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Topical Review

Membrane Potentials and the Mechanism of Intestinal Na^+-Dependent Sugar Transport

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I. Introduction

Our current understanding of the functional characteristics of Na^+-coupled transport systems has been established with the use of a wide variety of model experimental systems and for several different solute transport systems. Different species have been utilized and work aimed at Na^+-coupled systems in tissues other than intestine, especially the renal tubule, has often been considered by analogy to point toward similar functional properties for the intestinal epithelium. This diversity of models and extrapolation of related data bases has been highly useful and is the vital force behind most scientific investigation in the biological sciences.

At the same time, the diversity of exploration methodology and resultant complexity of a unified interpretation of all intestinal transport data renders a concise summary of properties more difficult. On the one hand, it is tempting to build analogy among related systems to establish useful paradigms for further consideration. On the other hand, it must always be remembered that there will be some differences, not always subtle, when comparing data derived from different tissues or even when comparing functional properties of intestinal tissue among different species. In addition, with new insights regarding function of Na^+-coupled transport systems, earlier work may prove to lack appropriate controls, which makes it difficult to provide easy or accurate interpretation of that work in the new context.

Our review of the functional characteristics of the intestinal Na^+/sugar co-transporter is not meant to be entirely comprehensive of work in the field for the 30-year interval since Crane [9, 12] first suggested a link between Na^+ and sugar transport in the intestine. In fact, excellent reviews are available summarizing early [10, 46, 102] and later work [34, 48, 107] in the field, and the reader is referred to them for comprehensive treatment of work through 1984. Aside from the brief capsule history of the evolving conceptual aspects which follows this section, we have chosen to focus primarily on work related to the role of membrane potentials in the energetics, kinetics and mechanism of Na^+-coupled sugar transport. We have summarized a number of considerations that need careful attention before the functional properties for Na^+-coupled systems can be assessed accurately. Keeping these precautions in mind, we then summarize work in which attempts were made to circumvent the pitfalls. Our effort focuses especially on work aimed at evaluating the role which Na^+ gradients and membrane potentials play in the molecular mechanism of intestinal Na^+-coupled sugar transport. In doing so, we build in part on the review of functional characteristics presented by Semenza et al. in 1984 [107].

II. Conceptual Evolution: Energetics of Na^+-Coupled Transport Systems (1959–1980)

A. The Na^+ Gradient

In the decade following Crane's first proposal of Na^+ flux providing a thermodynamic driving force for concentrative sugar transport in intestinal tissue

Key Words sodium-dependent sugar transport · membrane potential · coupling stoichiometry · phlorizin · sugar transport · energetics of Na^+-coupled transport

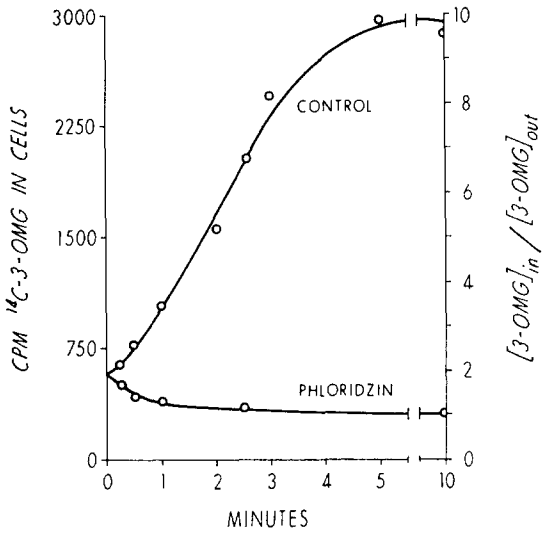

Fig. 1. Accumulation of 3-O-methylglucose by isolated chick intestinal cells with an imposed reversed Na^+ gradient (i.e., $Na_i^+ > Na_o^+$). At time zero, cells that had been loaded to 40 mM intracellular Na^+ and 2.5 mM ^{14}C-3-OMG were diluted into a medium with 20 mM Na^+ and 1.25 mM ^{14}C-3-OMG. The cells did not establish a normal Na^+ gradient ($Na_i^+ < Na_o^+$) until approximately 2 min had elapsed. (Reprinted with permission from *Biochemistry* **9**:3669–3678, copyright 1969 American Chemical Society)

[9, 12] most experimental work was aimed at confirming Na^+ dependence, substrate specificity and kinetic properties of the sugar transport system in various species and experimental model systems. Ideas relating to Na^+ being required for "active" sugar transport and its action to alter either K_m or V_{max} (or both) for these systems became established [11, 28, 102]. The role of Na^+ in "driving" transport of other organic solutes such as amino acids was established [13, 19, 125], and recognition of Na^+ coupled transport for amino acids in most living cells became recognized (first reviewed in ref. 102). The renal tubule was recognized as another site specialized for Na^+-dependent sugar transport during this same time interval [66].

Despite a growing recognition of Na^+-coupled transport as a new class of transport systems and their nearly ubiquitous occurrence in animal cells, certin fundamental "gaps" remained with respect to a complete understanding of their function. These gaps related in particular to whether the flow of Na^+ into cells down a gradient of chemical potential could provide adequate energy to account for the observed magnitude of sugar and amino acid gradients [36, 38, 44, 45, 99].

The fact that Na^+-dependent transport activity could be monitored as an electrical current flow (of Na^+) across an epithelial syncitium had been established in the mid-sixties [75, 103, 104]. This led to recognition of a possible role for an electrical driving force acting on Na^+-coupled systems, but hard evidence demonstrating this possibility was elusive. The fact that unidirectional transmural flux of amino acids across intact intestinal tissue does not change when the transmural potential is altered over a 50-mV range [21, 84] represented a serious complicating factor. This led early investigators to conclude that the thermodynamic driving force represented by the membrane potential is not manifested in the transport kinetics and therefore might not be an important factor in governing the function of Na^+-coupled transport systems. This conclusion seems to have been important in focusing early attention on transport models in which electrical driving forces were overlooked or ignored as part of the kinetic driving force acting on the system. Only later did it become clear that changes in transmural potential do not cause corresponding changes in cellular membrane potential across the brush border [25]. By then a collective "mind-set" had been established favoring mechanisms involving a single kind of driving force (i.e., ion flow down a gradient of chemical potential). It soon became clear, however, that sugar and amino acid accumulation against a concentration gradient can occur under conditions where the Na^+ chemical gradient could not have provided sufficient thermodynamic energy to explain the magnitude of the organic solute gradient [36, 38, 44, 45, 99]. An example is shown in Fig. 1. It was suggested that the flow of another ion, such as K^+, might be coupled directly to the transport system as an additional energy source [10, 17, 18]. Influx of Na^+ and efflux of K^+ on the ion-coupled transport system in equal stoichiometry would provide a model in which two ion fluxes contribute to the thermodynamic driving force with no net charge transfer and hence no thermodynamic role for the membrane potential. However, no definitive evidence favoring K^+ coupling could be established [57]. Nevertheless, some additional driving force must have been operative in these instances either to energize the Na^+-coupled transport system, or to provide active transport by a parallel pathway.

B. The Membrane Potential

No evidence for active sugar transport by any system other than those involving Na^+ dependence could be defined, however. Insight to this energetic paradox was finally provided by work with ascites cells (*see* Fig. 2) when it was shown that valinomycin-induced diffusion potentials for K^+ can enhance the magnitude of amino acid gradients established

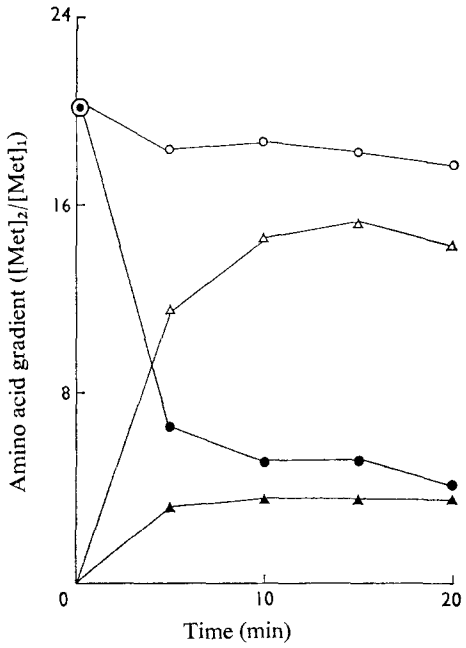

Fig. 2. Net flux of methionine in ATP-depleted ascites cells driven by imposed K^+ diffusion potentials. In two cases, the cells were loaded with methionine prior to start of the flux measurements (circles). In the other two cases, ^{14}C-methionine was added at time zero (triangles). Open symbols represent cases in which $[K^-]_i$ was much higher than $[K^+]_o$. Filled symbols are for cases where $[K^+]_i$ and $[K^+]_o$ were approximately equal. Valinomycin was present in all cases to confer high K^+ permeability. When an interior negative membrane potential was maintained the cells could either establish a methionine gradient (open triangles) or sustain the methionine gradient (open circles). A depolarized membrane potential (filled symbols) led to loss of methionine gradient-forming capacity. (From reference 91, reprinted by permission from *Biochem. J.* **140:**383–393, copyright (c) 1974, The Biochemical Society, London)

by those cells [27, 91]. Subsequent work with brush border membrane vesicles prepared from intestinal [85] and renal [6, 98] epithelial cells and with intact intestinal cells [7, 8, 54] showed that the phenomenon is common to most Na^+ coupled systems (Figs. 3 and 4). Studies of this sort leave little doubt that both chemical and electrical potential gradients for Na^+ provide thermodynamic driving forces that are manifested in the kinetic properties of the transport system. Relatively recent work by Lapointe et al. [68] and Smith-Maxwell et al. [111] demonstrates that over the physiological range of electrical potentials, the Na^+-coupled sugar transport system in *Necturus* and chick intestine exhibit a near-linear current-voltage relationship. These observations confirm the importance of the electrical driving forces as a determinant of Na^+-coupled transport kinetics and hence of the net transport capability of the system.

The flow of Na^+ down a gradient of both chemical and electrical potential (i.e., a gradient of electrochemical potential) can readily provide sufficient energy to establish the 4- to 10-fold solute gradients that had been observed for most experimental systems until that time [3]. For those instances where concentrative transport had been observed in the absence of a favorable chemical gradient for Na^+, an unrecognized favorable electrical potential must have accounted for continued sugar accumulation [38, 44, 45, 99]. Indeed, given the magnitude of Na^+ gradients and membrane potentials measured for intestinal epithelial cells under physiological conditions, it can be calculated that maximal theoretical sugar gradients as high as 30- to 40-fold can be established, for systems with a 1 : 1 coupling ratio between Na^+ and sugar fluxes, before the thermodynamic limit is reached [55, 101] (Fig. 5).

In many circumstances, a consideration of transport energetics has ended with an observation that a given sugar or amino acid gradient is within the thermodynamic limit that could be provided by the electrochemical gradient of Na^+ established by the cell population being considered [3, 36, 101]. This can lead to an inadequate exploration, not only of transport energetics, but also the details of transport stoichiometry and attendant clues relating to the molecular mechanism of transport, as described below.

C. Sugar Fluxes by Other Routes Complicate the Energetic Analysis

Inquiry occurring in the last ten years exemplifies some of the important considerations that must be taken into account before a complete analysis of Na^+-coupled transport energetics and properties can be accomplished. One of the important lessons from this interval relates to the fact that an evaluation of thermodynamic energy adequacy for Na^+-dependent flux pathways requires an understanding of cellular fluxes for the solute in question that occur by *all* cellular routes. [51]. To the extent that passive flux routes exist that are independent of the Na^+ coupled transport system, they will compromise the gradient forming capacity of those systems that otherwise act to establish a sugar gradient. The degree of compromise can be dramatic. For instance, consider a not unusual situation in which a solute is transported into a cell by an active transport system that accounts for 90% of the total influx and by a parallel passive system accounting for the remaining 10% (Fig. 6*A*). If the passive system does not show saturability at moderate solute concentrations, the cell can only establish a 10-fold concen-

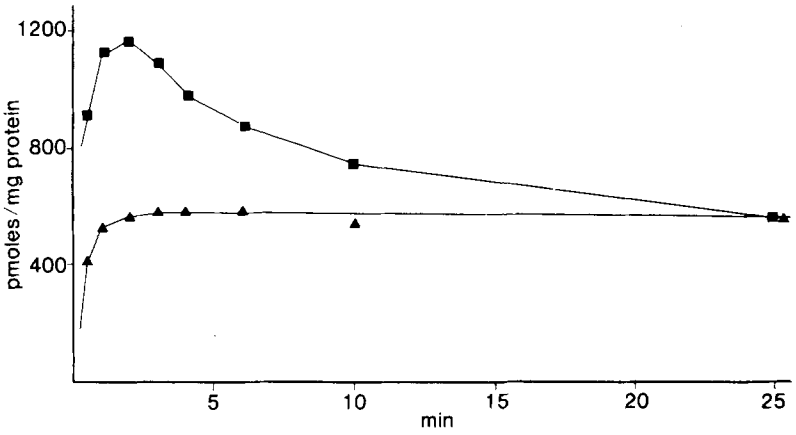

Fig. 3. Accumulation of ^{14}C-glucose induced by a K^+ diffusion potential in brush-border vesicles from rabbit intestine ($[K^-]_i > [K^+]_o$ in both cases). Valinomycin was added in one case to induce an interior negative diffusion potential (square symbols). (From reference 85, with permission)

