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Effect of K^+ and K^+ Gradients on Accumulation of Sugars by Isolated Intestinal Epithelial Cells

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Summary. The role played by transmembrane K^+ gradients in providing an energy input for Na^+-dependent monosaccharide transport systems was evaluated with the use of isolated intestinal epithelial cells. Experimentally imposing a K^+ gradient in a sense reversed from normal did not lead to extrusion of sugar from cells which had been pre-equilibrated with ^{14}C-3-OMG, even in situations where a reversed Na^+ gradient was also imposed. Furthermore, cells preloaded with K^+ have no better ability to accumulate 3-OMG than do cells depleted of K^+, when the two populations are compared under identical incubation conditions. Fluxes of K^+ associated with the sugar carrier could not be detected in terms of suspected sensitivity to agents which immobilize the sugar carrier. In addition, fluxes of sugar in response to imposed K^+ gradients were not demonstrable in cells de-energized by preincubation with DNP, no matter in which direction the K^+ gradient was imposed. Finally, the severe inhibitory effects of K^+ on Na^+-dependent sugar transport by the cells disappears in de-energized cells, despite the fact that Na^+-dependent carrier-mediated sugar entry still occurs. All of these facts are difficult to reconcile with a significant role for cellular K^+ gradients in supporting active sugar transport as envisioned by the ion gradient hypothesis. We have suggested instead a fundamental Na^+-dependent energy transductive event which depends on ATP, and which can generate a membrane-bound energized intermediate which serves to support a variety of active transport events. An analogy is drawn between this concept for animal cell plasma membranes and the better documented phosphotransferase system for sugar transport described for certain microorganisms.

A role for potassium ion in the events associated with sodium-dependent transport systems for sugars and amino acids has been clearly recognized for more than a decade [3, 25, 30, 31]. Christensen and Riggs first detected a possible functional role for K^+ on the basis of their studies with isolated ascites cells [9–11]. They observed that when certain amino acids are accumulated by ascites cells, a significant loss of cellular potassium occurs which in some cases is stoichiometric with the amount of nonelectrolyte

taken up. This led them to suggest that cellular K^+ might be exchanged for extracellular amino acid by means of a carrier which has binding capability for both species [30]. They further suggested that the gradient of K^+ ion normally maintained by living cells might provide the energy input for amino acid accumulation against a concentration gradient. In this regard, they envisioned K^+ ion combining with the carrier in a manner which diminishes its affinity for amino acid. An asymmetry in carrier affinity for amino acid would be created at the inner and outer membrane surfaces due to the asymmetric potassium distribution. Amino acid would tend to distribute across the membrane until the degree of saturation of the carrier is equivalent at both surfaces at which point cellular amino acid concentration must be greater than extracellular concentration. Riggs, Walker and Christensen [30] recognized that similar considerations might apply to the cellular sodium gradient, only in a sense converse to that just described for potassium. That is, sodium might act to enhance carrier affinity for amino acid at the outer membrane surface, but dissociate at the inner surface and thus decrease affinity. In this case, a net influx of sodium on the amino acid carrier would be expected; a prediction which the authors could not verify experimentally in all of the situations examined. For this and other reasons they ascribed primary importance to the K^+ ion gradient in supporting active amino acid transport.

Subsequent work by numerous investigators has indicated that Na^+ is most likely the ion species of prime importance [4, 12, 13, 14, 20]. Therefore, most recent studies have tended to focus on the role of sodium and the transmembrane sodium gradient. (For an excellent review *see* Ref. [34].) On the other hand, some of these studies, including our own, have shown that significant active accumulation of amino acids or sugars may occur in normally energized cells, even when the cellular sodium gradient is reversed from normal [21, 23, 24, 29, 33]. This may imply that a direct input of metabolic energy to the substrate carrier can occur and supersede the unfavorable sodium gradient. Another possibility is that a normal potassium gradient still existed which compensated for the reversed sodium asymmetry. The latter possibility implies a major role for cellular K^+ gradients in maintaining the ion-dependent transport systems.

For this reason, we decided to try to detect a functional role for transmembrane potassium gradients in supporting active accumulation of sugars by isolated intestinal epithelial cells. This paper is a description of that work and indicates that the accumulation of sugar we previously observed with reversed Na^+ gradients is not likely to have been supported by a normally maintained K^+ gradient.

Materials and Methods

Suspensions of epithelial cells were prepared from intestinal tissue of chicks 1 to 6 weeks of age by treatment with hyaluronidase, followed by gentle mechanical agitation as described in detail previously [22]. The standard isolation medium contained 80 mM NaCl, 20 mM Tris-Cl (pH 7.4), 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml bovine serum albumin (BSA) and sufficient mannitol to produce a total osmolarity of 300 milliosmolar. In certain situations, described below, the Na^+ concentration was decreased and an osmotically equivalent amount of mannitol or KCl was substituted. In other cases, KCl was substituted for mannitol to produce media with normal Na^+ but elevated K^+ concentrations. Isolated cells were held in polyethylene vessels immersed in crushed ice until ready for use. In some cases a 10-min preincubation was allowed in media of altered ion composition or in the presence of metabolic inhibitors in order to produce cell populations with intracellular ion concentration which differed from normal.

In some cases, Na^+ and K^+ content of the cell populations was determined. Aliquots (1 ml) of the population were subjected to millipore filtration, the filter surface and cell pellet were washed with 10 ml of ice-cold isotonic mannitol, and the filter and adhering cells extracted with 5 ml of 5% TCA. Appropriately diluted aliquots of the extract were subjected to flame photometry, and Na^+ or K^+ concentrations determined by comparing to standards of known concentration. Corrections for possible ion binding to the filters were made by filtering media of identical composition, but without cells. The filters were extracted and the extracts assayed by the same procedure used for samples with cells. The "blank values" so obtained were uniformly low and were subtracted from the appropriate cell samples.

Uptake of radioactive 3-OMG and Rb^+ by the cells was monitored by millipore filtration techniques identical to those described earlier [22]. ^{86}Rb and ^{14}C-3-OMG were obtained from Cambridge Nuclear and New England Nuclear Corporation, respectively.

Results

Our first approach toward evaluating the significance of cellular K^+ gradients in Na^+-dependent transport systems for monosaccharides was similar to those already described for evaluating the Na^+ gradient [23]. That is, we attempted to preload cells with ^{14}C-3-OMG to a distribution ratio of unity, and then monitor the effects of imposing unfavorable ion gradients on the ensuing flux of sugar. Accordingly, cells were preincubated at 37 °C for 1 min with 1.25 mM ^{14}C-3-OMG in the usual isolation medium which contains 80 mM Na^+. This short preincubation allows sugar to penetrate the cell and reach a concentration in the cell water at least equal to that used in the medium. The cell population was then returned to the ice bath for at least 10 min. We have shown that these conditions allow moderate loading of the cells with Na^+ (cell $[Na^+] \simeq 50$ mM). Following the loading interval, 1 ml of cells was suddenly introduced into 3.0 ml of normal medium or Na^+-free medium at 37 °C. In the latter case, an osmotically equivalent amount of either mannitol or K^+ replaced the Na^+. Extracellular Na^+ concentration became 20 mM due to dilution of Na^+ in the suspending

