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Energetic coupling of Na-glucose cotransport

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(1) Energetic coupling in Na-linked glucose transport in renal brush border membrane vesicles has been studied in terms of various carrier models differing with respect to reaction order (random vs. ordered), and to rate limitation of steps within the routes of carrier-mediated solute transfer (translation across the membrane barrier vs. binding/release between carrier and bulk solution). (2) By computer simulation it was found that effective energetic coupling requires the leakage routes to be significantly, if not predominantly, rate-limited by their (barrier-crossing) translatory steps. This does not apply to the transfer route of the ternary complex, as coupling is possible whether or not this route is rate-limited by the translatory step. (3) The system transports glucose in the absence of Na^+ (uniport) and the unidirectional flux is stimulated by unlabeled glucose on the trans side (negative tracer coupling). It is concluded that glucose binds to the carrier on either side without Na, as would be consistent with either a random system or one mode of ordered system with mirror symmetry (glucose binds before Na) but inconsistent with either mode of glide symmetry. The tracer coupling appears to indicate that the rate coefficient of carrier-mediated glucose transfer exceeds that of the empty carrier. (4) The Na-linked zero-trans flow of glucose in either direction is strongly trans-inhibited by Na. This consistent with a random system in which Na blocks or retards the translocation of the glucose-free carrier, thereby reducing 'slipping' through an internal leakage route. It is also consistent with the above mentioned ordered system, (i.e., in the absence of Na-transport without D-glucose) if it is assumed that trans Na interferes with the dissociation of the ternary complex, thereby slowing the release of glucose. (5) Minimum equilibrium exchange of glucose is stimulated in the presence of Na. This appears to indicate that Na expands the flow density of carrier-mediated glucose transfer. This expansion does not result from a 'velocity effect' (the ternary complex moving faster than the binary glucose carrier complex), as Na fails to stimulate maximum equilibrium exchange. It can instead be accounted for by an 'affinity effect' (the affinity of the carrier for glucose being increased by Na) as Na depresses the Michaelis constant of equilibrium exchange. (6) The data support the assumption that energetic coupling in a random system of Na-linked glucose transport is brought about by two kinds of effects: (a) Obstruction of the internal leakage route through the glucose-free Na-carrier complex (slipping), or (b) by expansion of the flow of the ternary Na-glucose-carrier complex by positive cooperativity. In the specified ordered system, the two effects reduce to one, as they both result from the failure of Na to bind to the glucose-free (empty) carrier. (7) Whereas the random model is consistent with all experimental observations, the ordered system appears to be inconsistent with some observations, and consistent with others only under improbable assumptions, for instance that the final release of glucose be slow enough to limit the overall transport rate. Though a rigorous proof may still be missing, the presented evidence appears to strongly favor the random-model. (8) Kinetic data alone do not tell to which extent any of the transfer routes is rate-limited by (barrier-crossing) translatory (T) steps and by binding/release (B) steps. It can be shown theoretically, however, that effective energetic coupling requires the leakage routes, i.e., those with either glucose alone or Na alone, to be significantly, if not predominantly rate-limited by their (barrier-crossing) translatory (T) steps. It is plausible, but cannot be fully ascertained yet, that this also applies to the (transport-effective) transfer route of the ternary complex.

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Introduction

Although the Na-linked D-glucose transport has been studied since 1960 [2], the kinetic mechanism of this transport system is not yet clear. Hopfer and Groseclose [3] postulated that Na^+-D-glucose cotransport follows an ordered iso bi-bi mechanism, Crane and Dorando [4], Semenza et al. [5], and Turner and Silverman [6] proposed a random iso bi-bi mechanism.

In this paper we have further characterized the kinetics of the D-glucose transport system in brush border membranes from pig kidney.

Part of these results have been presented in preliminary form [1].

Materials and Methods

Brush border membrane preparation

Pig kidneys were obtained in a local slaughterhouse immediately after killing of animals. Brush border membrane vesicles (BBM) were isolated from pig kidney cortex by a calcium precipitation method described by Vannier et al. [7] and modified in this laboratory [8]. In the final membrane fraction, the brush border membrane marker enzymes alkaline phosphatase (EC 3.1.3.1) and leucine aminopeptidase (EC 3.4.11.1) were enriched 9–12-fold, and the activity of the basal lateral marker Na,K-ATPase (EC 3.6.1.3) was reduced 8–11-fold as compared with the original homogenate. Final pellets were resuspended in vesicle buffer containing 100 mM mannitol, 20 mM Hepes and titrated to pH 7.4 with Tris. Protein concentrations of these suspensions were 20–30 mg/ml.

Vesicles were stored at −70 °C; and prior to use, they were rapidly thawed at 37 °C in a water bath and homogenized five times through a 23 gauge needle.

Protein and enzyme assays

Protein was determined after membrane precipitation with ice-cold trichloroacetic acid (10%) by a modified Lowry procedure, with bovine serum albumin as a standard [9]. Alkaline phosphatase (EC 3.1.3.1) and Na,K-stimulated ATPase (EC 3.6.1.3) were determined as described by Berner and Kinne [10]. Leucine aminopeptidase (EC 3.4.11.1) was spectrophotometrically determined by measuring the *p*-nitroanilide formed from the hydrolysis of L-leucine-*p*-nitroanilide at 405 nm [11].

Zero trans flow (initial steady state)

The zero trans influx rate was determined by mixing 10 μl of membrane suspension (0.08–0.12 mg of protein) prepared in vesicle buffer (100 mM mannitol, 20 mM Hepes titrated to pH 7.4 with Tris) with 100 μl of incubation medium. The incubation medium contained 5 μCi D-[6(n)-^{3}H]glucose (30 Ci/mmol) and the desired amount of D-glucose, NaCl or KCl in the vesicle buffer.

Uptake was terminated after 8 seconds of incubation at 25 °C by the addition of 1 ml ice-cold stop solution, containing 100 mM mannitol, 0.1 mM phlorizin, 20 mM Hepes-Tris and 150 mM NaCl (pH 7.4). The 8-s incubation time was chosen, as linearity of the time curve was observed up to about 12 s. The stop solution – containing the reaction mixture – was immediately transferred with a Pasteur pipette onto a Millipore filter (0.45 μm pore size) kept under suction. The filter was washed once with 4 ml ice-cold stop solution. The radioactivity remaining on the filters was analyzed by standard liquid scintillation counting techniques. All media and solutions used for isolation and transport of vesicles were filtered through a Millipore filter (0.22 μm pore size) immediately before use to avoid bacterial contamination.