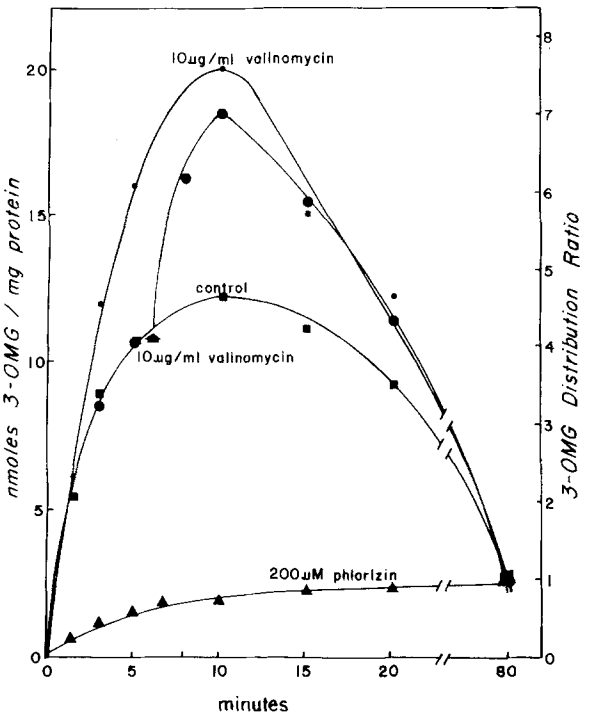

Fig. 4. Formation of 3-OMG gradients in isolated chick intestinal epithelial cells driven by valinomycin-induced K^- diffusion potentials. $[K^+]_i > [K^+]_o$ in all cases. Valinomycin was present in one case from the start of the experiment, added after 9 min in a second case, or absent in the control. (From reference 55, with permission of the publisher)

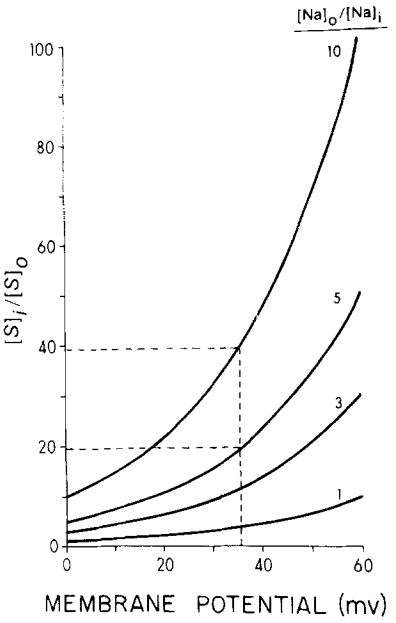

Fig. 5. Theoretical sugar gradients for a cotransport system with 1 : 1 coupling stoichiometry as a function of Na^+ gradient and membrane potential. The area bounded by the dashed lines shows the expected maximal sugar gradients for the steady-state driving forces typically maintained by intestinal epithelial cells. (From reference 47, with permission of the publisher)

tration gradient of solute before the intracellular concentration is high enough so that efflux via the passive system is equal in magnitude to influx via the active pathway (Fig. 6*B*). If any backflux is occurring on the active transport system, an even lower steady-state sugar gradient will be established. This is true regardless of the *theoretical* thermodynamic gradient forming capacity of the active transport system. This being the case, the magnitude of a steady-state cellular solute gradient is often more a function of passive, "leak" transport pathways than it is a measure of the thermodynamic driving forces acting on the active system. This is an intuitively simple lesson, but one which has often been overlooked in the history of evaluating Na^+-dependent transport energetics.

In the case of intestinal sugar transport, Na^+-dependent accumulation of sugar by the enterocyte

is opposed by passive "leaks" of sugar from the cell (i) via a serosally localized transport system that catalyzes a facilitated diffusional transfer and (ii) via a nonmediated diffusional pathway. Some sugars such as 3-O-methylglucose (3-OMG), satisfy all three flux routes as shown schematically in Fig. 7*A*. When 3-OMG is accumulated by these cells, they usually establish a 10- to 15-fold sugar gradient, at which point efflux via the two passive systems accounts for at least 90% of the total efflux [48, 49, 51, 59] (Fig. 7*B*). Inhibitors of the facilitated diffusion transport system are known (e.g., phloretin, flavones, theophylline and cytochalasin B) [58, 59, 89], any of which will block a part of the sugar efflux and allow the active transport system to create a higher steady-state sugar concentration (i.e., sugar gradient). Under some conditions with such inhibitors, enterocytes can establish 70-fold gradients of 3-OMG before a steady state is achieved [59]. Alternatively, accumulation of sugars such as α-MG that do not satisfy the serosal carrier can be evaluated. Gradients as high as 70 to 100-fold have been observed for α-MG [59]. Even in these circumstances, a high proportion of total steady state sugar efflux occurs by some route other than by backflux on the Na^+-dependent transport system. This can be demonstrated by adding phlorizin to a cell population which has established a steady state gradient of α-MG. Despite the fact that phlorizin will block Na-dependent sugar transport in either direction (i.e., influx or efflux) the α-MG is released at a rate that is nearly equal to the Na^+-dependent α-MG unidirectional influx (Fig. 8). This high rate of diffusional efflux implies that the cells would establish markedly higher sugar gradients if the outward leak could be inhibited. Because there is a good match between the observed diffusional efflux and that calculated from the measured value for intercellular sugar concentration, it is not likely that a significant fraction of the cellular sugar is bound or otherwise unavailable for efflux events. The important point to be made is that the gradient forming capacity of a cell population does not provide much insight to the thermodynamic capability of the operative active transport system except in the rather unlikely situation where other flux routes are absent. Only in the absence of any "leaks" will a Na^+-coupled transport system establish a steady-state sugar gradient that is indicative of the thermodynamic driving forces acting on the system. The magnitude of the steady-state gradient (i.e., difference in chemical potential for sugar) in the absence of leaks will be a measure of the difference in electrochemical potential for Na^+ that exists across the membrane [50, 53]. Stated differently, when "leak" pathways are operative, the observed steady-state sugar gradient

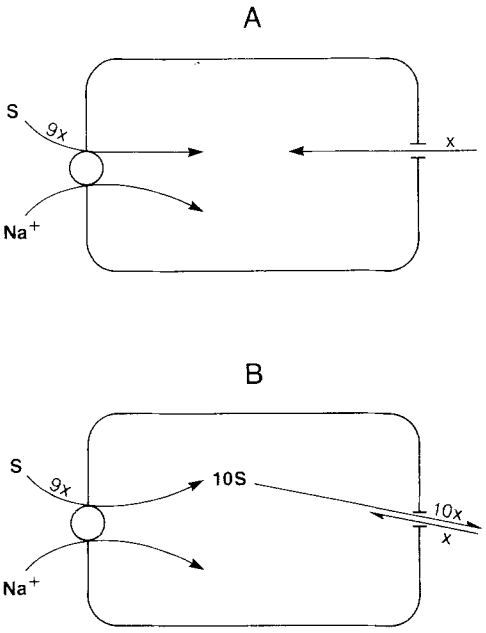

Fig. 6. (*A*) Unidirectional influx diagram for a hypothetical cell in which 90% of the solute enters via a Na^+-coupled transport system and 10% via a diffusional pathway. (*B*) Steady-state flux diagram for the same cell as shown in *A* after formation of a 10-fold solute gradient. Note that the total influx (10×) is matched by the diffusional efflux. No higher gradient can be achieved. If any efflux occurs via backflux on the Na^+-coupled carrier, an even lower steady-state gradient would be expected

must be well below the theoretical gradient one would calculate for a given coupling stoichiometry. As mentioned earlier, for a 1 : 1 coupling stoichiometry, the best estimate of $\Delta\tilde{\mu}_{Na^+}$ for enterocytes predicts maximal sugar gradients of about 30-fold [55, 101]. For the usual circumstances in which leak pathways are appreciable, observed sugar gradients of less than 30-fold do *not* imply that the Na^+ : sugar coupling stoichiometry is unity. As described below, the "leaks" can often mask a true stoichiometry that is greater than unity.

The above facts pointed to a need for careful reevaluation of Na^+-coupled transport energetics. Seventyfold sugar gradients observed for α-MG clearly are not compatible with the transport energy being derived from 1 : 1 flow of Na^+ and sugar down the $\Delta\mu_{Na^+}$ that isolated enterocytes are believed to maintain. This in turn implies that the correct coupling stoichiometry is greater than 1 : 1 or that some other transport route with greater thermodynamic capability for gradient formation is also available. Actually, the latter alternative is not an attractive one in the sense that a transport system with 1 : 1 coupling will catalyze a net efflux of sugar from the cell once the thermodynamic limit for such a system

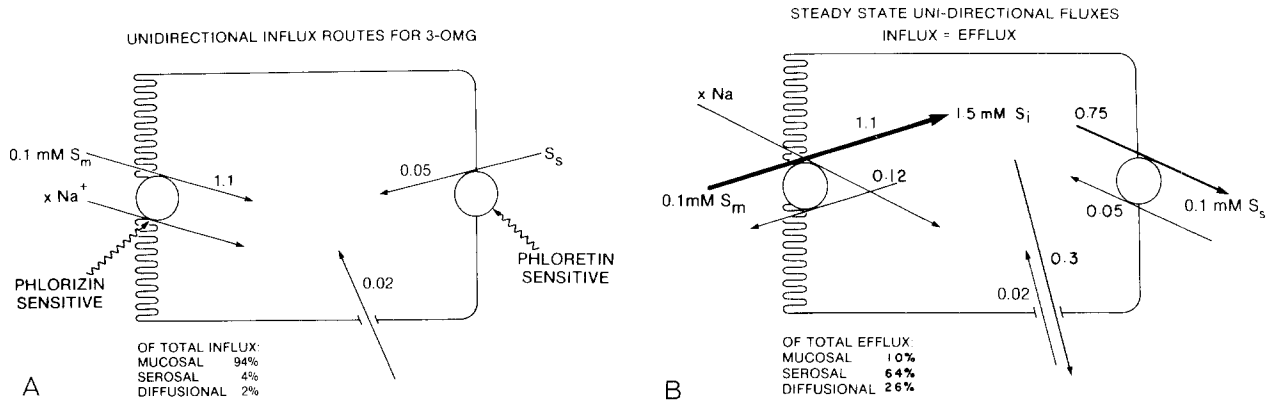

Fig. 7. (*A*) Observed unidirectional 3-OMG influx routes for isolated intestinal epithelial cells. About 6% of the total influx occurs via diffusional and facilitated diffusional pathways. (*B*) Observed steady-state sugar fluxes for the cells shown in *A*. The cells establish a 15-fold gradient for 3-OMG at which point 90% of the efflux occurs via the passive transfer pathways and only 10% via backflux on the Na^+-coupled transport system. The true gradient-forming capability of the Na^+-dependent system is severely limited by the sugar "leak" routes. (From reference 47, with permission of the publisher)

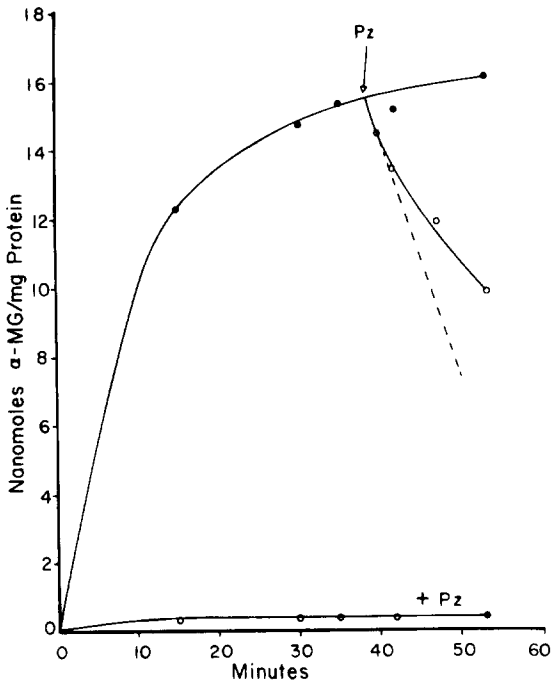

Fig. 8. Efflux of α-MG induced by addition of phlorizin to cells that had established a steady-state sugar gradient. The measured initial efflux (dashed line) represents loss of sugar by all routes other than the Na^+-coupled pathway. (From reference 61, with permission of the publisher)

has been exceeded by the gradient forming capacity of an energetically more competent system operating in parallel. It is always important to remember that two transport systems in the same cell with different energetic competency do not both contribute to solute gradient formation at a steady state that is above the energetic capability of one system. In that situation, the system with the poorest gradient forming capacity compromises the steady-state gradient in a manner similar to that described earlier for a passive leak pathway (Fig. 9*A* and *B*). It is also important to recognize that for a transport system in which a higher coupling order is possible (e.g., 2 Na^+ : 1 sugar) any flux catalyzed via a 1 : 1 transfer by the same carrier represents an "internal" leak pathway that will compromise the inherent gradient forming capability of the transport system.

III. Coupling Stoichiometry: Direct Measurements

A. The Importance of Controlling the Membrane Potential

The first attempts at measuring the stoichiometry of coupling between Na^+ and sugar fluxes by Na^+-dependent transport systems were reported for rabbit intestinal tissue in 1969 [28]. An intact tissue preparation was utilized and transmural solute fluxes were monitored. The ratio of sugar-induced Na^+ flux to Na^+-induced sugar flux was found to be 1 : 1, which was taken as an accurate measure of the transport stoichiometry. An important systematic error is inherent to many approaches in which coupling stoichiometries are determined by direct measurement of coupled solute fluxes, however. The error relates to the fact that the function of any Na^+-coupled transport system usually causes a net charge transfer across the cell membrane and a concomitant change (depolarization) in membrane potential [25, 75, 86, 97, 103, 104, 113, 128]. The

change in membrane potential is nearly instantaneous and will in turn cause a change in the rate of transfer of an ion by *any* route that is rheogenic. For Na^+, the depolarization induced by sugar transport will cause a decrease in influx of Na^+ by other rheogenic routes. Because sugar-induced Na^+ influx is measured as the difference between influx in the *presence* of sugar and basal Na^+ flux when sugar is *absent*, a problem is introduced related to the fact that the basal Na^+ flux (flux of Na^+ by all routes other than that occurring via the Na^+-dependent sugar carrier) does not remain constant when sugar is added to the system. The calculated sugar-induced Na^+ flux (total minus basal) will be underestimated because too high a basal flux value is subtracted due to the often unrecognized effect of the membrane depolarization on this parameter.

