Alanine and Sodium Fluxes Across Mucosal Border of Rabbit Ileum

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ABSTRACT Unidirectional influxes of L-alanine and Na from the mucosal solution into the epithelium of in vitro rabbit ileum have been determined. In the presence of 140 mM Na, alanine influx is approximately 2.2 μmoles/hr cm^2, but is inhibited if the NaCl in the mucosal solution is replaced by choline Cl, Tris-Cl, mannitol, LiCl, or KCl. Although alanine influx is strongly dependent upon Na in the mucosal solution, it is uninfluenced by marked reduction of intracellular Na pools. In addition, alanine influx is unaffected by intracellular alanine concentration. Na influx is markedly inhibited by the presence of Li. Evidence is presented that Na transport across the mucosal border cannot be attributed to simple diffusion even though the net flux across this surface is in the direction of the electrochemical potential difference.

The active transport of a variety of nonelectrolytes by animal cells appears to be dependent upon extracellular Na (1). For example, mucosal strips from rabbit ileum will accumulate L-alanine in an unbound, osmotically active form when incubated in a solution containing 140 mM Na. Removal of Na from the incubation medium has two effects on this process: (*a*) the rate of net alanine uptake is markedly reduced; and (*b*) the ability of the tissue to achieve an intracellular alanine concentration greater than that in the incubation medium is abolished (2). While these observations certainly implicate Na in the alanine transport process, they shed little light on the mechanism of the Na-amino acid interaction. Thus, inhibition of accumulation could result from a decreased influx into the tissue, an increased efflux out of the tissue, or a combination of both. Further, the preparations employed in studies of intestinal accumulation of nonelectrolytes do not distinguish between solute movements across the mucosal and across the serosal membranes of the cells.

In order to obtain information which would permit a more definitive insight into the interaction between Na and amino acid transport in intestine,

we have developed methods for the direct, simultaneous measurement of the unidirectional influxes of Na and amino acid from the mucosal bathing solution into the cells. The present communication describes these methods and the effects of cation replacement on the influxes of L-alanine and Na into the epithelium. The following paper is concerned with the kinetics of Na and amino acid influx, and with a model which is consistent with the observations on the interaction between Na and L-alanine transport.

METHODS

Male and female white rabbits, which had been maintained on normal food intake, were sacrificed by intravenous injection of pentobarbital. A section of terminal ileum was resected, opened along the mesenteric border, and washed free of

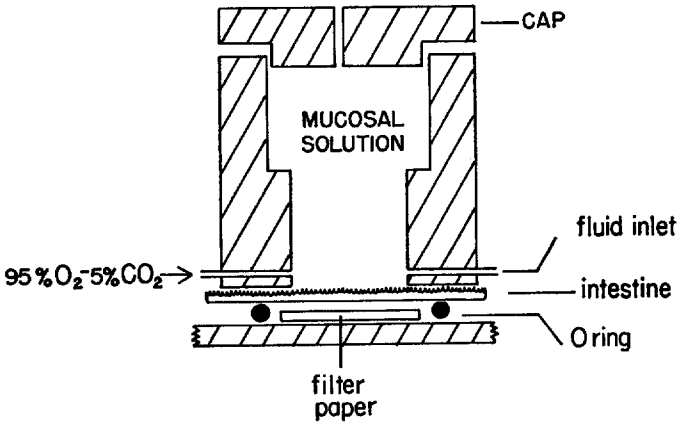

FIGURE 1. Schematic of a portion of the apparatus used for measurement of unidirectional influx of solutes from mucosal solution into intestinal epithelium. A single port is shown; the complete apparatus contained a row of four such ports.

intestinal contents with normal buffer. A segment of intestine, approximately 14 cm long, was then mounted mucosal surface up in the apparatus shown schematically in Fig. 1. In each port, 1.13 cm^2 of mucosal surface was exposed to a bathing solution which was stirred by a fine stream of humidified 95% O_2–5% CO_2. The serosal surface of the tissue rested on moistened filter paper and was not exposed to the mucosal solution. After mounting, the tissue was preincubated at 37°C with either a modified Ringer solution (NaCl, 140 mM; $KHCO_3$, 10 mM; K_2HPO_4, 1.2 mM; KH_2PO_4, 0.2 mM; $CaCl_2$, 1.2 mM; and, $MgCl_2$, 1.2 mM; pH 7.2) or a solution in which all or part of the NaCl was replaced with the isosmotic equivalent of choline Cl, KCl, Tris-Cl, LiCl, or mannitol. Unless otherwise stated, the *preincubation solution*[1] did not contain L-alanine or glucose. After 30 min, the preincubation solution

[1] *Preincubation solution* will refer to the buffer solution to which the tissue was exposed for 30 min prior to the determination of influx. *Test solution* will refer to the solution containing ^3H, ^{14}C, and ^{22}Na from which uptake was determined.

was removed and the appropriate *test solution* containing L-alanine-^{14}C (uniformly labeled, New England Nuclear Corp., Boston, Mass.), ^{3}H-inulin (methoxy-inulin-^{3}H, New England Nuclear Corp.) and ^{22}Na (New England Nuclear Corp.) was rapidly injected via one of the fluid inlets. The test solution was removed after 10 to 60 sec and the port was flushed with cold (4°C) isotonic mannitol solution (0.3 M) which was injected and immediately removed via a second inlet. The time interval from the injection of the test solution to the injection of the cold mannitol solution was taken as the duration of exposure of the mucosal surface to isotope. The mannitol wash served to terminate this exposure both by diluting the remaining test solution and by sudden cooling of the tissue.[2] The exposed 1.13 cm^2 of tissue was then cut out with a steel punch, washed briefly (3–5 sec) in cold 0.3 M mannitol, blotted, and extracted in 0.1 N HNO_3. Initially, the tissues were extracted for 24 hr but subsequent experiments showed that more than 98% of the radioactivity was extracted within 2 hr so that extraction periods between 2 and 24 hr were employed in later experiments.

