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Hexose Regulation of Sodium-Hexose Transport in LLC-PK$_1$ Epithelia: The Nature of the Signal

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Summary. We have shown previously that the concentration of glucose in the growth medium regulates sodium-coupled hexose transport in epithelia formed by the porcine renal cell line LLC-PK$_1$. Assayed in physiological salt solution, the ratio of the concentration of α-methyl glucoside (AMG) accumulated inside the cell at steady state to its concentration outside, and the number of glucose transporters, as measured by phlorizin binding, was inversely related to the glucose concentration in the growth medium. In this study, using a cloned line of LLC-PK$_1$ cells, we provide evidence that the difference in AMG concentrating capacity is the result of a regulatory signal and not simply due to a selection process where the growth of cells with enhanced glucose transport is favored by low glucose medium or vice-versa. By adding glucose to conditioned medium (collected after 48 hr incubation with cells and therefore containing less than 0.1 mM glucose), we demonstrate that the signal in the growth medium is indeed the concentration of glucose rather than another factor secreted into or depleted from the medium. Fructose and mannose, two sugars not transported by the sodium-dependent glucose transporter, can substitute for glucose as a carbohydrate source in the growth medium and have a modest glucose-like effect on the transporter. Growth in medium containing AMG does not affect the transporter, indicating that the regulatory signal is not a direct effect of the hexose on its carrier but involves hexose metabolism.

Key Words cultured epithelia · hexose transport · transport regulation

Introduction

LLC-PK$_1$ is a continuous epithelial cell line derived from porcine kidney [5]. The cells grow with their basal surface oriented toward the supporting substrate and form confluent epithelia with many characteristics of the proximal tubule [6, 12–14]. In particular, they exhibit a sodium-coupled hexose transport system in their apical plasma membrane [1, 7, 9, 10] with properties similar to the apical plasma membrane D-glucose transporter identified in the late proximal tubule [8, 9, 16, 17]. The ability of LLC-PK$_1$ cells to transport α-methyl glucose (AMG), a nonmetabolized glucose analogue, develops slowly with time after seeding, reaching a maximum after 3 to 4 weeks [4, 9, 11]. These changes presumably reflect increasing expression of the sodium-coupled hexose carrier. Recently we have shown that sodium-dependent hexose transport in this cell line is affected by the concentration of glucose in the growth medium [10]. Epithelia grown in medium containing low concentrations of glucose develop a much higher transport capacity for AMG than cells grown in medium containing a high concentration of glucose. Furthermore, this phenomenon is reversible. When epithelia grown in high glucose medium are switched to low glucose medium, or vice versa, the transport capacity of the epithelium for AMG increases or decreases accordingly. These changes, like the development of the sodium-coupled hexose transport system itself, are slow, requiring more than 48 hr before significant effects on AMG transport capacity are apparent. Phlorizin binding measurements indicate that it is the number of sodium-dependent glucose transporters that is affected; epithelia grown in low glucose have more carriers than epithelia grown in high glucose [10]. In the present study, we investigate the mechanism underlying this phenomenon. We provide evidence that the difference in AMG transport capacity is the result of a regulatory signal and not simply due to a selection process where the growth of cells with enhanced glucose transport is favored by low glucose medium or vice-versa. Furthermore, we show that the regulatory signal is the glucose concentration itself rather than the presence or absence of some other factor secreted into or depleted from the medium as a result of the glucose concentration. Finally, we rule out the possibility that the regulatory role of D-glucose is due to

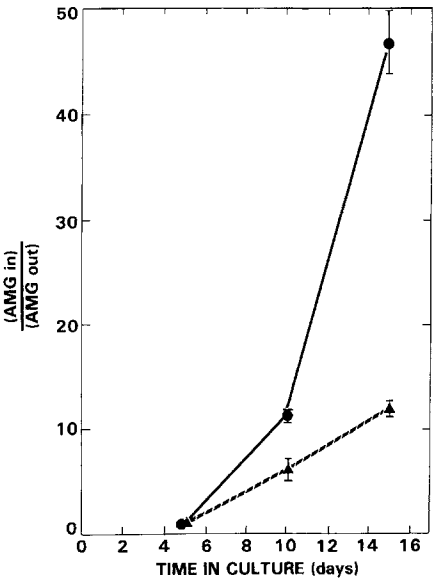

Fig. 1. Steady-state AMG-concentrating capacity of a cloned cell line as a function of time in culture. The cells were grown in media containing 5 mM (●) or 25 mM (▲) glucose

