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Characteristics of Amino Acid Accumulation by Isolated Intestinal Epithelial Cells

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Summary. Accumulation of neutral amino acids by isolated chick epithelial cells has been studied and the results discussed in terms of the ion gradient model, and a model invoking a direct input of metabolic energy. The cells establish four- to eightfold concentration gradients of amino acids at an extracellular concentration of 1 mM. The accumulation is sodium-dependent, inhibited by high extracellular potassium concentrations, and is sensitive to a variety of metabolic inhibitors. Also, amino acid uptake is depressed by actively transported sugars, and certain other amino acids, and is stimulated by phloridzin.

Cells equilibrated with valine and loaded with 30 to 40 mM intracellular sodium begin immediately to actively accumulate valine when suddenly introduced to media containing 20 mM sodium. The cells establish a threefold gradient of amino acid during the interval when intracellular sodium is higher than extracellular sodium.

Amino acid accumulation and ^{22}Na efflux were monitored simultaneously in cells treated with phloridzin. While phloridzin causes a 30% stimulation of amino acid uptake, no variation in the rate of ^{22}Na efflux or the steady-state level of ^{22}Na maintained by the cells can be detected. Similarly, either 2.5 mM glucose or 2.5 mM 3-O-methylglucose cause approximately a 50% inhibition of 1 mM valine uptake, but no detectable change in steady-state cellular ^{22}Na content. Several aspects of the data seem inconsistent with concepts embodied in the ion gradient hypothesis, and it is suggested that a directly energized transport mechanism can better accommodate all of the data.

The mechanism by which the cell membrane of intestinal epithelial cells transduces metabolic energy and utilizes it for active accumulation of sugars and amino acids has been the subject of a wide variety of independent investigations. Basically, two different views have been presented with regard to the nature of the energy input for intestinal nonelectrolyte transport events. Models of the type first proposed by Riggs, Walker and Christensen [22] and Crane and his colleagues [3–5] envision a mechanism in which

no direct expenditure of ATP occurs at the locus of the sugar or amino acid carrier. Instead, ATP is thought to be used at a separate locus to drive the so-called sodium pump, which acts to establish a transmembrane sodium gradient which, in turn, functions as an energy reservoir for support of substrate accumulation. The sugar or amino acid gradients observed are thought to be generated by membrane carriers which can bind Na^+ in a manner which concomitantly increases the affinity of the carrier for non-electrolytes [4]. The carrier is postulated to have very different affinities for its substrate at the inner and outer membrane surfaces due to the marked difference in availability of Na^+ at the two sites. The net flux of substrate would be expected to be inward on such a carrier until the degree of saturation of the carrier with substrate is equal at each surface. At this point, a concentration gradient of substrate will have been established, due to the aforementioned difference in affinity at each locus. Energy in the form of ATP is required only to maintain the transmembrane Na^+ asymmetry via active Na^+ extrusion from the cell. It is important to recognize that the site of Na^+ extrusion need not even be at the brush border pole of the cell. Indeed, considerable evidence has been reported which indicates that the Na^+-extrusion mechanism of intestinal epithelium occurs primarily at the lateral-serosal boundary (*see* review by Schultz & Curran [26]).

On the other hand, some recent evidence indicates that a more direct input of metabolic energy may be required for Na^+-dependent transport systems [9, 11, 20, 24, 25]. Potashner and Johnstone [20] and Schafer and Jacquez [25] in particular have shown that ascites cells can apparently actively accumulate certain amino acids when the usual transmembrane Na^+ gradient is absent or even reversed from normal. We have demonstrated the same phenomenon for Na^+-dependent sugar accumulation by isolated intestinal epithelial cells [11]. The isolated cell preparation offers a significant advantage in that intracellular ion and substrate concentration can be much more precisely defined than is possible with more intact tissue preparations containing several cell types with differing functional capabilities.

In view of the possible significance of these observations to the nature of the energy input mechanism functioning in support of the transport processes, the system warrants further study. Therefore, we decided to investigate another Na^+-dependent transport system with the isolated cell preparation, and to compare its characteristics with those of sugar transport. Sodium-dependent amino acid transport was chosen in light of recent evidence from our laboratory demonstrating significantly higher influx rates for valine than for actively transported sugars [14]. This finding implies that

valine entry might consequently be more responsive than sugar entry is to imposed sodium gradients.

This report describes the features of valine accumulation by the isolated intestinal cells and includes a consideration of the effect of experimentally manipulating the intracellular sodium concentration. The following two papers consider the role of K^+ and K^+ gradients in sugar or amino acid accumulation by the cells, and the significance of the interaction observed between the transport systems for sugars and amino acids [12, 13].

Materials and Methods

Isolated intestinal cells were obtained from 2- to 8-week-old White Leghorn chicks by the procedure reported by Kimmich [10]. Usually, the medium included 20 mM Tris-Cl (pH 7.4), 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 80 mM NaCl, 1 mg/ml bovine serum albumin (BSA), and 100 mM mannitol. Cells were isolated in this medium with 1 mg/ml hyaluronidase added. Media containing other sodium concentrations were prepared by adjusting the NaCl and mannitol concentrations so that the same total osmolarity was maintained. Lower potassium concentrations were produced by replacing K_2HPO_4 with Tris-PO_4. To increase the potassium concentration, KCl was added and an osmotically equivalent amount of mannitol removed. BSA (Cohn fraction V) and hyaluronidase (Type 1) were obtained from Sigma Chemical Co., St. Louis, Mo.

The isolated cells were kept at ice temperature until they were used. In some experiments, a preincubation interval at 37 °C was allowed, but in such cases the stock cell suspension was returned to the ice bath before the experimental phase. Experiments were initiated with the addition of 1 ml of cell suspension to 3 ml of the appropriate medium in a polyethylene beaker, held in a thermostated, oscillating waterbath maintained at 37 °C. Substrate, including radioactive tracer, was added to the medium, or in some cases to both medium and the concentrated cell suspension. Two hundred microliter serial samples were taken and filtered through Millipore filters (0.65 μ). The filters were rinsed with 5 ml ice-cold 150 mM NaCl, dried, placed in scintillation vials with scintillation cocktail (New England Nuclear Omnifluor in toluene), and counted for radioactivity in a Beckman LS-230 scintillation counter.

^{14}C- and ^{3}H-labeled compounds and $^{22}NaCl$ were obtained from Amersham-Searle. Experiments were typically run with 0.5 to 1 μC/ml of ^{3}H- or 0.1 μC/ml ^{14}C-labeled substrate, or 2 μC ^{22}Na in 1 ml cell suspension. Any variations in this format are noted in the text.

For experiments using cells preloaded with labeled substrate, cells were preincubated at 0 °C with the label for at least 10 min before use. Cells preloaded with ^{22}Na were preincubated at 37 °C with the label, and then were kept on ice for at least 10 min prior to the experimental phase. Double-label experiments were run with cells preloaded with both ^{3}H-labeled substrate and ^{22}Na. These cells were diluted into Na^+-free medium containing labeled substrate at the same concentration and specific activity as used during the preincubation. All experiments illustrated in the Results section represent typical data obtained from one of at least three separate experiments.

