) Time course of D-glucose uptake. Vesicles were preequilibrated as in Fig. 2. Transport medium consisted of 2 μM valinomycin, 7 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 100 μM amiloride, 100 mM KCl, 5 mM D-[6-^{3}H(*N*)]glucose, and 20 mM [^{22}Na]Cl (●) or D-mannitol (no NaCl) (■). Isosmolarity was maintained with D-mannitol buffered with 50 mM Hepes/Tris at pH 7.5. (*B*) Time course of sodium uptake. Vesicles were preequilibrated as described in Fig. 2. Transport medium consisted of 2 μM valinomycin, 7 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 100 μM amiloride, 100 mM KCl, 20 mM [^{22}Na]Cl, and either 5 mM D-glucose (●) or D-mannitol (no glucose) (■). Isosmolarity was maintained with D-mannitol buffered with 50 mM Hepes/Tris at pH 7.5.

DISCUSSION

The first experiments established that sodium-dependent D-glucose transport occurred in our preparation of intestinal brush border membranes in the absence of electrical and pH gradients. In addition, conditions were set for directly measuring simultaneous glucose and sodium cotransport.

Analysis of Figs. 2 and 3 indicates that simultaneous measurements of sodium-coupled D-glucose transport and D-glucose-stimulated sodium transport is possible. Further analysis reveals that at least two, and as many as four, sodium ions are involved in sodium–glucose coupled transport. A greater than 1:1 coupling ratio was measured both indirectly and directly, indicating that a 2:1 or greater sodium to glucose coupling ratio in rabbit intestinal brush border exists.

Measurement of the coupling ratio requires the determination of four separate fluxes, each of which has variation due to experimental error. Because calculation of the coupling ratio involves compounding each separate error, the resulting error is quite large, despite the fact that the error for each uptake is <5%. As a result, our estimate of the coupling ratio is subject to uncertainty. This is further complicated by the observation that extrapolation of the sodium uptake curves in Fig. 3 to zero time produces a positive uptake value. This phenomenon has been consistently observed in our laboratory and is thought to result from nonspecific membrane binding of ^{22}Na. However, such binding will not affect calculation of coupling ratios if the binding is equal under both uptake conditions—i.e., in the presence and absence of glucose.

Turner and Silverman (8), using renal brush border membrane vesicles, have used an equilibrium phlorizin binding technique to quantitate the relationship between phlorizin binding and sodium concentration. A value of 1.02 was calculated for the Hill coefficient (n) for sodium-dependent phlorizin binding, indicating that at least one sodium appears to be necessary for binding. Such data, when combined with the findings of the present study, may indicate that one sodium is necessary for glucose binding and one or more additional sodium ions are then required for glucose transport.

A greater than one transport coupling stoichiometry of sodium to organic solute has been measured in several systems. Stevens and Preston (21) indirectly estimated a 3:1 ratio of sodium to alanine influx in the marine invertebrate *Glycera dibranchiata*. A 3:1 sodium to succinate transport coupling ratio was measured by Wright *et al.* with renal brush border membrane vesicles (13). Since Goldner *et al.* (22) first described a 1:1 coupling stoichiometry of sodium to glucose, several efforts have been made to determine the sodium to glucose coupling ratio in other systems. A 1:1 stoichiometry has been suggested

by indirect means in renal (5) and intestinal (10) brush border membrane vesicles. However, a greater than 1:1 sodium to sugar ratio has been inferred in recent studies performed with brush borders prepared from a kidney cell line.* In addition, a 2:1 sodium to glucose coupling ratio has been measured directly with a double-label technique in the flatworm *Hymenolopsis dimunata* (23) and in isolated chicken intestinal cells by Kimmich and Randles (24). In the latter study, the simultaneous uptakes of ^{22}Na and ^{14}C-labeled 3-*O*-methyl-D-glucose were examined with and without phlorizin. When the phlorizin-inhibitable components of sodium and glucose uptakes were examined at each time point, a 2:1 sodium to glucose stoichiometry was found—in direct disagreement with other studies but consistent with our present observations. As stated previously, an exact quantitation of coupling stoichiometry can only be made during the linear uptake phase, which requires very rapid uptake times with isolated membrane vesicles. However, Kimmich and Randles (24) observed linear uptakes to 1 min in their system, probably reflecting on the extremely large size of the intestinal cells compared with that of brush border membrane vesicles. Thus, their figure may more accurately reflect the actual sodium to glucose coupling ratio than what was found in the present study.

Kinetic models such as those proposed by Schultz and Curran (1) based on one sodium ion cotransported with a molecule of organic substrate would necessarily have to be changed to be consistent with the present data and that of other investigators cited above. In addition, the thermodynamic potential energy available from the sodium gradient present between the cell exterior and interior may be greater than thought previously (25).

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