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Effects of Inhibitors on 3-O-Methylglycose Transport in Rabbit Ileum

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Summary. Previous studies (Goldner, Schultz & Curran, *J. Gen. Physiol.* 1969, **53**:362) have suggested a direct coupling between influxes of sugars and Na across the brush border membrane of rabbit ileum. Effects of several inhibitors, ouabain, cyanide, dinitrophenol and iodoacetate on 3-O-methylglucose fluxes were examined in an effort to obtain information about coupling of sugar transport to metabolism. The inhibitors virtually abolished net active sugar transport across the whole tissue but had less striking effects on sugar influx across the brush border membrane, particularly when the cells were prevented from gaining Na as a result of inhibitor action. However, substantial but incomplete inhibition of influx was observed when the cells were permitted to gain Na. Mucosal strips incubated with ouabain to elevate cellular Na extruded sugar against a concentration gradient when cell Na concentration exceeded that in the medium. Conversely, a small extrusion of Na from ouabain-poisoned cells was observed in the presence of an outwardly directed concentration gradient for sugar. These results provide further evidence of coupling between Na and sugar movement. Additional direct coupling of sugar movement to metabolism cannot be ruled out.

The active transport of sugars by in vitro preparations of small intestine depends on the presence of Na in the solutions bathing the tissue [19]. For example, in rabbit ileum, cellular accumulation [21] and net transmural flux [10] of 3-O-methylglucose (3-MG) are completely inhibited by removal of Na, and unidirectional influx of 3-MG across the brush border membrane is markedly reduced by the absence of Na in the solution bathing the mucosal surface [10]. In addition, Na influx across the brush border is stimulated by 3-MG. Comparison of the increment in Na influx with the influx of 3-MG indicates the presence of a coupled transport system in the brush border membrane that mediates the simultaneous influx of Na and

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3-MG at a 1:1 ratio at all Na concentrations tested [10]. These and a variety of observations on other preparations of intestine appear to be consistent with the hypothesis proposed by Crane [2] that active transport of sugars by intestine is driven by the Na concentration difference between the mucosal solution and the cytoplasm. However, Kimmich [14, 15] has recently questioned this hypothesis on the basis of his studies on sugar transport by isolated cells of chick intestine and has suggested a transport mechanism involving direct input of metabolic energy. Thus, in terms of the analysis of Kedem [13] (*see also* Katchalsky & Curran [12]), sugar transport may be coupled to Na flow or directly coupled to metabolism.

The present experiments were undertaken to examine the effects of several inhibitors on the influx of 3-MG across the brush border of rabbit ileum in an effort to obtain further information on the mechanism of sugar transport in this tissue. We have also examined the effect of concentration differences of Na and 3-MG on the net movement of these solutes between the cells and the bathing solutions.

Materials and Methods

The techniques used for the measurement of transmural flux [10], unidirectional influx [20], and net transfer in strips of mucosa [11] have been described previously. Briefly, terminal ileum from New Zealand white rabbits was removed after the animal had been sacrificed with sodium pentobarbital. Transmural fluxes were measured on pieces of ileum mounted as a flat sheet between Lucite chambers. Both sides of the tissue were bathed with a Ringer's solution containing 140 mM NaCl, 10 mM $KHCO_3$, 0.2 mM KH_2PO_4, 1.2 mM K_2HPO_4, 1.2 mM $MgCl_2$, and 1.2 mM $CaCl_2$ or with a Na-free solution in which all NaCl was replaced by choline chloride. Both bathing solutions contained 20 mM 3-MG; ^{14}C-3-MG was added to one solution and the steady-state rate of tracer appearance in the opposite solution was determined. Mucosal-to-serosal and serosal-to-mucosal fluxes were measured on adjacent pieces of tissue from the same animal. The inhibitors used were ouabain (10^{-4} M), NaCN (2×10^{-3} M), Na-iodoacetate (10^{-3} M) and 2,4-dinitrophenol (DNP) (10^{-4} M). Unidirectional influx across the brush border was determined on tissue mounted as a flat sheet in a chamber in which only the mucosal surface was exposed to the test solution [20]. The tissue was equilibrated for 30 min in the absence of the test sugar but in the presence of the appropriate inhibitor. The test solution contained 20 mM 3-MG, ^{14}C-3-MG and ^{3}H-inulin and was in contact with the tissue for 60 sec. The influx was estimated from the amount of ^{14}C-3-MG taken up by the tissue after correction for the amount of adherent medium determined by ^{3}H-inulin. Eight flux determinations were made in each experiment; four with an inhibitor and four controls. Results were expressed as a ratio of influx in the presence of inhibitor to control influx. Measurements of net transfer of 3-MG and Na between the cells and bathing medium were made using strips of mucosa obtained by scraping the tissue with a glass slide. The strips were incubated in Ringer's solution containing 10^{-4} M ouabain, ^{14}C-3-MG or ^{22}Na and ^{3}H-inulin. After 60 min, some of the strips were transferred to another flask containing a solution of different composition but with the same concentrations and specific activities of isotopes. In this way, concentration differences of