An underestimated sugar-induced Na^+ flux results in an underestimate of the true coupling stoichiometry. The more routes of rheogenic Na^+ transfer that are operative in an experimental system in which the membrane potential is unclamped, the larger the error in calculated coupling stoichiometry. For intact intestinal tissue, about 85% of the transmural tissue conductance occurs via tight junctional complexes between adjacent epithelial cells that have cation selective permeability [25]. Basal $^{22}Na^+$ fluxes are primarily paracellular through the tight junctional complexes, but occur in part via transcellular routes. Sugar transport induces a change in both fluxes due to depolarization of the epithelial cell membrane potential as well as depolarization of transmural potential across the tissue. For these reasons, measured coupling stoichiometries in intact tissue can be seriously underestimated if the cellular and transmural potential are not clamped and an *observed* value of 1 : 1 by itself implies a higher order actual stoichiometry. Values as low as 0.4 (Na : sugar) have been reported recently for intact epithelium using data uncorrected for changes in cellular or transmural potential [5].

From the foregoing discussion it should be clear than an accurate value for coupling stoichiometry can only be obtained when a correction is made for the sugar-induced change in basal Na^+ influx *or* when adequate precautions are taken to prevent any change in membrane potential from occurring during the period of flux measurement. In practice, the latter conditions are easier to achieve, and in 1980 the first report appeared in which attention was given to avoiding the systematic error otherwise associated with transport-induced changes in membrane potential [60]. That work utilized isolated chick enterocytes which were depleted of ATP and "clamped" at a potential near zero with the aid of high concentrations of K^+ on either side

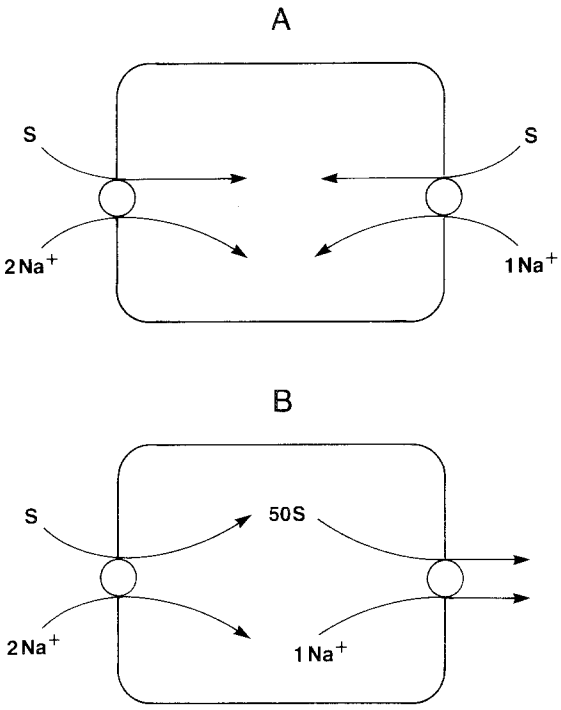

Fig. 9. (*A*) Schematic representation of sugar influx to a cell which has two Na^+-coupled sugar transport systems, one that exhibits 2 : 1 coupling stoichiometry and another with 1 : 1 stoichiometry. (*B*) Net flux of sugar via the two transport systems shown in *A* after the cell has established a 50-fold sugar gradient that exceeds the thermodynamic limit of the system with 1 : 1 coupling stoichiometry. The latter system can then only catalyze net efflux and compromise the gradient forming capacity of the system with higher order coupling

of the membrane and valinomycin added to confer a high degree of K^+ permeability. Under these conditions the K^+ equilibrium potential is the primary determinant of the membrane potential. The best possibility for creating an invariant potential during Na^+ and sugar flux measurements is to use relatively high intracellular and extracellular K^+ concentrations relative to other ions in the system (e.g., Na^+), which then dictates an experimental potential near zero millivolts (for $K_i = K_o$). Phlorizin was used as an inhibitor of the Na^+-coupled sugar transport system in order to define the magnitude of $^{22}Na^+$ and ^{14}C-sugar fluxes occurring via the sugar carrier. Ouabain was included in the incubation medium to minimize the possibility of the cellular Na^+ pump creating a membrane potential independent of that established by the K^+ equilibrium potential. Using this approach, the coupling stoichiometry in chick enterocytes proved to be 2 Na^+ : 1 sugar (Fig. 10).

A 2 : 1 coupling stoichiometry resolved any remaining energetic controversy surrounding ade-

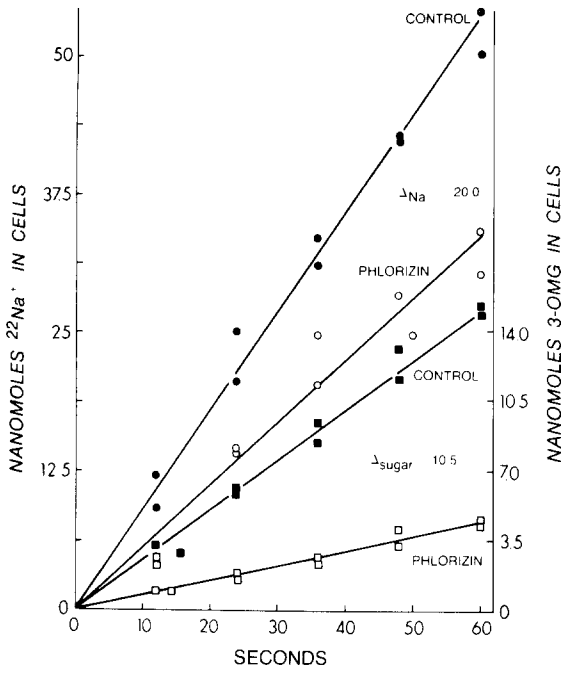

Fig. 10. Measurement of 2 : 1 coupling stoichiometry between Na^+ and 3-OMG in isolated chick intestinal cells. The cells were ATP-depleted and clamped near zero membrane potential with K^+ and valinomycin. Fluxes occurring via the Na^+-dependent transport system were identified by their sensitivity to inhibition by phlorizin. (From reference 47, with permission of the publisher)

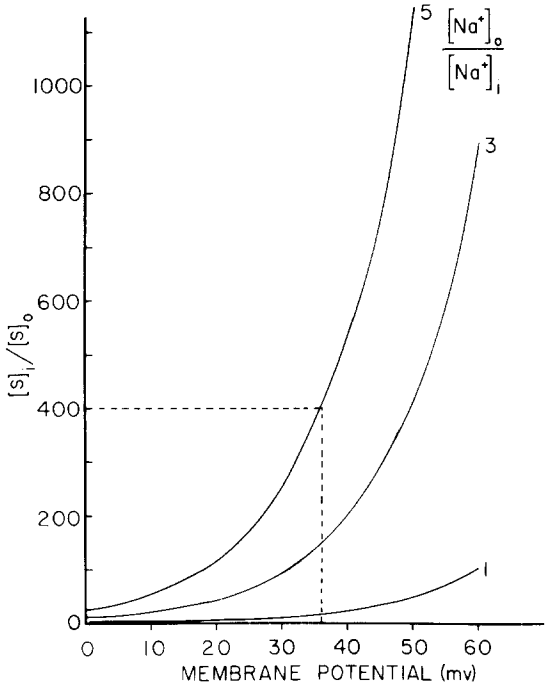

Fig. 11. Theoretical sugar gradients for a cotransport system with 2 : 1 coupling stoichiometry as a function of Na^+ gradient and membrane potential. The dashed lines indicate the expected maximal sugar gradient for the steady-state driving forces typically maintained by intestinal epithelial cells. (From reference 47, with permission of the publisher)

quacy of the $\Delta\tilde{\mu}_{Na}$ for supporting observed sugar gradients. The free energy available as an ion flows down a gradient of electrochemical potential is an exponential function of the number of ions transferred per sugar molecule. Consequently, the energy provided to a Na^+-dependent cotransport system is much greater for 2 : 1 coupling than it is for 1 : 1 coupling. The degree of amplification is dependent on the magnitude of the Na^+ gradient and membrane potential. For instance, intestinal cells maintaining a fivefold Na^+ gradient and a 36-mV membrane potential (interior negative) could establish a 400-fold steady-state sugar gradient in the absence of any "leak" pathways in contrast to the 35-fold gradient expected for 1 : 1 coupling (Fig. 11). These theoretical limits to gradient formation easily can accommodate the 70- to 100-fold sugar gradients that have been observed experimentally in several instances for this system [59, 61]. The difference between theoretical and observed gradients presumably reflects sugar "leakiness" occurring through routes that are not coupled to Na^+ transfer and which therefore compromise the gradient forming capacity otherwise achievable [50–53]. A diffusional influx that is only 1% of the Na^+-coupled influx would limit gradient formation to no greater than 100-fold (less if any backflux is occurring on the Na^+-dependent transport system).

It is important to remember that direct flux measurements of coupling stoichiometry will yield underestimates of the true stoichiometry if the membrane potential is not effectively clamped. In fact, when no attempt is made to clamp $\Delta\psi$ in ATP replete, metabolically competent chick enterocytes an apparent 1 : 1 coupling stoichiometry is measured [60] similar to that reported for various intact tissue preparations in the absence of clamping. The stoichiometries measured without voltage clamping are only circumstantially near 1 : 1, however, and it must be emphasized that they bear no meaningful relationship to the true coupling stoichiometry for the system under consideration. Nevertheless, the collective scientific mind-set has been so focused on the possibility of 1 : 1 coupling that some investigators have interpreted the *apparent* 1 : 1 stoichiometry measured without clamping as indicative of a variable stoichiometry that is unity under physiological conditions but becomes higher in the absence of a membrane potential (*cf.*, discussion of ref. 5).

In order to address the latter possibility, direct stoichiometry measurements of phlorizin-sensitive isotope fluxes in chick enterocytes have also been reported for cells clamped at an interior negative membrane potential. This was accomplished by utilizing a double diffusion potential created by an outward K^+ gradient (plus valinomycin) and an inward NO_3^- gradient. The stoichiometry remains at 2 Na^+ : 1 sugar under these conditions [62] (Fig. 12).

Stoichiometry measurements can also be used to assess whether partially loaded carrier forms can transfer Na^- or sugar across the membrane. For a system having 2 : 1 stoichiometry at physiological solute concentrations, any mobility of the ternary (NaCS) or binary (CS) carrier forms would be reflected by a decrease in stoichiometry at low Na^+ concentrations when these forms should predominate in frequency. Instead, the measured stoichiometry has been shown to remain constant at 2 Na^+ : 1 sugar over a Na^+ concentration range from 10 to 140 mM [62].

These data regarding coupling stoichiometry for chick enterocytes are more comprehensive than similar studies in other model systems. Only one Na^+-dependent sugar transport system has been implicated for these cells [93] and the data point toward a system that operates with fixed stoichiometry regardless of the concentrations of the two transported solutes or the magnitude of the membrane potential. These results tend to extend the analogy of kinetic characteristics often drawn between enzymatic and transport systems to include the concept of fixed mass action ratios for function, irrespective of the thermodynamic driving forces acting on the system.

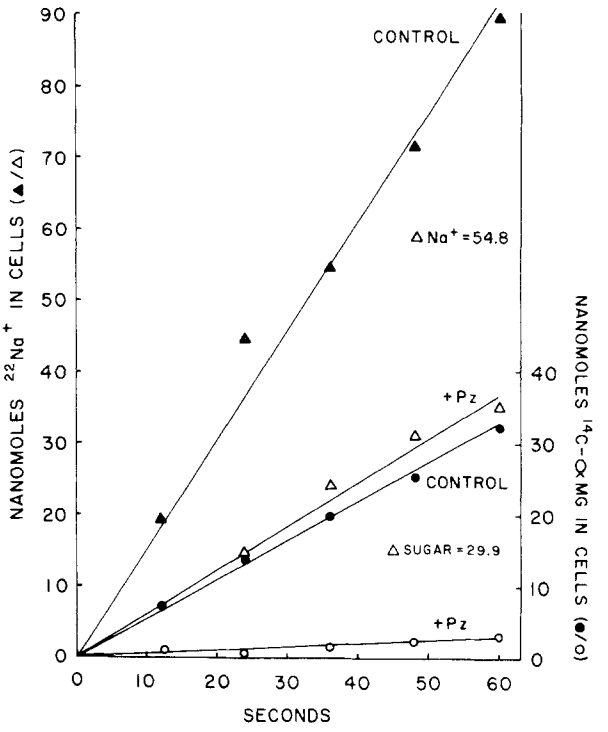

Fig. 12. Measurement of 2 : 1 coupling between Na^- and α-MG in isolated chick intestinal cells in which the membrane potential was clamped near −60 mV (interior negative) with K^+ plus valinomycin and NO_3^-. Phlorizin-sensitive fluxes were defined as those occurring via the Na^+-coupled transport system. (From reference 61, with permission of the publisher)

B. The Question of Multiple Na^+-Coupled Transport Systems

1. Intestinal Tissue—Kinetic Measurements

The question then arises as to how typical the results obtained with chick intestine are for Na^+-coupled sugar transport in other species or tissues. This question is not easy to answer because many studies do not give adequate attention to control of the membrane potential during kinetic and/or stoichiometric measurements. In addition, there is some evidence that suggests the possibility of two separate Na^+-dependent sugar transport systems functioning in intestinal tissue for certain species [16, 23, 24, 40–42] which can add further complexity of interpretation. It is clear, however, that a 2 : 1 coupling stoichiometry certainly is not unique to the chick intestine. Kaunitz et al. [40] have reported direct measurements of coupled solute fluxes in rabbit intestinal brush border membrane vesicles and concluded that two transport systems are involved, one of which requires two or more Na^+ ions coupled to the transfer of each glucose molecule. This direct measurement of coupled Na^+ and sugar fluxes is particularly noteworthy because it represents one of the only examples in which isotope flux ratios have been successful in vesicle preparations for detecting transfer of both solutes on the Na^+-dependent carrier. The authors used a relatively low Na^+ concentration (30 mM) and amiloride to limit Na^+ fluxes by the other routes. Valinomycin and K^+ was utilized to clamp the membrane potential at zero. The direct measurements were reinforced by kinetic measurements in which sugar flux at low sugar concentrations proved to be a sigmoidal function of Na^+ concentration. Best fit of the data to the Hill equation required a Hill coefficient of 1.9, which can be indicative of higher order (i.e., $> 1:1$) coupling stoichiometry. A sigmoidal J_S *vs.* [Na] relationship has often been the basis for suggesting 2 : 1 (or greater) coupling stoichiometry between Na^+ and sugar, although this kind of indirect data can have other explanations as summarized

elsewhere [93]. The Kaunitz et al. [40] data is also noteworthy in that it was derived from studies with rabbit tissue. The rabbit has been one of the prototypical animals used for investigating Na^+-coupled sugar transport since it was first utilized in the early experiments of Schultz and Zalusky [103, 104] and Goldner et al. [28]. The likelihood of a coupling stoichiometry greater than unity for rabbit intestinal tissue has also been suggested by Restrepo in experiments with isolated rabbit enterocytes [92]. The rabbit enterocytes establish a steady-state α-MG gradient of 90-fold, which is thermodynamically incompatible with 1 : 1 coupling for the $\Delta\mu_{Na}$ reported for such cells *in situ*. This fact, by itself, points toward 2 : 1 (or greater) coupling in the rabbit.