To study zero trans efflux brush border membrane vesicles were incubated at 25 °C during 90 min with a medium containing the desired amount of D-glucose, NaCl and KCl in the vesicle buffer. This incubation medium contained 1 μCi D-[6(n)-^{3}H]glucose (30 Ci/mmol) per μl of vesicles (20 mg protein/ml).

10 μl of the incubated vesicles were added to 1 ml incubation medium, containing the desired amount of non-radioactive D-glucose, NaCl and KCl. Efflux was stopped by adding 200 μl of the incubation medium to 1 ml of an ice-cold stop solution, rapid filtration followed as described above.

Equilibrium exchange

Brush border membranes were diluted with media containing the desired amount of D-glucose, NaCl and KCl in the vesicle buffer (100 mM mannitol, 20 mM Hepes titrated to pH 7.4 with Tris).

The vesicles were let at 25 °C during 90 min, 10 μl of the incubated vesicles were added to 100 μl of incubation medium containing 5 μCi of D-[6(n)-^{3}H]glucose (30 Ci/mmol) and the same concentration of glucose, NaCl and KCl in the vesicle buffer as inside the preloaded vesicles. The protein of the vesicles in this incubation medium was 0.08–0.12 μg.

Exchange was stopped by adding1 ml of an ice-cold stop solution. Rapid filtration followed as described above. Each value of the exchange rate ($^{ee}J_a$) was derived from the slope of label uptake with time according to the equation

$$^{ee}J_a = \ln\frac{a_\infty - a_t}{a_\infty} \cdot c \cdot t^{-1}$$

in which in a_t and a_∞ are vesicular label at time t and ∞, respectively, c the vesicular glucose in pmol per mg protein, and t the incubation time.

Chemicals

D[6(n)-^{3}H]Glucose was purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). All other chemicals used were obtained through Sigma Chemical Co. (Deisenhofen, F.R.G.) and were at least analytical grade quality.

Computer studies

Experimental data were compared with theoretical models using a Hewlett-Packard 2621B computer working with a BASIC program, which contained the equations of the initial rates from the eight models considered (v.i.).

Results

Theoretical models

Some theoretical considerations, described in more detail elsewhere (Centelles et al., in preparation), have been devoted to the question of 'rate-limitation' of certain transport steps, in particular to the question whether and to which extent it is justified to treat all (barrier-crossing) translatory steps of the various carrier species as 'rate-limiting'. This means whether these steps are slow enough to permit the interactions between the transported solute species and their binding sites to maintain 'quasi-equilibrium'. The main results are briefly discussed here in terms of the general model depicted in Fig. 1. This model applies to a random and any kind of ordered mechanism as well,

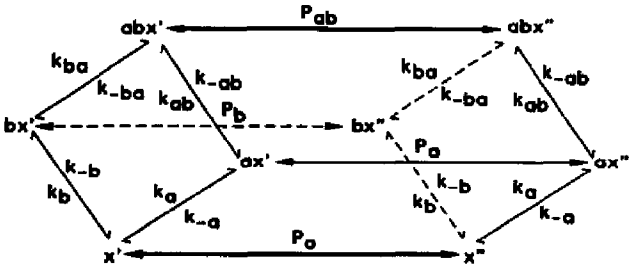

Fig. 1. General model of Na-glucose cotransport. The model consists of translatory (barrier-crossing) steps (T-steps) one for each of the carrier species, and of binding/release rate reactions (B-steps) of the various carrier-substrate complexes. The coefficients of the T-steps are denoted by P_0, P_a, P_b, and P_{ab}, for the empty carrier (x), the binary glucose-carrier complex (ax), the binary Na-carrier complex (bx) and the fully loaded, ternary carrier complex (abx), respectively. The coefficients of the 'on- and off' reactions (B-steps) are denoted by k_i (on) and k_{-i} (off), respectively, according to the following scheme:

$$k_a \cdot a \cdot x \rightleftharpoons k_{-a}\, ax$$

$$k_b \cdot b \cdot x \rightleftharpoons k_{-b} \cdot bx$$

$$k_{ab} \cdot ax \cdot b \rightleftharpoons k_{-ab} \cdot abx$$

$$k_{ba} \cdot bx \cdot a \rightleftharpoons k_{-ba} \cdot abx$$

The superscripts ' and " refer to cis- and trans-side of the barrier, respectively.

depending on the relative magnitude of the binding parameters. The stoichiometry of glucose and Na is taken to be 1 : 1 throughout according to Ref. 12 as was confirmed by our own experiments.

In its random form it consists of four translatory steps (T-steps), one for each barrier-crossing carrier species (x, ax, bx, and abx), and of four binding/release reactions (B-steps) on each side of the barrier, which refer to formation and splitting of the carrier species. The rate coefficients of the T-steps are denoted by P_i etc., and those of the B-steps, by k_i and k_{-i} etc. for binding and unbinding, respectively. Asymmetry is neglected as it would not add relevant information in the present context. The mediated transfer of the solutes (a and b) between the cis and trans side may occur via three separate routes, depending on the intermediate carrier species involved: a separate one for each solute and a coupled one for both. The two separate routes represent internal leak routes, whereas the coupled one is energy-conserving. It is seen that each route connects a T-step in series with B-steps, one or more on each side of the barrier.

As to the kind of steps that rate-limit the various routes we may as a first approach divide the many possible models into two groups: (1) the B-models in which all routes are rate-limited by the B-steps, and (2) the T-models, in which all routes are rate-limited by their T-steps. In the B-models the translatory steps are assumed to be fast enough to maintain 'quasi-equilibrium' between cis- and trans-distribution of the translatable carrier species concerned. In the T-models the B-steps are assumed to be at quasi-equilibrium. To study energetic coupling we may further subdivide each of these groups according to the carrier species that are permitted to cross the barrier. We may obtain the following border line cases. (1) Only the ternary complex (abx) and the empty carrier (x) are crossing, P_a and P_b being 0 (models B_0 and T_0). (2) Besides abx and x also the binary glucose carrier complex (ax) is crossing, P_b being 0 (models B_1 and T_1). (3) Besides abx and x also the binary Na-carrier complex (bx) is crossing, P_a being 0 (models B_2 and T_2) and (4). All four carrier species are crossing the barrier (models B_3 and T_3) (Fig. 2).