Protein content of the cell suspension was determined by the Biuret method [15], with an appropriate correction made for the BSA included in the medium.

Intracellular space was determined to allow the calculation of intracellular substrate concentrations. An aliquot of cell suspension was mixed with the impermeable marker,

^{14}C-polyethyleneglycol, and centrifuged. By counting aliquots of the supernatant and of the pellet resuspended in cold medium, the amount of extracellular water trapped in the pellet could be calculated. Total pellet water was determined by taking the difference between wet and dry weights of the pellet. The difference between total pellet water and extracellular water is taken as a measure of intracellular water. Values of 2 to 3 μliters/mg cell protein are typically obtained. Cellular concentration of substrate was calculated from the counts/minute of substrate taken up, specific activity of the labeled substrates and cellular volume according to the following formula:

$$[S]_{in} = \frac{\text{cpm taken up}}{(\text{spec. act.})(\text{cell volume})},$$

where specific activity is expressed in cpm/μmole and cell volume as μliters intracellular water. The distribution ratio of the substrate is simply the ratio of cellular concentration to that in the suspending medium.

Distribution ratios for inhibited cells were calculated assuming that cell volume does not change significantly in the presence of metabolic inhibitors. While this assumption may not be completely valid, our earlier studies indicated that no significant changes in cell volume could be detected due to treatment with DNP or phloridzin [10]. Still, it is logical to expect that some cell swelling may occur, particularly in those situations where ion transport is inhibited by ouabain, DNP, oligomycin, or rotenone. Therefore it is possible that steady-state levels of substrate or Na^+ are slightly over-estimated when inhibitors were employed. Such over-estimates do not materially affect the conclusions drawn as will be discussed following presentation of the data.

Results

Initially, a series of experiments was performed to determine the general characteristics of amino acid accumulation by the isolated cells. Valine accumulation by the cells has been demonstrated previously [10], but not considered in detail. Fig. 1 illustrates the effect of several agents on valine uptake by cells suspended in the standard incubation medium, as compared to a control noninhibited case. Distribution ratios were calculated as described in Materials and Methods. The uncoupler dinitrophenol (DNP) allowed uptake to the point where cellular and medium valine concentrations were equal. An overshoot of the final steady state is typically observed which apparently reflects a very brief period of active transport which can occur before the cellular energy reservoir is completely discharged. Similar overshoots have not been observed for sugar accumulation by the cells [10], but the rate of entry of sugars is only about one-third that for valine [14] and overloading probably does not occur in the short interval before DNP exerts its maximal effect. If the cells are preincubated for 5 min with DNP, no overshoot occurs. Rotenone, an electron transport inhibitor, and oligomycin, which blocks oxidative phosphorylation, inhibited uptake to the same degree as that shown for DNP, although the delay in onset of maximal

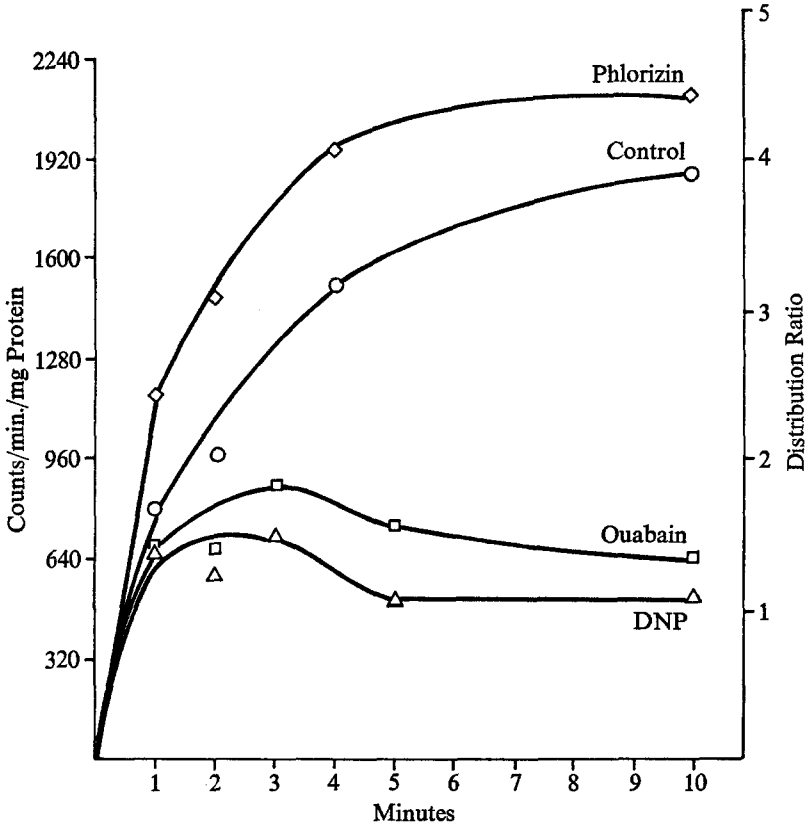

Fig. 1. Effect of inhibitors on 1.1 mM valine uptake. Uptake was followed as described in the text, in the presence of 250 μM dinitrophenol (DNP), 250 μM ouabain, 200 μM phloridzin, or in the absence of inhibitors. Uptake with 10 μg/ml oligomycin or 100 μM rotenone was the same as that with 250 μM DNP

inhibition is frequently absent when oligomycin is used (*see* Fig. 4). Ouabain, an agent thought to act specifically on active Na^+ transport, also is highly inhibitory as indicated. These inhibitors have identical effects on cellular uptake of leucine and alanine, neutral amino acids which are thought to share the same carrier [18]. In contrast to all of these inhibitors, phloridzin actually exerts a stimulatory effect on valine uptake. This effect of phloridzin, a potent inhibitor of sugar transport, has been reported previously for valine [10], and we have also observed it with other neutral amino acids.

If DNP is effective in preventing active accumulation of valine, but allows entry to the point where the cellular concentration of valine matches that in the medium, then the data of Fig. 1 indicate that the cells generate at least a fourfold concentration gradient in the control case. Calculated