Measurements of 2 : 1 coupling in intestinal tissue are not restricted to rabbit and chicken enterocytes. Kaunitz and Wright [41] have reported direct measurements of coupling stoichiometry in the 2.0–2.3 range in brush border vesicle preparations from bovine intestine. A coupling ratio greater than unity was also suggested by kinetic measurements relating to sigmoidicity of the sugar flux *vs.* Na^+ concentration relationship in bovine vesicles. Again, the authors concluded that two different transport systems are operative in this tissue, only one of which exhibits higher order stoichiometry. We will return to the implications related to the stimultaneous function of two systems with differing stoichiometry in terms of the reported kinetic parameters for each in a later section.

Lapointe et al. [68] have concluded that Na^+ : sugar coupling stoichiometry in *Necturus* intestine is 1 : 1. Their conclusion was based on electrophysiological measurements in which the reversal potential of the Na^+-coupled sugar transport system was determined. This was defined as the membrane potential at which no phlorizin-sensitive current could be detected when galactose was present in the bathing medium. The measured reversal potential allowed them to calculate the intracellular sugar concentration at which the chemical and electrical driving forces acting on the transport system were in equilibrium and to compare these values with intracellular sugar concentrations measured in steady-state uptake experiments utilizing isotopic galactose. Their measured intracellular galactose concentrations were 50–100% higher than the theoretical limits calculated from several potential measurements at two different extracellular galactose concentrations, if 1 : 1 coupling was assumed. The authors attempted to explain this discrepancy on the basis of galactose having been present at both surfaces of the tissue so that serosal efflux was decreased and the measured tissue galactose concentration was higher than would have otherwise been observed in the absence of serosal sugar. This is not an adequate explanation. Serosal efflux was still occurring and will compromise the gradient forming capability of the serosal Na^+-dependent transport system as summarized earlier. This in turn means that in the complete absence of serosal efflux the thermodynamic limit to sugar gradient formation for 1 : 1 coupling would have been exceeded by an even greater factor than the twofold multiple already noted by the authors. For these reasons, one must conclude that the Na^+ : sugar coupling stoichiometry for *Necturus* intestine exceeds unity. If the transport system in *Necturus* transfers an integral number of Na^+ ions with each sugar molecule, then the reported data require a coupling stoichiometry of at least 2 Na^+ : 1 sugar.

A coupling stoichiometry greater than unity has also been reported for sugar transport in cestode integument [90], for succinate or citrate transport in renal tubule brush borders [136], for alanine transport in marine invertebrates [112] and toad oocytes [39], for aspartate/glutamate transport in intestine [129, 130] renal [26] and *Halobacterium* cells [67], and for proline transport in rabbit intestine [113]. These collective observations suggest that 2 : 1 coupling for Na^+-dependent transport systems occurs in many different tissues and for a variety of organic solutes. For intestinal sugar transport, the weight of evidence favors 2 : 1 coupling in all or most instances where careful attention has been given to controlling and evaluating all of the relevant parameters. It should be noted, however, that 1 : 1 coupling has been carefully documented using reversal potential measurements for alanine transport in pancreatic acinar cells [73]. To our knowledge, similar work for evaulating the Na^+ : alanine coupling stoichiometry in intestinal tissue has not been reported. Part of the difficulty in evaluating the magnitude of coupled Na^+ and alanine fluxes is the lack of a specific inhibitor for the transport system catalyzing the coupling. Further details regarding renal transport stoichiometry are given in the following section.

2. Renal Systems—The Static-Head Procedure for Measuring Coupling Stoichiometry

Other reports of 2 : 1 coupling stoichiometry for sugar transport have been derived from renal tissue. These studies were pioneered by Turner and Moran who introduced a static-head technique as a new approach for measuring cotransport stoichiometry [118]. This procedure involves loading a brush border vesicle (or cellular) preparation to equilibrium with Na^+ and ^{14}C-sugar. The loaded vesicles are

then diluted into a medium such that extravesicular ^{14}C-sugar concentration is lower and the extravesicular Na^+ concentration is higher than the respective intravesicular concentrations. At the "static head" for the imposed Na^+ and sugar gradients there will be no net flux of ^{14}C-sugar via the Na^+-dependent carrier because the difference in chemical potential for sugar across the membrane will be equal in magnitude and opposite in sign to the difference in electrochemical potential for Na^+ acting on the sugar carrier. Because the $\Delta\tilde{\mu}_{Na}$ acting on the carrier is a function of the coupling stoichiometry, the Na^+ gradient imposed at the "static head" can be used to calculate the stoichiometry. In thermodynamic terms, at the static head

$$\Delta\tilde{\mu}_{Na} = \Delta\mu_S \tag{1}$$

or

$$n\left(RT \ln \frac{Na_o}{Na_i} + F\Delta\psi\right) = RT \ln \frac{S_i}{S_o} \tag{2}$$

where n is the coupling stoichiometry, $\Delta\psi$ is the membrane potential, R is the ideal gas law constant, T is the temperature in °K, i and o represent intracellular and extracellular concentrations for Na^+ and sugar (S), respectively. Operationally, it is very important to keep the membrane potential from changing during the trial dilutions into media with various Na^+ concentrations. For this reason the potential must be effectively clamped just as it is in the case of direct flux measurements of coupling stoichiometry. If $\Delta\psi$ can be clamped at zero, the algebraic relationship simplifies to

$$n = \frac{\ln\left(\frac{S_i}{S_o}\right)}{\ln\left(\frac{Na_o}{Na_i}\right)} \tag{3}$$

at the static head.

In practice, one experimental run is done with no Na^+ in the system but with the usual intravesicular sugar concentration and dilution factor in order to impose the standard S_i/S_o used for the trial runs with Na^+. Efflux of sugar in the case without Na^+ defines the loss of sugar by passive flux pathways unrelated to the Na^+-dependent transport system. One then tests for the experimentally imposed Na^+ gradient that will give the same rate of sugar efflux as observed in the absence of Na^+. Too high a Na^+ gradient causes net influx on the Na^+-dependent system, and cellular sugar loss will be too slow relative to that observed for Na^+-free conditions. Conversely, a Na^+ gradient that is too low will cause too high an efflux due to backflux of sugar on the Na^+-dependent carrier in addition to that occurring by passive flux routes. If the membrane potential is not effectively clamped, imposing the inward Na^+ gradient will create a Na^+ diffusion potential that is depolarizing (i.e., interior more positive). This in turn would require that a higher Na^+ gradient be maintained to achieve the static head than if the potential had remained constant. The net result would be an underestimate of coupling stoichiometry, just as is true for direct flux stoichiometry measurements for cases where the potential is not clamped.

Theoretically, the static-head approach offers the possibility of measuring coupling stoichiometry for high affinity-low capacity transport systems where direct measurements can be problematic because the sugar-coupled Na^+ flux is small relative to total Na^+ flux by all routes. The limitation to static-head stoichiometry measurements occurs when Na^+-coupled sugar fluxes are small relative to sugar fluxes by passive routes. For this reason, it is valuable to use α-MG as a test sugar because it is not a suitable substrate for the passive facilitated diffusion sugar transport system associated with the basolateral boundary of epithelial cells [61]. The static-head approach requires that passive influx of sugar by routes other than the Na^+-dependent system remain constant with and without Na^+ in the medium. It also assumes that the Na^+-coupled system is totally inoperative in the absence of Na^+. While this has not been evaluated for most tissues, it seems to be true for chick enterocytes [62], and the assumption may be generally valid as discussed earlier in analogy with enzymatic systems. Neither direct nor static-head approaches work when two or more Na^+ coupled transport systems with differing stoichiometry are operative in the same cell type, unless one system can be selectively inhibited.

By utilizing static-head methodology, Turner and Moran [119] have identified two different Na^+-coupled sugar transport systems in two separate segments of the rabbit renal tubule. One is a high-capacity, relatively low-affinity system (K_m = 6 mM; 10 nmol · min^{-1} · mg $protein^{-1}$) that is localized predominantly in brush border membranes from epithelial cells derived from the outer cortex (early proximal tubules) of rabbit kidney. It has a Na : sugar coupling stoichiometry of 1 : 1 [118]. The other system is a high-affinity, low-capacity carrier (K_m = 0.35 mM; V_{max} = 4 nmol · min^{-1} · mg $protein^{-1}$) that is localized in the outer medulla (late proximal tubules) of the kidney [119]. The medullary transport system exhibits a 2 : 1 coupling stoichiometry. This system also differed from the cortical system in having a binding site with nearly 35-fold lower

phlorizin affinity (51 *vs.* 1.5 μM) [120]. There is also a high-affinity phlorizin binding site in vesicles from the renal medullary epithelium, but it does not seem to be an integral part of the sugar transport system with 2 : 1 coupling stoichiometry [120]. Turner and Moran's work represents the most rigorous documentation of 2 Na^+ : 1 sugar coupling stoichiometry for a part of the renal sugar transport capability in terms of careful attention to control of the membrane potential and choice of methodology. Moran et al. [82] and Lever [74] have reported 2 : 1 coupling stoichimetry for a cultured renal cell line (LLC-PK_1 cells), but their interpretation was based on a sigmoidal relationship between sugar flux *vs.* extracellular Na^+ concentration. Such sigmoidicity can be characteristic of 1 : 1 coupling under certain circumstances [93]. In particular, it is impossible to discriminate between sigmoidicity relating to cotransport of two or more interacting Na^+ ions (i.e., interaction between binding sites) versus a situation where Na^+ interacts with the system, but is not transported (e.g., a Na^+activation site in addition to a transport site). Misfeldt and Sanders [77, 78] concluded that LLC-PK_1 cells have a coupling stoichiometry of 2 Na^+ : 1 sugar on the basis of a comparison of transmural fluxes of Na^+ and glucose across a filter bearing a confluent layer of the cultured cells. This approach is less satisfactory in that glucose transported across the brush border boundary will not necessarily be released at the serosal boundary in the same proportion to Na^+ as the flux ratio occurring during mucosal Na^+-sugar cotransport. The authors could find no evidence for release of glucose metabolites by their cultured cells so they concluded that metabolism of glucose was not a serious problem in terms of leading to ambiguity in interpretation of their results. However, intestinal epithelial cells are known to have a very high glycolytic activity [14, 43]. Metabolites of glucose would presumably be retained by the cell due to their ionic character so it is unlikely they would be detected in the suspension medium. Also, these authors made no attempt to control transmural or cellular potential during the flux measurement despite a low "tissue" resistance (~200–600 Ω) indicative of leaky tight junctional complexes that would allow large uncoupled Na^+ fluxes. Nevertheless, 2 : 1 coupling for LLC-PK_1, cells seems to be the true stoichiometry as evidenced by the kinetic characteristics and the magnitude of α-MG gradients observed in the careful experiments of Amsler and Cook [1, 127] and Moran et al. [82, 83]. This stoichiometry suggests that LLC-PK_1 cells express only the sugar transport system that is chracteristic of the late proximal tubule (i.e., outer renal medulla).

3. Further Considerations Regarding Discrimination Between One or Two Na^+-Coupled Sugar Transport Systems in Intestine

For the most part, "static-head" measurements of coupling stoichiometry have not been reported for intestinal tissue. An exception to this generalization originated in Quammes's laboratory for membrane vesicles prepared from rat tissue. Using KSCN and valinomycin to clamp the potential, Freeman and Quamme [24] described static-head experiments that were indicative of a coupling stoichiometry greater than 1.5. Because kinetic experiments had suggested the likelihood of at least two separate Na^+-dependent transport systems for their vesicle preparation they evaluated the coupling stoichiometry over a range of sugar concentrations that would allow transport predominantly by one or the other system, depending on the concentration chosen. In every case they concluded that the stoichiometry is greater than unity. These observations, along with those already summarized, point toward the likelihood that the primary Na^+-coupled sugar transport system in intestinal tissue is one with a 2 : 1 coupling stoichiometry. This likelihood brings to mind an early suggestion by Scriver et al. [105] in which they suggested that a low capacity renal sugar transport system is similar to the predominant sugar transport system expressed in the intestine. They proposed that glucose-galactose malabsorption syndrome represents a genetic defect in this system, which causes consequent pronounced defects in gut sugar absorption, but minimal effects on renal sugar transport. Conversely, familial glycosuria represents a genetic defect in the predominant renal sugar transport system, but this syndrome has little or no effect on intestinal sugar transport. The genetic evidence therefore suggests that glucose-galactose malabsorption reflects a malfunction of a sugar transport system with 2 : 1 coupling stoichiometry while glycosuria is a result of improper function of the 1 : 1 coupled renal system.

Semenza [107] and Hopfer [35] have reviewed the information pointing toward two separate intestinal sugar transport systems, and we will not repeat their summary. A precaution will be added. Two systems are often suggested on the basis of kinetic studies in which sugar flux is monitored as a function of Na^+ or sugar concentrations. The best fit to the data obtained is often an algebraic relationship with two Michaelis-Menten terms suggesting transport via parallel saturable systems for which separate K_m and V_{max} values can be calculated. Consider, however, that the kinetic data is often collected with no attempt to clamp the membrane

potential at a fixed value over the Na^+ and sugar concentration range studied. In other cases an attempt is made to clamp the potential, but there is no independent means of assessing that the clamp is successful for the entire range of experimental conditions evaluated.