The two kinds of border line models, B-models and T-models, have been theoretically compared as to the effectiveness of coupling by computer simulation. As criterion of coupling we used the 'accumulative' effect, i.e., the maximum static head distribution ratio of the substrate (a) at a given (fixed) ratio of the electrochemical activities of the driver ion (b), cis side over trans side, of the transporting membrane [13].

The results are listed in Table I. It is seen that with all B-models, except B_0, coupling is poor or absent, whether or not there is positive cooperativity between the two solutes ($r > 1$). As a consequence, all pure

TABLE I

Accumulative effect of coupling (computer simulation)

The table gives the Haldane ratios (maximum static head distribution) of substrate (a) at a fixed ratio of the electrochemical activities of the driver ion ($b'/b'' = 10$) for the various B- and T-models, as described in the text, with and without positive cooperativity ($r = 1$ and 25, respectively). The (composite) transfer coefficients of each route are arbitrary but numerically equal for the corresponding B- and T-models.

Model	Maximum static head accumulation ratio	
	$r = 1$	$r = 25$
B_0	10.0	10.0
B_1	1.1	1.3
B_2	1.9	1.8
B_3	1.0	1.0
T_0	10.0	10.0
T_1	5.9	9.7
T_2	4.7	4.7
T_3	2.8	4.6

B-models, except B_0, can be dismissed. By contrast, coupling is substantial with the corresponding T-models, even though the overall transfer coefficients of each are numerically identical with those of the corresponding B-model. Noteworthy is the strong enhancement of coupling by cooperativity ($r = 25$) in the T-models only. We may infer that energetic coupling in the system requires that leakage routes, if existent, be significantly rate-limited by their (barrier-crossing) translatory steps. This does not apply to the transfer of the ternary complex as coupling appears to be optimal in both the B_0- and the T_0-model.

In the following the mechanism of coupling is experimentally investigated with the main emphasis to characterize the interaction of glucose and sodium.

Experimental

Glucose carrier interaction in the absence of sodium

The kinetic parameters of Na-free glucose transport were determined for zero trans flow and for equilibrium exchange. For the zero trans uptake, vesicles were incubated for 8 s with media that contained variable concentrations of D-[^{3}H]glucose and 100 mM KCl. It was found that saturable glucose transport takes place in the absence of Na (uniport). As within experimental error its maximum rate is the same as that in presence of Na (Table II), this uniport appears to use the same mechanism as does Na-glucose cotransport. It appears no to be due to a contamination by the basolateral glucose transporter, as cytochalasin does not inhibit (unpublished observation). The unidirectional flux of glucose uniport is strongly transstimulated by unlabeled glucose (Fig. 5), as appears to indicate that the transfer of the glucose carrier complex is faster than that of the unloaded carrier ($P_a > P_0$). It can be inferred that glucose binds to the carrier on either side without Na.

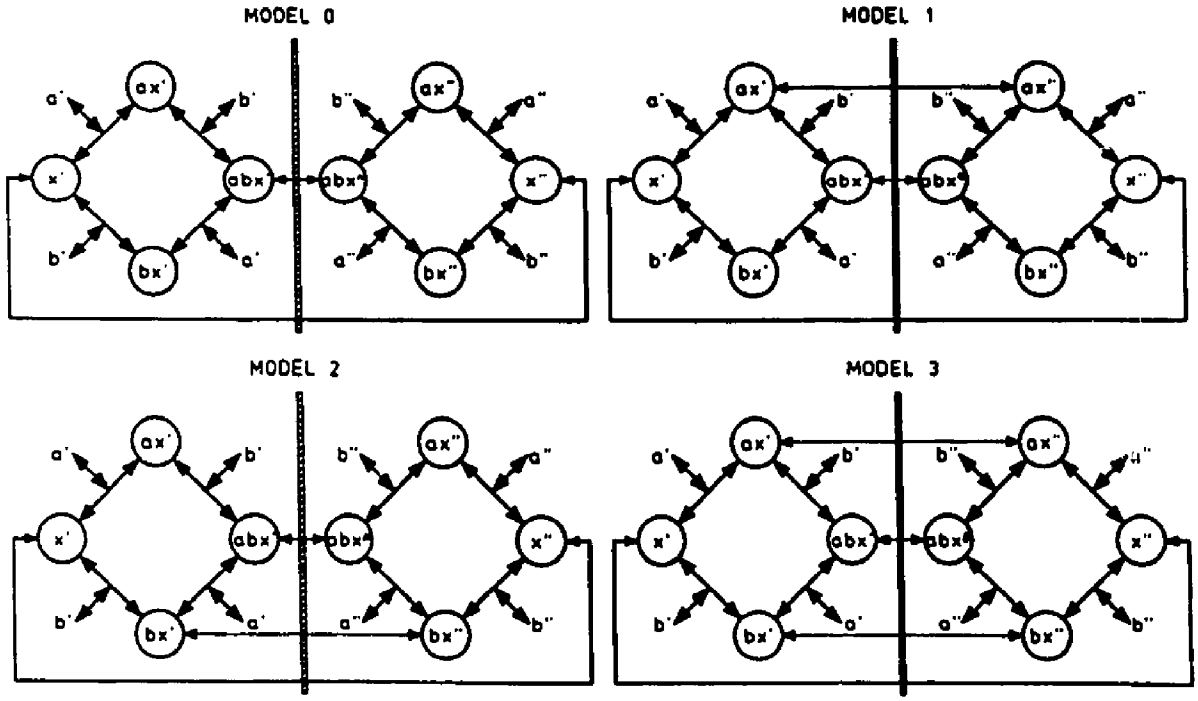

Fig. 2. Models for Na-D-glucose cotransport. a, b and x are D-glucose, Na and the transporter, respectively. The four B models refer to rapid translation and a slow interaction with the transporter. The four T models refer to rapid interaction with the transporter and a slow transport.