TABLE I

ISOTOPE SEPARATION IN TRIPLE LABEL EXPERIMENTS*

Contents of standard	^{3}H assay (Channel I)	^{14}C assay (Channel II)	^{22}Na assay (Channel III)
^{3}H, ^{14}C, ^{22}Na	6533	6291	16242
^{3}H, ^{14}C	6508	6414	7
^{3}H, ^{22}Na	6525	20	16116
^{14}C, ^{22}Na	−1	6363	16172

* Assay is given as counts per minute attributable to ^{3}H, ^{14}C, and ^{22}Na in channels I, II, and III respectively, after correction using channel ratio values obtained with standards containing each of these isotopes alone.

Aliquots of the tissue extract and the test solution were assayed for ^{3}H, ^{14}C, and ^{22}Na simultaneously using a liquid scintillation spectrometer (Nuclear Chicago, Model 680). The uptakes of L-alanine-^{14}C and ^{22}Na by the tissue were calculated after correction for the inulin-^{3}H "space." Since previous studies have shown that inulin does not permeate the intestinal cell (2, 4), the inulin space is a measure of the volume of adherent test solution which was not removed by the mannitol washes. This volume was usually between 0.3 and 1.0 μl.

Reliability of Methods

The determinations of L-alanine and ^{22}Na uptakes depend upon the reliability of the simultaneous assay for ^{3}H, ^{14}C, and ^{22}Na and on the assumption that all the ^{14}C and ^{22}Na which crosses the mucosal membrane is retained within the tissue during the test period. The accuracy of the separation of ^{3}H, ^{14}C, and ^{22}Na is illustrated in Table I where assays of standards containing these three isotopes in various combinations are given. Similar examples of the reliability of the assay method were obtained

[2] Since the Q_{10} for alanine influx is 4.5 (3), sudden cooling of the tissue to 4°C should abruptly halt this process.

from a series of eight observations in which ^{22}Na was not included in the test solution but the data were processed as if this isotope were present; this calculation provides a measure of the extent to which ^{3}H or ^{14}C may be "mistaken" for ^{22}Na. The calculated ^{22}Na uptake in these experiments averaged 0.2 μmole/hr cm^2, a value which is less than 2% of the average Na influx. Finally, experiments were performed using inulin-^{3}H and inulin-^{14}C in the test solution; the ^{14}C space and ^{3}H space did not differ significantly. These results indicate that any increase in the alanine-^{14}C/inulin-^{3}H ratio in the tissue extract above that of the test solution can be completely attributed to alanine uptake by the tissue and is not influenced by differential quenching of medium and extract or other counting artifacts.

L-Alanine and Na are transported by rabbit ileum from the mucosal to the serosal solution[3] (5). Thus, if any of the ^{14}C or ^{22}Na which crossed the mucosal membrane during the 60 sec exposure left the tissue across the serosal surface, spuriously low influx values would be obtained. The likelihood of this occurring to any significant extent is reduced by the fact that the serosal tissues (i.e. submucosa, muscularis mucosa, muscle layers, and the serosal lining) form a diffusion barrier to the transmural movements of these solutes. Studies of the transmural fluxes of L-alanine-^{14}C and ^{22}Na have shown that 25 to 45 min are required before a steady-state isotope flux is obtained[3] (5). Nevertheless, to rule out the possibility of a significant loss of isotope due to transmural transport, the underlying filter paper was assayed in several experiments. The ratios of ^{14}C to ^{3}H and ^{22}Na to ^{3}H in the filter paper extracts indicated that the content of L-alanine and Na could be accounted for by contamination with the test solution which occurred during removal of the tissue; no statistically significant "excess" content of L-alanine-^{14}C or of ^{22}Na was detected.

Treatment of Experimental Data

In most experiments, two adjacent lengths of terminal ileum were used. Each was clamped in an apparatus which consisted of four adjacent perfusion ports of the type shown in Fig. 1. The rubber "O" ring isolates each individual area of tissue and prevents leakage between ports. Thus, the results of alterations in the preincubation and test solutions could be compared with control results obtained on tissue from the same animal. In over 200 experiments involving more than 400 pieces of tissue under control conditions, there was relatively little variation in Na and L-alanine influxes from animal to animal. Nonetheless, small differences in fluxes resulting from changes in the experimental solutions were most apparent when comparisons were made on tissue from the same animal. Further, whenever possible, control and experimental studies were performed using alternating ports to minimize systematic differences which might result from differences in the portion of intestine or, more important, from variations in the amount of stretch applied while mounting the two segments of tissue. Whenever available, data from paired analyses are presented. In order to obtain some estimate of the variation in the mounting procedure, dry weights were determined after extraction on a series of 98 tissues. The dry weight averaged 7.0 $\pm$ 1.6 mg/cm^2 (SD) and a frequency plot of the data showed a normal distribution.

The processing of the raw data from the triple-label experiments, and the sta-

[3] Field, M., S. G. Schultz, and P. F. Curran. 1967. *Biochim. Biophys. Acta*, **135**:236.

tistical analyses of the final data were performed with an IBM 1620 digital computer. All errors are expressed as standard errors of the mean unless otherwise stated.

