Effects of Monovalent Cations on the Sodium-Alanine Interaction in Rabbit Ileum

Implication of anionic groups in sodium binding

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ABSTRACT H, K, Rb, and Li inhibit Na-dependent alanine influx across the brush border of rabbit ileum. Kinetic analysis indicates that H and K behave as competitive inhibitors of influx so that increasing the concentration of H or K in the mucosal solution is kinetically indistinguishable from decreasing the Na concentration. In addition the coupling between alanine and Na influxes is markedly reduced at pH 2.5. With the exception of H and Li, none of these monovalent cations significantly affects carrier-mediated alanine influx in the absence of Na indicating that their inhibitory effects are largely restricted to the Na-dependent fraction of influx. Increasing H concentration from 0.03 to 3 mM does not affect influx in the absence of Na but markedly inhibits influx in the presence of Na. Li significantly enhances alanine influx in the absence of Na. Ag, UO_2, and La also inhibit the Na-dependent fraction of alanine influx. These findings suggest that anionic groups having a pK_a of approximately 4 are involved in the interaction between Na and the alanine-carrier complex; present evidence implicates carboxylate groups however, phosphoryl residues cannot be ruled out. The previously proposed kinetic model for the Na-alanine interaction has been extended to accommodate these effects of H and other monovalent cations. The mechanistic and physiological implications of these findings are discussed.

The interaction between Na and the influx of alanine across the brush border of rabbit small intestine has been examined in considerable detail and a model consistent with the kinetics of this interaction has been presented (1). According to this model, alanine combines first with a component of the brush border to form a binary complex. This binary complex can either cross the membrane or it can combine with Na to form a ternary complex that then crosses the membrane. Kinetic data indicate that the dissociation constant of the binary complex is much greater than that of the ternary complex. Thus, Na enhances influx because it promotes the formation of a more stable complex; the greater the stability of the ternary complex compared to that of the

binary complex, the greater will be the stimulatory effect of Na. Other studies have suggested that the degree to which amino acid influx is stimulated by Na is influenced by the charge of the bound amino acid; the sequence of relative Na dependence is anionic amino acid > neutral amino acid > cationic amino acid. These observations suggest that a cationic amino acid can partially mimic the action of Na and that the Na–amino acid interaction at the brush border involves electrostatic interaction with anionic groups (2, 3).

An important feature of the kinetic model is the preferred sequence of formation of the ternary complex; that is, alanine must combine with the membrane component first and Na can only combine with the membrane component after the binary complex has been formed. Any significant deviation from this pathway would lead to quantitative predictions that are inconsistent with the experimental data.[1] This obligatory pathway suggests that it might be possible to selectively inhibit the binding of Na, and thereby the stimulatory effect of Na on alanine influx, without significantly affecting alanine influx via the binary complex. Such inhibition would not only provide additional support for the model, but could also yield information on the nature of reactive groups and forces involved in the binding of Na to the binary complex. The feasibility of this approach is supported by previous observations that metabolic inhibitors have no effect on the influx process (5). Thus, the effects of pH, heavy metals, etc. cannot be attributed to interference with metabolic pathways but must be due to direct interaction with the transport process and/or other properties of the mucosal membrane. We will present evidence that the interaction between Na and alanine influx across the brush border can be selectively inhibited by increasing the concentration of H or several other monovalent cations in the mucosal solution and that reactive groups having a pK of approximately 4 are involved in the formation of the Na–amino acid–carrier complex. In addition the effect on alanine influx of a number of group-reactive agents and heavy metals is reported.