TABLE II

The kinetic standard parameters of D-glucose transport at varying Na-activity (experimental)

Upper part: Zero trans flow (glucose and Na present on cis side only). Lower part: Equilibrium exchange (glucose and Na present on both sides). The maximum rate of equilibrium exchange, in contrast tot hat of zero trans flow, shows a non linear, but highly significant decrease ($P < 0.0027$) with the addition of Na. The Michaelis constant of both zero trans flow and equilibrium exchange decreases with increasing Na, but more so and highly significantly ($P < 0.0027$) in the latter. J_{max}/K_m of equilibrium exchange increses with the addition of Na^+ with high significance ($P < 0.0027$). In the upper part the increase in Na^+ in the medium should be accompanied by a change in electrical membrane p.d. owing to different permeabilities of Na^+ and K^+. These permeabilities are not precisely known but based on the assumption that the relative permeabilities of Na^+, K^+ and Cl^- are similar to those reported by Gunther et al. [21] for rabbit intestine, namely 0.67:1.25:1, the p.d. can be crudely estimated to rise from 0 at $[Na^+] = 0$ to about -7.5 mV at 100 mM Na^+, corresponding to an increase of the electrochemical activity coefficient ($\varepsilon = \exp[-F\Delta\psi/2R_1]$) from 1 to 1.15. Hence, the $^0J_{max}$ could maximally rise by 15% as is within experimental error of this table.

Zero trans flow ($n = 5$), all values ± S.E.			
$[Na^+]$ (mM)	$^0J_{max}$ (pmol/mg per s)	K_m (mM)	J_{max}/K_m (10^3 ml/g per s)
0	36.5 ± 0.4	3.3 ± 0.3	11.1 ± 1.1
10	40.8 ± 0.3	2.8 ± 0.1	14.6 ± 0.5
25	37.1 ± 0.5	1.8 ± 0.2	20.6 ± 2.5
50	34.9 ± 0.3	1.3 ± 0.2	26.8 ± 4.6
100	43.3 ± 0.5	1.0 ± 0.2	43.3 ± 7.8
Equilibrium exchange ($n = 6$), all values ± S.E.			
$[Na^+]$ (mM)	$^{eq}J_{max}$ (pmol/mg per s)	K_m (mM)	J_{max}/K_m (10^3 ml/g per s)
0	23.5 ± 0.3	7.1 ± 0.1	3.3 ± 0.1
10	18.9 ± 0.4	2.0 ± 0.1	9.5 ± 0.4
80	18.4 ± 0.4	1.7 ± 0.05	10.8 ± 0.3
100	16.7 ± 0.5	1.7 ± 0.2	9.8 ± 1.2
Change between 10 and 0 mM Na^+			
	−4.7 ± 0.5 ($P < 0.0027$)	−5.1 ± 0.1 ($P < 0.0027$)	6.2 ± 0.4 ($P < 0.0027$)

Glucose carrier interactions in the presence of sodium

Fig. 3 shows a Lineweaver-Burk plot for the D-glucose zero trans flow in the presence of different Na concentrations. Vesicles were incubated for 8 s with media that contained D-[^{3}H]glucose and a NaCl + KCl concentration of 100 mM.

The kinetic parameters obtained are summarized in Table II, upper part. It is seen that with increasing the Na-cis concentrations, the K_m of D-glucose zero trans flow diminishes, whereas the maximal velocity was constant (39 ± 4 pmol/mg per s). The ratio J_{max}/K_m (minimum rate coefficient) increases.

These results can be formally interpreted as 'competitive stimulation', which in this context may mean that the co-ion stimulates transport by increasing the affinity of the carrier for the substrate, rather than the velocity of the transfer.

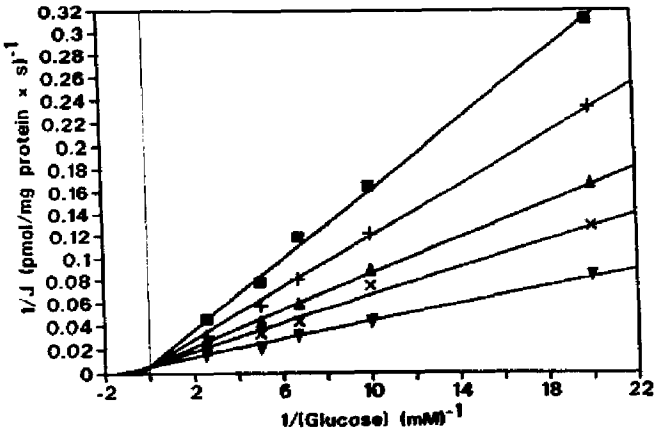

Fig. 3. Lineweaver-Burk plot for D-glucose initial uptake (zero trans). Vesicles were incubated for 8 seconds with D-[^{3}H]glucose and (■) 0 mM NaCl; (+) 10 mM NaCl; (▲) 25 mM NaCl; (×) 50 mM NaCl or (▼) 100 mM NaCl. KCl was added to the incubation media to a final chloride concentration of 100 mM.

More detailed information about the coupling mechanism can be obtained from the corresponding standard parameters of equilibrium exchange. To study equilibrium exchange, vesicles were preloaded for 90 min with 0.1 mM D-glucose in the presence of either 50 mM NaCl or 50 mM KCl. The time curves are shown on Fig. 4. As expected, a stimulation with Na was observed. The standard parameters of equilibrium exchange are listed in Table II, lower part. It is seen that the addition of Na to the Na-free system decreases both the maximum rate and the Michaelis constant, but increases the minimum rate coefficient (J_{max}/K_m). These changes are highly significant ($P < 0.0027$) after the addition of only 10 mM Na, but tend to level off at

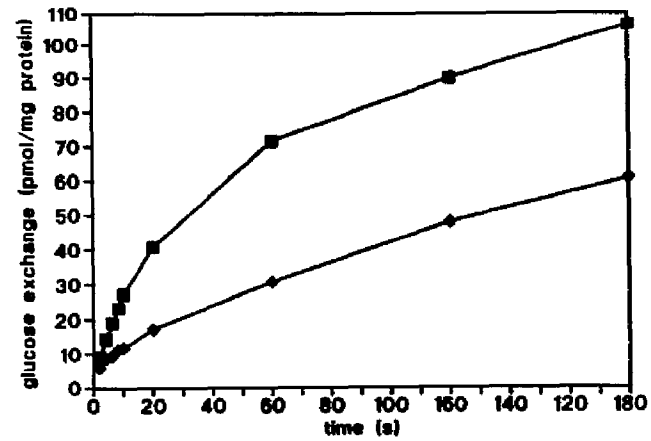

Fig. 4. D-Glucose equilibrium exchange, dependence with time. Vesicles were preloaded with 0.1 mM D-glucose and (■) 50 mM NaCl or (♦) 50 mM KCl. Incubation media were identical, but contained in addition 5 μCi D-[^{3}H]glucose. One representative experiment is shown.