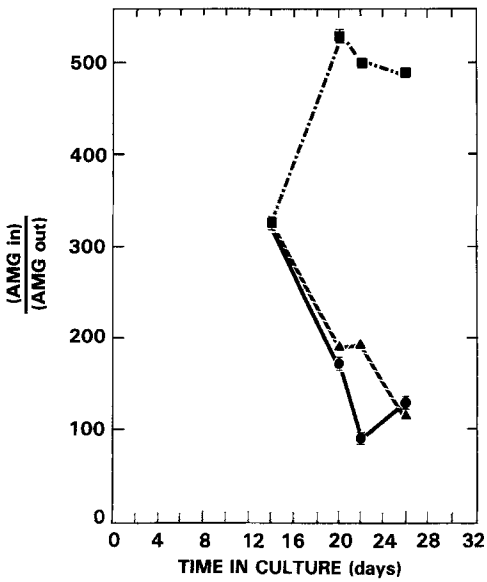

Fig. 2. Regulation of AMG-concentrating capacity in fresh and conditioned media (medium collected after exposure to cells for 48 hr). Epithelia grown for 14 days in medium containing 5 mM glucose were switched to different media: (▲) switched to fresh medium containing 25 mM glucose, (●) switched to conditioned medium supplemented with 25 mM glucose, and (■) continued growth in medium with 5 mM glucose

a direct effect of transported substrate on the carrier.

Materials and Methods

Cells were grown in cluster-12 wells as previously described [9, 10]. We assayed sodium-coupled hexose transport by measuring the steady-state concentrating capacity for AMG as described earlier [9, 10]. Briefly, on the day of the experiment the epithelia were rinsed with phosphate buffered saline (PBS) and then incubated with PBS containing 0.1 mM [^{14}C]-AMG and 0.1 mM [^{3}H]-raffinose (an extracellular marker) on a mechanical shaker at room temperature for 5 hr until a steady-state condition had been achieved. The incubation media were sampled and the cells rinsed and then solubilized using 0.5% Triton X-100 in water. Samples for counting radioactivity and for protein determination were taken. Cell volume was measured using the nonmetabolizable sugar 3-O-methyl glucoside (3OM) that does not accumulate in these cells. Glucose concentration was measured using a Beckman glucose analyzer. The AMG concentrating capacity is expressed as the steady-state ratio of AMG accumulated in cell water to that in the incubation medium. To estimate the number of cells in a plate the epithelia were suspended by trypsin and the cells were counted with a hemocytometer. The data points illustrated in the figures are the mean and standard deviation determined from three wells. Results of representative experiments are shown.

Materials

[^{14}C]-AMG, 3OM and [^{3}H]-raffinose were obtained from New England Nuclear (Boston, MA). Unlabeled raffinose and 3OM were from Cal Biochem. (La Jolla, CA). Uridine and unlabeled monosaccharides were from Sigma Chemicals (St. Louis, MO). Other chemicals were of the highest purity available from commercial sources. A cloned cell line of LLC-PK$_1$ was kindly given to us by Dr. John Cook of Oak Ridge, TN. The cells were cloned by repeated limiting dilution by Dr. Kurt Amsler in Dr. Cook's laboratory. The cells were grown in medium containing 25 mM glucose for two passages and then subcultured into two different media in Cluster-12 wells.

Results

In previous studies [10] we have observed that the change in AMG concentrating capacity of LLC-PK$_1$ epithelia in response to a shift in the concentration of glucose in the growth media is not evident for at least 48 hr. A slow response such as that might result if the glucose concentration in the medium selects cells from a heterogenous population. To evaluate this possibility, we used a cloned line of LLC-PK$_1$ cells. Figure 1 shows concentrating capacity of the cloned cell line as a function of days in growth media prepared to contain 5 or 25 mM glucose. As we have previously shown for the parent cell line [10], cloned cells grown in 5 mM glucose attained a much higher concentrating capacity than the same cells grown in medium containing 25 mM glucose. Thus, it is unlikely that growth medium with a low concentration of glucose acts merely by selecting against cells with a low number of glucose transporters.