RESULTS

Unidirectional Alanine and Na Influxes

The cumulative uptake of alanine-^{14}C and ^{22}Na by the tissue is plotted as a function of time of exposure to a test solution containing 5 mM alanine and 140 mM Na in Fig. 2. Both uptakes were determined simultaneously on

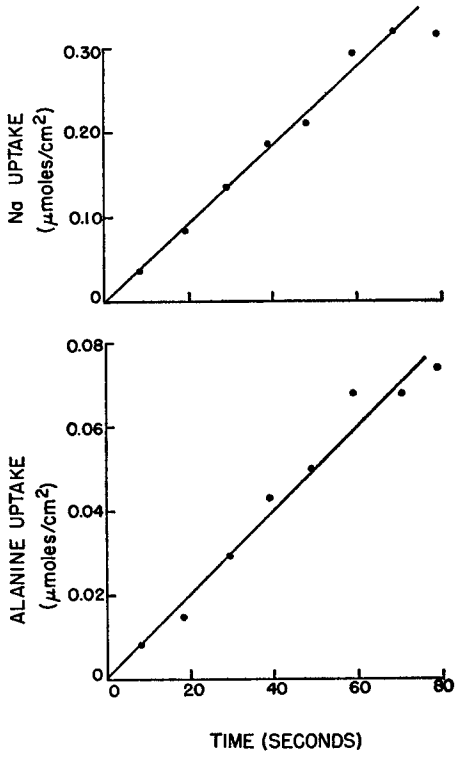

FIGURE 2. Uptake of alanine-^{14}C and ^{22}Na from mucosal (test) solution as a function of time. The concentrations were Na, 140 mM, alanine, 5 mM.

tissue from the same animal using each of the eight ports for an exposure of different duration. The uptake of each isotope is linear, within experimental error, for at least 60 sec and corresponds to a alanine-^{14}C influx of 3.6 μmoles/hr cm^2 and a ^{22}Na influx of 16.1 μmoles/hr cm^2. The observation that uptake is linear for more than 60 sec indicates that there is no significant back-flux of tracer during this period; thus these data are a true measure of unidirectional influx. Assuming that 1 cm^2 (serosal area) of ileum contains approximately 30 μl of intracellular water,[4] a maximum of 10 mM or 20% of the intracellular

[4] The height of mucosal cells of rabbit ileum is approximately 20 μ. Assuming that the villous infoldings increase the area 20-fold, the total volume of mucosa lining 1 cm^2 (serosal area) of intestine is 0.040 cm^3, and the water content (approximately 75%) is 30 μl.

Na exchanges in 60 sec. The maximal intracellular concentration of alanine achieved after 60 sec is 1 to 2 mM; this is 20–40% of the alanine concentration in the test solution and only 2–5% of the steady-state intracellular alanine concentration achieved by mucosal strips of rabbit ileum (2). The observation that the lines shown in Fig. 2 extrapolate close to the origin suggests that the uptake of tracer is abruptly halted by the cold mannitol wash and that negligible amounts of tracer are lost from the tissue during the washing and blotting procedures. If a significant loss of tracer occurred during these procedures, the amount lost would have to be a constant fraction of the total uptake in order for the lines to extrapolate to the origin; this possibility is considered unlikely. Further, these data suggest that binding of ^{22}Na or alanine-^{14}C to the tissue surface does not contribute significantly to the calculated uptake; such a process should be completed rapidly and would shift the extrapolated lines away from the origin toward a positive intercept on the ordinate.

Effect of Na on Alanine Influx

A series of experiments was carried out to examine the effect of Na on alanine influx. Tissues were preincubated for 30 min in either the normal Ringer solution, which will be referred to as "Na-Ringer's" (140 mM Na), or in a Na-free medium obtained by replacing all the NaCl with choline Cl ("choline-Ringer's"). The influxes of Na and alanine were then determined using either Na-Ringer's or choline-Ringer's as the test solution. In all experiments, the underlying filter paper was moistened with the solution used for preincubation. Previous studies (2) have shown that the intracellular Na concentration of mucosal strips of rabbit ileum incubated in Na-Ringer's is approximately 50 mM, and that after 30 min in choline-Ringer's the intracellular Na concentration is only 7–10 mM. These data may not be applicable, however, to the present experiments since they were obtained using a different preparation. In order to estimate the effect of choline-Ringer's on cell Na under conditions similar to those of the present experiments, everted sacs, prepared by the method of Wilson and Wiseman (6), were incubated at 37°C in either Na-Ringer's or choline-Ringer's. After 30 min, the sacs were removed and dipped into a 0.3 M mannitol solution at 4°C in order to remove adherent incubation medium. They were then blotted gently, and the mucosa was stripped off and analyzed for Na content using previously described methods (2). In eight experiments, the Na content of mucosa exposed to Na-Ringer's averaged 0.76 ± 0.11 μmole/mg dry weight compared with 0.24 ± 0.04 μmole/mg dry weight for sacs exposed to choline-Ringer's. Assuming that the intracellular Na concentration in the control tissue was 50 mM, the concentration in the tissue incubated in Na-free media was only 17 mM.[5]

[5] Since this value is uncorrected for Na present in the extracellular spaces underlying the mucosal cells, primarily the villous cores, it is an overestimate of the intracellular Na.