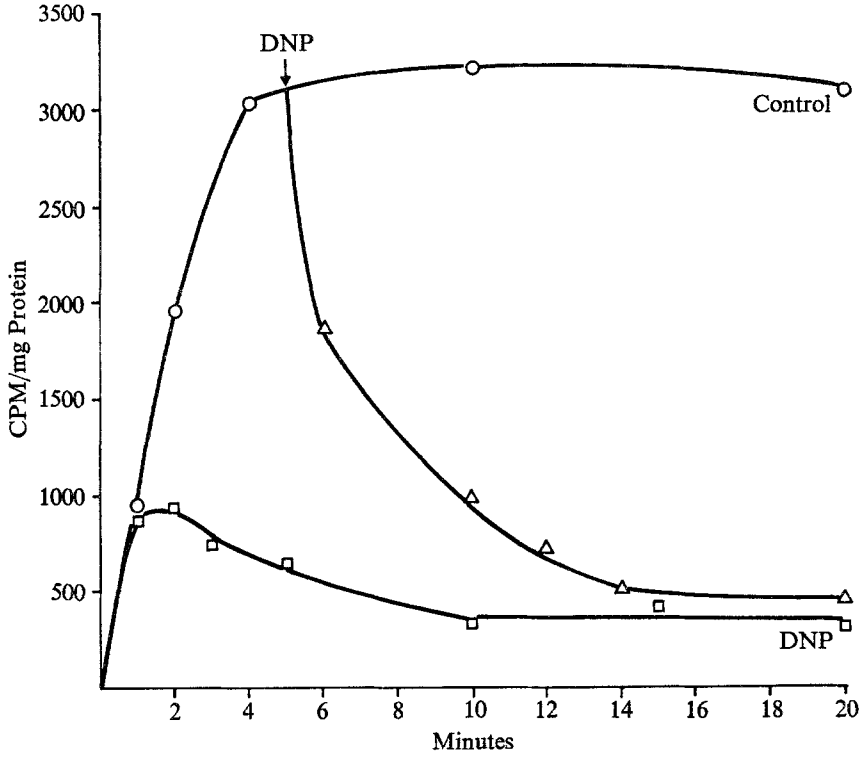

Fig. 2. Effect of 250 μM DNP on 1 mM valine uptake. DNP was added at the beginning of the experiment (□—□) or at 5 min (△—△)

distribution ratios confirm this impression. Further evidence for the existence of such a gradient is shown in Fig. 2. An interval of valine accumulation was allowed, and then DNP was introduced to prevent energy-dependent entry. The fact that previously accumulated valine is rapidly lost from the cell indicates that a concentration gradient of valine had been established prior to the addition of the inhibitor. The efflux continues until the same steady-state level of valine is reached as would have been established had DNP been present from the start. Again, the final steady-state amount of valine retained by the cells in the presence of DNP represents the amount expected for a distribution ratio of unity. The rapid efflux of labeled amino acid in this experiment also suggests that the accumulated valine is present in an unbound, readily diffusible form.

Another property of valine transport which has been demonstrated in more intact tissue preparations, is inhibition by other amino acids [18] and by certain sugars [17]. The same phenomenon can be observed for the

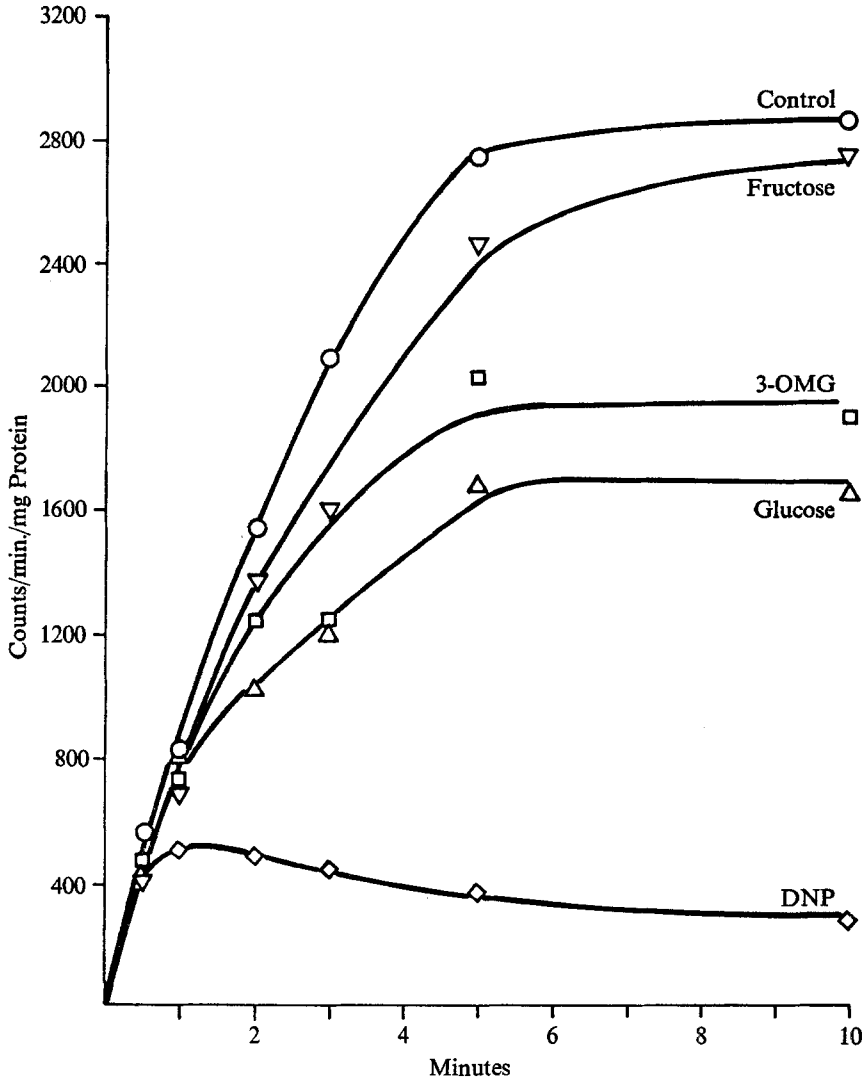

Fig. 3. Effect of sugars (2.5 mM) on 1 mM valine uptake

isolated cells. Fig. 3 shows that both metabolized (glucose) and nonmetabolized (3-O-methylglucose) actively transported sugars are effective inhibitors of valine uptake. As little as 2.5 mM glucose inhibited the active uptake of 1 mM valine by 45% and 3-OMG was nearly as effective. This is in contrast to data obtained by Newey and Smyth [17], which indicates that glucose is noninhibitory in preparations of rat jejunum. Fructose, which is metabolized but not actively accumulated by the gut [7], has no effect on valine uptake or is only slightly inhibitory (less than 10%).