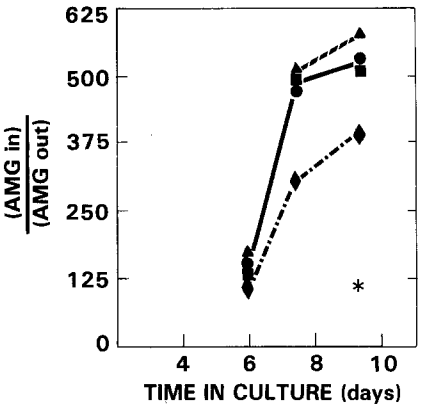

Fig. 3. Effect of glucose concentration on the AMG-concentrating capacity. Epithelia were grown (from day 0) in media containing: 5 mM glucose + 5 mM uridine (♦, ▲) and 5 mM uridine (●, ■), and 25 mM glucose (*). Some epithelia (♦, ●) were fed every 8 to 12 hr; the others were fed every 48 hr

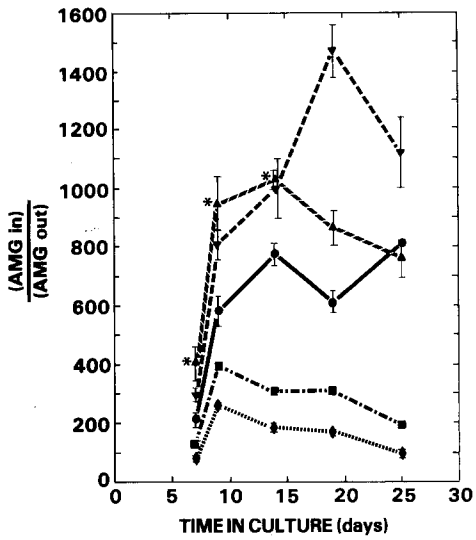

Fig. 4. The effect of different substrates in the growth medium on the AMG-concentrating capacity. 5 mM glucose (▲), 25 mM glucose (♦), 25 mM glucose + 5 mM uridine (■), 5 mM uridine (▼), 25 mM mannose (●), and 5 mM glucose + 5 mM uridine (*)

To identify the factor in the growth medium responsible for the regulation of glucose transport, we compared the effect of fresh medium with that of conditioned medium. The conditioned medium used in these experiments initially contained 5 mM glucose and was collected from LLC-PK_1 epithelia after 48 hr in culture. Consequently, it contained less than 0.1 mM glucose. It should also contain or be depleted of any other factors that signal the epithelium to develop more hexose transporters. Epithelia grown in the usual fashion in 5 mM glucose for 14 days were switched into either conditioned medium supplemented with 25 mM glucose or into fresh medium containing 25 mM glucose. As depicted in Fig. 2, conditioned medium supplemented with 25 mM glucose caused the same suppression of the AMG concentrating capacity as fresh medium containing 25 mM glucose. Since the conditioned medium should contain (or lack) any substance that was secreted into or depleted from the medium by the cells, we conclude that glucose, the only supplement, is the cause of the suppression of concentrating capacity observed in this experiment.

In an attempt to quantitatively relate the concentrating capacity of the cells to the concentration of glucose in the medium throughout the growth period, we used frequent feeding as a means of keeping the glucose concentration within known limits (albeit not constant). Epithelia were assigned to two groups. One group was fed with medium containing 5 mM glucose, the other with medium containing no glucose. Half the epithelia within each group were fed every 8–12 hr, the other as usual, every 48 hr. The concentration of glucose in the medium was monitored throughout the experiment. The glucose concentration in medium prepared to contain 5 mM and exchanged every 48 hr decreased below 0.1 mM after 15 hr (data not shown). By feeding the cells every 8–12 hr with medium containing 5 mM glucose, the concentration of glucose in the medium was maintained above 1 mM. All media in this experiment were supplemented with 5 mM uridine, which has been shown to support cell growth in the absence of glucose or other carbohydrates in the growth medium [3]. Fig. 3 depicts the result of this series of experiments. There is no difference between the concentrating capacity of epithelia fed in the customary fashion (every 48 hr) with 5 mM glucose and those fed with glucose-free medium containing uridine regardless of how often the latter were fed (cells were in media containing zero or less than 0.1 mM glucose most of the time). However, when the glucose concentration in the growth medium was kept above 1 mM by frequent feeding, the AMG concentrating capacity was lower. Cells which were grown in 25 mM glucose (where glucose concentration in the medium was always above 5 mM) attained even lower AMG-concentrating capacity, which indicates that the regulatory effect of the concentration of glucose is monotonic: the higher the concentration of glucose in the medium the lower the AMG-accumulating capacity achieved.

