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THE STATIC HEAD METHOD FOR DETERMINING THE CHARGE STOICHIOMETRY OF COUPLED TRANSPORT SYSTEMS

APPLICATIONS TO THE SODIUM-COUPLED D-GLUCOSE TRANSPORTERS OF THE RENAL PROXIMAL TUBULE

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The static head method for determining the charge stoichiometry (the number of moles of charge translocated per mole of substrate) of a coupled transport system is presented. The method involves establishing experimental conditions under which a membrane potential exactly balances the thermodynamic driving force of a known substrate gradient. The charge stoichiometry can then be calculated from thermodynamic principles. In contrast to the usual steady-state method for determining charge stoichiometry in cell suspensions and vesicle preparations, the static head method is applicable to systems which are not capable of maintaining a constant membrane potential over time. The charge stoichiometries of two renal sodium coupled D-glucose transporters previously identified in brush-border membrane vesicle preparations from the outer cortex (early proximal tubule) and outer medulla (late proximal tubule) are determined. The charge stoichiometries of these transporters are in good agreement with their sodium/glucose coupling ratios arguing against the possibility that glucose transport is coupled to ions other than sodium in these membranes.

Introduction

Secondary active tranport systems are used by a variety of cell types and intracellular organelles to actively accumulate or extrude certain solutes [1–10]. The transport of substrate (S) from side 1 to side 2 of the membrane by such a coupled carrier may be energized by both the transmembrane activator (A) chemical gradient, $\Delta\mu_A = (RT/F)\ln(A_2/A_1)$, and the membrane potential, $\Delta\psi = \psi_2 - \psi_1$. The degree to which a cell or organelle can drive S against a concentration gradient will depend dramatically on n_A, the activator: substrate coupling stoichiometry (the number of moles of A translocated per mole of S), and q, the charge stoichiometry (the number of moles of charge translocated per mole of S). Thus these parameters are of obvious physiological significance. In addition n_A and q are of considerable

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Abbreviation: 10 mM Tris-Hepes: 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered with Tris to pH 7.4.

interest from a more fundamental point of view since they provide essential information for determining the mechanistic aspects of the transport process.

Although several procedures have been employed to measure activator : substrate coupling stoichiometries for secondary active transport systems [11], only one method has been routinely used to measure charge stoichiometries [12–16]. This method, which is discussed in more detail below, involves determining the steady-state accumulation ratio of substrate in response to a known membrane potential and thus is only applicable to systems capable of maintaining a constant membrane potential over sufficient time that such a steady-state can be reached. The purpose of this paper is to present a method for determining charge stoichiometries which is applicable to systems regardless of whether they are capable of maintaining membrane potentials. This method is based on the 'static head' principle which has been previously employed in our laboratory to measure activator : substrate coupling ratios [17–19]. This static head procedure is ideally suited to membrane vesicle systems most of which cannot be maintained in an energized steady-state over time. The theoretical and practical details of the method are given later in the paper.

The charge stoichiometries of two renal sodium-coupled D-glucose transporters are determined here as a demonstration of the use of the static head method. These two transporters have been identified in brush-border membrane vesicles prepared from renal outer cortex (early proximal tubule) and outer medulla (late proximal tubule), respectively [17,18,20]. The outer cortical transporter is a low-affinity system ($K_m = 6$ mM) with a sodium : glucose coupling stoichiometry of 1 : 1, while the outer medullary transporter has a high affinity ($K_m = 0.4$ mM) and translocates approximately two sodium ions per glucose molecule. It has been suggested that this arrangement of sodium-coupled D-glucose transporters as a function of length along the proximal nephron allows the kidney to reabsorb glucose from the urine in an energy efficient fashion [18]. More specifically, the bulk of the glucose load is reabsorbed in the early proximal tubule by the outer cortical transporter at an energetic cost of one sodium ion per glucose molecule (the energetic cost to the cell is that required to pump the cotransported sodium back into the extracellular space) while the last traces of glucose are salvaged by the outer medullary transporter with the higher driving force and concommitant higher energetic cost associated with a 2 : 1 coupling stoichiometry. We find here that the charge stoichiometries for both these transporters are approximately equal to their sodium : glucose coupling ratios (i.e., $q = n_A$) indicating that the electric charge transferred per translocation event is that associated with the cotransported sodium ions. These results argue against the co- or counter-transport of other ions by these systems.