Na or 3-MG were set up and their effects on net fluxes between cells and medium could be determined. Samples of tissue were removed at 5-min intervals and intracellular 3-MG or Na concentration and cell water were determined by standard methods using ^{3}H-inulin to estimate extracellular space (*see* Ref. [11]). Details of solutions used are given under Results. All experiments were carried out at 37 °C.

Results

The unidirectional transmural fluxes of 3-MG under a variety of conditions are summarized in Table 1. Under control conditions with 20 mM 3-MG in both bathing solutions, a net flux of 0.89 μmoles/hr cm^2 from mucosa-to-serosa was observed. This net transport was almost completely abolished by DNP, cyanide, iodoacetate or ouabain, primarily as a result of a marked decrease in the unidirectional flux from mucosa-to-serosa. Only with ouabain was there still a significant net flux but it was less than 25% of the control value. The net active transport of 3-MG was also completely inhibited when both sides of the tissue were bathed in Na-free medium. Thus, ouabain, metabolic poisons, and Na removal are essentially equivalent in their effects on net transport and on the unidirectional 3-MG flux from mucosa-to-serosa.

A different pattern emerges, however, from studies of the unidirectional influx of 3-MG across the brush border from the mucosal solution into the cells. Results of these studies are summarized in Figs. 1 and 2 for two different conditions. In the experiments shown in Fig. 1, all tissues were preincubated for 30 min in Na-free choline medium (plus inhibitors) and influx was then measured from solution containing 140 mM Na. Under these conditions, the inhibitors had only relatively small, but statistically significant effects. The reduction in influx caused by Na removal was several times greater than those caused by the inhibitors. In these studies, the average

Table 1. Effect of inhibitors on 3-MG fluxes

Condition	J_{ms}	J_{sm} (μmoles/hr cm^2)	J_{net}
Control	1.14±0.04	0.25±0.04	0.89
Na-free	0.19±0.04	0.18±0.04	0.01
DNP	0.41±0.03	0.36±0.03	0.05
Cyanide	0.43±0.02	0.34±0.03	0.09
Iodoacetate	0.52±0.04	0.41±0.04	0.11
Ouabain	0.37±0.02	0.16±0.03	0.21

J_{ms} and J_{sm} denote mucosal-to-serosal and serosal-to-mucosal fluxes, respectively. Measurements were made on tissues from three to six animals under each condition and four flux periods of 20-min duration were used for each tissue.