The results of experiments in which the influence of intracellular (preincubation) and extracellular (test) Na on alanine influx was examined separately are given in Table II. Each set of data, comparing different preincubation or test conditions, was obtained from paired experiments in which both conditions were studied using tissue from the same animal. These data permit the following conclusions: (*a*) when Na is present in the test solution, alanine influx is independent of the preincubation conditions and is thus uninfluenced by a marked reduction in the intracellular Na content (A); and (*b*) a significant reduction in the alanine influx is observed whenever Na is omitted from the test solution regardless of the preincubation conditions (B and C). The most marked inhibition of influx is obtained when Na is omitted from both the pre-

TABLE II

EFFECT OF Na ON L-ALANINE INFLUX

Experiment	Principal cation		Alanine influx	n*	*p*
	Preincubation solution	Test solution	($\mu mol/hr\ cm^2 \pm SEM$)		
A	Na	Na	2.2±0.1	10	>0.8
	Choline	Na	2.2±0.2	11	
B	Choline	Na	1.9±0.2	12	<0.01
	Choline	Choline	0.6±0.1	12	
C	Na	Na	2.8±0.2	25	<0.01
	Na	Choline	1.9±0.2	22	

* *n* is the number of flux measurements.

incubation and the test solutions (B). Removal of Na from the test solution alone (C) leads to a significant reduction in alanine influx but the effect is not as marked as when the tissue was also preincubated in a Na-free solution. Since tissue preincubated in Na-Ringer's contains a fairly large Na pool and since, as discussed below, Na efflux out of the cell across the mucosal membrane is rapid, the failure to obtain a more marked inhibition in Experiment C may be due to recycling of cellular Na. That is, Na leaving the cell under the influence of a large concentration difference may provide, in part, the Na necessary for alanine influx.

The results given in Table II suggest that alanine influx is dependent upon extracellular Na but that it is virtually independent of the *bulk* intracellular Na pool. An alternative explanation for these results is that an important pool of intracellular Na is rapidly replenished during the course of the influx measurement. As shown in Fig. 3, the time course of alanine uptake by tissue preincubated in choline-Ringer's is essentially linear over the range of 20 to 60

sec. Thus, any replenishment would have to be completed by 20 sec during which time the intracellular Na concentration increases by less than 5 mM. While this possibility cannot be ruled out, it seems clear that the bulk intracellular Na pool does not significantly influence alanine influx.

In order to demonstrate that the inhibition of alanine influx observed in Experiment B (Table II) is the result of removal of Na rather than an inhibitory effect of choline in the test solution, experiments were performed in which Tris-Cl, mannitol, LiCl, and KCl were used to replace most of the NaCl. Because alanine influx is so low in the complete absence of Na, all but 22 mM of the NaCl was replaced with the other solutes, and the concentration of alanine in the test solution was elevated to 20 mM. Under these conditions,

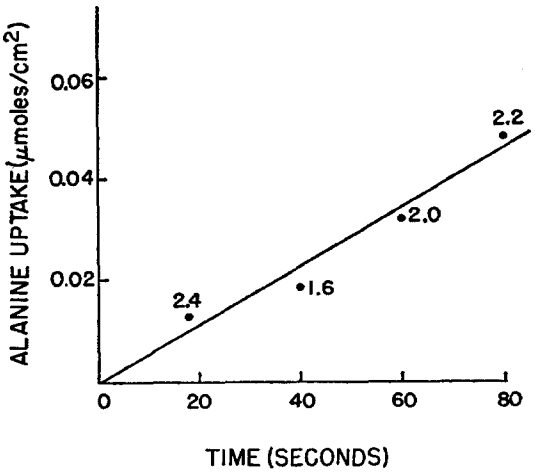

FIGURE 3. Time course of alanine-^{14}C uptake by tissue preincubated in Na-free (choline) medium; the test solution contained 140 mM Na. Alanine influx, calculated for each experimental point, is given in μmoles/hr cm^2.

the alanine fluxes are considerably larger and comparison of the effect of different substitutes for Na can be made more reliably. In all experiments with Tris, mannitol, and Li, the tissues were preincubated with choline-Ringer's so that the effects of other substitutes in the test solution alone could be examined. The results of these experiments are given in Figs. 4 and 5.

It is clear from the data of Fig. 4 that alanine and Na influxes in the presence of 118 mM Tris or 236 mM mannitol do not differ significantly from those observed when choline is used to replace Na. Replacement of Na with Li results in a small reduction (approximately 18%) in the alanine influx and a highly significant reduction in Na influx. These results differ, in part, from those of Bosačková and Crane (7) who have reported that sugar uptake by hamster small intestine is more markedly inhibited when mannitol or LiCl replaces all but 24 mM NaCl than when either Tris or choline is used as a substitute cation. In the experiments of Bosačková and Crane, uptake was measured after a 10 to 20 min incubation in the mannitol or Li medium, so that it is unclear

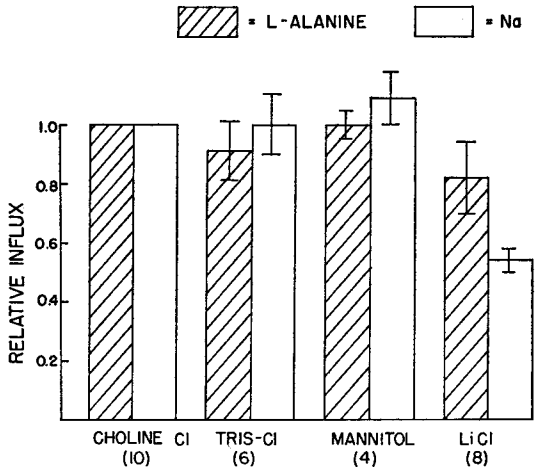

FIGURE 4. Comparison of the effects of different substitutes for Na on alanine and Na influxes. All test solutions contained 22 mM NaCl and 20 mM alanine. Data are expressed relative to the influxes observed when choline Cl is used to replace 118 mM NaCl. These fluxes averaged 4.3 ± 0.3 μmoles/hr cm² for alanine and 7.2 ± 0.5 μmoles/hr cm² for Na. Only the inhibition of Na influx by Li is statistically significant, $p < 0.01$. The number of observations is given in parentheses.