Experimental Procedures

Vesicle preparation and characterization

Brush-border membrane vesicles were prepared from outer cortical and outer medullary tissue from the kidneys of White New Zealand rabbits as previously described [20]. In both final vesicle fractions the activity of the brush-border membrane enzyme marker maltase (EC 3.2.1.20) was enriched approx. 12-times relative to the starting tissue homogenate, while the activity of enzyme markers for antiluminal membranes ($(Na^+ + K^+)$-ATPase, EC 3.6.1.3) and intracellular organelles (glucose-6-phosphatase, EC 3.1.3.9 and succinate dehydrogenase, EC 1.3.99.1) was less than one times that of the homogenate.

Flux measurements (rapid filtration technique)

Flux measurements were carried out as previously described [17,18,20]. The fast sampling apparatus described by Turner and Moran [20] was used for all points. The detailed composition of the various media used in each experiment are given in the figure legends. Buffer A (10 mM Tris-Hepes containing 100 mM mannitol) was used for the basis of all solutions. Valinomycin (12.5 μg/mg vesicle protein) was added to the vesicles as a stock solution of 25 mg/ml in ethanol. Tracer D-[^{14}C]glucose and L-[^{3}H]glucose were used at concentrations of 30 and 140 Ci/mol, respectively. The simultaneously measured 'uptake' of L-glucose was used the correct D-glucose uptake for

nonspecific effects such as binding and trapping by the membranes and filters. Since the vesicles are relatively impermeable to L-glucose [20] no significant flux of this ligand was detectable over the time period of the experiments (0–6 s).

Experimental points were carried out in triplicate (Fig. 3) or quadruplicate (Figs. 1 and 2) at 28°C. The error bars shown in the figures are the standard deviations on the points. The results of representative experiments are shown.

Materials

Radioactively labelled ligands were obtained from New England Nuclear Corp. (Boston. MA). Other chemicals were of the highest purity available from commercial sources.

Principle of Method and Results

Consider a secondary active transport system which is tightly coupled, i.e., translocation of either substrate or activator alone via the carrier is not possible (the case of non-tightly coupled systems is considered in the Discussion). The thermodynamic condition that there is no net flux via the transporter, or equivalently that the thermodynamic driving forces for activator and substrate fluxes are balanced, is given by

$$\ln(S_1/S_2) = \frac{RT}{F}\left[\sum_A n_A \Delta\mu_A + q\Delta\psi\right] \qquad (1)$$

Here the sum is over all activators, i.e., over all substances co- or counter-transported with S via the carrier (In our convention. n_A is positive if A is co-transported with S and negative if A is counter-transported with S.).

When all activator species are at chemical equilibrium Eqn. 1 reduces to

$$\ln(S_1/S_2) = qF\Delta\psi/RT \qquad (2)$$

In analogy with the static head method for determining activator : substrate coupling ratios [11,17,18], the principle of the static head procedure for determining charge stoichiometries is to experimentally establish a given substrate gradient then vary $\Delta\psi$ until a situation is found where Eqn. 2 holds, i.e., until there is no net substrate flux via the carrier. In our experiments $\Delta\psi$ was generated by an intravesicular to extravesicular potassium (K) gradient in the presence of valinomycin. Thus