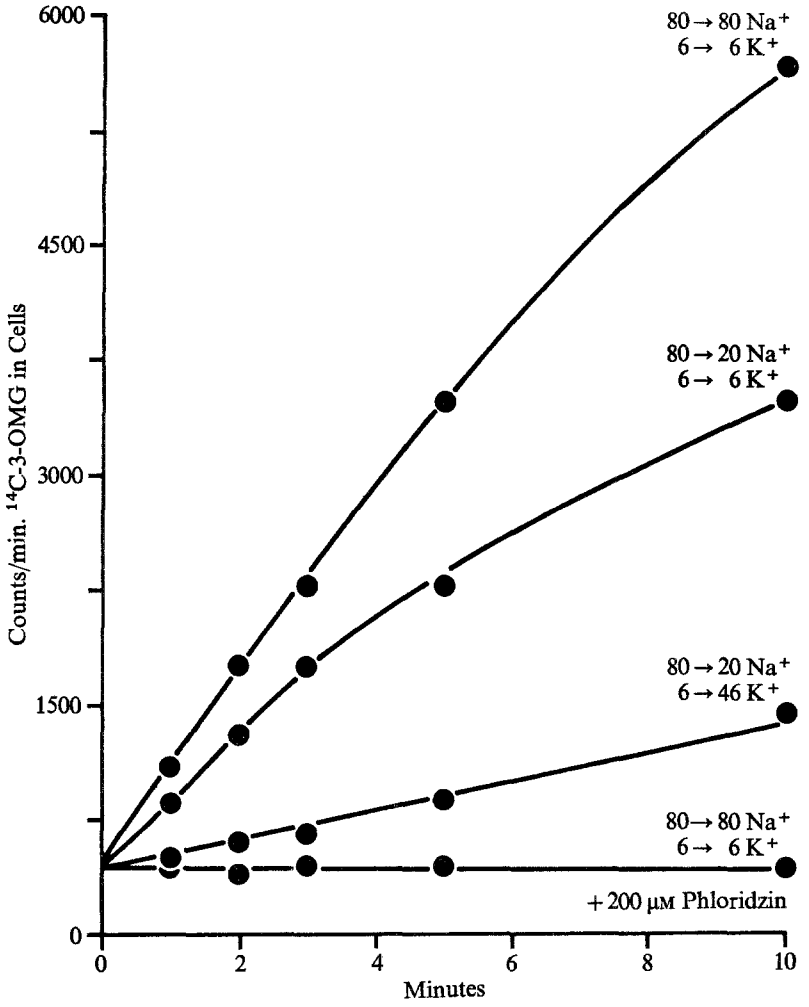


Fig. 1. Effect of imposing reversed Na^+ or Na^+ and K^+ gradients on accumulation of 1.25 mM ^{14}C-3-OMG by isolated intestinal epithelial cells from chick. The cells were pre-equilibrated with ^{14}C-3-OMG before imposing the ion gradients. The first number of each pair indicates the ion concentration during preincubation and the second number is the concentration of that ion during the final incubation. 15 mg of cell protein were used in a 4.0-ml incubation volume. Serial samples (200 μliters) were taken at the indicated intervals. Incubation temp. = 37 °C. Further details are given in the text

medium. K^+ remained at 6 mM in one case, and was elevated to 46 mM in the other. Results are illustrated in Fig. 1. Cells incubated at 80 mM Na^+ generated a sixfold concentration gradient of 3-OMG over the next 10-min interval. The cells which experienced an initially unfavorable Na^+ gradient also accumulated sugar as we have shown previously [23]. Furthermore, the

rate of accumulation was as rapid during the first 2 min of the experiment as at subsequent 2-min intervals although this is the period when the sodium gradient is reversed from normal [23]. Finally, even when both ion gradients are reversed from normal, additional active sugar accumulation is observed. The uptake commences immediately even though sugar *extrusion* would initially be expected if the energy for transport was derived from and dependent upon normally directed Na^+ and K^+ gradients. The fact that no net flux of sugar occurred in the presence of phloridzin indicates that the cells had attained equilibrium with respect to sugar distribution during the preincubation period. In contrast to the situation with intact intestinal tissue, phloridzin does not prevent passive fluxes of sugar by the isolated cells [22].

Of course, our interpretation of the data in Fig. 1 depends on whether we have actually succeeded in reversing the Na^+ and K^+ gradients. We have already considered evidence that the Na^+ gradient is reversed from normal under these conditions in an earlier publication [23], and will not deal with it further here. Data with regard to the K^+ gradient will be considered below and in the Discussion.

The fact that a markedly lower rate and extent of 3-OMG uptake occurs when unfavorable gradients of both ions are imposed compared to the case where only the Na^+ gradient is reversed does not necessarily indicate that a large portion of the energy for sugar transport is normally derived from the K^+ gradient. It may simply reflect the fact that the final K^+ concentration in the two experiments is quite different. There is no question that elevated concentrations of K^+ are very inhibitory to active intestinal sugar transport [5, 13, 23]. However, the basis of that inhibition does not seem to be related to the magnitude or direction of the K^+ gradient existing across the cellular membrane. Proof of the latter statement is best demonstrated by comparing situations where cellular K^+ concentrations have been manipulated, but sugar uptake monitored in media of equal K^+ concentration. A comparison of this type is shown in Fig. 2. A cell suspension was divided into two populations, one of which was preincubated in the absence of K^+ and the other at 80 mM K^+. Neither medium contained Na^+ ion in order to facilitate loss of K^+ from those cells suspended in the K^+-free medium. Absence of Na^+ also allowed the inclusion of ^{14}C-3-OMG in the preincubation medium. Under these conditions no active accumulation of sugar will occur, but the cells will readily equilibrate sugar between cellular and medium water. At time 0, aliquots of each population were transferred to media chosen so that the extracellular ion concentrations became 60 mM for Na^+ and 20 mM for K^+ in each case. Thus, each cell population experienced the same final ion

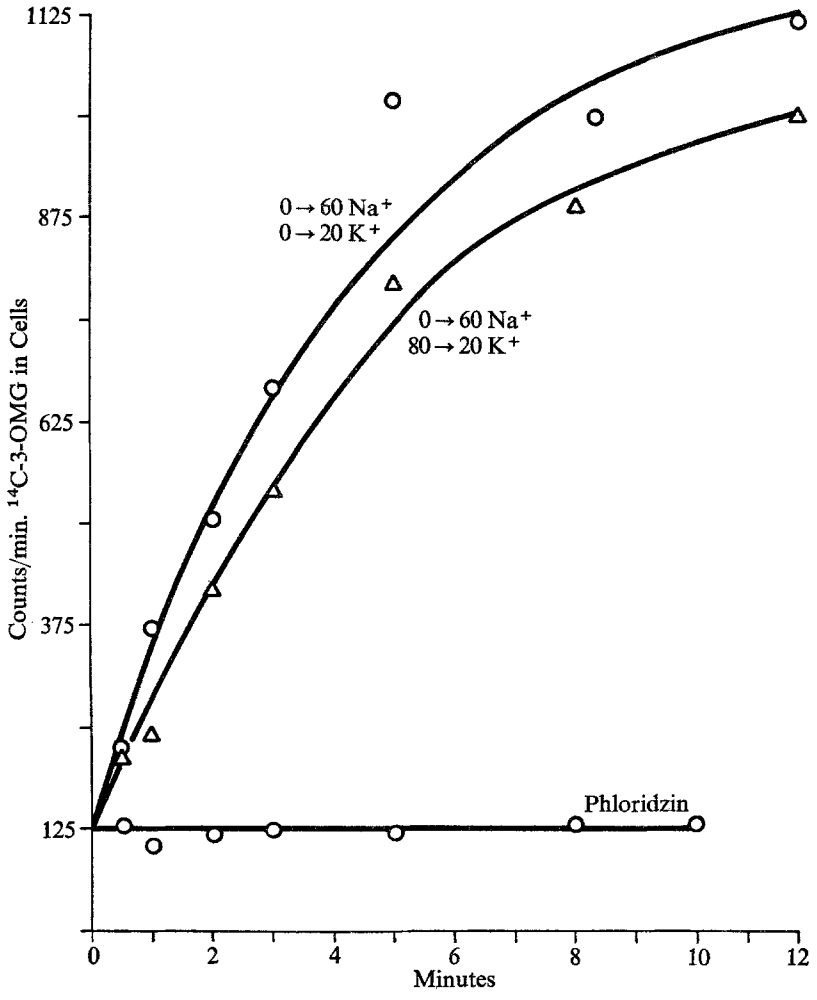


Fig. 2. Accumulation of 1 mM 3-OMG by K^+-loaded or K^+-depleted chick intestinal epithelial cells. Final concentration of Na^+ (60 mM) and K^+ (20 mM) was the same in each situation. Again, the first number in each pair indicates ion concentrations during a 10-min preincubation. 10 mg of cell protein were used in a 4.0-ml incubation volume. Phloridzin concentration = 200 μM, when present