whether the observed inhibitions are attributable to extracellular mannitol or Li, or to changes in the intracellular ion and water composition resulting from these prolonged incubations (2, 8). Further, because of the long time

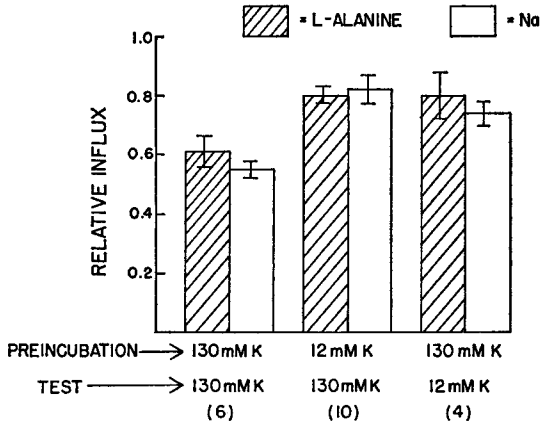

FIGURE 5. The effect of high K media on alanine and Na influxes. All values are expressed relative to the influxes observed in paired tissues when choline Cl was used to replace 118 mM Na. These fluxes averaged 4.8 ± 0.3 μmoles/hr cm² for alanine and 7.0 ± 0.4 μmoles/hr cm² for Na. The K concentrations of the preincubation and test solutions are given below each set of data. The number of observations is given in parentheses.

periods involved, the data of Bosačková and Crane represent net sugar accumulation rather than unidirectional influx across the mucosal border of the cell.

A series of experiments was performed in which the effects on alanine influx of a 30 min preincubation in K-Ringer's and of a 60 sec exposure to K-Ringer's were compared. As shown in Fig. 5, alanine influx is markedly reduced when KCl replaces all but 22 mM NaCl in both the preincubation solution and the test solution. On the other hand, when the tissue is preincubated in choline-Ringer's and the test solution contains 130 mM K, alanine influx is inhibited by only 19%. Conversely, when the tissue is preincubated in high K media, alanine influx from Na-Ringer's (12 mM K) is also reduced by 19%. These results suggest that the inhibitory effect of high K media on alanine influx may be composed of two independent components. The first is attributable to the effects on the tissue of prolonged exposure to a high K solution and is independent of the composition of the test solution. The second appears to be a direct effect of high extracellular K on the transport process.

Fig. 5 also shows that Na influx is inhibited in high K media, and that the fractional inhibition closely parallels that observed for alanine influx. Since, under these conditions, a large fraction of the Na influx is unrelated to alanine influx (3), these findings suggest that the inhibitory effect of K is not specific for alanine transport but that K exerts a more general action which affects at least two independent transport processes. A detailed analysis of the data of Figs. 4 and 5 is given in the subsequent paper (3).

Effect of Intracellular Alanine on Alanine Influx

Heinz and Walsh (9) have demonstrated that the influx of labeled glycine into Ehrlich ascites tumor cells is greatly increased if the cells are preloaded with this amino acid, or with other neutral amino acids which share the same carrier mechanism. This *transconcentration* effect has also been suggested by several investigators for sugar uptake by erythrocytes (10, 11). Recently, Johnstone and Scholefield (12) have demonstrated that although net uptake of L-methionine by ascites tumor cells is a Na-dependent process, the rate of exchange of labeled L-methionine with cells preloaded with unlabeled methionine is the same in the presence and absence of extracellular Na.

To evaluate the influence of intracellular alanine on alanine influx across the mucosal border of rabbit ileum, studies were performed using paired tissues which were preincubated for 30 min in either Na-Ringer's containing 5 mM alanine or in Na-Ringer's which did not contain alanine. Studies on mucosal strips of rabbit ileum (2) have shown that in the presence of 5 mM alanine a steady-state intracellular concentration of approximately 40 mM is achieved within 30 min. The influx of alanine into preloaded tissue (eight

tissues) averaged 2.2 ± 0.2 μmoles/hr cm² and did not differ significantly from the influx into tissue (eight tissues) which was not previously exposed to alanine, 2.6 ± 0.4 μmoles/hr cm². Similar results were obtained in the presence of 15 mM alanine where influx into control and preloaded tissue (four tissues) averaged 3.1 ± 0.4 μmoles/hr cm² and 2.9 ± 0.7 μmoles/hr cm², respectively. These data indicate that, within experimental error, alanine influx is not subject to a transconcentration effect and is uncomplicated by exchange diffusion at the concentrations used.