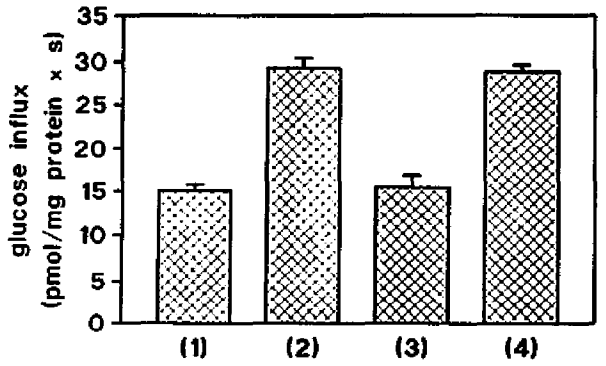

Fig. 5. Influx after 8-s incubation in media containing 10 mM D-[^{3}H]glucose and 150 mM KCl. Vesicles were preloaded with: (1) 150 mM KCl; (2) 10 mM D-glucose and 150 mM KCl; (3) 150 mM NaCl; or (4) 10 mM D-glucose and 150 mM NaCl.

higher Na, presumably owing to saturation of the carrier with Na. As will be discussed later, these Na-induced changes in equilibrium exchange can reasonably be explained as follows: the decrease of J_{max} appears to indicate that the ternary complex is slower than the binary glucose complex (negative velocity effect) ($P_{ab} < P_a$). The decrease of K_m appears to indicate positive cooperativity between the two ligands ($r > 1$) (positive affinity effect). The increase of the ratio J_{max}/K_m with both zero trans flow and equilibrium exchange is associated with a Na-induced increase in density of transport flow, but does not differentiate between the before mentioned velocity and affinity effects.

It should be mentioned that the data for zero trans flow and equilibrium exchange, respectively, were obtained each with different batches of membranes. Hence the fact that the maximum rates of the former exceed those of the latter is presumably not typical but rather accidental. On the contrary, whenever the corresponding parameters were obtained with batches of the same preparation, it was the other way round: maximum equilibrium exchange, especially at Na = 0, exceeded maximum zero trans flow, as exemplified on Figs. 5 and 6, and predicted by Eqns. D8 and D9.

To study the trans effect of Na^+ directly, D-glucose influx was measured after 8 s of incubation with a medium containing 50 mM NaCl, 50 mM KCl and 0.4 mM D-[^{3}H]glucose. Vesicles were preloaded for 90 min at 25 °C with 100 mM KCl or with 50 mM NaCl and 50 mM KCl (either in presence or absence of 0.4 mM D-glucose). The presence of Na within the vesicles (with or without D-glucose) diminishes the D-glucose initial uptake (Fig. 6A).

Similar results were obtained when D-glucose zero trans efflux was measured (Fig. 6B).

At low D-glucose (0.4 mM) trans glucose did not relieve Na trans inhibition of influx or efflux (Fig. 6). At higher (saturating) D-glucose concentration (10 mM) influx and efflux of Na-linked glucose, however, were both trans-stimulated by glucose (either in presence or in absence of 150 mM NaCl in the trans side) (Fig. 7). In other words, at low glucose zero trans flow exceeds equilibrium exchange, whereas at saturating glucose the opposite is true. This behavior will be interpreted in terms of Eqns. D8 and D9 in the Discussion.

To test whether Na transinhibits the Na-independent glucose flow, glucose uptake was studied with and without Na on the trans side. At first influx studies were performed, where D-glucose uptake was measured after 8 s of incubation with a medium containing 100 mM KCl and 0.4 mM D-[^{3}H]glucose. Vesicles were preloaded for 90 min at 25 °C with 100 mM KCl or with 50 mM NaCl and 50 mM KCl. No Na-transinhibition was observed, probably due to a rapid increase of D-glucose inside the vesicles, which through the mobile ternary complex could restore carrier to the outside, thus counteracting a potential blockade by Na. To avoid this complication, the effect of trans Na was studied on Na-free glucose efflux. Under these conditions the magnitude of the extracellular space would prevent a significant rise in trans glucose concentration. The anticipated trans inhibition of Na-free glu-

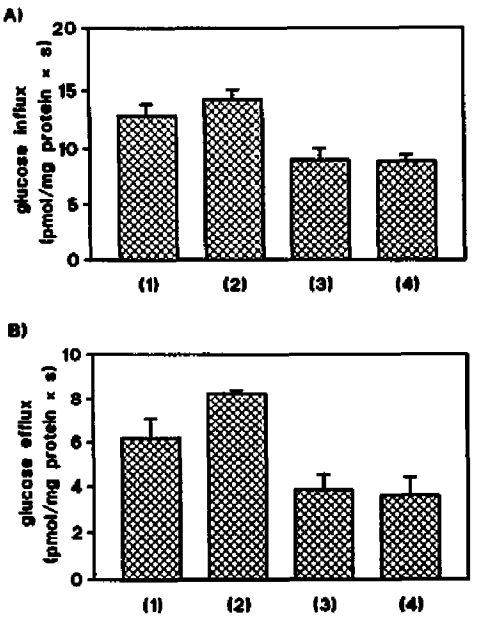

Fig. 6. Influx and efflux after 8 s of transport. (A) Influx studies were performed with media containing 0.4 mM D-[^{3}H]glucose, 50 mM NaCl and 50 mM KCl; and vesicles were preloaded with: (1) 100 mM KCl; (2) 0.4 mM D-glucose and 100 mM KCl; (3) 50 mM NaCl and 50 mM KCl; or (4) 0.4 mM D-glucose, 50 mM NaCl and 50 mM KCl. (B) Efflux studies were performed with media containing: (1) 100 mM KCl; (2) 0.4 mM D-glucose and 100 mM KCl; (3) 50 mM NaCl and 50 mM KCl; or (4) 0.4 mM D-glucose, 50 mM NaCl and 50 mM KCl; and vesicles preloaded with 0.4 mM D-[^{3}H]glucose, 50 mM NaCl and 50 mM KCl. In the first two columns of both A and B an electrical membrane p.d., if estimated on the same basis at that in Table II, can be expected to attain −4.5 mV, corresponding to an electrochemical activity coefficient of 1.07. The resulting increase in these flows by at the most 7% would be within experimental error.