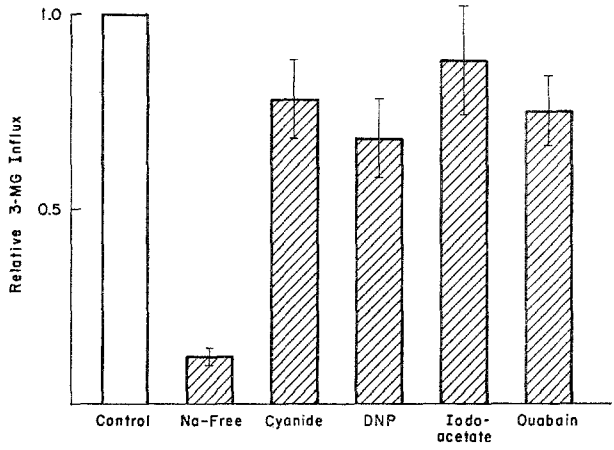

Fig. 1. Effect of inhibitors on 3-MG influx across the brush border. Tissues were preincubated for 30 min in Na-free choline medium (plus inhibitor) and influx was measured from solution containing 140 mM Na except for the Na-free case in which influx was measured from choline medium. Bars indicate ± 1 SEM

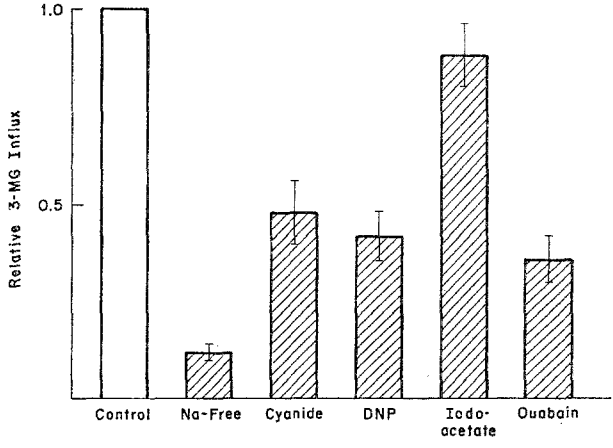

Fig. 2. Experiments identical to those shown in Fig. 1 except that the preincubation solution contained 140 mM Na in all cases except the "Na-free" condition

3-MG influx under control conditions was 1.62 μmoles/hr cm^2. In the experiments shown in Fig. 2, tissues were preincubated for 30 min in normal Ringer's solution (plus inhibitors) and 3-MG influx measured from the same solution. All inhibitors except iodoacetate caused a substantial reduction in influx. However, in each case the influx in the presence of inhibitor was still significantly higher than the value observed in Na-free solution. In control tissues, the mean value of influx for all experiments was 1.54 μmoles/hr cm^2. Thus, as previously reported [10], preincubation of the tissue

in Na-free choline medium does not alter the influx of 3-MG when it is measured from a solution containing 140 mM Na.

The results shown in Figs. 1 and 2 indicate that metabolic poisons and ouabain do not completely inhibit the primary active step in 3-MG transport (the influx process) although these agents do completely inhibit net active transmural transport (Table 1). These observations would be consistent with the "Na-gradient hypothesis" but certainly do not prove it. In an effort to obtain further information on this point, we performed experiments examining the effect on net 3-MG movement of a Na concentration difference between the mucosal cells and the external solution.

As described under Materials and Methods, strips of mucosa were incubated in Ringer's solution containing 10 mM 3-MG and 10^{-4} M ouabain. After allowing a sufficient time for cellular Na and 3-MG to equilibrate with the external solution, the strips were transferred to a Na-free solution (all Na in the Ringer's replaced by K) containing the same 3-MG concentration. Fig. 3 summarizes the changes in cell water, 3-MG content of the cells and the cellular concentration of 3-MG observed after transfer of the tissues. There is clearly a net extrusion of 3-MG since cellular 3-MG content decreases significantly. This net transport takes place against a concentration difference since the 3-MG concentration in cell water falls well below that in the external medium.