concentrations and transition in Na^+ concentration. However, one population was relatively depleted of cellular K^+ while the other retained normal amounts. The rate and extent of 3-OMG uptake by each group was nearly identical. These cells with the elevated internal K^+ concentration showed no better ability to accumulate 3-OMG than the K^+-depleted group. In fact, in most cases the low K^+ cells exhibited slightly faster sugar transport rates, in contrast to what would be expected if normally directed K^+ gradients

Table 1. K^+ content of cells preincubated in Na^+-free media containing 0 or 80 mM K^+

Time elapsed after transfer of cells to media with 60 mM Na: 20 mM K^+ (min)	n moles K^+/mg cell protein	
	Cells preincubated at 0 mM K^+	80 mM K^+
0	33	85
1	28	88
3	45	88
5	50	—
8	63	88
10	75	69

play a significant role in supporting uptake of the nonelectrolyte. In some cases, phloridzin was added to the incubation medium. No flux of sugar was detected in these instances with either cell population indicating that the cells had indeed equilibrated with sugar before the experimental phase was initiated.

The K^+ content of cells from each of the populations described above was examined more closely by means of flame photometry measurements as described in Materials and Methods. The data are shown in Table 1. Extracts of cells preincubated with K^+ initially have nearly 3 times more K^+ than those incubated in K^+-free medium. For the 0 time analysis, the two cell populations were added to the final incubation medium ($K^+ = 20$ mM) before samples were taken for analysis so that any extracellular fluid trapped by the pellet during filtration will not account for the observed difference. In fact, even after 8-min incubation in media of identical ion composition (Na = 60 mM, K = 20 mM) there is a distinct difference in K^+ associated with the cells in the two groups.

Loss of cellular K^+ during periods of rapid amino acid accumulation is a characteristic feature of ascites cells as we pointed out in the Introduction. Eddy [17] has reported that the transmembrane K^+ gradient maintained by those cells is an important determinant of the magnitude of the glycine gradient which they can establish. Potassium gradients may also be an important contributory factor to the generation of sugar gradients by intestinal tissue. On the basis of kinetic evidence, Crane, Forstner and Eicholz [13] have concluded that K^+ can compete with Na^+ for a cation binding site on the sugar carrier of hamster intestine. This conclusion was reinforced by the earlier observation of Bosackova and Crane [5] which indicated that K^+ inhibits influx of ^{22}Na into hamster intestine, a part of which was thought to occur on the sugar carrier. Crane *et al.* [13] have

demonstrated that the K^+-bound carrier has poor affinity for sugar, and they suggested that an asymmetry in K^+ distribution across the brush border membrane might amplify the asymmetry in carrier affinity induced by cellular Na^+ gradient. However, Schafer [34] has recently emphasized that mere maintenance of a K^+ gradient cannot in itself serve to provide energy for support of transport. If the K^+ gradient is to provide an important energy source, the transport mechanism must act to dissipate the gradient and thus release the potential energy which it represents. If K^+ binds directly to the sugar carrier as Crane *et al.* [13] have suggested, the easiest mode of dissipation to envision would simply involve movement of the K^+-carrier complex to the outer membrane surface where it can dissociate K^+ and re-associate with Na^+ to regenerate the high affinity carrier. Several observations suggest that if this viewpoint is accurate, the carrier-mediated K^+ efflux must be rather substantial in magnitude. For instance, it has thus far been impossible to demonstrate a *net* increase in intestinal cell sodium content during periods of active sugar or amino acid transport in *normally energized* tissue preparations (i.e., tissue not previously treated with metabolic inhibitors) [2, 15, 26, 28, 37]. This is true despite the fact that marked increases in unidirectional influx of Na^+ are easily demonstrable [16, 19], and indicates that increases in active Na^+ extrusion must be keeping pace with the additional Na^+ being delivered to the cell on the substrate carrier. However, Na^+ extrusion is thought to be at least partially linked to K^+ accumulation. Therefore, one might expect sugar or amino acid transport to lead to gains in cellular K^+. Instead, K^+ content of intestinal tissue does not change during Na^+-dependent solute transport [2, 15, 26, 28, 37], which implies that efflux of K^+ on the nonelectrolyte carrier must have occurred which can compensate for the expected increase in cellular K^+ associated with the Na^+ pump. If this interpretation is accurate, agents which prevent mobility of the substrate carrier should prevent carrier-mediated K^+ loss, and allow the cell to generate larger K^+ gradients than normally possible. We have investigated this possibility with the use of phloridzin, a glycoside which seems to form an abortive complex with the sugar carrier [7]. Uptake of ^{86}Rb was monitored rather than ^{42}K due to the longer half-life of the former isotope. We have shown that Rb^+ mimics K^+ in every aspect of its effects on sugar or amino acid transport by the intestinal cells, as has also been shown for intact tissue preparations. Fig. 3 shows that phloridzin does not enhance the Rb^+ gradient generated by the cells in the presence of 1 mM 3-OMG, and in fact produces a slight but reproducible inhibition of Rb^+ transport. The concentration of phloridzin used (0.25 mM) was sufficient to totally inhibit active sugar accumulation by the cells.

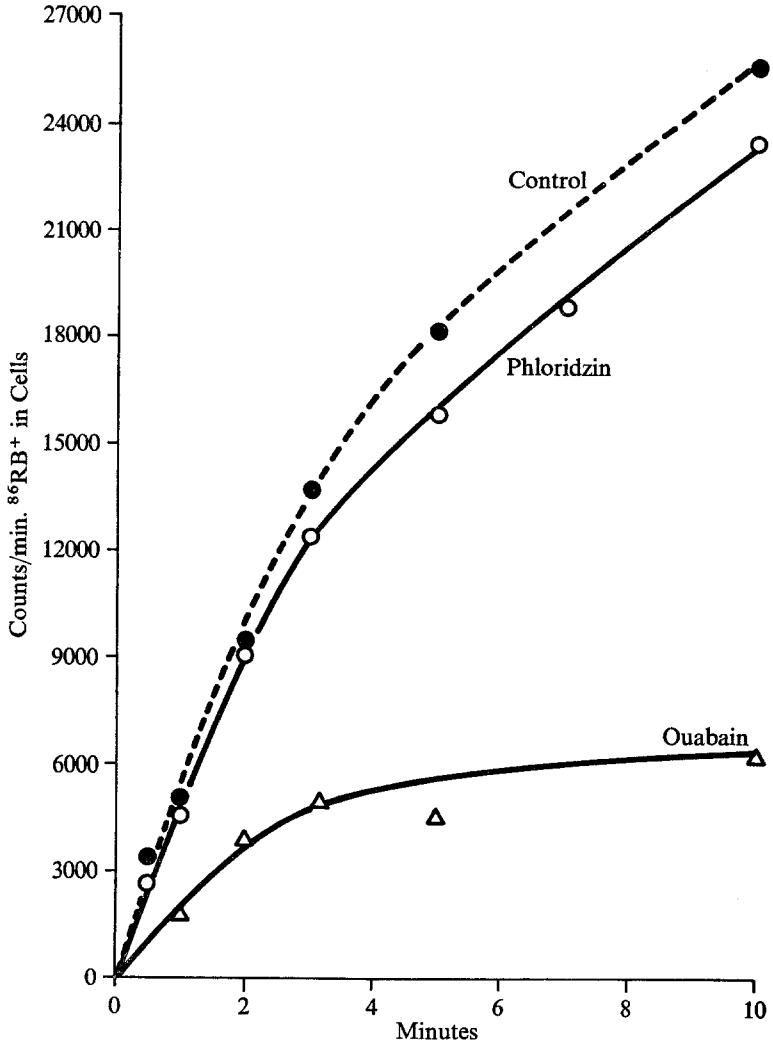


Fig. 3. Effect of phloridzin (200 μM) and ouabain (125 μM) on the accumulation of ^{86}Rb by isolated intestinal epithelial cells