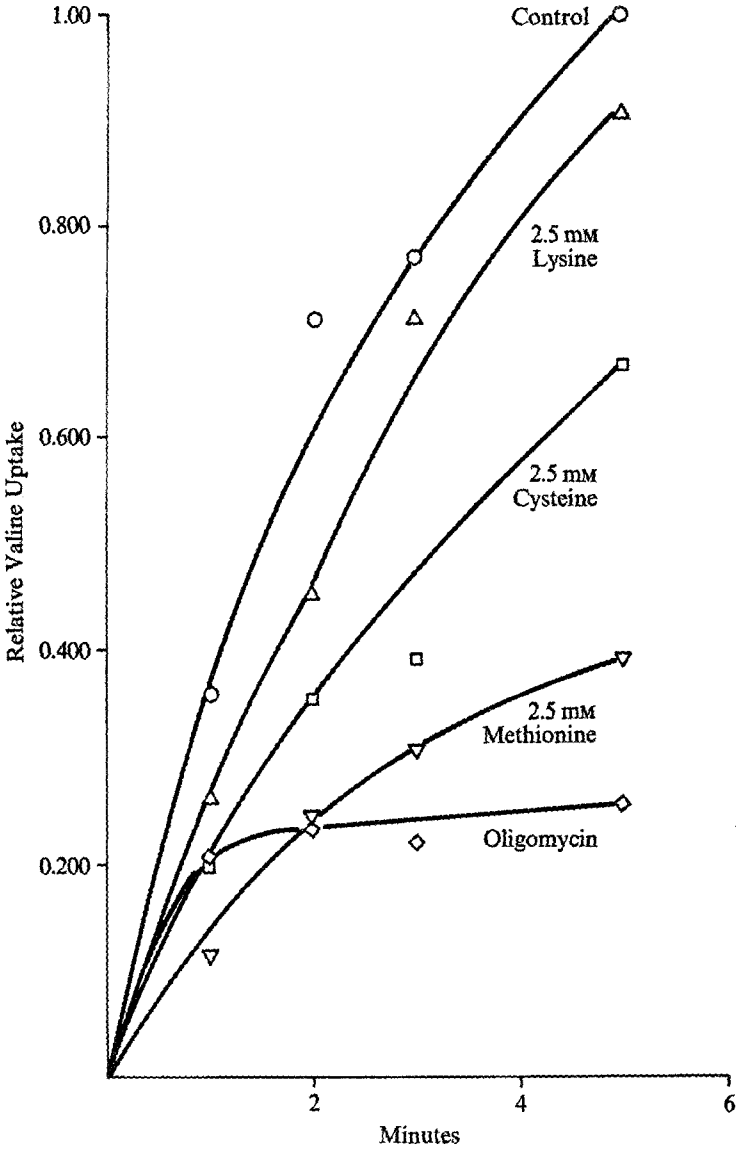

Fig. 4. Effect of other amino acids (2.5 mM) on 1 mM valine uptake. Data is expressed as the amount of valine uptake at each point relative to the amount taken up by the control at 5 min

Fig. 4 illustrates the effect of a variety of amino acids on valine accumulation. In each case, the concentration of valine was 1 mM and that of the inhibiting amino acid was 2.5 mM. Proline, α-amino-isobutyric acid, arginine, and glutamic acid which are not shown, all inhibit to the same

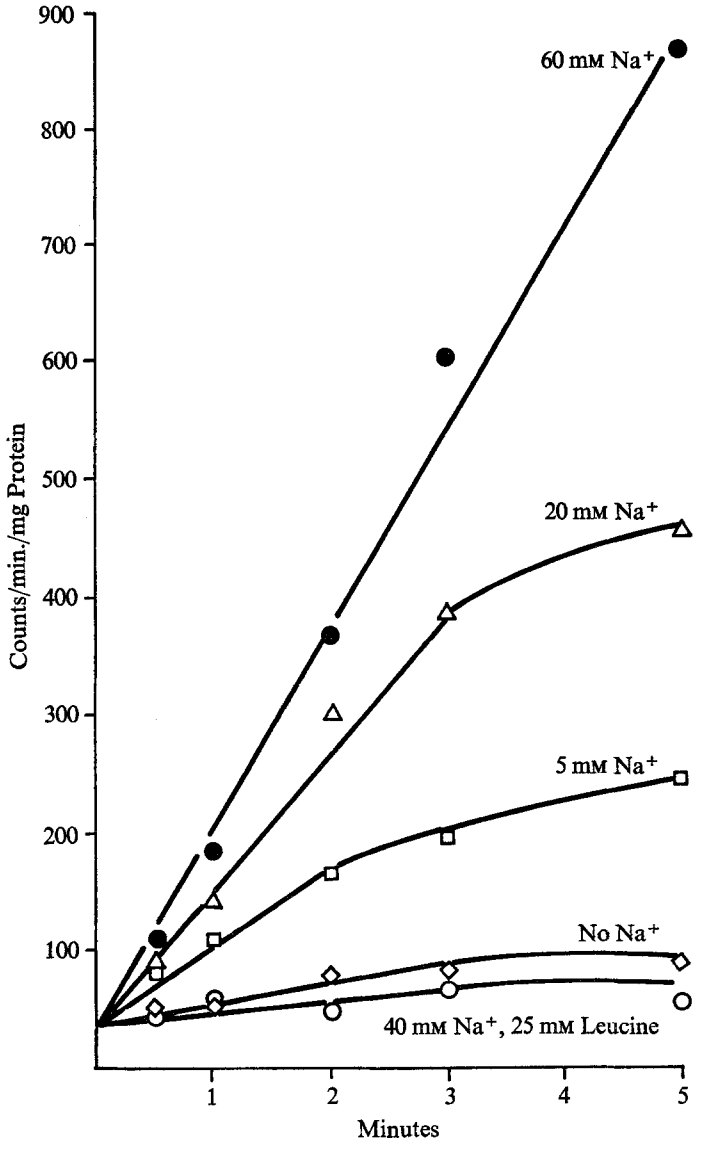

Fig. 5. Sodium-dependence of 0.25 mM valine uptake. Cells were isolated in medium including 80 mM Na^+ and 6 mM K^+. The isolated cells were then washed 3 times and resuspended in 6 mM K^+, sodium-free medium. One ml of these cells was added to 3 ml medium with sufficient sodium in it to give a final sodium concentration of 5 mM, 20 mM, 40 mM, or 60 mM as indicated in the figure, and ^{3}H-valine (1 mM) uptake was measured. As described in the text, valine entry in the presence of 25 mM leucine was used to determine diffusional entry. Medium containing 25 mM leucine was corrected for osmolarity by removing mannitol

degree as lysine. Cysteine inhibits active accumulation by 45% as indicated, and leucine exerts an effect equal to that shown for methionine. Nonactive uptake of 1 mM valine in the presence of oligomycin is also shown to allow comparison of the degree of uptake in each case with uptake observed in a fully inhibited state.

One of the most significant aspects of intestinal amino acid transport systems is their dependence on sodium ion [6, 28]. As shown in Fig. 5, this dependence is also found with the isolated cell system. In this experiment, cells were isolated in the standard 80 mM sodium medium, and were washed several times and resuspended in sodium-free medium. They were then introduced to media with the desired Na^+ concentration for the incubation portion of the experiment. If the cells are isolated in sodium-free medium, the dependence of amino acid uptake on sodium is not restored or is severely decreased, and little active accumulation of valine occurs. After isolation in the presence of sodium, the cells can be kept on ice in sodium-free medium and will respond to the addition of sodium.

A Lineweaver-Burke type plot was constructed with Na^+ as the variable substrate, and using the initial entry rates determined from Fig. 5. Entry of 1 mM valine in the presence of 25 mM leucine was used as a correction for diffusional entry. This concentration of leucine appears to be sufficient to block carrier-mediated valine entry [13, 14]. The difference between the entry rates in the absence of leucine and those observed with 25 mM leucine present was taken as the rate of carrier-mediated entry. Initial rates calculated in this way were used to determine the K_T^{Na} for valine. A value of 21 mM was obtained, which is similar to that determined for galactose (24 mM) by Kimmich [10].