Similar experiments were carried out to examine the effects of a concentration difference of 3-MG on net Na movement in ouabain-poisoned cells. The tissues were preincubated in Ringer's solution containing 40 mM 3-MG and 10^{-4} M ouabain. After equilibration, they were transferred to a solution identical in all respects except that it contained no 3-MG. The results are summarized in Fig. 4. The initial cellular Na concentration (144 mM) was nearly identical to that in the medium (140 mM) but during the first 5-min period after transfer there was a small but statistically significant net movement of Na out of the cells. As indicated by the concentration ratio, this Na extrusion occurred against a concentration difference.

Several attempts to carry out the converse experiment of causing 3-MG to accumulate in poisoned cells as a result of a Na concentration difference were unsuccessful. Initially, we preincubated cells in Na-free medium to deplete cellular Na. However, under these conditions 3-MG enters the cells very slowly [10] and when the tissues were transferred to Na medium containing the same concentration of 3-MG, the external 3-MG concentration was higher than the internal concentration. Under these conditions, any observed 3-MG entry could be ascribed to diffusion. Attempts to estimate what the cellular 3-MG concentration would be after the initial incubation

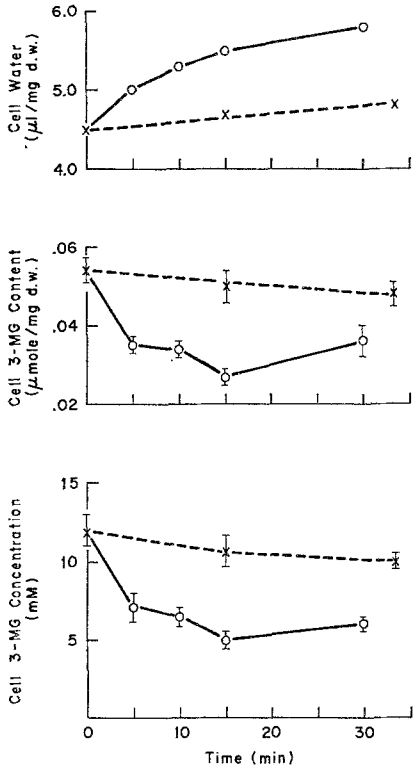

Fig. 3

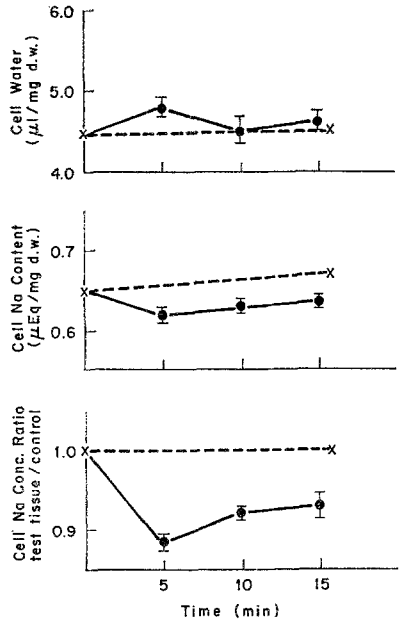

Fig. 4

Fig. 3. Extrusion of 3-MG from mucosal cells caused by a Na gradient. Ouabain-poisoned strips of mucosa were preincubated in solution containing 140 mM Na and 10 mM 3-MG. At zero time they were transferred to Na-free KCl medium containing 10 mM 3-MG. Dashed lines indicate control tissues that remained in the initial preincubation solution. Each point is the average of six determinations and bars indicate ± 1 SEM

Fig. 4. Extrusion of Na from mucosal cells caused by a 3-MG gradient. Ouabain-poisoned strips of mucosa were preincubated in solution containing 140 mM Na and 40 mM 3-MG. At zero time they were transferred to solution containing 140 mM Na but no 3-MG. Dashed lines indicate tissues that remained in the initial preincubation solution. Each point is the average of six determinations and bars indicate ± 1 SEM

and to adjust the concentration in the second incubation solution also proved unsuccessful. In five experiments, tissues were preincubated in Na-free choline medium containing ouabain or cyanide and 10 mM 3-MG. Some tissues were then transferred to medium containing 140 mM Na and some to new Na-free medium; both solutions contained 4 mM 3-MG plus ^{14}C-3-MG at the same specific activity as the original incubation solution. The ratio of cellular 3-MG content in tissues transferred to Na-free medium to control values averaged 0.97 ± 0.05 for the 30 samples taken over a 15-min

period following transfer. This ratio averaged 1.08 ± 0.04 for tissues transferred to Na medium. Thus, an inwardly directed Na gradient appeared to induce a small net entry of 3-MG into the cells, but in no case were we able to demonstrate a clear-cut net entry of 3-MG against a concentration difference.