Consider further that Na^+-coupled sugar transport itself tends to depolarize the membrane potential, and, to the extent that depolarization occurs, transport is self inhibitory. Alternatively, changing the Na^+ concentration over a wide range can induce diffusion potentials that alter the magnitude of Na^+-coupled sugar flux. Finally, different degrees of Na^+ loading of vesicle or cellular systems can induce pronounced transinhibition effects on sugar influx. Loading of the intravesicular or intracellular compartment will occur at a rate that is dependent on the extracellular Na^+ concentration employed so that the degree of transinhibition will vary when studied over a wide range of Na^+ concentrations. For all these reasons it is possible to have a single transport system provide kinetic information that may suggest simultaneous function of two or more separate systems.

An example is illustrated in Fig. 13. In this example a single Na^+-coupled transport system, like that described for the chick enterocyte, is considered to be the only route of sugar entry to the cell. The system is highly dependent on membrane potential. If sugar flux is studied at a fixed physiological membrane potential ($\Delta\psi = A$), a saturable flux *vs.* sugar concentration relationship like that shown in the top panel of Fig. 13 would be obtained. At a lower membrane potential ($\Delta\psi = B$) (less interior negative), the V_{max} for transport remains constant but the K_m shifts to a markedly higher value (lower line in the top panel of Fig. 13). The middle panel of the figure illustrates a situation in which control of the membrane potential is inadequate over the range of sugar concentrations studied, but instead shifts from value *A* at low sugar concentrations to value *B* at higher concentrations. The flux relationship that would be found experimentally under these conditions is given in the lower panel of Fig. 13. At low sugar concentrations, where the potential was maintained, a relationship similar to that given by the early part of curve *A* will be obtained. As the potential diminishes at higher sugar concentrations, however, the sugar influx will rise more slowly and finally take on the appearance of curve *B*. The best fit to the observed flux relationship is the summation of two separate Michaelis-Menten type relationships similar to those shown in the top panel. The interpretation of the observed fit would be that two transport systems had been operative

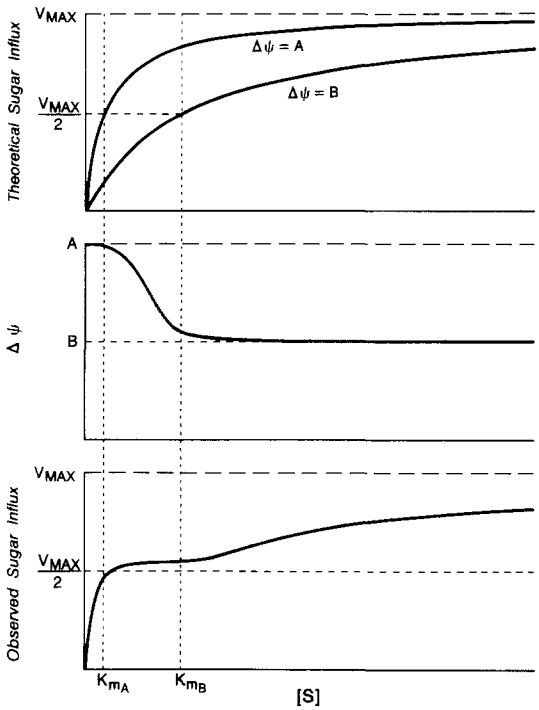

Fig. 13. *Top panel*: Expected flux *vs.* sugar concentration relationships for a Na^+-coupled system that obeys Michaelis-Menten kinetics. The relationship is shown for two different values of membrane potential (*A* and *B*) and it is assumed that the potential has an effect on the K_m of the system, but not the V_{max} (*see* reference 95). For each individual curve, the potential is assumed to be constant. *Middle panel*: The effect of sugar concentration on the membrane potential maintained by a cell population. It is assumed that the potential depolarizes from value *A* to value *B* due to entry of Na^+ on the Na^+-dependent sugar carrier. *Lower panel*: Expected relationship between sugar flux and sugar concentration for a single Na^+-coupled transport system when the change in membrane potential shown in the middle panel is taken into account. Note that the flux relationship for the transport system is now similar to that which would be expected for function of two separate transport systems with differing K_m

rather than a single potential-dependent system with an unrecognized change in potential induced by the spectrum of experimental conditions. Similar arguments can be made if Na^+ is the variable substrate. To the extent that diffusional flux routes are also operative (as is always the case), resolution of the kinetic data will be even more complex leading to a higher likelihood that a single transport system will be mistakenly interpreted as two (or more) systems.

It is usually not possible to decide whether kinetic data indicating the presence of two separate systems operating in parallel is in reality a reflection of a single system with inadequate control of the membrane potential during the flux measurements.

Table 1. Effect of Na^+ concentration and membrane potential on phlorizin binding to rabbit renal microvillus membranes[a]

[Na^+]	[Anion]	Phlorizin bound
0	(none mannitol)	0.71
120	SO_4^{2-}	7.87
120	Cl^-	10.92
120	SCN^-	14.38

[a] Data taken from reference 4, with permission.

We call attention to the latter possibility because of the relative scarcity of reports in which careful attention is given to control the potential or where a stable potential can be verified independently.

IV. Phlorizin Binding

A. General Properties

Phlorizin, a glycoside produced by certain species of the apple family, is a potent, highly selective, competitive inhibitor of the Na^+-coupled sugar transport system in intestinal and renal tissue [15, 108]. Because its potency is indicative of a high affinity interaction with the Na^+-sugar transporter, it is logical to expect that phlorizin might be a useful tool for selective identification of the carrier protein. Phlorizin binding has been used as a measure of the number of sugar carrier molecules (i.e., transport sites) associated with a particular cell or membrane preparation. Early experiments of this type were instrumental in showing that phlorizin binding is Na^+ dependent (Table 1) and that transported sugars compete for the site to which phlorizin is bound [109]. The binding affinity for phlorizin is markedly enhanced by Na^+, but the binding capacity is not changed. These observations suggest that phlorizin is binding to the sugar site on the carrier and are consistent with other observations showing that, in general, Na^+ increases the sugar binding affinity of the carrier (i.e., decreases the K_m for sugar transport) [16, 41, 93]. Recent work from Wright's laboratory using fluorescent molecules that bind to the transport protein indicate that Na^+ induces a conformational change in the carrier [87, 88. 132, 133]. Collectively, these observations suggest that Na^+ induces a specific carrier conformation that has a higher affinity than the Na^+-free conformational state for sugar molecules.

B. Carrier Turnover Number

By comparing the capacity of high affinity phlorizin binding sites to the V_{max} for sugar transport, one can calculate the turnover number for the Na^+-coupled sugar transport system. Such calculations typically provide a value between 5–125 per second depending on the temperature and solute concentrations employed during the influx measurements [74, 92, 96, 107, 133]. Values at the high end of the range are characteristic of measurements at 37°C [74, 93], while the lower values usually reflect flux measurements taken at room temperature [96, 107, 133]. Phlorizin binding that is specific to the Na^+-sugar cotransporter is usually identified by the Na^+-dependence, high affinity (K_D = 0.1–1 μM), and/or inhibition by high concentrations of glucose [4, 96, 115]. The magnitude of the turnover number for the cotransport system calculated in this manner is similar to values calculated for Na^+-dependent alanine transport in pancreatic acinar cells [37], the H^+-coupled galactoside transport system in intact *E. coli* cells [135], and in membrane vesicles prepared from the same organism [134]. Such low values are more consistent with those that might be expected for rate-limiting conformational events in a macromolecular system [30] than for diffusion of solutes via a membrane pore or channel that typically exhibit much higher values (10^6–10^8 sec^{-1}). The observation that temperatures below the lipid phase transition temperature inhibit the Na^+-coupled transport systems suggests the occurrence of conformational events in a membrane-embedded component rather than channel-like properties, also. Nevertheless, the Na^+-dependent sugar transporter has certain properties characteristic of channels, as will be discussed in more detail in a later section.

C. Effects of the Membrane Potential: Carrier Translocation *vs.* Na^+-Well Concepts

A notable characteristic of phlorizin binding to either intestinal or renal epithelial preparations is that it exhibits a marked dependence on the magnitude of the membrane potential [4, 74, 96, 114, 115, 116, 122]. Interior negative potentials induce two- to threefold greater phlorizin binding than when no potential is imposed. The usual interpretation of such results is to assume that phlorizin is "titrating" a carrier conformation in which the sugar binding site is accessible to extracellular (or extravesicular) phlorizin molecules. Phlorizin is a relatively large, hydrophilic molecule, which is believed to be rela-

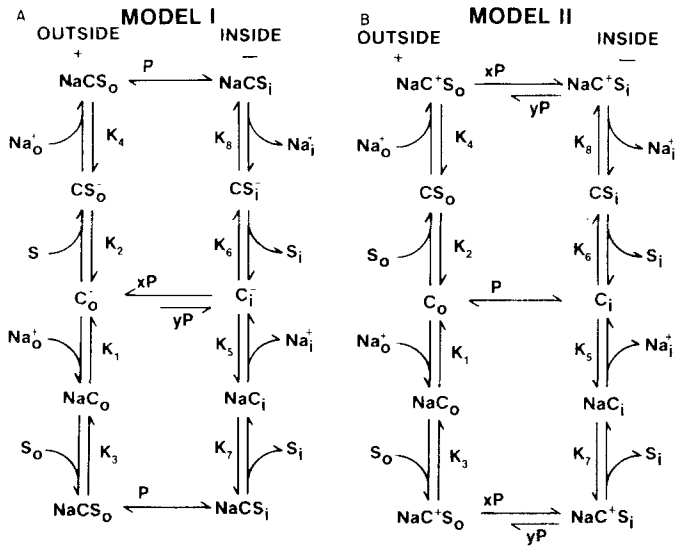

Fig. 14. Two models for Na^+-coupled transport systems with 1 : 1 coupling stoichiometry in which translocation of the carrier is potential dependent. In *Model I* the free carrier is presumed to bear a single anionic charge and to respond to the potential. In *Model II* the free carrier is presumed neutral, but the Na^+-loaded carrier now bears a charge and is potential responsive. (From reference 54, with permission of the publisher)

tively impermeant to the membrane bilayer. In the absence of a potential, it is proposed that sugar (phlorizin) binding sites on the carrier have a predominant orientation that is toward the interior of the membrane bounded compartment and which are therefore "shielded" from access. Interior negative potentials are envisioned as inducing a transition or "translocation" of these sites to an outward-facing orientation or conformation. This conceptual idea is usually depicted by models in which the rate-limiting step for transport is designated by a potential-dependent translocation event as shown schematically in Fig. 14. The general concept is an important element in the potential-dependent "gated-channel" model described by Kessler and Semenza [42, 116], which itself originated in part from their early work describing the potential dependence of phlorizin binding. They proposed an anionic "gate" as a part of the carrier protein that can change orientation in response to changes in the polarity and magnitude of the membrane potential as shown in Fig. 15. Because sugar transport can be inhibited by DCCD under conditions indicative of chemical reactivity with a carboxyl group, it was proposed that a carboxyl residue might be an integral part of the potential-responsive gate or translocation event [126]. The DCCD-sensitive site is not protected by high Na^+ concentrations, but the authors still favored the carboxylate group as playing a role in the Na^+ binding site. Turner [117] has identified a second type of carboxyl site in the renal Na^+-dependent sugar transporter that is protected by sugar or phlorizin, but not by Na^+. This carboxylate therefore seems related to function of the sugar binding site. It is always tantilizing to speculate that anionic sites might be instrumental in allowing the Na^+ cation to bind to the sugar carrier (i.e., be part of the Na^+ "site" or part of the access route to such a site), and this idea is an important aspect of the "gated-channel" model [42] even though the experimental evidence provides no direct support for a Na^+ site role for the carboxylate.

These concepts should be considered in conjunction with certain other observations relating to phlorizin binding. For instance, Aronson [4] and Toggenburger et al. [116] have studied details of the membrane potential and Na^+ concentration dependence of phlorizin binding by renal and intestinal brush border membrane vesicles, respectively. They showed that binding of phlorizin is dependent on both the membrane potential and on Na^+ concentration while phlorizin dissociation is Na^+-dependent but potential independent. Two possibilities were considered in order to explain the potential dependence of binding. The first is one we have already summarized regarding a possible potential-dependent conformational change (translocation) of the carrier to an outwardly oriented form which can then bind Na^+ and phlorizin [116]. The other envisioned a possible role for the potential in promoting Na^+ binding to the carrier. This "Na^+ well" concept is analogous to the "H^+-well" concept that Mitchell [79, 80] and Maloney [76] have proposed to describe the molecular basis of chemiosmotic energy coupling events occurring during oxidative phosphorylation. The Na^+-well alternative was considered but ruled out by Aronson [4] on the basis of his observation that indicated that debinding of phlorizin from the carrier is Na^+ dependent, but potential independent (*see* Fig. 16). He

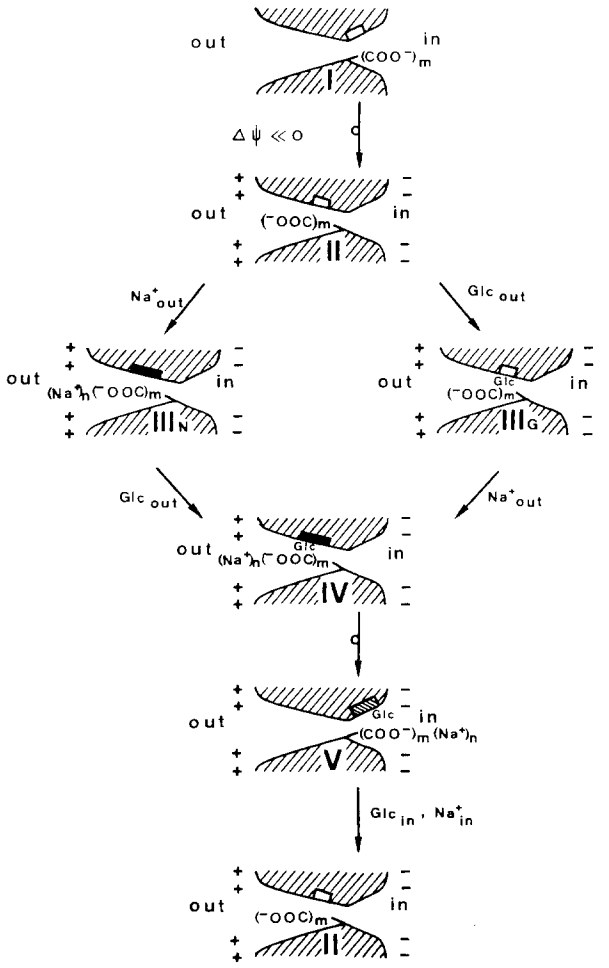

Fig. 15. Schematic representation of the potential-responsive "gated channel" model of Kessler and Semenza (from reference 42, with permission). The "gate" is presumed to be a part of the membrane spanning portions of the transport protein and to undergo a conformation alteration from inward-facing to outward-facing states in response to an interior negative potential