The pronounced inhibitory effect of high extracellular potassium on amino acid transport has been known for many years [2, 4, 8]. The same phenomenon can be demonstrated for the isolated cells, as shown in Fig. 6. These experiments were performed at a sodium concentration of 20 mM and at 1 mM valine. Active valine accumulation is progressively more inhibited as the potassium concentration is raised from 1 to 76 mM, reaching 70% inhibition at the highest potassium concentration tested.

In addition to studying the direct effects of sodium and potassium concentrations on valine accumulation, we also attempted to determine the role of cellular sodium gradients on valine accumulation. The cells were incubated with ^{22}Na at 37 °C for 10 min, and returned to 0 °C. 1.0 mM ^3H-valine was then added and the cells allowed to stand an additional 10 min. The total sodium concentration was 80 mM and potassium was 1 mM during the loading phase. Following the preincubation, a 1-ml sample of cells was

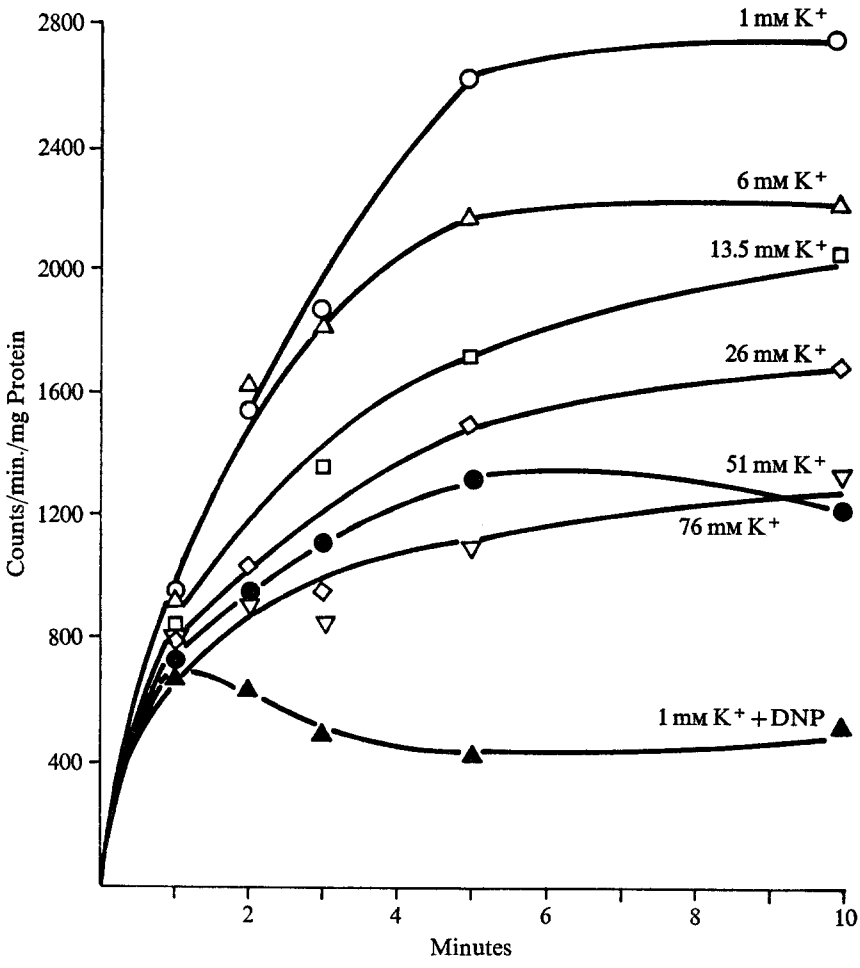

Fig. 6. Effect of elevated K^+ concentrations on accumulation of 1 mM valine by isolated intestinal cells. Medium Na^+ concentration was 20 mM in this case

introduced to 3 ml of sodium-free medium at 37 °C to begin the experiment. ^{3}H-valine was also present in this medium at the same concentration and specific activity as it was in the preincubation. The dilution caused the medium sodium concentration to fall to 20 mM while the specific activity of the ^{22}Na remained constant.

In some cases, DNP was used to inhibit active sodium extrusion. It can be seen in Fig. 7*a* that as the concentration of DNP is increased from 50 mM to 300 μM, the final steady-state concentration of ^{22}Na maintained by the cells increases. At a DNP concentration sufficient to inhibit valine uptake by approximately 50% (70 μM), an effect on steady-state ^{22}Na levels is

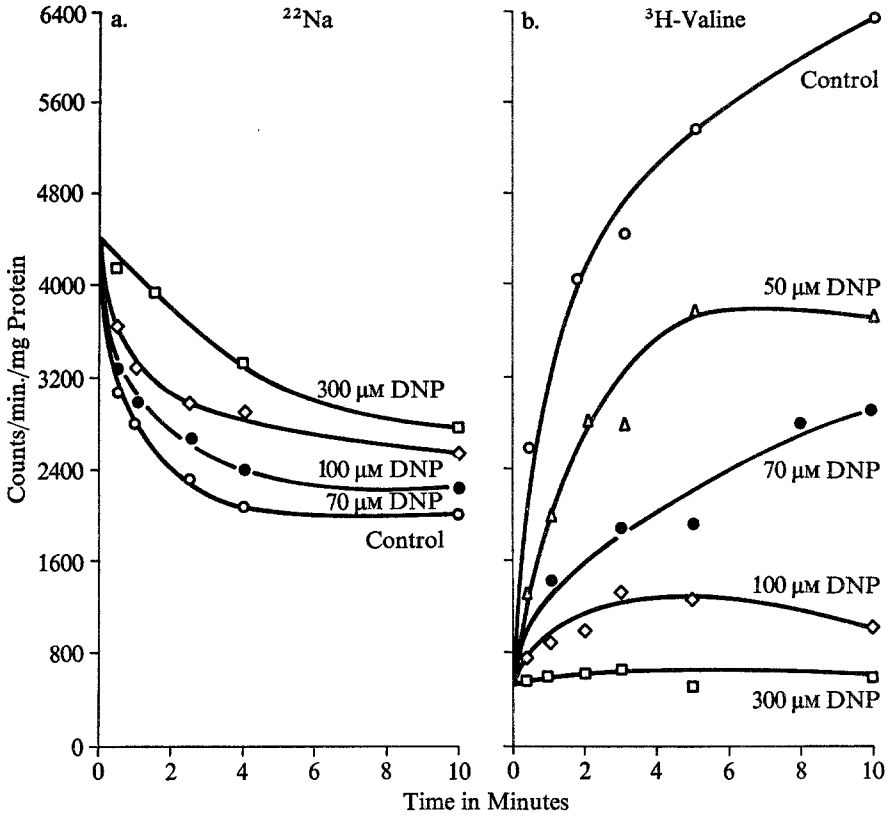

Fig. 7. Effect of various DNP concentrations on ^{22}Na extrusion and ^{3}H-valine accumulation by isolated intestinal epithelial cells. Cells were preloaded with ^{22}Na by preincubating at 37 °C for 10 min at 80 mM Na^+. Following their return to 0 °C, ^{3}H-valine was added to the stock suspension and an additional 10 min allowed for valine equilibration, before initiating the experiment. Final [Na^+] during the experiment = 20 mM

easily detectable. A small increase in the steady-state level of ^{22}Na maintained by the cells is sometimes observed at 50 μM DNP, but the effect is not always reproducible.