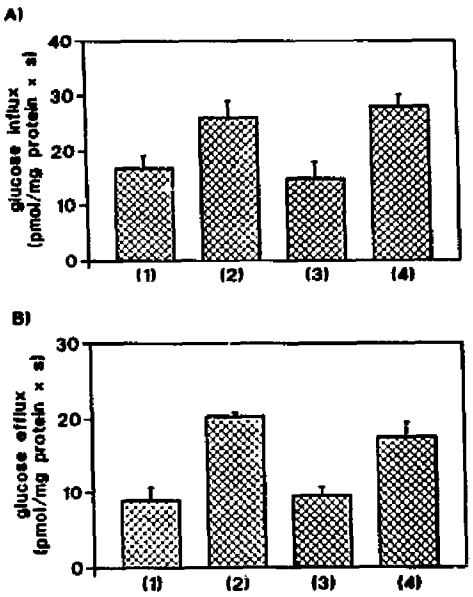

Fig. 7. Influx and efflux after 8 s of transport. (A) Influx studies were performed with media containing 10 mM D-[^{3}H]glucose and 150 mM NaCl; and vesicles were preloaded with: (1) 150 mM KCl; (2) 10 mM D-glucose and 150 mM KCl; (3) 150 mM NaCl; or (4) 10 mM D-glucose and 150 mM NaCl. (B) Efflux studies were performed with media containing: (1) 150 mM KCl; (2) 10 mM D-glucose and 150 mM KCl; (3) 150 mM NaCl; or (4) 10 mM D-glucose and 150 mM NaCl; and vesicles preloaded with 10 mM D-[^{3}H]glucose and 150 mM NaCl. In columns 1 and 2 of A and B, an electrical p.d., estimated on the same basis as the corresponding one in Table II, of about −7.5 mV, corresponding to an electrical activity coefficient of 1.15 can be expected to increase the flow values by about 15% or less. This increase, though beyond experimental error, would strengthen the conclusion drawn from this figure, namely that equilibrium exchange here exceeds zero trans flow.

cose efflux by Na was indeed observed: While without Na on the trans side the efflux was 1.2 ± 0.1 S.D. pmol/mg per s protein, in the presence of trans Na (50 mM NaCl + 50 mM KCl), it was significantly reduced to only 0.8 ± 0.1 S.D. pmol/mg per s ($P < 0.01$).

Discussion

General

The present system of the Na-linked glucose transport may be represented by the diagram in Fig. 1.

In its simplest 'random' form it consists of four parallel translatory steps according to the number of (mobile) carrier species that cross the barriers. In its 'ordered' form, one of these steps is missing as the corresponding binary complex does not significantly form.

The kinetic equations of such transport systems have usually been based on the assumption that the rate of each transfer route is limited by the (translatory) T-steps, the binding and release reactions (B-steps) being fast enough to maintain 'quasi-equilibrium' of binding. This assumption is generally considered most probable, but it cannot be excluded that some subpathways are significantly rate-limited by B-steps. Since however, the T-steps and B-steps cannot be separated kinetically, in analogy to certain (bi-bi-iso) enzyme reactions [14], the equations derived under the assumption that only T-steps are rate-limiting, should formally not depend on this assumption, but rather have a more general validity, provided that the parameters are reinterpreted accordingly.

For instance in kinetic transport studies, the velocity coefficients (P_i) do not refer to translatory (T) steps only, nor do the apparent affinities (K_i) necessarily refer to binding and release reactions only. They are instead 'composite parameters', each of which being potentially a function of both translatory and binding/unbinding rate coefficients. In the following they will be called 'transfer' parameters and be marked by a bar, e.g., $\bar{P}_a$, $\bar{K}_a$, respectively.

If it thus may be irrelevant kinetically which of several steps in series predominantly limits the overall rate of a particular transfer route, this may not so be in energetic coupling.

Energetic coupling between the two flows in the above system requires that internal leakage routes, i.e. those through the incomplete (binary) complexes, ax and bx, are repressed relative to the energy conserving routes, i.e. those involving the empty carrier (x) and the fully loaded (ternary) one (abx).

It had been shown previously that a model in which all translatory (T)-steps are at quasi-equilibrium is incapable to account for any energetic coupling [15].

Table I shows that coupling is also absent or poor whenever only one of the two leakage routes is rate-limited by B-steps only, whereas it is seizable in the corresponding T-models, in which the same leakage routes are rate-limited by T-steps, even though the overall transfer coefficients are kept equal in both kinds of models. Accordingly, the corresponding models B_1, B_2, and B_3 can be dismissed because they do not allow effective coupling. It follows that for effective coupling both leakage routes must be significantly, if not exclusively rate-limited by their translatory steps.

Na-free glucose transport (uniport)

Mediated glucose transport in this preparation is possible in the absence of Na^+ (uniport), for instance if Na^+ on both sides is replaced by K^+, which does not interact by symport or antiport with this glucose transport system [16]. The unidirectional flux of glucose of this system is strongly transstimulated by (unlabelled) glucose (Fig. 5) (negative tracer coupling). Accordingly, equilibrium exchange of this uniport exceeds the corresponding (zero trans) transport of glucose.

The transstimulation cannot be attributed to electrical potentials, as ion gradients are absent and as glu-

cose transfer is neutral. It can instead be interpreted to indicate that the transfer coefficient of the glucose carrier complex ($\bar{P}_a$) distinctly exceeds that of the empty carrier (P_0). Since P_a (see Eqn. D3) cannot exceed the translatory coefficient P_a, P_0 must be distinctly smaller than the latter.