To test whether growth in the complete absence of glucose would evoke a higher AMG-concentrating capacity than growth in 5 mM glucose, we measured the AMG-concentrating capacity of epithelia fed medium without glucose but supplemented with 5 mM uridine for over 2 weeks (the time AMG-concentrating capacity reaches a plateau). Figure 4

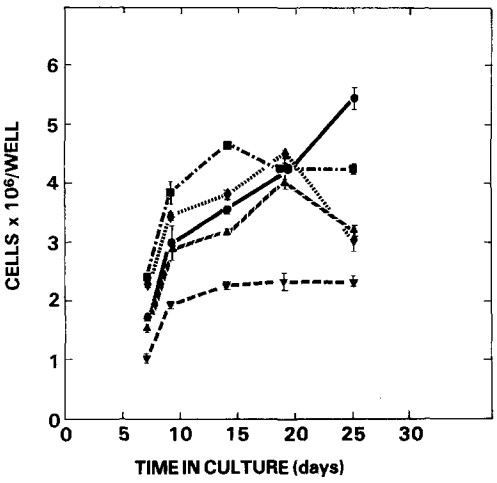

Fig. 5. Density of cells in different growth media. Number of cells per well (4 cm^2) as a function of time in culture. Data from experiment shown in Fig. 4

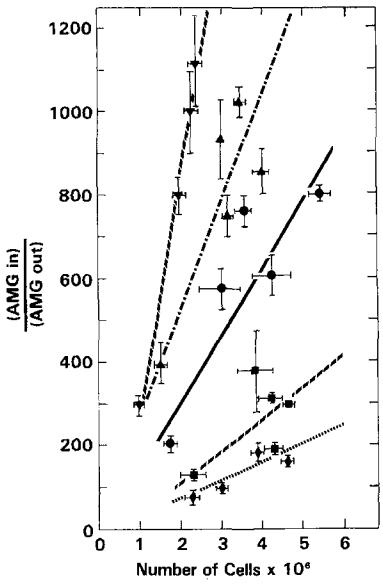

Fig. 6. The AMG-concentration capacity as a function of cell density. Data from Figs. 4 and 5. Lines drawn by linear regression analysis yield the following values for the accumulating capacity to 10^6 cell ratio (mean ± SE): 5 mM glucose, 643 ± 61 (▼); 25 mM glucose, 46 ± 6 (◆); 25 mM glucose + 5 mM uridine, 83 ± 17 (■); 5 mM uridine, 233 ± 34 (▲); 25 mM mannose, 143 ± 14 (●)

shows the concentrating capacity of cells as a function of days in culture in different media. Cells grown in 25 mM glucose exhibit the lowest AMG-concentrating capacity, whereas those grown in 5 mM glucose the highest. When uridine is added to medium containing 25 mM glucose it elicits a significant increase in the concentrating capacity of the epithelia. In contrast, when added to medium containing 5 mM glucose uridine does not affect the concentrating capacity of the epithelia over the first 2 weeks. The concentrating capacity of cells fed with uridine alone (5 mM) is not significantly different from the concentrating capacity of cells fed with 5 mM glucose alone, at least for the first 2 weeks after seeding.