The steady-state concentration of sodium maintained by the cells at the highest DNP concentration is taken as the point at which the sodium activity of the cell matches that of the medium. It is apparent that in the absence of an inhibitor, the rate and extent of sodium extrusion is greater than when DNP is included (Fig. 7). Approximately 1 min is required for noninhibited cells to extrude sodium to the steady-state level maintained by the inhibited cells. That is, approximately 1 min elapses before cellular sodium in control cells falls to the level present in the medium. During this interval, while the

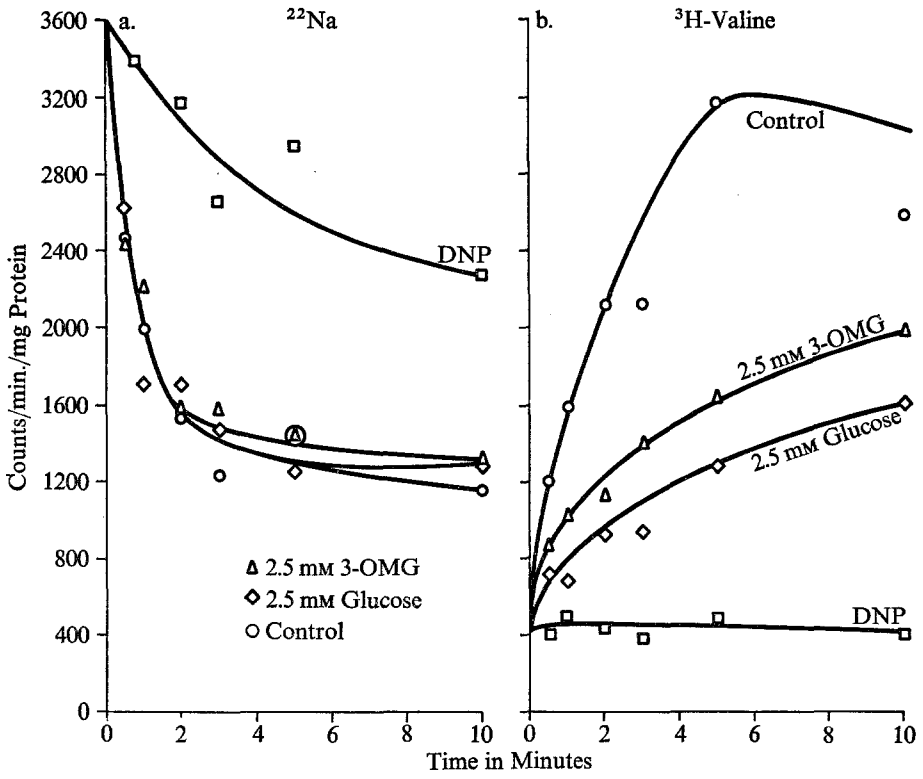

Fig. 8. Effect of 3-OMG and glucose on ^{22}Na extrusion and ^{3}H-valine accumulation by isolated intestinal cells. Experimental format was the same as described for Fig. 7. Cells were added to media containing the required sugar concentration at time 0

sodium gradient of the cell is reversed from normal, valine is rapidly accumulated against its electrochemical gradient (Fig. 7*b*). This finding is in contrast to the extrusion of valine predicted by the concepts implicit in the sodium gradient hypothesis. At the end of 1 min a more than threefold gradient of valine is established, and the rate of accumulation during the initial minute is more rapid than at subsequent intervals when a normally directed sodium gradient has begun to be re-established. Absence of a ^{3}H-valine flux in cells treated with high DNP concentrations (300 μM) indicates that the cells had indeed been preloaded to equilibrium before the final incubation.

In another series of experiments, ^{22}Na efflux was monitored in cells actively accumulating valine in the presence or absence of actively transported sugars. As seen in Fig. 8*a*, 2.5 mM glucose has no effect on the steady-state level of ^{22}Na maintained by the cells in the presence of 1 mM valine.

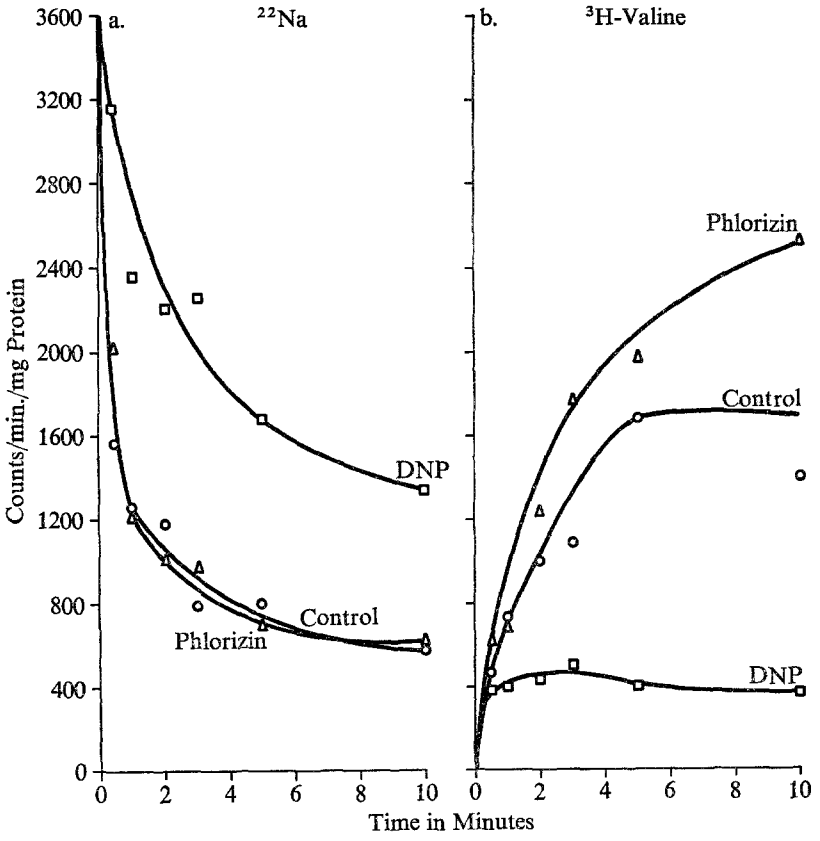

Fig. 9. Effect of phloridzin on ^{22}Na extrusion and ^{3}H-valine accumulation by isolated intestinal cells. The cells were preincubated at 37 °C for 10 min at 80 mM Na (+^{22}Na), but no subsequent equilibration with ^{3}H-valine was allowed. Cells were diluted into Na-free medium with ^{3}H-valine to initiate the experiment. Final $[Na^+]=20$ mM. Phloridzin and DNP were used at concentrations of 200 μM, when present

The same glucose concentration is sufficient to inhibit active 1 mM valine accumulation by 45% (Fig. 8*b*). Similar results were obtained with 3-OMG, an actively transported, but nonmetabolized sugar.