Zero trans uniport and equilibrium exchange of glucose by a symmetric system can most simply be described by the equation

$$^{0}J_a = \frac{\bar{P}_a \cdot P_0 \cdot a'}{2P_0\bar{K}_a + (P_0 + \bar{P}_a)a'} \quad \text{(D1)}$$

$$^{cc}J_a = \frac{\bar{P}_a \cdot a'}{2(\bar{K}_a + a')} \quad \text{(D2)}$$

As mentioned before, the bars over P_a and K_a are to indicate that these parameters are composite, i.e., composed of translatory and chemical rate coefficients, which cannot be separated directly by kinetic means. For instance for a symmetric system

$$\bar{P}_a = \frac{P_a \cdot k_{-a}}{2P_a + k_{-a}} \quad \text{(D3)}$$

and

$$\bar{K}_a = K_a \quad \text{(D4)}$$

Hence, we cannot tell from these observations to which extent for instance $\bar{P}_a$ is predominantly determined by P_a and by k_{-a}, respectively. Provided that the interaction between substrate and carrier is similar to that between substrate and enzyme a plausible estimate can be made: Accordingly, the association coefficient (k_a) should be in the range between 10^7 and 10^9 s^{-1} [17]. Since the dissociation constant of glucose in the present system has been estimated to be about 30 mM [6] the corresponding k_{-a} would be above $3 \cdot 10^5$ s^{-1}. This would be more than 3000-times higher than the transfer coefficient, which is considered to be in the range of 100 s^{-1}. Accordingly, the rate of glucose transfer by this system should be almost entirely limited by the translation step (P_a). It should be kept in mind, however, that in transport systems the ligands in free solution might be separated from their carrier sites by 'access channels', the diffusional resistance of which might considerably diminish the on- and off-coefficients, though probably not to the range of transfer coefficients.

This uniport is not a separate mechanism independent of cotransport, as at high (saturating) glucose the addition of Na does not increase the glucose transport.

Negative tracer coupling of this system indicates that glucose can be bound by the carrier on either side without the presence of Na^+, as would exclude any ordered system in which Na^+ has to be bound prior to glucose ('Na first on'). It also appears to exclude any kind of 'glide symmetry' as this would not permit Na-free glucose binding on both sides. Hence, only one type of ordered system is consistent with the results of glucose uniport, namely that glucose gets on before Na, and gets off after Na. Any subsequent mention of 'ordered' system will refer to this system only.

Na-transinhibition

The glucose-free transfer of Na with the glucose carrier represents another route of internal leakage.

Na-linked glucose transport under zero-trans conditions, in either direction is strongly inhibited by raising Na on the trans side (Na-transinhibition) (Fig. 6). Also zero-trans uniport of glucose is inhibited by trans Na^+.

For a random system Na-transinhibition can be interpreted to indicate that Na blocks, or retards, the translocation of the glucose-free carrier, thereby interfering with its return to the cis side. In other words, the mobility of the binary Na-carrier complex (P_b) is zero or at least much smaller than that of the empty carrier (P_0) [18]. This 'negative velocity effect' contributes to the energetic coupling between the two flows of substrate and Na, respectively, as it prevents internal leakage by 'slipping' [13].

Also in an 'ordered' system Na-transinhibition should in principle be possible. But since the binary Na-carrier complex does not form, the inhibition has to be explained differently: For instance trans Na could interfere with the dissociation of Na from the ternary complex and hence with the release of glucose at the trans side which can take place only after sodium has been released. Against this kind of Na-transinhibition, and hence against this ordered system, the following arguments can be raised:

(1) This Na-transinhibition will become manifest only if the dissociation coefficient of the binary complex (k_{-a}) is small enough to significantly limit the overall transport rate. It will be recalled, however, that this coefficient has been estimated to be probably too large for this purpose, though this cannot be tested directly by kinetic means.

(2) This Na-transinhibition, as it depends on the translocation of the ternary complex, should not affect Na-free glucose transport (uniport). It has, however, been shown that Na transinhibits glucose uniport, at least in the outward direction (v.s.).

(3) There is at least indirect evidence that Na binds to the carrier without glucose. First, Lin et al. [19] demonstrated that the glucose carrier can be blocked by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), an agent interacting with tyrosine residues, and that this blockage can be prevented by Na^+. Furthermore, the experiments with the inhibition of this transport system by Ca have been interpreted in terms of 'slipping', i.e. by the mobilization of an (existent) Na-carrier complex.

This implies prior binding of Na to the glucose-free carrier [18] (unless one postulates that Ca converts an ordered system into a random one).

It would appear from these considerations that the ordered model is less probable than the random one.

Energetic coupling between glucose flow and Na flow is accounted for by both the random system and the ordered system, but the underlying mechanism is somewhat different. As will be shown, the random system involves two separate and mutually independent effects of Na, the ordered system, by contrast only a single effect.

In the 'random' system one effect of Na would be to immobilize the substrate-free carrier, thereby obstructing a dissipative internal leak route [20]. The resulting contribution to the energetic coupling, however, is rather weak and incomplete, and could hardly account for the full extent of coupling observed. An additional effect of Na to promote coupling is indeed indicated by the experimental observation that for equilibrium exchange the ratio (${}^{ec}J_{max}/K_m$) ('relative minimum equilibrium exchange') of glucose is stimulated by the presence of Na (Table II). As equilibrium exchange is independent of glucose-free carrier translocation, this stimulation should indicate increased density of mediated glucose transfer across the barrier. It does not tell, though, to which extent the density increase results from an increased translatory rate of the carrier ($P_{ab} > P_a$, velocity effect) and to which extent from an increased binding of glucose to the carrier (abx > ax, affinity effect, due for instance to positive cooperativity of binding). Each of these effects would apply to both a random and an ordered system. Their separation is rather simple for the (random) T model (in which the translocation of the ternary complex is rate-limited by the translatory step): Here, the velocity effect should show also in the maximum equilibrium exchange, whereas the affinity effect should only appear in the Michaelis constant, each after the addition of Na, would follow from the following equations for equilibrium exchange in a symmetric system:

$$ {}^{ec}_{max}J_a = \frac{\bar{P}_a K_b + \bar{P}_{ab} \cdot r \cdot b}{2(K_b + r \cdot b)} \qquad \text{(D4)} $$

$$ {}^{ec}K_m = \frac{K_b + b}{K_b + r \cdot b} K_a \qquad \text{(D5)} $$

Asymmetry is ignored, as would make the equations more complicated without fundamentally changing results.

Experimentally, it has been found (Table II) that Na decreases both the maximum equilibrium exchange and the corresponding Michaelis constant. The former would indicate that Na decreases, rather than increases, the mobility of the glucose-carrier complex, and thus rules out a (positive) velocity effect. Effective coupling would, therefore, call for a strong affinity effect, which is confirmed by the diminishing effect of Na on the Michaelis constant of equilibrium exchange (Table II).