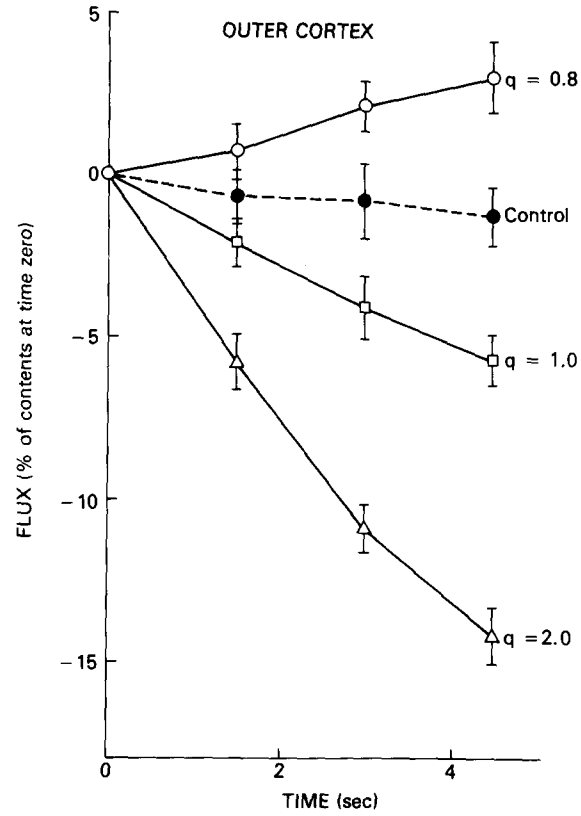

Fig. 1. The charge stoichiometry of the renal outer cortical brush-border membrane D-glucose transporter. Outer cortical brush-border membrane vesicles were prepared in Buffer A containing 120 mM K_2SO_4, 20 mM Na_2SO_4, 1 mM labelled D- and L-glucose, and valinomycin. The incubation media were such that the final extravesicular concentrations of Na_2SO_4 and labelled glucose were 20 mM and 0.25 mM, respectively, and such that the final extravesicular concentrations of K_2SO_4 were 20 mM (○——○, $q = 0.8$), 30 mM (□——□, $q = 1.0$) and 60 mM (△——△, $q = 2.0$). The incubation media were isoosmotic with the vesicles, the extra osmolarity being made up with mannitol. In the control run (●— — —●) 20 mM Na_2SO_4 was replaced by 60 mM mannitol in all media and the final extravesicular K_2SO_4 concentration was 30 mM. In additional studies (not shown) for both outer cortical and outer medullary vesicles we have established that the control run is not significantly affected by the extravesicular K_2SO_4 concentrations employed in our experiments. The q values shown on the figure are the charge stoichiometries which would be predicted were that run to result in static head conditions. The stereospecific flux of D-glucose has been expressed as a percentage of the total (equilibrium) intravesicular glucose at time zero.

$\Delta\psi$ is given to a good approximation by the potassium diffusion potential, i.e., $\Delta\psi = (RT/F)\ln(K_1/K_2)$, and Eqn. 2 simplifies to

$$S_1/S_2 = (K_1/K_2)^q \quad (3)$$

The charge stoichiometry q can be calculated directly from this equation using the values of S_1, S_2, K_1 and K_2 which result in static head conditions.

The practical details of the static head procedure can be more easily appreciated by considering an actual example. In the experiment illustrated in Fig. 1 outer cortical brush-border

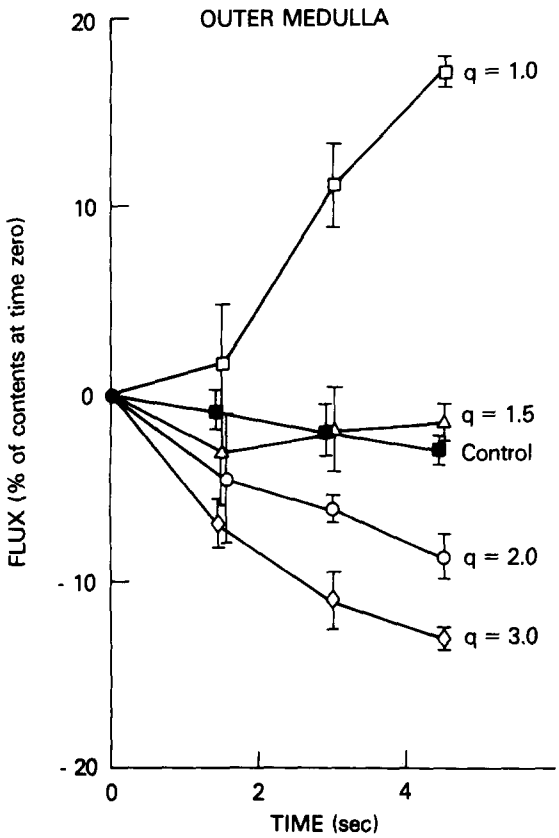

Fig. 2. The charge stoichiometry of the renal outer medullary brush-border membrane D-glucose transporter. The protocol for this experiment was identical to that of Fig. 1 except that the final extravesicular concentration of labelled glucose was 0.2 mM and the final extravesicular K_2SO_4 concentrations were 24 mM (□———□, $q = 1.0$), 41 mM (△———△, $q = 1.5$), 54 mM (○———○, $q = 2.0$) and 70 mM (◇———◇, $q = 3.0$). The control run (■———■) was carried out at 54 mM extravesicular K_2SO_4.