Fluxes of Rb^+ associated with the sugar carrier have not been demonstrable in two related experiments illustrated in Figs. 4 and 5. Cells were preincubated with 0.2 mM DNP in order to discharge energy reserves. In one case, the cells were loaded with Rb^+ at a concentration of 80 mM and suddenly diluted to a final Rb^+ concentration of 20 mM to initiate efflux of the ion (Fig. 4). In the other situation, inward flux of Rb^+ was monitored as cells were introduced to a medium containing 80 mM Rb^+ following pre-

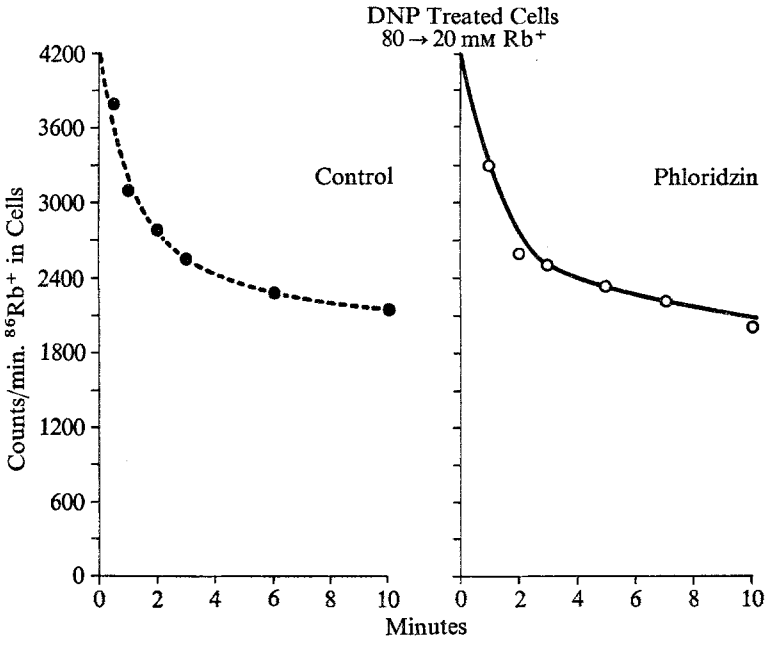


Fig. 4. Effect of 200 μM phloridzin on the efflux of ^{86}Rb from isolated intestinal epithelial cells treated with DNP. The cells were preloaded with ^{86}Rb during a 10-min preincubation at 80 mM Rb^+ and 200 μM DNP. The experiment was initiated by diluting (1:4) the cells into Rb-free incubation medium. Iso-osmolarity was maintained with mannitol

incubation in a Rb^+-free medium (Fig. 5). In neither case was it possible to detect an effect of phloridzin on the observed Rb^+ fluxes, even though it is possible to show that phloridzin retains its ability to decrease sugar fluxes in the DNP-treated cells (Figs. 6, 7, and 9).

It is possible that Rb^+ fluxes associated with the sugar carrier were not detected in the previous experiments because they represent too small a portion of the total Rb^+ flux to be recognized experimentally. We will consider this possibility further in the Discussion.

If Na^+ and K^+ are able to interact with the sugar carrier to alter its affinity for substrate as Crane *et al.* [13] have suggested, it should be possible to generate sugar gradients in cells incapable of metabolic energy production if an asymmetry of Na^+ or K^+ can be created across the cell membrane. In fact, gradients of glycine have been induced in ascites cells and red blood cells pretreated with metabolic inhibitors by imposing inwardly directed Na^+ gradients [17, 39, 40]. Better nonelectrolyte accumulation was observed if gradients of both Na^+ and K^+ were applied [17]. These observations have been widely cited as compelling evidence in favor of the sodium-gradient hypothesis.

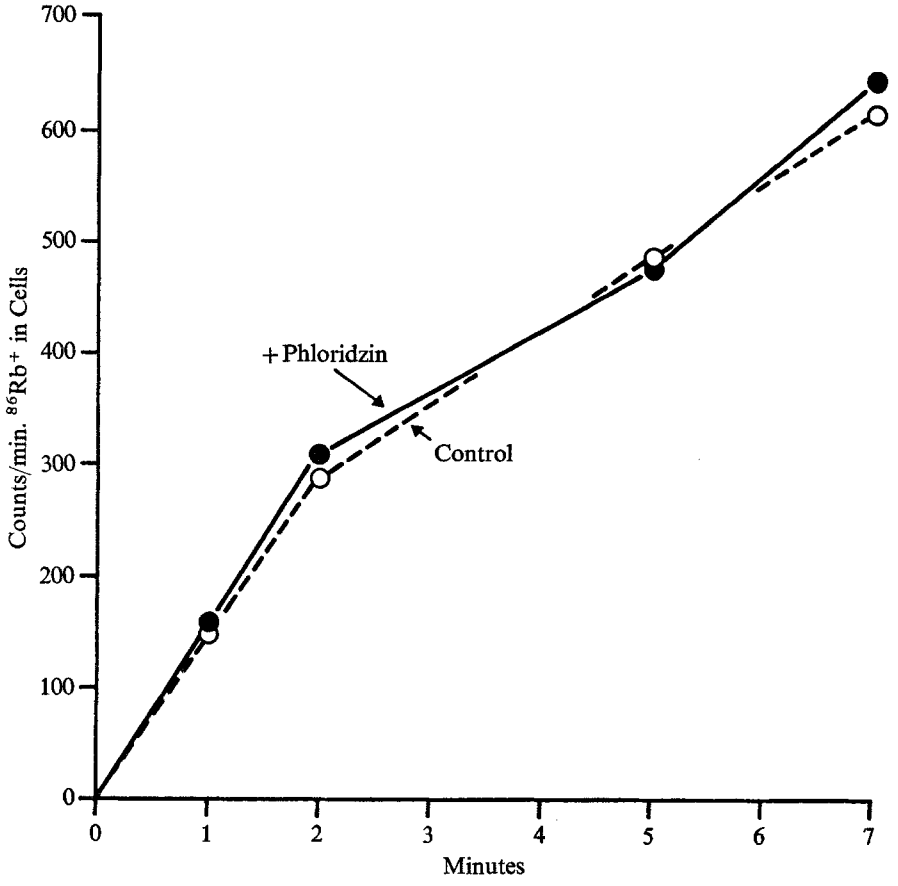


Fig. 5. Effect of 200 μM phloridzin on the influx of 80 mM ^{86}Rb into DNP-treated isolated intestinal epithelial cells