DISCUSSION

The importance of Na in the transmural transport and accumulation of sugars and amino acids by small intestine (2, 3, 13–18) and kidney (19, 20) and in the accumulation of amino acids by a variety of cells (21–26, 37) is well established. Two hypotheses have been suggested to explain this Na requirement. The first was proposed by Crane to account for the Na-dependent entry of sugars into hamster intestinal cells (27, 1), and was subsequently extended by others, with some modifications, to explain amino acid transport by small intestine (17, 28), pigeon erythrocytes (25), and striated muscle (24). This hypothesis maintains that Na and the nonelectrolyte combine with a carrier at the mucosal border to form a ternary complex which traverses the cell membrane. Since a similar complex is presumed to mediate efflux across the mucosal border, accumulation of sugars and amino acids is attributed, in part, to the difference between intracellular and extracellular Na concentrations. The second hypothesis was proposed by Csáky in order to explain the ubiquitous nature of the Na requirement for nonelectrolyte transport (29). He suggested that intracellular Na is required for the coupling of metabolic energy to the carrier systems for sugars, amino acids, and other nonelectrolytes, and that removal of Na from the external medium inhibits active transport due to depletion of intracellular Na pools. The major differences between these two hypotheses are: (*a*) the first proposes a requirement for extracellular Na and the second for intracellular Na; (*b*) the first suggests that sugar or amino acid uptake is not directly coupled to metabolic energy whereas the second suggests a direct Na-dependent link between metabolic energy and the nonelectrolyte uptake processes; and (*c*) a coupled entry of Na and the nonelectrolyte is postulated by Crane's model while, according to Csáky's model, the entries of Na and nonelectrolyte into the cell may be entirely independent processes. Both hypotheses are consistent with the observation that ouabain inhibits sugar and amino acid transport (footnote 3 and references 2, 17, 20, 24, 30–32); the first attributes this to the fact that ouabain abolishes the Na concentration difference (2), whereas the second maintains that the energy link is inhibited, directly (29) or indirectly (33), by the digitalis glycosides.

Although these two hypotheses have generated considerable discussion, no

direct, compelling evidence has been previously presented for or against either of them. Our reasons for this conclusion follow:

1. Although there is good evidence that the mechanisms for amino acid and sugar transport are located at the brush border (4, 34) and that Na is required in the solution bathing this surface (13, 35), studies on the effect of Na on nonelectrolyte accumulation by intestinal tissue have not distinguished between the mucosal and serosal membranes of the epithelial cells. Since net accumulation is the result of bidirectional fluxes across each of these two different membranes, the previously reported results cannot be attributed unequivocally to a specific effect of Na at the brush border. Studies of transmural fluxes or of concentration differences produced in everted intestinal sacs suffer from the same shortcomings; the results depend on events at both the mucosal and serosal borders of the cells and interpretations in terms of processes which take place at the mucosal border alone may be misleading.

2. Previous studies have been concerned with the effect of Na on net accumulation of nonelectrolytes by intestinal tissue. Since this process may be slowed by either an increased efflux from the tissue or a decreased influx into the tissue (across either the mucosal or serosal membranes), an unequivocal interpretation of these data in terms of mechanisms is not possible.

3. All previous attempts at evaluating the effects of cation replacement on sugar or amino acid transport by intestine have involved exposure of the tissue to altered medium for as long as 30 min. Incubation of intestinal tissue in Na-free media results in a marked reduction of intracellular Na pools within 15 min and virtually complete depletion of these pools by 30 min (2, 8, 17). Thus, the results of such experiments cannot be attributed to changes in the ionic composition of either the extracellular or intracellular compartment alone. This point is especially important since it is directly concerned with one of the important differences between the two hypotheses outlined above.

In the present investigation, we have examined the effect of Na on the unidirectional influx of alanine across the mucosal border of the cell. These experiments were carried out during a period sufficiently brief that intracellular ionic composition was not greatly affected by changes in the composition of the mucosal solution (test solution). The results indicate that alanine influx is markedly inhibited by removal of Na from the mucosal solution, but is unaffected by a large reduction in the bulk intracellular Na concentration. Since similar inhibition is observed when Na is replaced by a variety of solutes, the effect appears to be due to Na removal rather than to the solute chosen to replace Na, and the role of Na in this process appears to be highly specific. These findings are consistent with the model proposed by Crane but are difficult to reconcile with Csáky's hypothesis for the role of intracellular Na in nonelectrolyte transport.

In 1952, Christensen and Riggs (36) reported that replacement of NaCl with KCl inhibited glycine uptake by Ehrlich ascites tumor cells. Although these authors, in a subsequent report (37), raised the possibility that glycine entry may be coupled to Na influx, they presented a number of arguments which favored an inhibitory role for extracellular K. It is clear from the studies of Kromphardt et al. (21) that much of the effect observed by Christensen and Riggs can be attributed to the fact that glycine accumulation by ascites cells is a Na-dependent process. Several investigators have, however, reported specific inhibitory effects of high K media on both sugar (7) and amino acid accumulation (20, 24) in excess of that which can be attributed simply to replacement of Na. Thus, the data of Bosačková and Crane (7) indicate that, in the presence of 24 mM Na, the uptake of sugar by hamster intestine when K is used to replace Na is reduced by 75% compared to the uptakes observed when either Tris or choline is used as a substitute cation. This marked inhibitory effect of high K media has led to the suggestion that K competes with Na for a cation-binding site on the sugar carrier and that the affinity of the mechanism for nonelectrolyte efflux from the cell is reduced by the combined effects of a low intracellular Na concentration and a high intracellular K concentration (see Fig. 6 of reference 1). The present results do not provide strong support for such a role of K in amino acid transport. Although the presence of 130 mM K in the test solution alone inhibits alanine influx to a greater extent than can be attributed solely to Na replacement (Fig. 5), this inhibition is considerably smaller than that observed by Bosačková and Crane and it is not specific for alanine influx. While these differences may reflect differences between animal species and/or differences between the mechanisms of sugar and amino acid transport, an alternative explanation is possible. As shown in Fig. 5, exposure of the tissue to a high K preincubation solution for 30 min results in a parallel inhibition of Na and alanine influxes which appears to be independent of the K concentration of the test solution. Thus, in the experiments of Bosačková and Crane, and others (20, 24), in which the tissue was exposed to high K media for relatively prolonged periods, the specific effects of replacement of extracellular Na with K may have been exaggerated. The mechanism by which prolonged exposure of tissue to high K media inhibits alanine and Na influxes is unclear but it may be related to the marked swelling of intestinal (2, 7) and renal (20) tissues observed under these conditions.