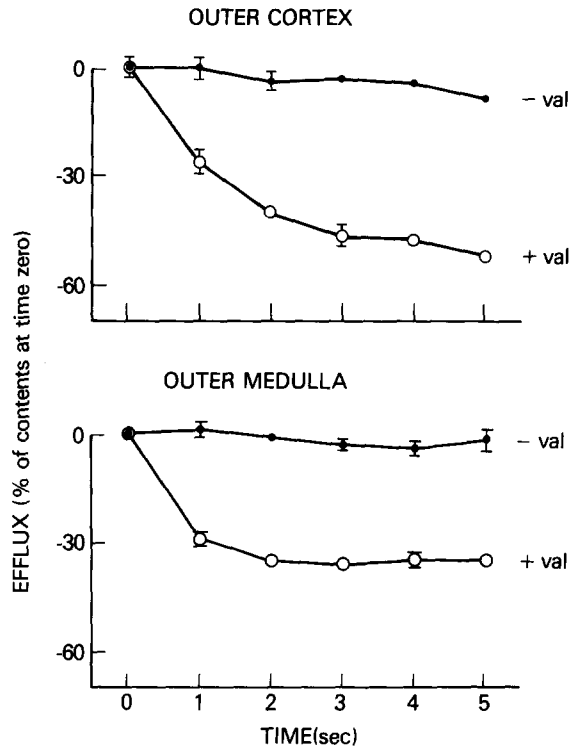

Fig. 3. The effect of a potassium chemical gradient on D-glucose flux in outer cortical and outer medullary brush border membrane vesicles. Vesicles were prepared in Buffer A containing 50 mM sodium gluconate, 100 mM mannitol and 0.1 mM labelled D-glucose. with (○———○) or without (●———●) valinomycin (an equivalent amount of ethanol was added to vesicles without valinomycin). The incubation medium was the same solution (including labelled glucose) with 100 mM mannitol replaced by 50 mM potassium gluconate. 100 μl of incubation medium was added to 20 μl of vesicles.

membrane vesicles were prepared in a medium containing 1.0 mM labelled glucose, 120 mM K_2SO_4 and 20 mM Na_2SO_4. At time zero 20 μl of these vesicles were diluted isoosmotically with 100 μl of incubation media whose composition was such that the final extravesicular concentrations of Na_2SO_4 and labelled glucose were 20 mM and 0.25 mM, respectively, and the final extravesicular K_2SO_4 concentration was 20, 30, or 60 mM. The glucose retained in these vesicles was measured as a function of time at the various K^+ concentrations (see Fig. 1). Thus the flux of glucose due to a 4 : 1 glucose concentration gradient was monitored at sodium equilibrium in the presence of membrane potentials due to 6 : 1, 4 : 1 and 2 : 1 potassium gradients. A control run was carried out in

the absence of sodium to measure efflux of glucose via unrelated sodium-independent pathways. The static head condition is characterised by that external potassium concentration which causes the test points to superimpose on the control. Here the outwardly directed potassium gradient produces a (negative inside) membrane potential which exactly balances the driving force due to the outwardly directed glucose gradient. Fig. 1 illustrates that static head conditions occur at an outwardly directed potassium gradient slightly greater than 4:1 ($q \approx 0.9$). A similar experiment for outer medullary brush border membrane vesicles is shown in Fig. 2. Here a 3:1 potassium gradient is required to balance a 5:1 glucose gradient ($q \approx 1.5$).