We have obtained somewhat different results with the isolated intestinal cells. Cells were isolated in mannitol medium and pretreated for 10 min at 37 °C with DNP to deplete them of ATP and Na^+. This interval is sufficient to totally inhibit active sugar accumulation [14]. ^{14}C-3-OMG was included in the preincubation medium to allow equilibration of sugar between cell water and medium. The experiment was initiated by introducing a 1-ml aliquot of cells to 3 ml of 80 mM Na medium so that the final Na^+ concentration during the incubation was 60 mM. A 3.5-fold gradient of 3-OMG developed within 5 min as shown in Fig. 6. Phloridzin completely prevented the inward sugar flux. In fact, a slight loss of sugar was observed for the phloridzinized cells indicating that the cell suspension may have been loaded slightly beyond a distribution ratio of unity during the preincubation. The

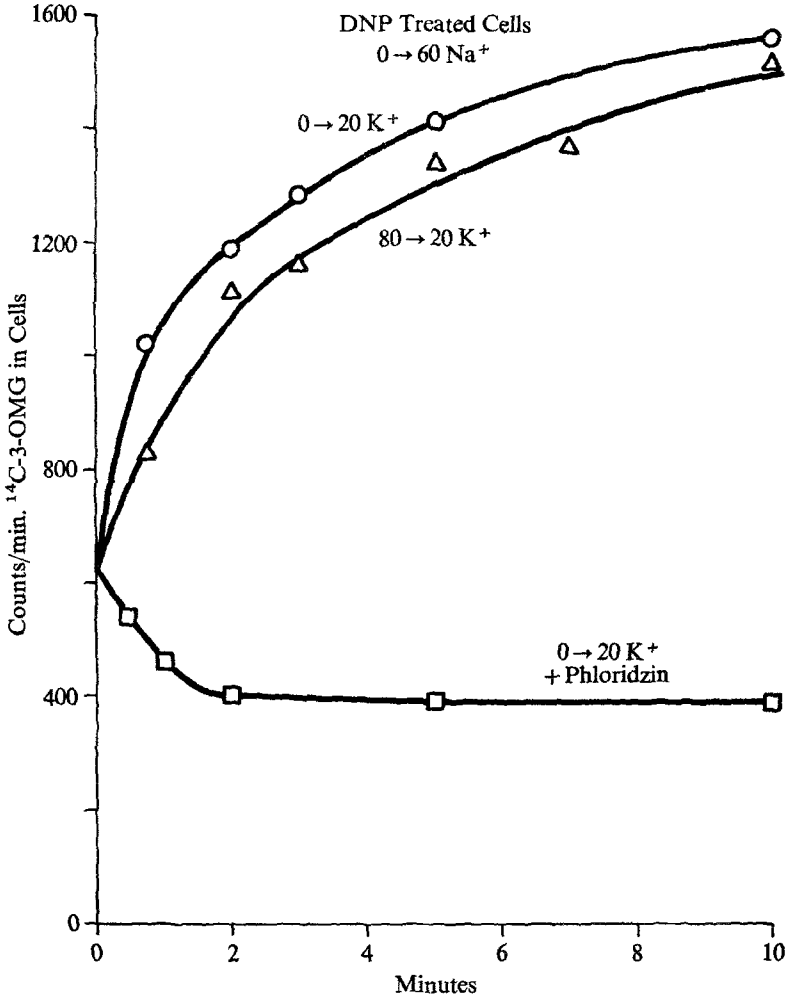


Fig. 6. Comparison of ^{14}C-3-OMG influx in K^+-depleted and K^+-loaded intestinal epithelial cells. The cells were preincubated with 200 μM DNP and pre-equilibrated with ^{14}C-3-OMG. A 60-mM Na^+ gradient was imposed in each case, and final K^+ concentration in the incubation medium = 20 mM. Phloridzin was used at a concentration of 200 μM when present

concentration of DNP employed in both the preincubation and experimental phases of the experiment was the same as that shown previously to inhibit active transport of 3-OMG to the same extent as phloridzin. Thus, it seems quite likely that the sugar gradient observed in this situation is a result of the imposed Na^+ asymmetry. On the other hand, if a normally directed K^+ gradient was also imposed, neither the rate nor extent of 3-OMG accumulation was enhanced. In the latter case the preincubation medium included

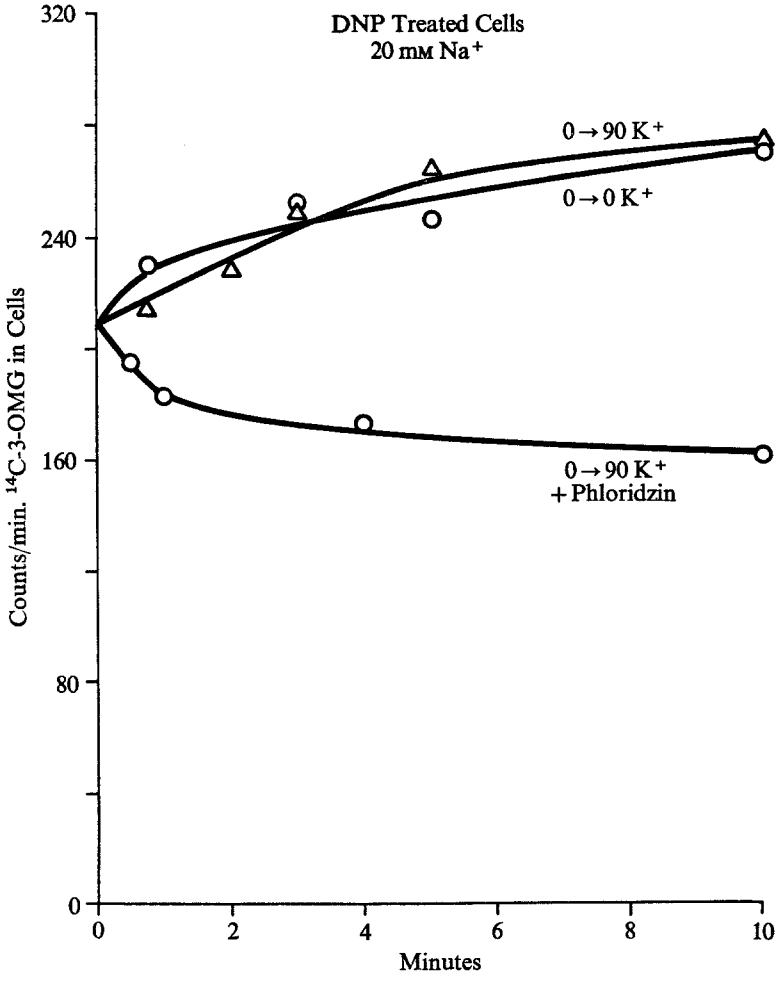


Fig. 7. Effect of imposing a reversed K^+ gradient on ^{14}C-3-OMG flux in cells pretreated with 200 μM DNP and equilibrated with ^{14}C-3-OMG. [Na^+] was maintained at 20 mM during both preincubation and incubation

80 mM K^+ and cells were diluted into K^+-free medium so that the final K^+ concentration was 20 mM. This data is also presented in Fig. 6. Note that the final ion concentration in both experiments was the same for both Na^+ and K^+. If cellular K^+ were an important determinant in the mechanism for sugar accumulation one would expect the K^+-preloaded cells to establish a 3-OMG gradient more rapidly and of greater magnitude than those cells with only a Na^+ gradient imposed.