Discussion

There is general agreement that active sugar transport by in vitro preparations of intestine is influenced by Na concentration in the bathing solutions. However, the mechanisms underlying this Na dependence are perhaps less clear. Crane [2] initially proposed that it was a reflection of the fact that the energy for sugar transport was supplied by the Na concentration difference between the mucosal solution and the cytoplasm. The results of Goldner, Schultz and Curran [10] seemed consistent with this view, particularly since they demonstrated that influx of 3-MG across the brush border of rabbit ileum was associated with a 1:1 increment in Na influx. However, Kimmich [14, 15] has recently reported that isolated cells from chicken intestine can accumulate sugars in the absence of or even against a Na concentration difference. He has proposed that the transport process in the brush border is driven by an ATPase system that is sensitive to Na and K and has suggested a specific model in which a high-energy intermediate in a Na, K-ATPase system can be utilized to drive sugar (and amino acid) transport in addition to (or instead of) monovalent ion transport[1]. These two hypotheses can be expressed clearly in terms of an analysis of sugar flux using the approach of Kedem [13] based on nonequilibrium thermodynamics. According to this analysis, net sugar flux J_s across the brush border would be given by

$$J_s = \frac{\Delta \mu_s}{R_{ss}} - \frac{R_{s(\mathrm{Na})}}{R_{ss}} J_{\mathrm{Na}} - \frac{R_{sr}}{R_{ss}} J_r \qquad (1)$$

in which $\Delta \mu_s$ is the difference in chemical potential between mucosal solution and cytoplasm, J_{Na} is Na flux and J_r is the rate of the appropriate metabolic reaction. R_{ss} is the resistance coefficient of the membrane for sugar diffusion and the coefficients $R_{s(\mathrm{Na})}$ and R_{sr} are measures of coupling of sugar flow to Na flow and to metabolism, respectively. According to Eq. (1), active sugar transport (i.e., flow against a difference in chemical potential)

1 The specific scheme proposed by Kimmich requires the presence of a Na, K-activated, ouabain-sensitive ATPase in the brush border membrane. This point seems to be in some dispute. Fujita, Mitsui, Nagano and Nakao [7] found very little ouabain-sensitive ATPase in the brush border of rat intestine while Quigley and Gotterer [16] report significant amounts in their preparations from rat intestine.

could be caused by coupling of sugar flow to Na flow or directly to metabolism or possibly to both. If the "Na-gradient" hypothesis is correct, $R_{sr}=0$ and if Kimmich's hypothesis is correct, $R_{s(Na)}=0$. However, in the latter case, R_{sr} would be a function of Na concentration since active transport is dependent on Na concentration.

The present results provide some information on the possible applicability of such a scheme to the sugar transport system in rabbit ileum. According to Kimmich's hypothesis, poisoning with ouabain or with metabolic inhibitors should have effects on sugar transport equivalent to removal of Na. Table 1 shows that this is true in one respect; Na-free solutions, DNP, cyanide, iodoacetate and ouabain all cause nearly complete inhibition of net transmural active transport of 3-MG. However, the effects of these treatments on the primary step in active sugar transport, influx across the brush border membrane, are not equivalent. As shown in Fig. 1, Na removal causes a 90% reduction in 3-MG influx while the various poisons cause an inhibition of only 20% or less. Thus, in these experiments in which the tissue was prevented from gaining Na by preincubation in Na-free solution (plus the appropriate inhibitor), the poisoned tissue continues to display a large Na-dependent influx. These results would argue against the concept that the sole effect of Na removal is to inhibit the operation of an ATPase that requires Na (and is sensitive to ouabain) or in terms of Eq. (1), that the only effect of Na is on the coefficient R_{sr}.