Fig. 9 illustrates the effect of 200 μM phloridzin on efflux of ^{22}Na and accumulation of ^{3}H-valine. As we have reported, a significant stimulation of valine uptake is produced by phloridzin. However, phloridzin does not cause a corresponding increase in the magnitude of the cellular Na^+ gradient. In fact, no change in either the rate or extent of ^{22}Na extrusion has been detected in a large number of experiments, one of which is shown in Fig. 9*a*. If amino acid uptake is driven solely by a normally directed sodium gradient, then increased valine uptake should be reflected by a greater than normal

sodium gradient. Therefore, one might expect a more extensive sodium efflux from phloridzin-treated cells and a lower final steady-state cellular Na^+ concentration.

In a similar series of experiments, both the sodium and potassium gradients were imposed in a direction reversed from normal. Cells were isolated and suspended in medium containing 80 mM Na^+ and 6 mM K^+. They were then added to a sodium-free, high-potassium medium. Dilution was such that the sodium concentration fell to 20 mM and the potassium concentration became 90 mM. These concentrations, as compared with measured cellular levels of K (40 to 50 mM) and Na (30 mM) are sufficient to cause the sodium concentration gradient to be directed outward and the potassium inward. Again, valine was actively accumulated during the initial part of the experiment. The low level of accumulation in the presence of 90 mM potassium is the result of the inhibitory effect of potassium mentioned previously. Even though the K^+ inhibition is severe, there is a clearly demonstrable accumulation of valine above equilibrium levels at early time points. Equilibrium valine levels were determined in this case by pretreating cells prepared in the manner just described with oligomycin and then adding them to media including the same concentration of oligomycin. The equilibrium valine concentration determined by this method was identical to that determined with DNP, and again no efflux of valine from the cells was observed despite the unfavorable ion gradients.

Discussion

The data described above show that amino acids are actively accumulated by the intestinal epithelial cell preparation, and that the accumulation has the same general properties that characterize amino acid transport in more intact tissue preparations. Clearly, amino acid uptake by these cells is sodium-dependent, potassium-sensitive, and inhibited by a variety of metabolic inhibitors and by the presence of other actively transported amino acids or sugars. However, these characteristics alone do not allow one to discriminate between the two basic models that have been suggested for the nature of the energy input acting in support of transport.

According to most current concepts, sodium-dependent transport may require no direct expenditure of metabolic energy. Instead, transport is thought to be driven by the energy inherent in the transmembrane sodium gradient. Energy in the form of ATP is required to maintain the sodium

asymmetry, but is thought not to be used directly at the substrate transport site.

Alternatively, other models propose a more or less direct participation of ATP. One such model is that previously proposed [11] as a result of work from this laboratory on active sugar transport. In this case, it was suggested that the source of energy might be a high-energy intermediate derived from ATP hydrolysis which could be tapped to drive several active transport systems including those for sugars, amino acids, and monovalent ions. The direct energy input was suggested in view of evidence similar to that presented here, which indicated that active sugar transport could occur in spite of an experimentally imposed reversed sodium gradient [11].

According to the model for directly energized transport, the inhibition of valine transport by sugars described above, would represent an increased cellular energy drain by means of a more rapid depletion either of cellular ATP, or of a more restricted energy pool represented by the energized intermediate [11]. On the other hand, interaction between transport systems might also represent competition for a limited energy supply in the form of the transmembrane sodium gradient, as has been suggested by Read [21]. Each of the inhibitors described could exert its action by discharging either cellular ATP pools or the existing sodium gradient. The observed inhibition by potassium is equally ambiguous, and might reflect either discharge of the postulated energized intermediate or a competition between potassium and sodium for a common carrier site. Crane, Forstner and Eicholz [4] have suggested an ion binding site associated with the substrate carrier which can accept either sodium or potassium. They have suggested that sodium acts to confer high substrate affinity on the carrier, while potassium might act in a converse sense to markedly decrease affinity and consequently to inhibit transport.

Certain aspects of amino acid transport in these cells, however, are difficult to reconcile with the concepts of the ion-gradient hypothesis. For instance, just as we have shown for the accumulation of sugars [11], active amino acid uptake by the isolated cells can occur in the presence of an outwardly-directed sodium gradient. A system in which the energy for transport is derived wholly from the cellular ion gradients, should exhibit substrate fluxes which are totally dependent on the direction of the imposed ion gradients. Substrate flux should always be in the direction of the sodium gradient and counter to the potassium gradient. In the absence of ion gradients, accumulation of substrate against a concentration gradient should not be possible. As noted in the results, not only was there no extrusion of pre-equilibrated valine while the ion gradients were reversed from their

normal directions, but significant active accumulation occurred during this interval. More significantly, as much as a two- to threefold valine gradient was generated during the first minute of the experiment, when cellular sodium concentration exceeded that in the medium. These gradients are somewhat greater than those observed for sugars within the same interval, and apparently reflect the faster entry rates associated with valine entry [13, 14].

It is important to recognize the fact that in dealing with isolated intestinal cells the polarity of function is difficult to ascertain in contrast to more intact tissue preparations. One must continually be aware of the fact that serosal-lateral membranes as well as the brush border surface, are exposed to the incubation medium, and observed net fluxes represent a composite of individual fluxes across all of the exposed surfaces. The question arises as to whether fluxes associated with boundaries other than the brush border can materially affect interpretation of the data described above. The possibility seems unlikely for several reasons. Schultz, Curran, Chez and Fuisz [27] have demonstrated by analysis of unidirectional and transmural fluxes that the serosal-to-cell influx of 5 mM alanine is only 15% of the unidirectional influx across the brush border of rabbit ileum. Extracellular Na^+ has no significant effect on the serosal influx rate. Munck and Schultz [16] have shown that serosal-to-cell influx of 2 mM leucine is less than 3% of the mucosal-to-cell influx using the same tissue. Using a somewhat different approach, Alvarado [1] has reported that serosal influx rates constitute only a small part of total observed influx in hamster intestine. While comparable data for chick intestine is not available, there is no compelling reason to suspect that serosal influx will be a greater proportion of the total for that species. Furthermore, if the serosal membrane fluxes are passive (nonactive), they could not account for the active accumulation which we have observed in the experiments described above. While it is possible that active transport occurs at the lateral-serosal boundary, any such event must be Na^+-dependent (Fig. 5), and ouabain sensitive (Fig. 1), and therefore itself suspect for coupling to the Na^+ gradient. Consequently, imposition of a reversed Na^+ gradient would also be expected to lead to extrusion of valine at these cellular sites, in contrast to the net accumulation actually observed.