Fig. 7 shows that it is also not possible to generate a net flux of sugar if only a K^+ gradient is imposed. In this case, cells were equilibrated with

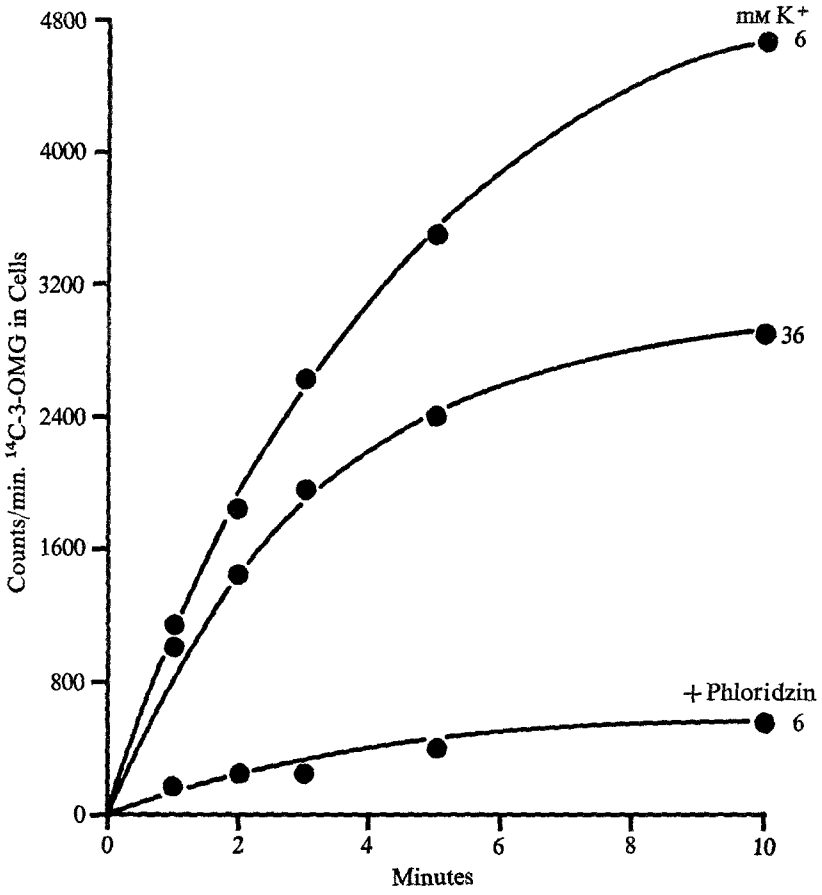


Fig. 8. Effect of 36 mM K^+ on accumulation of ^{14}C-3-OMG by normally energized isolated intestinal epithelial cells. Cells were preincubated at 0 Na^+ and 6 mM K^+ prior to the experiment. Na^+ concentration = 40 mM during the experiment

^{14}C-3-OMG in a low Na^+ and K-free medium in the presence of DNP. At the start of the experiment they were introduced to a K^+-free medium or media containing 90 mM K^+. Sodium concentration remained at 20 mM in both cases. Again no difference in rate or direction of sugar flux was observed, even though an extrusion of 3-OMG from those cells introduced to high K^+ media would be expected if elevated K^+ concentration can modify carrier affinity as has been suggested. Elevated extracellular K^+ would have created an asymmetry in carrier affinity at the two membrane surfaces (low affinity outside; high affinity inside) and produced a net outward flux.

In no case yet examined have we been able to produce a net flux of 3-OMG by applying either an inward or outwardly directed gradient of K^+ in cells depleted of their energy reserves by preincubation with DNP.

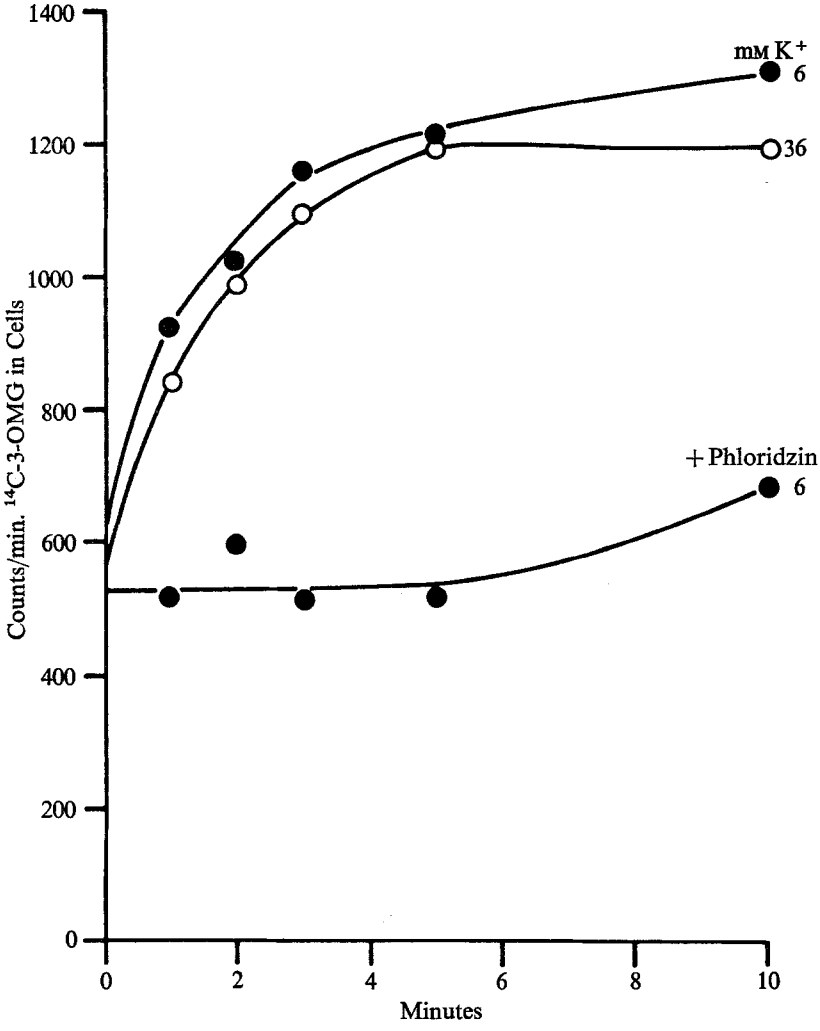


Fig. 9. Effect of 36 mM K^+ on ^{14}C-3-OMG accumulation by de-energized isolated intestinal epithelial cells. Cells were de-energized by preincubation with 200 μM DNP at 0 Na^+ and 6 mM K^+. Incubation was performed at 40 mM Na^+ and 200 μM DNP. When present, phloridzin was used at 200 μM

Neither does intracellular K^+ concentration seem to modify the initial rate of sugar accumulation in normally energized cells as shown in Fig. 2. Yet, K^+ ion is clearly inhibitory toward Na^+-dependent solute transport systems, both in this tissue, and intestinal tissue from other species which have been examined in this regard. What mechanistic role can be ascribed to K^+ as an inhibitor in these instances? A possible clue may be provided by the data illustrated in Figs. 8 and 9 in which the relative inhibitory effectiveness of K^+ on 3-OMG uptake is compared for normally energized and DNP-

treated intestinal cells. At the rather modest sodium concentration employed (40 mM), 36 mM K^+ inhibits 40% of the phloridzin-sensitive sugar entry in the untreated cell population (Fig. 8). On the other hand, cells de-energized by a 10-min preincubation with DNP exhibit a phloridzin-sensitive sugar entry which is remarkably insensitive to increased K^+ concentration. For the case shown in Fig. 9, 36 mM K^+ produced only a 10% inhibition. It should be emphasized that the entry observed in the non-phloridzinized cells is highly Na^+-dependent. In light of the fact that phloridzin inhibition and Na^+ dependence are rather specific characteristics of carrier-mediated sugar entry events associated with the brush border, we must conclude that the carrier is much less sensitive toward potassium ion when cells are depleted of their energy reserves, than in the control situation when normal energy coupling events are possible. In fact, it is possible that the DNP-treated cells are not totally depleted of energy, since the sugar content of the non-phloridzinized cells is clearly greater than that attained in the presence of phloridzin, even near the end of the experiment when the cells are maintaining a steady-state level of sugar concentration. If a residual output of metabolic energy does occur, the small effect of K^+ observed might be attributed to interaction with energy coupling events rather than direct interaction with the sugar carrier. The significance of this implication will be considered below.