The situation is, however, made somewhat more complicated by the results shown in Fig. 2. In these experiments, preincubation was carried out in Na-containing medium so that poisoned tissue gained substantial amounts of Na. In this case, treatment with ouabain, cyanide or DNP caused a substantially larger inhibition of 3-MG influx. The effect of iodoacetate, however, was again minimal. In spite of the large inhibition, some Na-dependent influx remains in the presence of ouabain, cyanide and DNP but under these conditions the effects of Na removal and addition of inhibitors are more nearly equivalent.

The reasons for the greater inhibition of influx observed for the studies shown in Fig. 2 compared to those in Fig. 1 is not known precisely. The effect could be caused in part by altered or more effective action of the inhibitors in the presence of Na. Thus, the changes in cellular energy stores could be different for the two conditions of preincubation but we have no evidence regarding this possibility at present. In addition, it is necessary to explain the observation that ouabain, with a very different mode of action than cyanide and DNP, also shows a similar change in effectiveness while iodoacetate does not.

All these effects could be explained, however, if the greater inhibition of influx in the experiments shown in Fig. 2 were caused by the increase in cell Na during preincubation and to cell swelling. According to the model for the sugar transport system in rabbit ileum developed by Goldner *et al.* [10], an increase in cell Na should cause a reduction in sugar influx across the brush border particularly under conditions in which no sugar is present in the cells. (*See* Schultz & Curran [19] for a detailed discussion of this point.) In a previous study on the effects of inhibitors on alanine influx across the brush border, we found that swelling of the cells caused a decrease in influx [1]. Since the cells swell when treated with inhibitors in the presence of Na [21], this effect may also contribute to the observed decrease in sugar influx. These possibilities seem to be supported by the observation that iodoacetate has little effect under these conditions. Studies on strips of mucosa showed that in the presence of 10^{-3} M iodoacetate, the mucosal cell Na concentration increased by less than 10 mM over a 30-min period (which is the usual preincubation period for influx measurements). In contrast, mucosal strips treated with 10^{-4} M ouabain showed a rise in cellular Na of nearly 50 mM in 30 min. This observation thus suggests that the increased inhibition observed when preincubation is carried out in the presence of Na may be caused, at least in part, by a rapid increase in cellular Na concentration. The reason for the minimal effect of iodoacetate on cell Na over a 30-min period is unknown, but longer incubation did lead to a substantial effect on cell Na. With regard to this point, it is important to note that the studies of transmural flux (Table 1), in which iodoacetate has a marked effect, were carried out over 120 min, a much longer time period.

These results seem somewhat difficult to reconcile with the scheme suggested by Kimmich but they certainly do not rule it out. In particular, the mode of action of a high-energy intermediate could be such that inhibition would have relatively small effects on the influx process; that is, the energy contribution could be used to maintain a low efflux across the brush border thus giving rise to the observed active transport. However, the present results also reinforce a point made by Goldner *et al.* [10]; the primary site of Na action on 3-MG influx is on the external side of the membrane. That is, depletion of cell Na by preincubation in Na-free solution does not appreciably affect 3-MG influx measured from a Na-containing solution. On the other hand, Kimmich's model would appear to suggest that the primary site of Na action should be on the cytoplasmic side of the brush border since it is cellular Na and not extracellular Na that activates Na, K-ATPase in red blood cells [9]. Clearly, a more detailed knowledge of the transport system will be necessary before these points can be clarified.