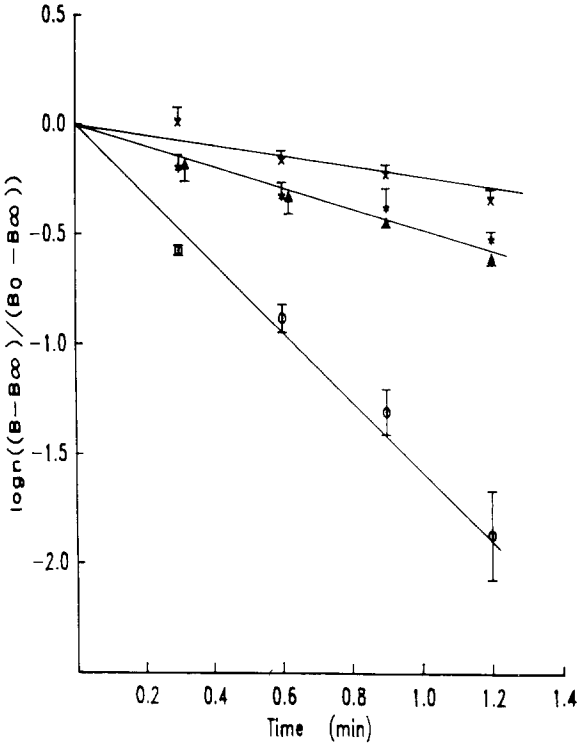

Fig. 16. Dissociation of phlorizin from chick enterocytes at different values of membrane potential or Na^+ concentration. Rapid dissociation occurred at 5.4 mM Na^+ (○—○). At 300 mM Na^+, dissociation was slow (×—×). The other two lines show dissociation for two different membrane potentials (▲—▲ = $\psi <$ 0; *—* = ψ = 0) at a Na^+ concentration of 152 mM. (From reference 96, with permission)

reasoned that potential-dependent Na^+ binding would require debinding of Na^+ to be also potential-dependent. Because phlorizin debinding is not potential dependent (even though it is dependent on Na^+ concentration) it was impossible for Aronson to defend the Na^+-well concept, and he opted for a potential-dependent translocation model [4].

It is noteworthy that the Aronson observations were interpreted at a time when the coupling stoichiometry between Na^+ and sugar was believed to be 1 : 1. Because it is unclear which portion of the renal tubule he utilized in his vesicle preparation, it is also unclear what stoichiometry should be considered when interpreting the data. A hyperbolic relationship between phlorizin and Na^+ concentration was taken as an indication of 1 : 1 coupling, but this relationship can also be consistent with certain models involving 2 : 1 coupling (*cf.*, ref. 93). For instance, if only one Na^+ need bind prior to phlorizin binding then a hyperbolic binding *vs.* Na^+ relationship could occur. Indeed, a number of investigators have deduced a Na^+-first, phlorizin-second binding sequence on the basis of the *rate* of phlorizin binding being Na^+ dependent [4, 78, 116]. Semenza et al. [107] have even suggested the possibility that phlorizin might be an inhibitor because it binds after one Na^+ and *prevents* the binding of the second Na^+ (for a 2 : 1 coupled system). Lever [74] came to a similar conclusion in studies with LLC-PK_1 cells. The partially occupied carrier was considered to be incapable of further reactivity, other than dissociation of bound solutes.

With these ideas in mind, Restrepo and Kimmich [96] evaluated phlorizin binding in the chick enterocyte, a model system with known 2 : 1 coupling stoichiometry and for which there is no evidence of multiple Na^+-dependent sugar transport systems. These investigators found certain properties similar to those described for the renal system.

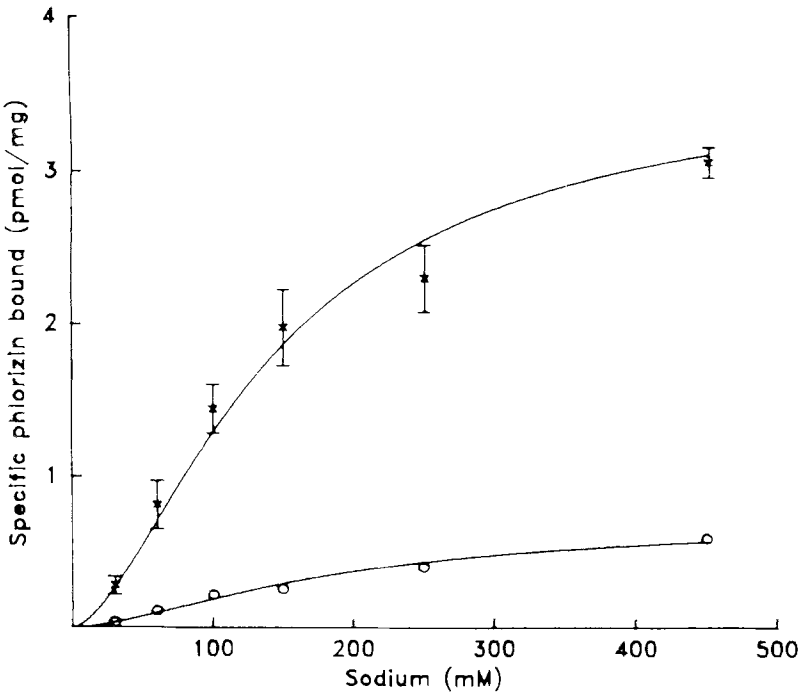

Fig. 17. Relationship between phlorizin binding and Na^+ concentration in chick intestinal cells. *—* = 0.9 μM phlorizin and O—O = 0.095 μM phlorizin (From reference 96, with permission)

Phlorizin binding is dependent on Na^+ concentration as well as on membrane potential. Phlorizin debinding is dependent on Na^+ concentration but not on the membrane potential. On the other hand, the phlorizin binding *vs.* Na^+ concentration relationship is sigmoidal, indicative of the 2 : 1 coupling stoichiometry for sugar transport for this system (Fig. 17). Taken together, the authors concluded that the data are consistent with either the potential-dependent translocation concept or with a model involving potential-dependent Na^+ binding, as described below.

For the latter case, a Na^+ site on the carrier protein is envisioned as embedded "within" the membrane matrix. Access to the site is via a rather narrow restricted channel such that a Na^+ ion "feels" a part of the electric field represented by the membrane potential in making its way to the site. Sodium binding would therefore be potential dependent in a manner analogous to that described by Läuger in his theoretical treatment of potential-dependent ion binding events [69–73] (*see* Fig. 18). Potential-dependent binding of the initial Na^+ ion could then induce a conformational change that facilitates binding of a phlorizin (or sugar) molecule, consistent with the many observations indicating that Na^+ enhances affinity (K_m) of the carrier for phlorizin or sugar. Finally, a second Na^+ binds, but if it binds near the outer surface of the membrane it need not be a potential-dependent event. When the fully loaded carrier (2 Na^+ and a phlorizin molecule)

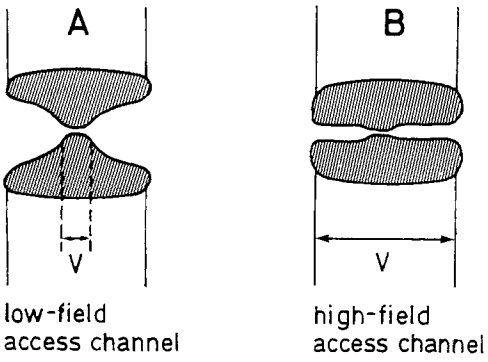

Fig. 18. Schematic representation of two types of membrane channels. In *A*, the access route to the principal flux barrier is a wide vestibule such that an ion diffusing to the barrier would not be highly subject to the membrane potential. The principal drop in potential is envisioned as occurring over a relatively restricted fraction of the membrane width. In *B*, however, the access route to the flux barrier is via a narrow channel with "architectural" features that are highly specific to the ion in transit. The electric field represented by the membrane potential spans the entire membrane, and an ion moving to and from the channel barrier would be driven in part by electrical forces (ion-well behavior). (From reference 71, with permission)

dissociates, if the last Na^+ bound is the first to dissociate then phlorizin debinding would be Na^+-dependent, but potential independent, as observed. The remaining Na^+ can then dissociate in a potential-dependent manner, but this, of course, need not modify the rate at which the earlier dissociating

phlorizin molecule debinds. It must be emphasized that the phlorizin binding data are also consistent with a potential-dependent translocation model, so that on the basis of evidence cited thus far, either Na^+ binding or carrier translocation can be defended as the basis of the potential-dependent event.

V. Transport Kinetics

A. Clamping the Membrane Potential

From the foregoing it should be clear that accurate interpretation of coupling stoichiometry, phlorizin binding, and kinetic data for Na^+-coupled transport systems is absolutely dependent on adequate control of the membrane potential during the appropriate flux or binding measurements. Too often this fact is overlooked, or assumptions are made regarding the adequacy of a supposed voltage clamp for which there is not independent support. Potassium gradients imposed with valinomycin present are usually assumed to provide a K^+ equilibrium potential for the entire range of Na^+ concentrations used in exploring the characteristics of a Na^+-dependent system. The assumption may or may not be valid, depending on the magnitude of the K^+ gradient employed, the amount of valinomycin utilized and the concentration (and relative permeabilities) of the other ions in the system [64]. Because the coupled transport system itself represents a permeability route for Na^+, the degree to which the K^+ equilibrium potential has been achieved will not always be as satisfactory at high Na^+ and sugar concentrations as it is at lower concentrations. A procedure is required for determining rapidly whether a given approach has been truly successful in creating a stable voltage for all of the experimental conditions involving comparisons of kinetic or binding data.

B. Evaluating Efficacy of the Voltage Clamp

1. Fluorescent Dyes

Two approaches for addressing this problem have been reported. In one, fluorescent carbocyanine dyes were employed in order to detect potential changes that might have been induced by the experimental conditions [100, 131]. These cationic dyes are known to distribute across cell membranes in a manner that reflects the magnitude of the membrane potential [22, 33, 110]. The fluorescence yield of intracellular or intravesicular dye is quenched for reasons that are poorly understood, but which may relate in part to aggregation of dye molecules or binding to cellular components [29, 32, 56]. A decrease in fluorescence therefore represents hyperpolarization of the membrane potential and an increase implies depolarization. Using this technique, Schell et al. [100] concluded that their voltage-clamping procedure (valinomycin plus K^+) in rabbit brush-border vesicles was adequate. The stability of membrane potentials as assessed in this manner was an important factor in their interpretation of kinetic data, which suggested that two sugar transport systems are operative in bovine and rabbit intestine with differing Na^+ : sugar stoichiometry. One system was detected with relatively high capacity (K_m = 80 μM; V_{max} = 8 nmol · min^{-1} · mg^{-1}) and a hyperbolic sugar flux *vs.* Na^+ relationship that suggested a 1 : 1 coupling stoichiometry. Another low capacity system (K_m = 10 μM; V_{max} = 0.5 nmol · min^{-1} · mg^{-1}) was found with a sigmoidal flux *vs.* Na^+ dependence, suggesting a higher order coupling stoichiometry.

Their conclusion is somewhat compromised by direct measurements of coupling stoichiometry that are reported in a related paper [41]. These experiments were performed under conditions in which the transport system with 2 : 1 coupling stoichiometry should have provided no more than 10% of the total sugar influx (1 mM glucose). Nevertheless, the directly measured coupling stoichiometry proved to be 2.2 for the bovine vesicles and over 3.0 for the rabbit vesicles. If these measurements are correct, it can be calculated that the coupling stoichiometry for the higher order system must be 10 : 1 (Na : sugar) or greater. This seems unrealistically high and raises questions regarding accuracy of the measured kinetic constants for the two transport systems. One possibility is that the carbocyanine dyes do not respond rapidly enough to detect potential changes occurring within the short time interval required for determining kinetic parameters in vesicle preparations. This explanation implies in turn that the K^+-valinomycin voltage clamp may not be as adequate for the vesicle system as is generally assumed. The possibility needs further evaluation. Suffice it to say that if a change in potential did occur, then the indication of two separately functioning transport systems could be in error for the reasons described earlier.

2. Influx of Lipophilic Ions

Procedures that depend on equilibrium (or steady state) distribution of fluorescent dyes or lipophilic ions are, in general, not satisfactory for detecting rapid changes in membrane potential that can occur

Table 2. Sugar-induced changes in TPP^+ influx (a sensor of $\Delta\psi$) in valinomycin/K^+ "clamped" intestinal cells[a]

Medium	TPP^+ influx	α-MG influx
Mannitol	0.527	—
+15 mM α-MG	0.463	31.2
+15 mM α-MG and 136 mM NO_3^-	0.570	31.1

[a] From reference 95, with permission.
Influx values are given in units of nM · mg protein^{-1} · min^{-1}.

during transport kinetic studies. These sensors require too long to come to equilibrium such that an imposed potential may partially dissipate during the attempted measurement. On the other hand, the *rate* of uptake of a lipophilic ion can be a reliable index of the driving forces acting to create the flux (i.e., chemical and electrical potentials) [53, 64]. If these driving forces do not change during the measurement interval, then the unidirectional influx of the ion should remain constant. Theoretically, one might expect the influx of an ion that is moving independently of any transport system to obey the Goldman flux equation as a function of potential, and in fact this theoretical relationship has been demonstrated for ^{14}C-tetraphenylphosphonium (TPP^+) influx into isolated chick enterocytes [53, 65, 94, 95]. In practice, however, no matter what precise function of potential is involved, a linear influx for an ion should indicate constancy of the driving forces involved during the interval of measurement, including that provided by the membrane potential. To the extent that the indicator ion is bound (or otherwise sequestered) in the intracellular compartment, the likelihood of backflux (due to rising concentration inside) diminishes and these events will extend the interval of linear influx (assuming constancy of the driving forces). The same factors that limit the use of lipophilic ions when steady-state distribution is employed to ascertain membrane potential, therefore, improve the results obtained when unidirectional influx is considered.