Discussion

On the basis of the data described above, it appears unlikely that cellular K^+ gradients play a major role in directly determining the direction or magnitude of sugar fluxes generated by Na^+-dependent transport systems. An important objective of this work was to try to establish whether normally maintained K^+ gradients might account for the active uptake of sugar we reported previously in situations where unfavorable Na^+ gradients had been imposed [23]. The most direct approach for evaluating this possibility is to study sugar fluxes in cell populations in which both ion gradients have been reversed from normal. If active uptake continues in the latter situation as suggested by the data in Fig. 1, a significant role for the K^+ gradient can be ruled out. It is difficult, however, to be certain that the K^+ gradient is actually reversed in this situation, as we have already pointed out. If it is assumed that cellular K^+ is unbound and uniformly distributed in the cell water, it can be calculated that an average cellular concentration of approximately 15 mM was present initially in those cells preincubated at 0 °C and at an extracellular $[K^+]$ of 6 mM. In the experiments illustrated in Fig. 1, we made use of 46 mM extracellular K^+ to achieve concentrations higher

than the calculated initial cellular levels. It is important to recognize at this point that an asymmetry in carrier affinity for substrate at inner and outer membrane surfaces can in itself lead to generation of concentration gradients of the substrate [35]. Therefore, if an asymmetry in carrier affinity for 3-OMG can be created by the imposed asymmetries in Na^+ and K^+, as suggested by Crane *et al.* [13], an extrusion of sugar from the cells should have been observed in this situation, in contrast to the active entry observed. The data must be viewed with caution, however, in light of the difficulty involved in accurate determination of cellular water and the possibility of non-uniform cellular K^+ distribution. Both problems can lead to serious errors in calculating intracellular K^+ concentration at the inner surface of the brush border membrane.

The basic approach of monitoring sugar accumulation while a reversed K^+ gradient is imposed is further limited by the fact that elevated K^+ levels are known to severely inhibit sugar uptake [5, 23, 25]. Active accumulation is progressively more difficult to detect as K^+ concentrations are raised, yet relatively high K^+ concentrations must be used to significantly reverse the K^+ gradient. While these facts might suggest that substrate accumulation is inhibited as the K^+ gradient becomes smaller in magnitude, that interpretation would imply a very significant role for the K^+ gradient as an energy input to the transport mechanism. A major role for the K^+ gradient was not indicated by the other experiments already described, as discussed below.

More significance may be attached to the comparison of sugar uptake by two cell populations with different cellular K^+ contents, but incubated under identical conditions as depicted in Fig. 2. Under these conditions there can be no question that cells preincubated at 80 mM K^+ will contain more K^+ than those preincubated in K^+-free media; and the data of Table 1 verify this prediction. Yet, the K^+-loaded cells have no greater ability to accumulate 3-OMG than the K^+-depleted cells, nor is the initial rate of uptake increased. It should be mentioned that intestinal tissue incubated at elevated K^+ concentrations for prolonged intervals has been shown to undergo a rather pronounced degree of swelling [37]. It is possible that such swelling can produce a nonspecific decrease in Na^+-dependent solute transport capability which might mask a positive effect exerted by the favorable K^+ distribution. While we cannot rule out this possibility, it would seem extremely fortuitous that the opposing changes in transport are of such a magnitude to exactly cancel one another. Also, the initial amount of ^{14}C-3-OMG contained in the pre-equilibrated cells is identical in the two populations in contrast to what would be expected if significant swelling

had occurred in one case and not the other. Finally, both populations maintain *the same* steady-state amount of ^{14}C-3-OMG throughout the course of the experiment in the presence of phloridzin. Significant volume changes in either population would lead to differences in the amount of equilibrated solute. For these reasons we feel that changes in cell swelling cannot adequately explain the data presented in Fig. 2. Moreover, we feel that the data implies a minimal role for the K^+ gradient in providing energy for support of the sugar transport events. The latter implication is supported by the fact that we were unable to detect Rb^+ fluxes associated with the sugar carrier, as evidenced by lack of phloridzin sensitivity (Figs. 3–5). The notion that phloridzin forms an immobile complex with the Na^+-dependent sugar carrier is based on kinetic information which indicates that phloridzin binds to a site at or near the sugar carrier [1], yet is unable to induce the phenomenon of counter-transport [7]. Counter-transport of one actively transported sugar induced by another was observed under the same conditions [7]. If a nonmobile carrier-phloridzin complex is indeed formed, any Rb^+ flux mediated by the sugar carrier should disappear when phloridzin is added. In contrast, we were unable to demonstrate any effect of phloridzin on cellular Rb^+ fluxes under any of the conditions employed. We have already mentioned the possibility that Rb^+ fluxes mediated by the sugar carrier might not be demonstrable if they represent only a small fraction of the total fluxes involved. However, the total rate of Rb^+ influx calculated from the control curve in Fig. 3 is 11.5 nmoles $\times$ min^{-1} $\times$ mg protein^{-1}. The rate of influx of 3-OMG under these conditions is 2.5 nmoles $\times$ min^{-1} $\times$ mg protein^{-1} [24]. Therefore, the sugar carrier could potentially cause a Rb^+ efflux equal to 20% of the rate of Rb^+ influx if the stoichiometry of interaction between sugar carrier and Rb^+ is 1:1 and Rb^+ leaves the cell with each cycle of the carrier. Recall that the sugar transport mechanism must act to dissipate the K^+ gradient if the gradient is to serve as an important energy input [34], and it is this possibility which we are trying to evaluate. Furthermore, if normally directed K^+ gradients are responsible for maintaining sugar transport in those situations where unfavorable Na^+ gradients had been imposed [23], an efflux of K^+ (Rb^+) with virtually every carrier cycle does not seem totally unrealistic for providing energy at the required rate. If phloridzin can prevent a Rb^+ efflux equal to 20% of the influx rate, it should induce a net increase in the Rb^+ gradient maintained by the cells, in contrast to observation (Fig. 3).

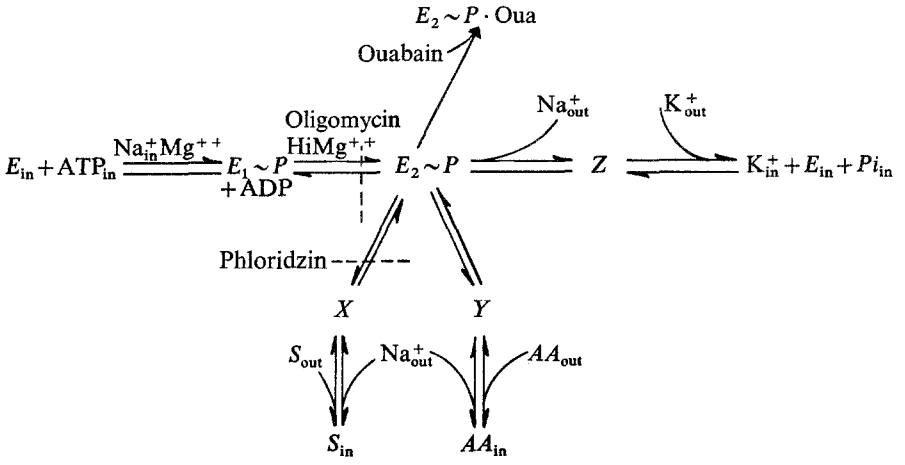
An even stronger case can be stated for the data illustrated in Fig. 5. In the presence of DNP, the energy-dependent Rb^+ influx disappears, and total influx is only 4.0 nmoles $\times$ min^{-1} $\times$ mg protein^{-1} even though extra-

cellular Rb^+ was elevated to 80 mM. Any Rb^+ flux mediated by the sugar carrier should comprise a larger fraction of the total flux under these conditions, yet no sensitivity to phloridzin could be detected. It might be claimed that the K^+-carrier complex suggested by Crane *et al.* [13] is immobile and therefore noncontributory to cellular K^+ (Rb^+) fluxes, but in that event K^+ could only act to reduce the magnitude of the sugar gradient generated (*see* Goldner, Schultz & Curran [19], for a discussion of the effect of changes in carrier mobility on Na^+-dependent sugar transport in rabbit), and never serve as an important energy reservoir.