The different growth conditions result in the formation of epithelia with different population densities, which also change with age. To evaluate the correlation between cell density and the AMG-concentrating capacity attained in these studies, we followed the change in cell number as a function of the carbohydrate concentration in the growth medium and time after subculture. In Fig. 5, we plot the number of cells per well as a function of time in the experiment depicted in Fig. 4. The number of cells per well increases with the concentration of carbohydrate in the medium. Regardless of composition of the medium, the density of cells in a well reaches a plateau at approximately the same time as the AMG-accumulating capacity. In Fig. 6, the concentrating capacity attained in the epithelia described in Fig. 4 is plotted as a function of the number of cells per well from Fig. 5. For each growth condition the epithelia attained a different concentrating capacity despite comparable cell density. However, in each medium there is a linear relationship between cell density and the AMG-concentrating capacity achieved: the higher the cell density the greater the AMG-concentrating capacity. This relationship appears to be independent of cell age; as shown in Fig. 5, in some cases cell density actually decreased with cell age.

Does the regulation involve a direct effect of the substrate on its carrier or is it a process in which the glucose concentration serves as a primary trigger? To address this question we replaced glucose in the growth medium with the metabolizable sugars mannose and fructose, which are not transported by the sodium-dependent hexose transporter [15], and AMG, which shares the transporter but is not metabolizable. The AMG-concentrating capacity achieved with 25 mM mannose is higher than the concentrating capacity the cells reached when fed with medium containing the same concentration of glucose (Fig. 4). Figure 7, shows the result of an experiment in which epithelia grown for 13 days in 25 mM glucose were switched to media containing either 5 mM glucose, 25 mM mannose, or 25 mM fructose or continued in 25 mM glucose. Clearly, mannose and fructose, sugars that are not substrates for the sodium coupled glucose transporter [15], had a modest effect like that of glucose in down regulating the AMG-concentrating capacity.

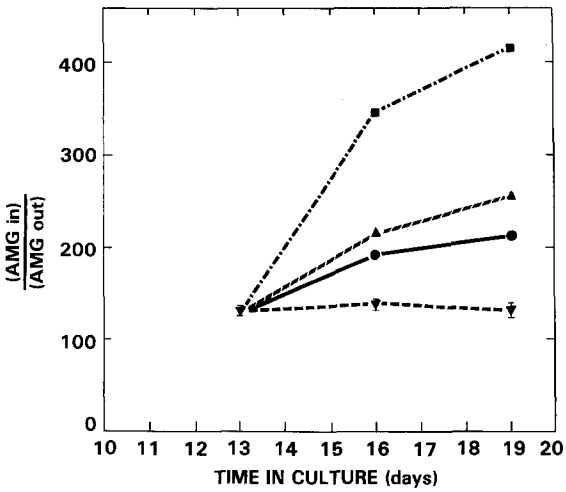

Fig. 7. AMG-concentrating capacity of epithelia grown for 13 days in medium containing 25 mM glucose and then switched to media supplemented with different hexoses. (●) 25 mM fructose, (▲) 25 mM mannose, (■) 5 mM glucose, and (▼) continued in 25 mM glucose

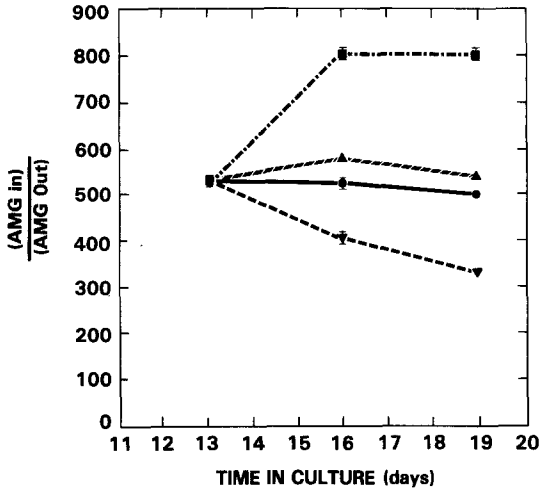

Fig. 8. AMG-concentrating capacity of epithelia grown for 13 days in medium containing 5 mM glucose and then switched to media supplemented with different hexoses: (●) 25 mM fructose, (▲) 25 mM mannose, (▼) 25 mM glucose, and (■) continued in 5 mM glucose

In a similar experiment (Fig. 8), we observed that switching epithelia grown in 5 mM glucose to media containing 25 mM mannose or 25 mM fructose results in a decrease in their concentrating capacity, which was, however, less than the decrease in epithelia switched into 25 mM glucose. Thus the absence of substrate-carrier interaction did not prevent the regulatory effect.