The results presented in Figs. 3 and 4 also seem consistent with our previous concepts regarding the Na-sugar interaction. In ouabain-poisoned cells, an outwardly directed Na concentration difference caused a net extrusion of 3-MG against a concentration difference. Similarly, an outwardly directed gradient of 3-MG caused a small but statistically significant extrusion of Na against a concentration difference. These results are similar to those reported previously [5, 11] for alanine and Na movements in rabbit ileum and to those reported by Crane [3] for 6-deoxyglucose movement in hamster intestine. Unlike the case for the alanine studies, we do not at present have sufficient information to show that the extrusions of 3-MG and Na take place mainly across the brush border membrane. However, if we assume that they do, the above results suggest that the sugar transport system is at least partially reversible and that in poisoned tissue a Na concentration difference can provide sufficient energy to cause net "active" extrusion of sugar, presumably via the coupled co-transport system we have previously proposed. In terms of Eq. (1), $R_{s(\mathrm{Na})} \neq 0$ since, in poisoned tissue an outwardly directed J_{Na} can cause a flow of sugar against $\Delta \mu_s$. Although the effect of a sugar gradient on Na movement was small, it is consistent with these concepts. Thus, a complete analysis of such a system using nonequilibrium thermodynamics would yield an equation for Na flow J_{Na} having the form

$$J_{\mathrm{Na}} = \frac{\Delta \tilde{\mu}_{\mathrm{Na}}}{R_{\mathrm{Na(Na)}}} - \frac{R_{\mathrm{Na}(s)}}{R_{\mathrm{Na(Na)}}} J_s + \cdots. \tag{2}$$

In other words, an outwardly directed sugar flow should cause an outward Na flow against $\Delta \tilde{\mu}_{\mathrm{Na}}$.

Although the present results seem reasonably consistent with our previous model [10] and with the concept that energy required for sugar transport by intestine can be provided at least in part by a Na concentration difference, they do not prove the concept nor do they rule out direct involvement of metabolic energy. We have at present no explanation for the fact that we were unable to cause accumulation of 3-MG in poisoned cells when external Na was higher than internal Na. As indicated, there are certain technical difficulties with this experiment that we were unable to overcome in a satisfactory manner. On the other hand, we cannot rule out more fundamental explanations related to properties of the transport system itself and further experiments are clearly required. Kimmich's observations [14, 15] of cellular uptake of galactose and 3-MG against a concentration difference in the absence of or against a Na concentration difference are certainly not consistent with coupling to Na as the sole mechanism of

active sugar transport. However, before reaching a final conclusion, it would be of value to have further information on effects of K.

We have previously observed [10] that K has rather striking effects of 3-MG influx; it inhibits influx and appears to act as an antagonist to Na. Thus, a K concentration difference (high in the cells and low outside) could also lead to an accumulation of sugar in the cells, as suggested by Crane [4]. Effects of K concentration differences on amino acid accumulation have been demonstrated in ascites cells [6]. A second factor that should be considered is the possible influence of the electrical potential difference (PD) between the mucosal solution and the cytoplasm [8]. Since the transport site appears to combine with Na ion as well as the sugar molecule, there should be a difference in net charge between the combined and uncombined site. Under these conditions, the rates of transfer across the brush border membrane should be influenced by PD. The observations of Rose and Schultz [17] and White and Armstrong [22] indicate that this force is not negligible since they found an average PD of approximately 35 mV (mucosal solution positive) in rabbit and bullfrog intestine, respectively, when the mucosal solution was a normal Ringer's. There are thus a variety of points that will have to be further clarified before we understand the sugar transport system in intestine and there are several apparently conflicting observations that must be incorporated into any model of the system. For example, it is entirely possible that both the Na gradient and a metabolic process are involved in the energy input to the transport system as indicated by Eq. (1). Such an arrangement might well provide an explanation of results that appear to be in conflict with one or the other of the concepts regarding energy supply. It is of interest to note in this regard that Schafer and Heinz [18], using a thermodynamic analysis similar to that discussed here, concluded that coupling to both ion movements and metabolism was involved in amino acid transport in Ehrlich ascites cells.

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