Kimmich et al. [53, 64, 93–95] have demonstrated the use of unidirectional influx measurements for ^{14}C-TPP^+ as a means of monitoring the adequacy of voltage clamping during a given experimental approach. Their results showed that even with K^+-valinomycin "clamped" cells, the addition of 15 mM sugar to the cell population can induce a change in the membrane potential [93, 94] (Table 2). This emphasizes the necessity for actually monitoring for $\Delta\psi$ changes rather than assuming adequate control during transport measurements. By combining outward gradients of K^+ (plus valinomycin) with inward gradients of a permeant anion (such as NO_3^- or SCN^-) they were able to define conditions in which the potential remained stable even at high sugar concentrations (Table 2). With this approach for effective voltage clamping during kinetic measurements of the Na^+-coupled sugar transport system in chick enterocytes, they were able to accomplish a careful evaluation of the kinetics and potential dependence for the system [94].

C. Transport Kinetics at Constant Potential

The initial experiments on the voltage-clamped chick enterocyte were performed at a membrane potential held constant near −50 mV (interior negative) [93]. Under these conditions, sugar influx exhibits a hyperbolic dependence on sugar concentration but a sigmoidal dependence on Na^+ concentration. The K_m for sugar transport is highly dependent on Na^+ concentration (4.2 mM at 136 mM Na and 15.2 mM at 40 mM Na^+) consistent with earlier results from other laboratories (although the Na^+ dependence is less pronounced in many of the other cases). The K_m for Na^+ dependence was likewise dependent on the sugar concentration (100 mM at 0.25 mM and 77 mM at 15 mM sugar). The ratio of V_{max} to K_m for sugar changes markedly with the Na^+ concentration. This fact rules out "ping-pong" mechanistic models in which the two transported solutes are bound alternately at opposite sides of the membrane to different carrier conformational states. This observation confirmed similar results by Kessler and Semenza [42] using vesicles from rabbit intestine.

The kinetic data obtained at constant potential were used to evaluate predictions derived from several different transport models [93]. Nine different models in which carrier translocation was considered to be the rate-limiting event were examined. Of those involving 2 : 1 coupling stoichiometry, only two gave an acceptable fit between predicted and observed flux relationships. One of these is for a situation in which Na^+ and sugar bind randomly and

the other for a $Na^+ : S : Na^+$ ordered binding sequence.

It is interesting to note that the data fit well with kinetic equations derived for a random binding transport model with 1 : 1 stoichiometry despite the pronounced sigmoidicity in the sugar flux *vs.* Na^+ concentration relationship [93]. This relates to the fact that even though there is a single *physical* binding site for Na^+, in a random binding model there may be two *functionally* distinct sites due to the possibility that the affinity for Na^+ changes, depending on whether sugar has already been bound. For the chick enterocyte the directly measured coupling stoichiometry proves that the correct value is $2Na^+ : 1$ sugar [52, 60, 62], so the 1 : 1-random model can be ruled out on that basis. These observations point out the danger in using kinetic flux relationships as the *sole* basis for defining transport stoichiometry even when the membrane potential has been adequately clamped. Only an accurately measured coupling stoichiometry can serve as a suitable basis for ruling out steady-state bi-bi models rigorously once a sigmoidal flux *vs.* Na^+ relationship is observed. All models with 2 : 1 coupling stoichiometry in which transport of the partially loaded carrier forms is allowed could be discarded on the previously mentioned grounds that the coupling stoichiometry remains constant at 2 : 1 as the Na^+ concentration is varied.

It has also been shown that the rate of $^{22}Na^+$ influx via the Na^+-dependent sugar carrier (i.e., sugar-dependent and phlorizin-sensitive $^{22}Na^+$ influx) increases as intracellular Na^+ concentration is elevated [62]. An increase in cellular sugar, on the other hand, has no effect on sugar influx via the carrier. These observations suggest that Na^+ is the first to dissociate at the inner face of the cell membrane. When cellular Na^+ is present, it can exchange with $^{22}Na^+$ on the fully loaded carrier to deliver isotope to the cell. In this mode the Na_2CS quaternary transport complex is serving to exchange extracellular isotopic Na^+ for intracellular nonisotopic ion. The high rate at which the exchange process occurs, relative to cases with zero intracellular Na^+ and sugar, implies that translocation of the fully loaded carrier is more rapid than translocation of the free carrier. Hopfer and Groseclose [35] and Kessler and Semenza [42] have noted a similar high rate of isotope exchange (relative to net flux rates at zero intracellular Na^+ and sugar concentrations) in rabbit intestinal brush border vesicles. They too suggested that translocation of the fully loaded carrier is rapid in comparison to translocation of the free carrier.

Kinetic studies of the chick cotransport system at constant potential (~50 mV interior negative) are consistent with a rapid equilibrium ter-ter system with either a random binding order of solutes or an ordered $Na : S : Na$ binding sequence [93]. If solute binding is random, then binding of one Na^+ prior to sugar must be highly preferred in order to account for the many observations indicating that the rate and capacity for phlorizin binding is Na^+ dependent. A completely random sequence would imply that phlorizin could bind in the absence of Na^+, although the affinity may be so poor for a Na^+-free carrier that it is difficult to detect. Either way, the data point toward preferred binding of one Na^+ to the transporter before phlorizin (or sugar) can bind. A degree of randomness for the latter two steps may be indicated by a phlorizin binding *vs.* Na^+ relationship that is hyperbolic in some instances [74, 78, 116] and sigmoidal in others [81, 96, 120]. It is interesting to note that some of the data reported for LLC-PK_1 cells in which the potential was not carefully clamped [74] are not in agreement with the sigmoidal relationship found for voltage clamped conditions [81]. The observations for unclamped conditions may imply more about preferred binding order of solutes than they do about binding stoichiometry, even though they have often been interpreted in the latter sense [74, 78, 116].

It is important to recognize that most kinetic analyses for Na^+-coupled transport systems assume that carrier translocation is the rate-limiting step. Certain of the kinetic models ruled out by Restrepo and Kimmich [93] such as the $N^+ : N^+ : S$ and $S : N^+ : N^+$ ordered ter ter models are only ruled out if this assumption is correct. This limitation is equally true for other analyses dependent on kinetic modeling that assume the translocation step is rate limiting. The assumption seems intuitively sound given that translocation involves a macromolecular conformational change which might be slow relative to binding-debinding events for much smaller solutes that are primarily limited by rates of diffusion. However, the assumption must be borne in mind as a limitation to full understanding of the kinetic data. Nuclear magnetic resonance studies of the anion exchange system in red blood cells suggest that translocation is rate limiting [20]. On the other hand, data obtained for valinomycin indicate that the rate constants for association or dissociation of K^+ are similar in magnitude to those for translocation of the ionophore complex [69].

D. Influence of the Membrane Potential on Transport Kinetics

Restrepo and Kimmich [94, 95] continued their kinetic analysis of the chick intestinal cotransport system by considering the change in transport function which occurs in response to defined changes in

the membrane potential. Unidirectional influx of ^{14}C-TPP^+ was used as the sensor of potential in conjunction with a calibration curve relating TPP^+ influx to K^+ diffusion potentials of defined magnitude. Using this technique, the quantitative relationship between membrane potential and Na^+-dependent sugar flux was determined at various Na^+ and α-MG concentrations. The results showed a high degree of $\Delta\psi$ dependence for the transport Michaelis constant, but a maximal velocity for transport that is independent of $\Delta\psi$ [95].

The data were evaluated with regard to several transport models. This analysis showed that for models in which translocation is the potential dependent step, the free carrier cannot be neutral. If the carrier is anionic, the transporter must be functionally asymmetric. These results are therefore reminiscent of the anionic "gated channel" concept proposed by Kessler and Semenza [42] which was based on qualitative relationships regarding the potential dependence of phlorizin binding and *trans*-inhibition effects for Na^+ and sugar in relation to the magnitude of the membrane potential. If translocation is the rate-limiting step, the kinetic analysis of potential dependence also showed that at zero potential the carrier is distributed across the membrane so that the fraction facing the extracellular milieu must be less than $\frac{1}{4}$ of the total carrier. An asymmetry of distribution of free carrier is necessary for *all* models involving an anionic carrier even when the translocation rate-limiting step assumption is relaxed. Models involving a neutral free carrier have been dismissed on the basis of (i) the potential dependence of the transport kinetics [95], (ii) the potential dependence of transinhibition effects for Na^+ and sugar [42], and (iii) the potential dependence of phlorizin binding (but only for an assumed 1 : 1 stoichiometry and if translocation is presumed to be rate limiting as well as the potential dependent step) [4].

A notable conclusion drawn from the quantitative relationships between membrane potential and transport kinetics reported by Restrepo and Kimmich [93–95] is that all of the data from chick enterocytes, as well as from other systems, can be accommodated by a Na^+-well model in which Na^+ binding is the potential dependent step. When translocation is assumed to be rate limiting, the kinetic data are fit equally well by the Na^+-well conceptual model and by potential-dependent translocation models. When the rate limiting translocation assumption is relaxed, the Na^+-well model provides the best fit of all models tested.

In order to discriminate between two of the better-fitting models (an ordered N : S : N ter-ter kinetic model and a random ter-ter model), Restrepo and Kimmich [96] turned to a consideration of phlorizin binding by chick enterocytes. This represents an inherently simpler system because the phlorizin-carrier complex cannot translocate across the membrane so that (with zero *trans* Na^+ and sugar concentrations) fewer carrier forms must be modeled. In addition to the characteristics mentioned earlier for phlorizin binding, the maximal binding capacity proved to be independent of $\Delta\psi$. However, at saturating Na^+ concentrations, it was noted that maximal phlorizin binding is dependent on the phlorizin concentration employed. Theoretical analysis of the two ter-ter models in question shows that only the random binding model can accommodate the latter fact [96]. If binding is ordered (i.e., Na : Pz : Na), all of the carrier would be converted to the fully loaded form (Na_2CPz) at high Na^+ concentrations due to Na^+-induced mass action in the last step leading to formation of the quaternary complex. This would occur over a wide range of phlorizin concentrations. On the other hand, if phlorizin can bind last, the carrier may be distributed between the Na^+-saturated form (Na_2C) and one which includes phlorizin (Na_2CPz) (Fig. 19). The total amount of phlorizin bound is dependent on phlorizin concentration in this case, as observed experimentally [96].

There is, however, a limitation to the assignment of a random binding model to the observation that maximal phlorizin binding is dependent on the phlorizin concentration. Some of the data were obtained at a phlorizin concentration (0.095 μM) which is about equal to the concentration of total binding sites. At this (and lower) phlorizin concentrations, maximal binding will, of course, depend on phlorizin concentration no matter which model is correct (random or ordered). For this reason, it is still possible that binding is fully ordered and in the sequence Na : S : Na. Under most circumstances the ordered binding sequence seems to be preferred, if not obligatory [62, 96]. At the moment, it is not possible to discriminate with certainty between an obligatory and a random binding order with a statistical preference for a Na : S : Na binding sequence.

Just as for sugar influx kinetics, all of the observations regarding phlorizin binding in the chick enterocyte can be explained equally well by a potential dependent Na^+-well model. The K_m for phlorizin binding is markedly dependent on the membrane potential (1.35 μM at a negative $\Delta\psi$ and 2.63 μM at $\Delta\psi = 0$) and on the Na^+ concentration (26 μM at 30 mM Na^+ and 0.9 μM at saturating Na^+). A notable feature of the chick system is that phlorizin binding is a sigmoidal (rather than hyperbolic) function of Na^+ concentration [96] (Fig. 17). This kinetic evidence indicating Na^+ cooperativity in a system with a known 2 : 1 coupling stoichiometry indicates that both Na^+ sites can be filled when

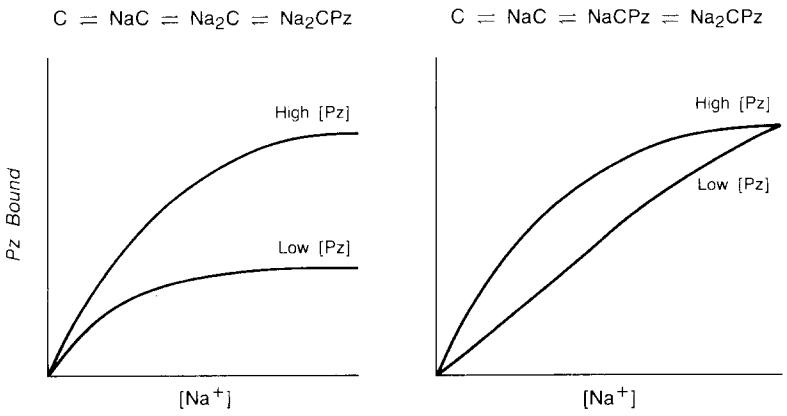

Fig. 19. An illustration of the expected Na^+ dependence of phlorizin binding for a random model in which phlorizin (*Pz*) can bind last *vs.* an ordered model in which Na^+ *must* bind last. In the former case, maximal binding will be dependent on the phlorizin concentration used for the binding experiments, as has been observed in at least one study [96]

phlorizin is bound even though the carrier complex cannot undergo translocation (*also see* similar data for LLC-PK_1 cells [81]. The suggestion that phlorizin might render transport impossible by blocking binding of a second Na^+ ion in a 2 : 1 coupled system [42] therefore seems unlikely for chick intestine, and for LLC-PK_1 cells.