In the experiments shown in Figs. 1 and 2 we have assumed that potassium is not an activator of brush-border membrane D-glucose transport and thus that the only energetic contribution of a potassium gradient to sodium-dependent D-glucose flux occurs via the membrane potential. The experiments shown in Fig. 3 provide strong evidence that this is indeed the case for both the outer cortical and outer medullary preparation. Here vesicles were prepared in the presence of sodium, mannitol and labelled glucose with or without valinomycin, then diluted into the same medium with mannitol replaced isoosmotically by potassium gluconate. The figure shows that glucose flux cannot be driven by a K^+ gradient alone although it is markedly affected by a valinomycin-induced potassium diffusion potential.

Discussion

This paper presents a new procedure for determining the charge stoichiometry of coupled-transport systems. Past determinations of charge stiochiometry in isolated cell or vesicle preparations are only applicable to systems which are capable of maintaining constant membrane potential over time [12–16]. In these experiments steady-state accumulation ratios of substrate in response to known (measured) membrane potentials are determined and q is calculated as above from Eqn. 3. This procedure is impractical for systems which cannot maintain membrane potentials since in this case such a sustained steady-state never occurs. Also this method assumes that the observed substrate gradient is in thermodynamic equilibrium with $\Delta\psi$. This assumption has recently been questioned [21] since it neglects the the existence of substrate leaks via pathways other than the coupled carrier (e.g., simple diffusion). If these leaks are significant, the observed accumulation ratio of substrate will represent a kinetic steady-state between the cotransporter and the leak rather than a thermodynamic equilibrium between the membrane potential and the substrate gradient. In this case the calculated value of q will be a lower limit on the true charge stoichiometry. As emphasized above the existence of leak pathways is explicitly taken into account in the static head method.

The calculation of q from steady-state accumulation ratios is also complicated by the existence of contaminating membranes, e.g., inactive membranes or membranes which do not contain the transporter of interest. The existence of these contaminants means that measurements of the membrane potential and the substrate accumulation ratio may not represent the actual values of these quantities for the fraction of the preparation of interest. Nor will such errors necessarily compensate for one another as has been suggested. Again this difficulty is avoided in the static head method since the membrane potential is known from the initial conditions and efflux from contaminating membranes is compensated for by the control run.

Although substrate leaks via pathways unrelated to the transporter of interest are taken into account by the static head method the existence of internal leaks, i.e., uncoupled fluxes of substrate via the transporter, can complicate the interpretation of results. In this case the value of q obtained from the static head experiment is the average charge translocated per substrate molecule rather than the net charge which would be translocated if the transporter operated in a tightly coupled mode.

In addition to the thermodynamic approach (static head or steady-state accumulation ratio methods) two other methods for determining substrate : activator coupling stoichiometries (n_A) have been employed [11]. These are (i) the direct method, where simultaneously measured substate-dependent activator fluxes and activator-dependent sub-

strate fluxes are compared and (ii) the activation method, where substrate flux is measured as a function of activator concentration and the results are interpreted in terms of a kinetic model (the Hill equation is typically used).

An electrophysiological version of the direct method has been used by several authors to measure charge stoichiometry in intact epithelia [22,23], however, no attempts to measure substrate flux and charge translocation directly in cell suspensions or vesicle preparations appear to have been attempted. As outlined in Ref. 24, the determination of charge stoichiometry using the activation method would be extremely difficult if not impossible at the present time owing to the complex way in which $\Delta\psi$ enters into the kinetic equations of cotransport models.

Recalling Eqn. 1 and the associated discussion we have that

$$q = Z_S + \sum_A n_A Z_A$$

where Z_A and Z_S are the charges on A and S, respectively. In the case of a tightly coupled system this equation provides a valuable means of checking the consistency of measured values of q and n_A and of ascertaining whether all of the participating activator species have been identified. Also, in the case of multivalent ions, Eqn 4 may be used to determine which charged forms of S and/or A are transported.

The charge stoichiometries determined in this paper for the outer cortical and outer medullary brush-border membrane D-glucose transporters confirm previous observations that proximal tubular D-glucose transport is electrogenic, involving the net transfer of positive charge. We demonstrate here that the charge stoichiometries of these two transporters ($q = 0.9$ and 1.5 for the outer cortical and outer medulary systems) are in good agreement with their previously determined [17,18] sodium : glucose coupling stoichiometries (1.0 and 1.8, respectively). These results provide strong evidence that no other ions are energetically involved in these transport processes.

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