Unidirectional Fluxes and Transmural Transport of Alanine and Na

Absorption across the intestinal epithelial layer involves transport across at least two barriers arranged in series, the brush border (the mucosal membrane) and the combined serosal and lateral membranes. These barriers not

only differ structurally, but they must also differ functionally in order for net transport across the cell layer to take place against a concentration difference. The forces responsible for transport across each of these boundaries must be known before the mechanism of absorption of any substance can be fully understood. Studies of amino acid accumulation in the serosal solution of everted sacs do not provide the necessary information, and kinetic treatments of either amino acid accumulation or net transport which consider the intestine as if it were a single membrane are difficult to interpret and may be misleading. The present results represent the first direct determination of unidirectional influx of L-alanine and Na across the mucosal border of intestinal epithelial cells and, together with the results of previous studies, permit a preliminary analysis of the steady-state fluxes across the two barriers.

Unidirectional and net fluxes of L-alanine across rabbit ileum have been determined by Field, Schultz, and Curran.[3] With 140 mM Na and 5 mM L-alanine in the mucosal and serosal solutions, the unidirectional alanine flux from mucosa to serosa averages 1.4 μmoles/hr cm^2 and the serosa to mucosa flux averages 0.1 μmole/hr cm^2; thus, there is a net flux of 1.3 μmoles/hr cm^2 from mucosa to serosa. When all NaCl in the mucosal and serosal solutions is replaced by choline Cl, the mucosa to serosa flux and the serosa to mucosa flux both average 0.15 μmole/hr cm^2 and the net alanine flux is not significantly different from zero. Assuming that the tissue and the two bathing solutions behave as a three compartment system, shown in Fig. 6 *a*, the steady-state fluxes from mucosa to serosa, J_{ms}, and from serosa to mucosa, J_{sm}, are related to the unidirectional fluxes across the mucosal and serosal membranes as follows (38):—

$$J_{ms} = \frac{J_{mc}\, J_{cs}}{J_{cm} + J_{cs}}$$

$$J_{sm} = \frac{J_{sc}\, J_{cm}}{J_{cm} + J_{cs}}$$

$$J_{\text{net}} = J_{ms} - J_{sm} = J_{mc} - J_{cm} = J_{cs} - J_{sc}$$

Thus, knowledge of J_{ms}, J_{sm}, and J_{mc}, is sufficient to evaluate the remaining three fluxes. Using the values for J_{ms} and J_{sm} given above, together with average values of 2.2 μmole/hr cm^2 for J_{mc} in Na-Ringer's, and 0.6 μmole/hr cm^2 for J_{mc} in choline-Ringer's, the unidirectional fluxes given in Fig. 6 *b* and *c* are obtained. The ratio of unidirectional alanine fluxes across the serosal membrane (J_{cs}/J_{sc}) is of the same order of magnitude as the ratio of intracellular to extracellular alanine concentrations observed in mucosal strips (2) so that these calculations do not assist in distinguishing between simple diffusion or carrier-mediated transport as possible mechanisms for the exit process.

If J_{cs}/J_{sc} were much greater than the concentration ratio, the exit mechanism could not be attributed to simple diffusion even though net movement of alanine is in the direction of the concentration difference. It should be noted that a flux analysis using the three compartment system shown in Fig. 6 *a* is based on the assumption that the subepithelial tissues do not present a major barrier to the movement of alanine. Studies of alanine fluxes across serosal strips indicate that this assumption is not entirely valid.[6] The use of a kinetic

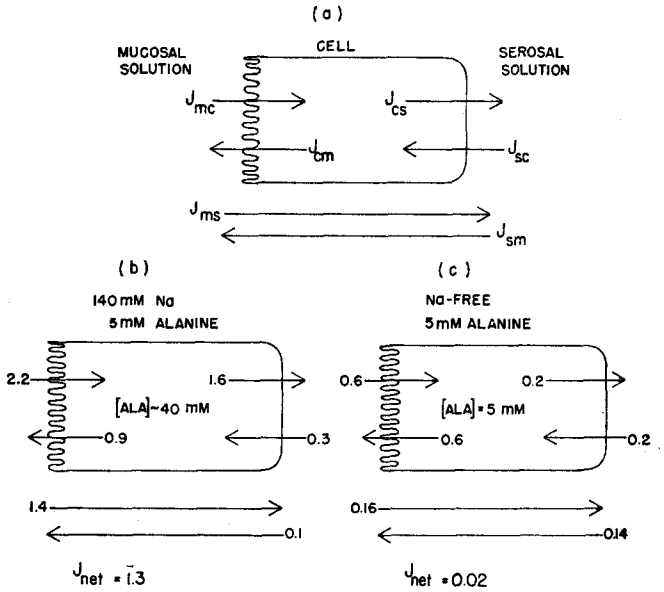

FIGURE 6. (*a*) Three compartment model for analysis of unidirectional fluxes across mucosal and serosal membranes of epithelial tissue. (*b*) Unidirectional alanine fluxes when [Na] = 140 mM. (*c*) Unidirectional fluxes in choline-Ringer's ([Na] = 0). In (*b*) and (*c*) the concentration of L-alanine in the mucosal and serosal solutions is 5 mM. Alanine fluxes are in μmoles/hr cm^2.

model which includes a serosal diffusion barrier results in different calculated values for the serosal fluxes and for the alanine concentration in the serosal tissues immediately adjacent to the serosal membrane, but does not significantly affect the agreement between the flux ratio and alanine concentration ratio.