Next we tested the effect of a high concentration of AMG on the development of the concentrating capacity. Epithelia were grown for 14 days in medium containing 25 mM glucose. At that time one third of the epithelia were switched to medium containing 5 mM glucose, one third to medium containing 5 mM glucose + 20 mM AMG, and the remaining cells continued in medium containing 25 mM glucose. Uptake measurements in epithelia growing in media containing AMG will show a false reduction in AMG-concentrating capacity. AMG accumulating in the cells interferes with the uptake measurements unless the excess AMG is eliminated from the cells preceding the assay for AMG-concentrating capacity. Therefore AMG was chased by incubating the epithelia with medium containing 25 mM glucose for 4 hr prior to the assay. As we showed previously, no change in the concentrating capacity is detectable in less than 48 hr after changing the medium from one concentration of glucose to another [10]. Thus, 4 hr of 25 mM glucose did not affect the measurements. We found no difference between the concentrating capacity of cells grown in media containing either 5 mM glucose or 5 mM glucose + 20 mM AMG (245 ± 12 and 258 ± 10, respectively). Thus a substrate-carrier interaction with substrate in high concentration during incubation with low glucose did not suppress the development of high AMG-concentrating capacity. In view of the effect of mannose and fructose and the absence of an effect of AMG on the regulatory process, we conclude that the regulation does not require a direct interaction of the sugar substrate with the carrier, but involves another mechanism.

Discussion

In this study we deal with two main questions concerning the regulation of the sodium-coupled glucose transport system of LLC-PK_1 epithelia in culture: (i) Is the concentration of glucose in the medium the factor responsible for the regulation of the transport system? (ii) Is the regulation mediated by the effect of the substrate on its carrier, or rather some other mechanism?

Before addressing these two basic problems, other questions inherent in the tissue culture technique have to be resolved. In many cases, by manipulating the culture medium, one selects cells with certain properties. The possibility that the concentration of glucose in the growth medium promotes the growth of cells with certain glucose transport characteristics is always a possibility that has to be taken into account. The fact that a cloned cell line manifested the same basic regulatory characteristic makes this possibility very unlikely. In addition, the fact that the phenomenon of up and down

regulation is completely reversible upon changing the growth conditions also supports this conclusion.

Population density is also known to affect cell properties [11]. In the present work we find a linear relationship between the population density and the concentrating capacity within each treatment: the higher the population density the greater the AMG-concentrating capacity achieved. On the other hand, the differences in the AMG-concentrating capacity between cells grown in media containing 5 or 25 mM glucose are not the result of differences in population density.

To explore the nature of the extracellular regulatory signal, we used a conditioned medium in which cells that expressed increased AMG-concentrating capacity had been grown. The results eliminated all metabolites but glucose as the primary effector in the regulation. The only difference between the medium that caused an elevation in the concentrating capacity and the medium that suppressed it was the presence of a high concentration of glucose in the latter. By using frequent feeding for replenishment of consumed glucose, three growth conditions were created: (i) the concentration of glucose in the medium was virtually zero, (ii) it was maintained between 5 and 1 mM, and (iii) it was between 25 and 5 mM. The three growth conditions result in three different AMG-concentrating capacities related inversely to the glucose concentrations in the media.

In nonpolarized cells, such as fibroblasts, there is evidence that the metabolic state of the cell plays a role in the regulation of glucose transport [2, 18]. Our observation that sugars that are not transported by the sodium-dependent glucose transporter do affect the AMG-concentrating capacity of LLC-PK_1 epithelia, whereas a sugar that is transported by this transport system but is not metabolized does not, indicates that the regulation of this transport system involves hexose metabolism rather than an interaction with the transporter itself. One reasonable candidate for a second messenger in this regulatory system is intra- or extracellular pH. Since LLC-PK_1 epithelia have a high rate of glycolysis, cells grown in high glucose acidify the medium more than cells grown in low glucose. However, hydrogen ion concentration cannot completely explain the regulatory effect of medium glucose concentration since media that contained mannose or fructose became as acidic as the medium containing high glucose, yet the concentrating capacity was significantly higher in mannose or fructose media.