We have already mentioned the possibility that cell swelling might occur in those situations where metabolic inhibitors were employed. Significant swelling seems unlikely in view of the fact that cells pre-equilibrated with substrate maintain the same steady-state level of substrate when introduced into media containing DNP (Figs. 7 and 8) or oligomycin (Fig. 10). Further-

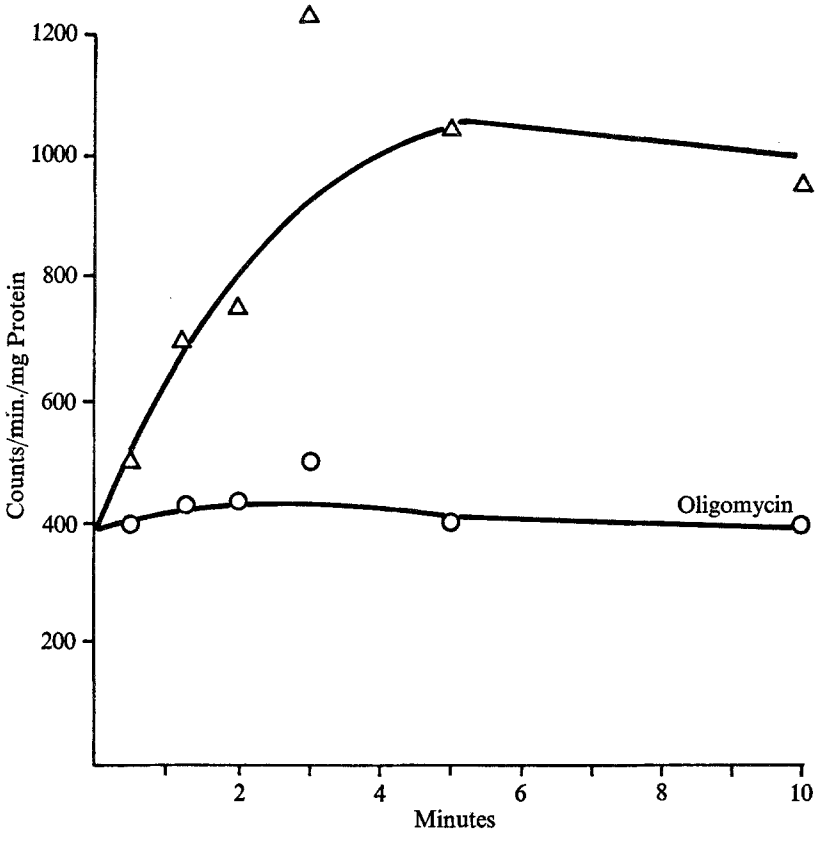

Fig. 10. Accumulation of 1 mM ^{3}H-valine by isolated intestinal cells in which reversed Na^+ and K^+ gradients had been imposed. Preincubation was at 80 mM Na^+ and 6 mM K^+. At time 0 the cells were introduced to Na^+-free, high K^+ medium. Final concentrations were 20 mM Na^+ and 90 mM K^+. The cells were also pre-equilibrated with 1 mM ^{3}H-valine. When present, oligomycin was used at a concentration of 5 μg/ml

more, if cells swell in the presence of DNP, the amount of ^{22}Na retained at the steady state (equilibrium Na^+ distribution) represents an overestimate of that expected for noninhibited (nonswollen) cells at equilibrium. Therefore, any tendency to swell in the presence of DNP would lead to an under-estimate of the time during which a reversed Na^+ gradient is maintained by the control cells. Errors due to inhibitor-induced swelling therefore would tend to minimize the net entry of valine observed during the interval in which Na^+ gradients are reversed from normal.

As we mentioned earlier, the interaction between sodium-dependent transport systems may be a reflection of competition for energy inherent in

the cellular sodium gradient. Co-transport of sodium with substrate is thought to produce a partial discharge of the sodium gradient, which consequently diminishes the transport of any other substrate deriving its energy from the same source [21]. We will examine this premise in detail in one of the following papers [13]. For the present discussion, however, it is important to recognize that there are certain inconsistencies between the work presented here, and predictions derived from a model for interaction based on transport-induced dissipation of the sodium gradient. As shown in Fig. 8, when 2.5 mM glucose is included in the incubation medium, the accumulation of 1 mM valine is inhibited by 50%. Despite the severity of this interaction, there is no detectable change in either the rate or steady-state level of ^{22}Na maintained by cells exposed to both glucose and valine as compared to those incubated with valine alone. While it is impossible to predict exactly the degree of dissipation of the sodium gradient expected for a given degree of transport inhibition, it seems certain that at least some decrease in the sodium gradient (steady-state ^{22}Na level) would be observed with a 50% inhibition of valine transport, if the energy input for transport is derived entirely from the sodium gradient. Indeed, 70 μM DNP produced about the same degree of inhibition of valine transport as 2.5 mM glucose, but the uncoupler caused a detectable change in steady-state ^{22}Na levels, in contrast to glucose.

Similar considerations may apply to the situation where phloridzin was observed to stimulate accumulation of valine. It seems possible that the phloridzin effect might be attributed to its action of blocking the mobility of the sugar carrier, and consequently preventing a slow, but continual influx of sodium by means of the sugar carrier. The sodium "leak" through the carrier would act to partially discharge the sodium gradient, and according to the sodium gradient hypothesis, would reduce the driving force for amino acid transport. In this case, one might expect that phloridzin-treated cells would maintain a better steady-state sodium gradient than untreated cells. In contrast, the data presented in Fig. 9 show that despite a 30% increase in the valine gradient maintained, no difference in the steady-state level of sodium maintained can be detected.

These data, therefore, are contrary to the basic concepts embodied in the ion gradient model. The direction of amino acid flux is not entirely dictated by the direction of the transmembrane ion gradients. Furthermore, significant changes in the valine gradient can be induced by actively transported sugars, or by phloridzin, which are not reflected by changes in the sodium gradient. For these reasons, we feel that a mechanism dependent on a more direct input of metabolic energy [10] must be considered as an

alternative to the sodium gradient hypothesis. However, it should be pointed out that in experiments involving measurement of sodium gradients, only the bulk cellular gradients can be monitored. It is possible that sodium-dependent transport of amino acids may be dependent on rather localized ion gradients established, for instance, in restricted areas of the cell near the brush border membrane surfaces. These could involve too small a part of the total ionic environment of the cell to be detectable. We consider this possibility unlikely since it implies a compartmentation of the brush border region of the cell which can restrict diffusion of Na^+ from more remote cellular regions. If such compartmentation exists, one would expect the diffusion barrier to likewise restrict diffusion of sugar to the cell interior. The elegant autoradiography experiments of Stirling and Kinter [29] tend to rule out any intracellular diffusion barrier to sugar near the brush border boundary. Also, in the absence of a diffusion barrier to Na^+ it has been calculated that cytoplasmic gradients of Na^+ would not be expected to exist for more than a few milliseconds [19]. An experimental approach toward evaluating the possibility of ion gradients existing in restricted regions of the cell near the brush border will be described in the third paper of this series [13].

It is also possible that the potassium gradient may play a much more significant role as an energy reservoir than is commonly recognized [23]. In most of the experiments described here, this possibility was ignored; and the following paper [12] is a detailed consideration of the role the potassium might play in sodium-dependent substrate transport. A detailed description of a modified model for a directly energized transport mechanism will be developed after consideration of the additional data.

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