VI. The Na^+-Well Concept

A. Potential Dependent K_m for Na^+

Kinetic modeling for any transport system involving three substrates, two membrane translocation events, at least eight different carrier states (with related association and dissociation rate constants) and one or more potential-dependent events is very complex. Nevertheless, certain features of a "Na^+-well" molecular event are implicit to the concept and should be experimentally demonstrable. For instance, if Na^+ binding to the carrier is indeed potential dependent then the K_m for Na^+ for supporting sugar influx must be a potential-dependent parameter. On the other hand, for a potential-dependent translocation model, the K_m for Na^+ will not necessarily vary with potential. The outward-facing carrier conformation will have an inherent thermodynamic affinity for Na^+. Potential-dependent translocation may change the amount of carrier in the outward conformation and hence its capacity for binding Na^+, but not the affinity that conformer has for Na^+. In contrast, potential-dependent Na^+ binding implies that a favorable potential will facilitate "driving" Na^+ to its binding site such that it becomes fully "loaded" at a lower Na^+ concentration than in the absence of a potential [63]. In essence, the potential is transforming a favorable electrical potential into a favorable chemical potential by increasing the effective Na^+ concentration (or more properly the chemical activity) at the ion binding site within the access channel that is partly embedded in the membrane matrix.

Recent observations show that the K_m for Na^+ in the chick enterocyte sugar cotransport system is markedly dependent on the magnitude of the membrane potential [63]. Half-maximal transport is achieved at 40 mM Na^+ with an interior negative potential (approximately −70 mV), but can only be achieved at extracellular Na^+ concentrations greater than 100 mM Na^+ in the absence of a potential. The V_{max} for transport is the same in the two cases, indicating that once the ion binding site has been filled, optimal transport rates can be achieved.

B. Potential Dependent K_i for Na_i^+

Equally important, the same study indicates that the role that intracellular Na^+ plays in the transport cycle is also modified by the membrane potential [63]. It has been recognized for many years that intracellular Na^+ (or sugar) can exert a pronounced inhibitory effect on the unidirectional influx of sugar via the phlorizin-sensitive cotransport system [16, 42, 102, 106]. These "*trans*" inhibition effects can be explained on the basis of the cellular solutes inducing formation of nonproductive carrier forms at the inner face of the membrane, thereby "removing" carrier available for supporting transport. One problem limiting easy interpretation of the magnitude of *trans*-inhibitory effects relates to the fact that the experimental procedures used to elevate cellular Na^+ also tend to depolarize the cell membrane potential. It is often difficult to know what part of an observed response is due to the increase in cell Na^+ and what part is due to concomitant depolarization of the potential. However, if the potential is experimentally maintained with the aid of imposed diffusion potentials after loading of the cell with Na^+, then the potency of intracellular Na^+ as

an inhibitor is greatly reduced [63]. Representative data illustrating this important point is shown in Fig. 20. Note that 50 mM intracellular Na^+ causes a 60% inhibition of sugar influx in the absence of a membrane potential but virtually no inhibition when the potential is maintained. Cellular Na^+ concentrations as high as 100 mM have little or no effect on the V_{max} for the transport system when an adequate interior negative potential is maintained. In contrast the V_{max} is decreased markedly by cellular Na^+ in depolarized cells.

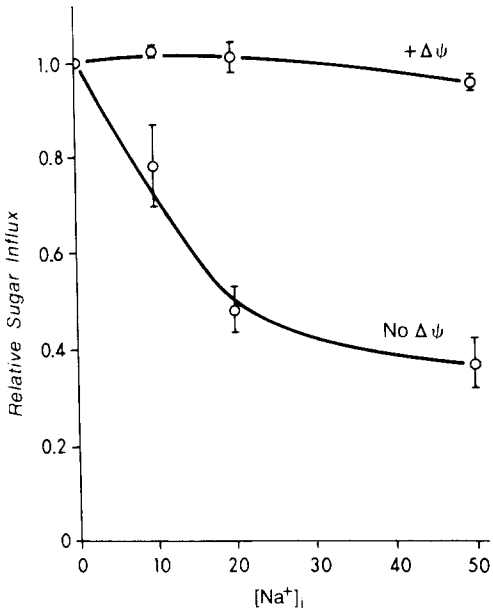

Fig. 20. Relief of Na^+-induced transinhibition of sugar influx by the membrane potential. An interior negative membrane potential caused a nearly complete reversal of transinhibition otherwise induced by 50 mM intracellular Na^+ when no potential was present. (From reference 63, with permission of the publisher)

C. Loss of Potential-Dependence at High Substrate Conditions

Behavior of the Na^+-coupled sugar transport system in response to changes in the membrane potential that are induced at high concentrations of Na^+ and sugar can also provide clues to the locus of a potential-dependent event in the transport cycle. For instance, if translocation of the fully loaded carrier is responsible for all or part of the potential dependence of the system then there should still be a potential response when the carrier is studied at saturating concentrations of the two substrates. Instead, Restrepo [92, 95] has shown that there is no detectable increase in ^{14}C-AMG influx at elevated sugar concentrations when a -60 mV (interior negative) diffusion potential is induced in avian enterocytes at two different extracellular Na^+ concentrations (36 and 136 mM). Much higher sugar concentrations were required at the lower Na^+ concentration (270 *vs.* 57 mM), but the V_{max} was the same within experimental error. These results also suggest a primary role for the membrane potential that facilitates substrate (Na^+) binding, but once carrier loading is complete there is little or no further mechanistic role for the the electrical potential. The recent demonstration that sugar-induced Na^+ currents can be studied by whole-cell recording electrophysiological techniques [111] should allow further exploration of potential dependence at various Na^+ and sugar concentrations under conditions where the potential can be maintained under rigorous control.

D. Speculations on Carrier Architecture

Taken together these facts are consistent with a transport model in which Na^+ binding to the sugar carrier at the outer surface of the cell membrane as well as Na^+ debinding at the inner surface are both potential-dependent events. The phlorizin binding data suggest additional features of the carrier architecture. Recall that the first Na^+ to bind is potential dependent as if it penetrates a substantial portion of the electric field across the membrane. The second Na^+ to bind is potential independent, suggesting little or no penetration of the field. If the two ions hold their position relative to the electric field across the membrane during the conformational change relating to the translocation event, then it is logical to expect that the last Na^+ to bind will be released through all or most of the electric field across the membrane and therefore debind (dissociate) in a potential-dependent manner. The first Na^+ bound would have a smaller (or no) potential dependence, having already traversed a part (or all) of the field.

We therefore envision a transport process that has both channel-like and carrier-like characteristics. Preferred binding sites for Na^+ ions and a sugar molecule provide those properties reminiscent of membrane carriers including saturability, competition by substrate analogs, low turnover number, transinhibition, counter flow, etc. At the same time, Na^+ ions may be moving into and exiting from binding sites via access routes reminiscent of channels including ion selectivity "filters," relatively high rates of ion entry or exit, and potential dependence. The channel-like aspects of the transport system may have certain properties analogous to those which have been defined for channel forming ionophores such as gramicidin [2]. These agents are ion selective due to the spatial separation of chemi-

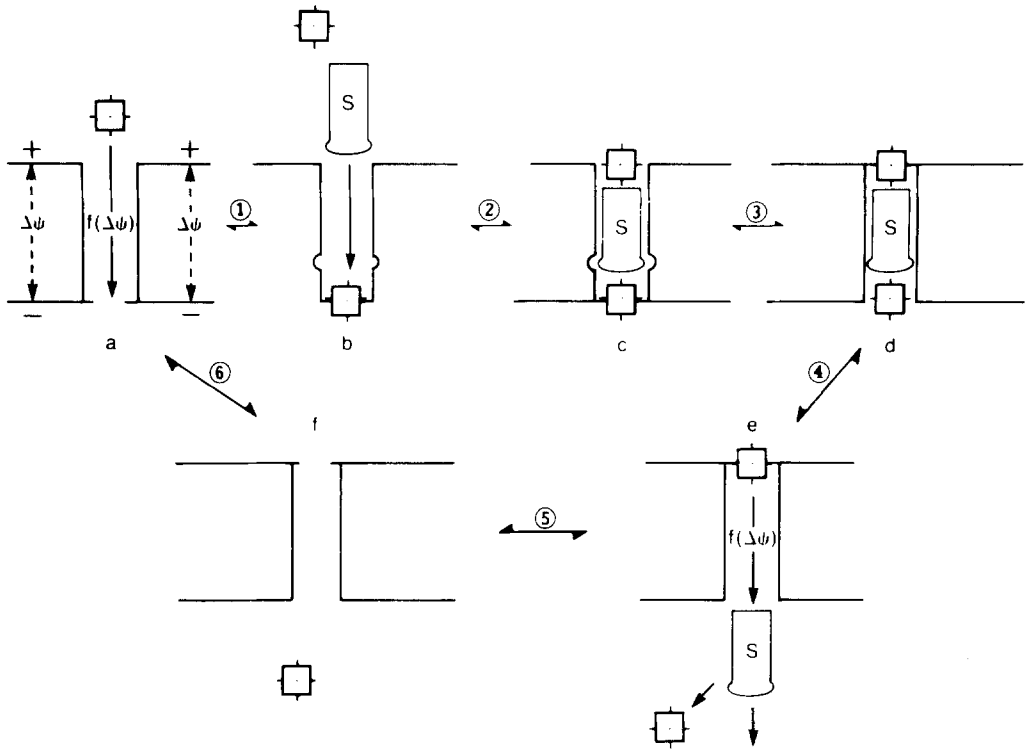

Fig. 21. Schematic representation for a model of Na^+-dependent sugar transport in which Na^+ binding-debinding events represent primary potential-dependent events. The first Na^+ bound (□) is envisioned as driven to a site deep in the membrane barrier (*a*) in a manner that is a function of $\Delta\psi$. Na^+ binding to this site probably induces a subtle conformational change that facilitates binding of sugar (*b*) followed by a second Na^+ binding near the extracellular surface of the membrane (*c*) such that the membrane potential is not "sensed" appreciably. The fully loaded carrier must undergo a conformational change that allows access of bound solutes to the intracellular compartment while at the same time excluding further entry of solutes from outside (*d*). Debinding of the first Na^+ could occur with limited or no influence of membrane potential, but release of the second Na^+ would be strongly potential dependent (*e*). A final conformational change of the unloaded carrier must occur (*f*) to initiate another cycle of solute entry at the outer surface of the membrane. As long as conformational events occur in a manner in which the Na^+ ions keep their relative position in the electric field represented by the membrane potential, carrier translocation need not be potential dependent. (From reference 63, with permission of the publisher)

cal groups in the channel interior which can replace some of the water molecules in the hydration shell of ions with appropriate radii [69]. Ion flux through the channel is single file. In the case of gramicidin, no more than two monovalent ions can occupy the membrane-spanning channel simultaneously, and the "sites" for highest frequency of occupancy are localized near the ends of the channel. A channel with two ions has a much greater tendency to lose an ion by "dissociation" than otherwise expected given the dissociation constant for a singly occupied channel. This suggests the possibility that the mutually repulsive electrostatic fields for the two ions at opposite ends of a gramicidin channel represents a thermodynamic driving force leading to dissociation of one of the ions [123, 124]. A conformational change in the gramicidin molecule induced by binding of the second ion may also be involved. Similar forces may be operative for Na^+-coupled cotransport systems when two (or more) similar ions participate in each transport cycle. It is tempting to speculate that electrostatic interaction between the two Na^+ ions might be the basis for the high chemical potential for a fully loaded sugar carrier that induces the transition to a lower energy state represented by an inward facing conformation. This carrier conformation would then be appropriate for dissociation of solutes to the cytosol. Dissociation events could be enhanced in rate by the action of the membrane potential to impel dissociating Na^+ ions through the remaining channel-like portion of the transport protein.

VII. Conclusion

A schematic representation of the events described above is shown in Fig. 21. A potential dependence

for binding the first Na^+ ion, but not the second, is shown in accordance with our data regarding phlorizin binding. For convenience, only the last Na^+ bound is shown dissociating in a potential-dependent manner, although the first to dissociate may also be potential dependent. The ions are shown retaining their positions relative to the electric field represented by the membrane potential during the carrier conformational change relating to translocation. The latter event would therefore be independent of the potential.

This conceptual model represents another way of viewing new data along with that which led Kessler and Semenza [42] and Semenza et al. [108] to consider certain channel-like properties of the Na^+ : sugar cotransporter. In both cases, it seems likely that several of the cotransporter α-helical sub-units that span the plasma membrane must be involved in the "channel" architecture [31]. Subtle conformational adjustments in this architecture undoubtedly occur in response to ion binding within the channel. These subtle events are undoubtedly more akin to "breathing" movements of adjacent portions of the macromolecule than they are to translational movements across the entire hydrophobic matrix. Whether the conformational change is the primary potential-dependent event or whether it is secondary to Na^+ ions being "driven" to and from their binding sites by (a part of) the electric field represented by the membrane potential is the key question. Because it seems unlikely that either Na^+ ion would be moved through the entire field by a conformational change occurring in a macromolecule embedded in the membrane, we favor ion binding-dissociation events as the primary ones dependent on membrane potential. Ion binding-dissociation and translocation events both might be potential dependent, however.

While our presentation has implied that passage of Na^+ and sugar occurs through a single channel, there is no reason to exclude the possibility of interactive separate pathways for the ions and organic solutes. Semenza et al. [108] have proposed a speculative model in which two separate but interacting "rails" were envisioned for the two kinds of solute species. Their proposal was based on a variety of observations with chemically reactive probes that identified different functional groups associated with sites on the transporter that could be blocked selectively by Na^+ or sugar. It is not yet possible to know whether such groups are part of a common channel or separate channels with conformational states that depend on events occurring in an adjacent one. In either case, Na^+ binding-dissociation events can be potential dependent.

We present this perspective for the functional aspects of the Na^+-coupled sugar transport system as a means of providing a fresh conceptual view for events that might occur at the molecular level. As we mentioned earlier, it is not possible to rule out mechanisms in which translocation is the only potential-dependent event. The challenge for future investigation is to design experiments that define whether ion binding-debinding or carrier translocation (or both) is the basis for potential dependence of these intriguing systems. Recognizing that 30 years have passed since the first model for Na^+-coupled transport systems was presented, I close with this quote from Thoreau:

"*This is a long, long story which should not be long, but it will take a long time to make it short.*"

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