Although aspects of the regulation of the sodium-coupled hexose transport have been established, additional studies are required for further understanding. For example, it is not clear whether the electrochemical gradient for sodium entry across the apical membrane, i.e., the driving force for this hexose transport, is also regulated. Furthermore, it remains to be determined whether regulation occurs in mature cells or only in dividing cells.

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References

1. Amsler, K., Cook, J.S. 1982. Development of Na^+ dependent hexose transport in a culture line of porcine kidney cells. *Am. J. Physiol.* **242:***C*94–*C*101
2. Bader, J.P., Brown, N.R., Ray, D.A. 1981. Increased glucose uptake capacity of Rous-transformed cells and the relevance of deprivation depression. *Cancer Res.* **41:**1702–1709
3. Burton, M.W., Reitzer, L.J., Kennell, D. 1981. The continuous growth of vertebrate cells in the absence of sugars. *J. Biol. Chem.* **256:**7812–7819
4. Cook, J.S., Amsler, K., Weiss, E.R., Shaffer, C. 1982. Development of Na^+ hexose transport *in vitro*. *In:* Membranes in Growth and Development. J.F. Hoffman, G. Giebisch, and L. Bolix, editors. pp. 551–567. A.R. Liss, New York
5. Hull, R.N., Cherry, W.R., Weaver, G.W. 1976. The origin and characteristics of a pig kidney cell strain LLC-PK_1. *In Vitro* **12:**670–677
6. Mills, J.W., Macknight, A.D.C., Jarrell, J.A., Dayer, J.M., Ausiello, D.A. 1981. Interaction of ouabain with the Na^+ pump in intact epithelial cells. *J. Cell Biol.* **88:**637–643
7. Misfeldt, D.S., Sanders, M.J. 1981. Transepithelial transport in cell culture: D-glucose transport by a pig kidney cell line (LLC-PK_1). *J. Membrane Biol.* **59:**13–18
8. Misfeldt, D.S., Sanders, M.J. 1983. Transepithelial transport in cell culture: Stoichiometry of Na/phlorizin binding and Na/D-glucose cotransport. A two-step, two-sodium model of binding and translocation. *J. Membrane Biol.* **70:**191–198
9. Moran, A., Handler, J.S., Turner, R.J. 1982. Na^+ dependent hexose transport in vesicles from cultured renal epithelial cell line. *Am. J. Physiol.* **243:***C*293–*C*298
10. Moran, A., Turner, R.J., Handler, J.S. 1983. Regulation of sodium coupled glucose transport by glucose in a cultured epithelium. *J. Biol. Chem.* **258:**15087–15090
11. Mullin, J.M., Weilbel, J., Diamond, L., Kleinzeller, A. 1980. Sugar transport in the LLC-PK_1 renal epithelial cell line: Similarity to mammalian kidney and the influence of cell density. *J. Cell Physiol.* **104:**375–389
12. Rabito, C.A. 1980. Phosphate transport by a kidney epithelial cell line. *J. Gen. Physiol.* **76:**20*a*
13. Rabito, C.A. 1981. Localization of the Na^+ sugar cotransport system in a kidney epithelial cell line (LLC-PK_1). *Biochim. Biophys. Acta.* **649:**286–297
14. Rabito, C.A., Karish, M.V. 1982. Polarized amino acid transport by an epithelial cell line of renal origin (LLC-PK_1). The basolateral system. *J. Biol. Chem.* **257:**6802–6808

15. Sanders, M.J., Simon, L.M., Misfeldt, D.S. 1982. Transepithelial transport in cell culture: Bioenergetics of Na, D-glucose coupled transport. *J. Cell Physiol.* **114:**263–266
16. Turner, R.J., Moran, A. 1982. Heterogeneity of sodium dependent D-glucose transport sites along the proximal tubule. *Am. J. Physiol.* **242:***F*406–*F*414
17. Turner, R.J., Moran, A. 1982. Further studies of proximal tubular brush border membrane D-glucose transport heterogeneity. *J. Membrane Biol.* **70:**37–45
18. Ullrey, D., Gammon, M.T., Kalckar, H.M. 1975. Uptake patterns and transport enhancements in cultures of hamster cells deprived of carbohydrates. *Arch. Biochem. Biophys.* **167:**410–416

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