An inconsequential role for the K^+ gradient in supporting active sugar entry is further suggested by the fact that imposed K^+ gradients have no effect in modifying sugar fluxes in cells depleted of ATP by treatment with DNP (Figs. 6 and 7). Lack of response toward K^+ in these instances apparently does not represent nonspecific loss of carrier characteristics induced by DNP, since the entry is still phloridzin sensitive, and increased fluxes in response to Na^+ are still demonstrable. In fact, the data of Fig. 6 shows that a 3.5-fold gradient of sugar can be generated when a Na^+ gradient is imposed. It seems likely that the sugar gradient produced in this situation is a direct result of the normally directed Na^+ gradient. If this interpretation is accurate, our earlier suggestion of a directly energized sugar carrier [23] must be modified to include a role for the Na^+ gradient. On the other hand, if the DNP-treated cells are incompletely depleted of energy, the sugar uptake might still represent a Na^+-dependent but directly energized transport process. This alternative is considered unlikely at present, but cannot be completely ruled out. In fact, the prolonged steady-state level of sugar accumulation observed in this case would not be expected if energy is derived from a steadily dissipating Na^+ gradient. The characteristics of sugar transport observed under these circumstances is under further investigation in our laboratory.

Finally, we regard the fact that the sugar transport system loses sensitivity to potassium ion in DNP-treated cells as having special significance. It suggests that the action of K^+ is dependent on the energy status of the cell in contrast to what would be expected if K^+ interacts directly with the sugar carrier. Our earlier proposal of a direct energy input to the sugar carrier supported by energized intermediates of the sodium pump [23], is entirely consistent with an indirect role for K^+, and a loss of K^+ sensitivity of the system in energy-depleted cells. The model we suggested is illustrated below in modified form (Scheme 1).

The diagram is intended simply to show an energy flow scheme for events associated with Na^+-dependent transport systems. $E_1 \sim P$ and $E_2 \sim P$ re-



Scheme 1. A possible mechanism illustrating direct energetic coupling between monovalent ion transport and Na^+-dependent metabolite transport

present the two phosphorylated intermediates of ($Na^+ \times K^+$)-activated ATPase which have been described [18, 36]. One of the energized intermediates ($E_2 \sim P$) is envisioned as playing a central role in a basic set of energy transduction events which function in support of a variety of membrane transport processes. X and Y represent unspecified membrane components (carriers?) which couple energy flow from $E_2 \sim P$ to the sugar and amino acid transport system, respectively. Z represents an analogous component which interacts with extracellular K^+ [presumable a part of the ($Na^+ + K^+$)-ATPase complex] and allows energy transduction for support of monovalent ion transport. Without specifying a detailed mechanism, we have indicated that X and Y exhibit a requirement for extracellular Na^+. It is at this locus that a transmembrane Na^+ gradient *may* provide an additional energy derived from $E_2 \sim P$ directly. This idea is consistent with our data indicating a modest concentration gradient of sugar can be generated by an imposed Na^+ gradient in DNP-treated cells. It likewise accommodates similar data derived from other cell types [17, 39, 40]. On the other hand, no role for K^+ is implied at the sugar carrier locus in agreement with the data presented in this paper. Potassium ion is proposed to inhibit nonelectrolyte transport indirectly by diverting energy from $E_2 \sim P$ for support of monovalent ion transport events thereby partially depriving energy flow toward sugar and amino acid transport events. Depletion of cellular ATP by DNP will prevent formation of the $E_2 \sim P$ energy donor and consequently the inhibitory effects of K^+ disappear. In an analogous manner, rapid transport of either sugars or amino acids would be expected to partially deprive

monovalent ion transport of $E_2 \sim P$. If such deprivation is of sufficient magnitude the rate of K^+ entry will be diminished and a net decrease of cell K^+ will be observed. Losses of cellular K^+ in this situation have been described for ascites cells as we mentioned in the Introduction. Concomitant net changes in cellular [Na^+] need not be necessary, and indeed have not been noted in normally energized tissue [2, 6, 15, 26, 28].

We recognize that our model predicts that some common elements for monovalent ion and nonelectrolyte transport exist in the mucosal boundary of the intestinal cell, in contrast to most current opinion [35]. It also predicts that cellular Na^+ can play an important role in directly energizing the substrate carrier, again contrary to some reports [19, 35]. However, some evidence exists for both concepts. Unidirectional influx of amino acids across the mucosal boundary of rabbit intestine occurs at nearly normal rates in Na^+-free medium if the tissue is preincubated in media with normal Na^+ concentrations [35]. It seems likely that cellular Na^+ is supporting the observed influx rates in this situation rather than extracellular Na^+ which has leaked by diffusion from cellular pools as has been suggested. Furthermore, if the tissue is preincubated in Na^+-free medium to deplete the cellular Na^+ reserves, the unidirectional influx rates decrease to a low level as we would predict. By the same token, ouabain has no effect on unidirectional influx rates of alanine across the brush border boundary of rabbit ileum, if the tissue has first been depleted of intracellular Na^+ by preincubation in choline [8]. A marked inhibitory effect of ouabain occurs, however, if cellular Na^+ has been maintained by preincubation in normal Ringer's [8]. Our model predicts ouabain sensitivity for that portion of influx which derives its energy directly from ATP expenditure and consequently on the generation of $E_2 \sim P$ by reactions dependent on cellular Na^+. A role for cellular Na^+ has also been detected for 3-OMG entry on the basis of unidirectional flux measurements [27].

Finally, the concept of several energy-dependent events deriving energy from a common membrane-bound energized intermediate is not a new one. We have previously emphasized the analogy between our ideas for energy coupling events at the cell membrane and those postulated for energy transduction during mitochondrial oxidative phosphorylation [23]. Recent developments in the study of bacterial sugar transport systems by Kundig and Roseman [27] and Roseman [32] indicate that a fundamentally similar mechanism may occur in certain bacterial species. For instance, in *E. coli* it has been demonstrated that a cytoplasmic protein (HPr) can be phosphorylated at the expense of phosphoenolpyruvate (PEP). Phosphorylated HPr can in turn phosphorylate a specific membrane protein which can apparently

energize a number of independent sugar specific proteins which function in monosaccharide transport [27]. In this case the free energy of the membrane-bound intermediate supports phosphorylation of the sugar in question as well as vectorial flux. Independent membrane components for each sugar entry system is suggested by the fact that each transport process is separately inducible. In fact, proteins specific for each system have been isolated [27], yet each can apparently derive energy from a single phosphorylated intermediate. It is reasonable to expect that similar independent transport events energized by a common membrane-bound energized intermediate may function in mammalian cells. In this light the interaction between Na^+-dependent transport systems for sugars and amino acids may have special significance. This possibility is considered in greater detail in the following paper.

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