A similar analysis may be applied to Na fluxes. Unidirectional Na fluxes across the mucosal and serosal surfaces of the tissue are given in Fig. 7 *a*. The Na influx (J_{mc}) in the absence of alanine averages 18 μmoles/hr cm^2 and the

[6] Measurements of L-alanine flux across serosal strips indicate that the permeability of this tissue is approximately 0.16 cm/hr. Since the serosal strip is approximately 0.2–0.4 mm thick, this permeability corresponds to a diffusion coefficient of 1–2 $\times$ 10^{-7} cm^2/sec, a value which is small compared with the diffusion coefficient of alanine in free solution (1.2 $\times$ 10^{-5} cm^2/sec (39)).

values for J_{ms} (9 μmoles/hr cm²) and J_{sm} (6 μmoles/hr cm²) are average values for the transmural Na fluxes in the presence of an electrical potential differences of 5 mv (serosa-positive) (5). Although the unidirectional fluxes across the serosal membrane are subject to the reservations discussed above, it is of interest to consider the implications of the Na flux ratio across the mucosal membrane. Using the previously determined value of 50 mM (2) for the intracellular Na concentration $[Na]_c$, and a value of -15 mv for the electrical potential difference across the mucosal membrane, $\Delta\psi$, (the sign refers to the

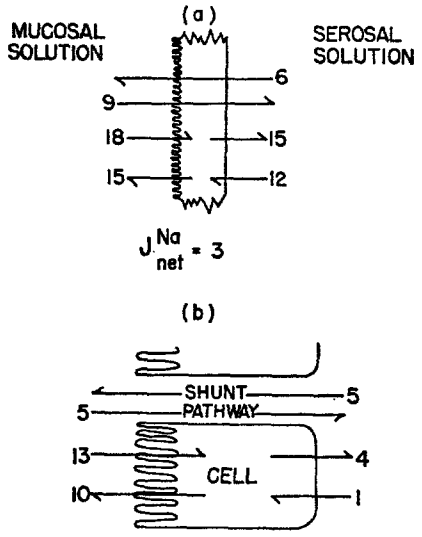

FIGURE 7. (*a*) Unidirectional Na fluxes across mucosal and serosal surfaces of rabbit ileum. (*b*) Unidirectional Na fluxes corrected for possible "shunt" pathway. Since the transmural electrical potential difference is small (5 mv, serosa-positive) the bidirectional movements through the shunt are approximately equal. Na fluxes are in μmoles/hr cm².

cell interior relative to the mucosal solution),[7] the Ussing equation (40) for passive ionic diffusion predicts a flux ratio of

$$\frac{J_{mc}}{J_{cm}} = \frac{[Na]_m}{[Na]_c} \exp\left\{\frac{-F\Delta\psi}{RT}\right\} = 4.9$$

(assuming that the activity coefficients for intracellular and extracellular Na are equal). The observed ratio of $J_{mc}/J_{cm} = 1.2$ indicates that the Na fluxes across the mucosal surface cannot be attributed to simple diffusion even though net Na flux is in the direction of its electrochemical potential difference. Instead, the observed flux ratio suggests the presence of either exchange diffusion or a mechanism which brings about an active extrusion of Na from the cell across the mucosal membrane. This analysis of the Na flux ratio across the mucosal border is complicated by the possibility that Na traverses the tissue via several parallel pathways. Thus, part of the unidirectional flux may actually cross the brush border and pass through the cells, while another

[7] Field, M., and P. F. Curran, unpublished observations.

portion of the flux may traverse a "shunt" pathway either between cells or through areas which are denuded. The maximum contribution of such a shunt pathway to J_{mc} cannot exceed the serosa to mucosa Na flux, 6 μmoles/hr cm^2, and is probably somewhat smaller since if these fluxes were precisely equal, J_{sc} would be zero. Fig. 7 *b* shows the values of the Na fluxes across the mucosal border corrected for a flux of 5 μmoles/hr cm^2 through a shunt pathway. The corrected flux ratio, 1.3, still does not meet the criteria for simple diffusion.

Two independent observations are consistent with the suggestion that Na transport across the mucosal border of the intestinal cell is not entirely the result of simple diffusion. The first is the finding that Li markedly inhibits Na influx and that this inhibition appears to be specific inasmuch as alanine influx is only slightly reduced by Li (Fig. 4). These observations are consistent with the hypothesis that Li interacts with a transport mechanism which is, at least in part, responsible for Na influx into the intestinal cell. Such interaction has been demonstrated in other systems (41, 42); however, further investigation is necessary before this can be established unequivocally for this preparation. Finally, Na-K–stimulated ATPase activity has been observed in brush border preparations of guinea pig (43) and hamster intestine (1). Such ATPase activity has been implicated in active cation transport mechanisms in a variety of tissues and its presence in the brush border of small intestine is consistent with the presence of a mechanism for active Na extrusion from the cell. Although this extrusion mechanism would not be required for net Na absorption, it may be part of a K-Na transport mechanism responsible for the maintenance of the high intracellular K concentration and low intracellular Na concentration (2) characteristic of the mucosal cells.

Note Added in Proof A model for salt and water transport across small intestine that includes a shunt pathway for passive movements of Na and Cl, and that is consistent with the analysis in Fig. 7 *b* has recently been proposed by Clarkson (*J. Gen. Physiol.* 1967. **50**:695).

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