In the 'ordered' system the binary Na-complex does not form so the energetic coupling comes about only through the flow density of the ternary complex. Analyzing of the experimental data in terms of the corresponding equations for maximum rate and Michaelis constant of equilibrium exchange (D6 and D7).

$$ {}^{ec}_{max}J_a = \frac{P_a K_{ab} + P_{ab} \cdot b}{K_{ab} + b} \qquad \text{(D6)} $$

$$ {}^{ec}K_m = \frac{K_{ab}}{K_{ab} + b} \qquad \text{(D7)} $$

we obtain the same results as for the random system: A positive velocity effect on the ternary complex is excluded and a high affinity effect is confirmed. In this respect the ordered system represents a limiting case of a random system in that both K_b and r become infinite, and P_{ab} zero. It would appear that at equal conditions the ordered system provides for more effective energetic coupling than does the random system, albeit less likely to occur.

To the extent that the transfer of the ternary complex is predominantly rate-limited by the B-steps, the situation becomes more complicated. A distinction between a velocity effect ($P_{ab} > P_a$) and an affinity effect ($r > 1$) is no longer meaningful, because both depend inversely on the same parameters, namely the dissociation coefficients of the ternary complex. Hence, if Na, owing to positive cooperativity, depresses these coefficients, it will at the same time enhance the binding of glucose (affinity effect), and diminish the rate of its transfer. As a result the two effects on energetic coupling may more or less cancel. The experimental observation that Na decreases both the maximum equilibrium exchange and the Michaelis constant would not contradict this model which cannot be safely excluded so far.

The equation of K_m in equilibrium exchange is identical for both the T-model and the B-model, whether in the random or in the ordered form. Hence, the strong decrease of this K_m with increasing Na^+ may safely be taken to indicate either strong positive cooperativity between Na and substrate, or an ordered reaction. Whereas from the above results P_{ab} appears to be smaller than P_a, less can be said about the relative magnitude of P_{ab} with respect to P_0, the translocation of the free carrier. Some approximate estimate could be derived from comparing equilibrium exchange with the corresponding zero trans flow, i.e. at

maximum and vanishing concentration, respectively, of substrate for the random and the ordered model. These may approximately be represented by the following equation, under the assumption that the binary complexes are at 'quasi-equilibrium' of binding:
Random maximum rates

$$\left(\frac{^{cc}J_a}{^{0}J_a}\right)_{max} = \frac{(P_0+\bar{P}_a)K_b+(P_0+\bar{P}_{ab})r\cdot b}{2P_0K_b+2P_0\cdot r\cdot b} \quad \text{(D8a)}$$

Random minimum rates

$$\left(\frac{^{cc}J_a}{^{0}J_a}\right)_{min} = \frac{2P_0K_b+(P_0+P_b)\cdot b}{2P_0K_b+2P_0\cdot b} \quad \text{(D8b)}$$

Ordered maximum rates

$$\left(\frac{^{cc}J_a}{^{0}J_a}\right)_{max} = \frac{(P_0+\bar{P}_a)K_{ab}+(P_0+\bar{P}_{ab})b}{2P_0K_{ab}+2P_0\cdot b} \quad \text{(D9a)}$$

Ordered minimum rates

$$\left(\frac{^{cc}J_a}{^{0}J_a}\right)_{min} = 1 \quad \text{(D9b)}$$

As to the relation between $\bar{P}_{ab}$ and P_0, Eqns. D8a and D9a predict for both the random and the ordered system that the ratio of maximum equilibrium exchange over maximum zero trans flow decreases with increasing b (Na) if P_{ab} is smaller than P_0. This prediction seems to be experimentally confirmed as may be inferred from Table II, which shows that maximum equilibrium exchange decreases with increasing b (Na^+), in contrast to maximum zero trans flow, which remains rather constant under these conditions. Accordingly, $\bar{P}_{ab}$, by whichever step it may be rate-limited, seems to be smaller than P_0. It cannot be too much smaller, though, because otherwise maximum equilibrium exchange could not exceed maximum zero trans flow (Fig. 6). As to 'minimum equilibrium exchange' Eqns. D8b and D9b predict a characteristic difference in behaviour between the random and ordered systems. Experimentally, it was found that equilibrium exchange at very low substrate concentration is distinctly smaller than the corresponding zero trans flow (Fig. 6). According to Eqn. D9b this is consistent only with the random system, where it would be in line with our assumption that Na inhibits the translocation of the glucose-free carrier (v.s.). So far, it seems that $\bar{P}_{ab}$ is of similar order of magnitude as P_0; that it is smaller than P_a but greater than P_b. It should be kept in mind, however, that the precision of the experimental results may not suffice to substantiate such an estimate. In addition, the zero trans measurements, in contrast to the equilibrium exchange measurements, are affected by membrane potential changes, which are presumably small but nonetheless may somewhat distort the results.

Although cooperativity appears to play a crucial role in coupling between glucose and Na flow a quantitative estimate of the cooperativity coefficient (r) is not possible at the present time, since we do not know the value of K_b, i.e. the dissociation constant of the binary carrier Na complex (in the absence of glucose), whether it is finite or infinite, as would be the case in an ordered system in which glucose binds before Na.

Conclusions

The data confirm the hypothesis that energetic coupling in this system of Na-linked glucose transport comes about by two effects:

(1) Preventing an internal leakage ('slipping') through the glucose-free Na-carrier complex route, either by immobilizing such a complex (random system) or excluding its formation (ordered system).

(2) Promoting the translocation of glucose through the ternary complex, either by strong cooperativity between Na and glucose with respect to binding to the carrier (random system), or by restricting Na-binding to the (binary) glucose-carrier complex (ordered system).

The two effects are distinct and mutually independent in the random system, but identical in the ordered system.

The kinetic data do not indicate whether the system is random or ordered. All data are consistent with a random system, but with an ordered system only under the improbable condition that the dissociation of the binary glucose-carrier complex is slow enough to limit the overall transport rate.

The kinetic data do not indicate to which extent the various transfer routes are rate limited by translation (T-steps) or by binding and release reactions (B-steps). All data are consistent with the first alternative, but effective energetic coupling requires that the leakage pathways, i.e., those of the binary complexes, with glucose or Na, respectively, are predominantly rate-limited by their translation steps. It is probable but uncertain whether the transfer rate of the ternary complex is predominantly limited by the translation step.

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