METHODS

Male and female New Zealand white rabbits, maintained on normal food intake, were sacrificed by intravenous injection of pentobarbital. A segment of distal ileum was resected, opened along the mesenteric border, and rinsed free of intestinal contents. The method for the determination of unidirectional alanine influx from the mucosal solution across the brush border into the epithelium has been described in detail (6). Briefly, the ileal segment is mounted in a special apparatus that permits exposure of defined areas of the mucosal surface of the tissue alone to a solution of desired composition; the serosal surface of the tissue rests on a piece of moistened filter paper. The mucosal surface of the tissue is exposed to a preincubation solution for 30 min at 37°C; stirring and oxygenation are accomplished by bubbling with a

[1] Statistical analysis indicates that the probability that alanine combines with the membrane site first is at least 50 times greater than the probability that Na combines first (4).

fine stream of 95% O_2–5% CO_2. The preincubation solution is then withdrawn and the tissue is exposed to a test solution containing alanine-^{14}C and inulin-^{3}H for less than 1 min.[2] The exposed tissue is punched out, washed briefly in ice-cold, isotonic mannitol solution, and extracted in 0.1 N HNO_3 for at least 4 hr. The extract and an aliquot of the test solution are assayed for C-14 and H-3 simultaneously using a liquid scintillation spectrometer. Since inulin does not cross the brush border (7, 8), the H-3 content of the tissue extract is a measure of the volume of adherent radioactive test solution that was not removed by the brief wash. As shown in Fig. 1, this inulin "space" is not influenced by the duration of exposure over the range 0.2 to 2 min. These data (typical of many experiments) indicate that (*a*) inulin does not significantly permeate the brush border, and (*b*) inulin equilibrates with the extracellular space rapidly; i.e., there is no significant space that is accessible to inulin by simple diffusion that is not equilibrated with inulin within 1 min. The C-14 content of the extract,

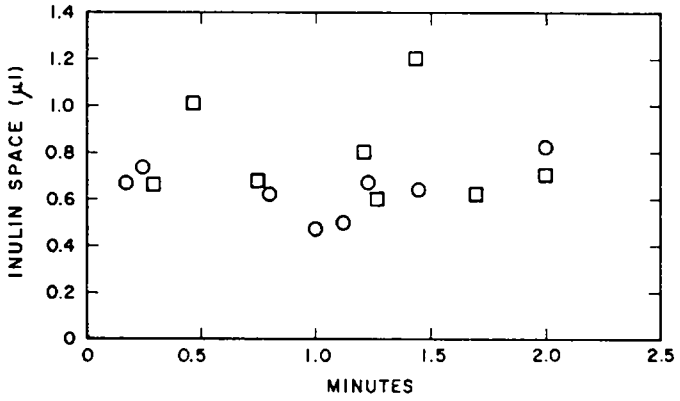


FIGURE 1. Inulin space as a function of time. Data from two experiments.

after correction for the volume of adherent test solution, increases linearly with time for at least 1.5 min and is a direct measure of unidirectional alanine influx across the brush border into the epithelium (6). The apparatus permits eight determinations of alanine influx on tissue from the same animal.

Unless indicated otherwise the preincubation and test solutions contained 140 mM NaCl, 10 mM $KHCO_3$, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM K_2HPO_4, and 0.2 mM KH_2PO_4 at pH 7.4. When a Na-free medium was desired, NaCl was replaced with choline chloride unless otherwise noted. The pH of the incubation medium was buffered above pH 6 using 10 mM Tris in place of bicarbonate and phosphate, or below 7 using 1.6 mM phthalic acid; both buffers were employed in separate experiments at pHs between 6 and 7 to determine whether any effects on influx could be attributed to the buffer per se. In both instances the final pH was obtained by addition of small amounts of 2 N hydrochloric acid or potassium hydroxide. The buffer capacity was sufficient so that the initial pH was not affected by exposure to the tissue. In the initial experiments, the tissue was preincubated for 30 min at the pH used to test

[2] In studies designed to determine simultaneous alanine and Na influxes, ^{22}Na was included in the test solution.

alanine influx. Subsequent experiments demonstrated that a 2 min preexposure to pH 2.5 was sufficient to elicit the complete pH effect so that this procedure was used routinely. Where the effects of heavy metals or group-reactive agents were determined they were included in the preincubation solution only.

All errors are expressed as standard errors of the mean.

RESULTS AND DISCUSSION

Effect of pH

The effect of pH on alanine influx is illustrated in Fig. 2. The tissue was preincubated at the indicated pH for 30 min and the pH of the test solution was

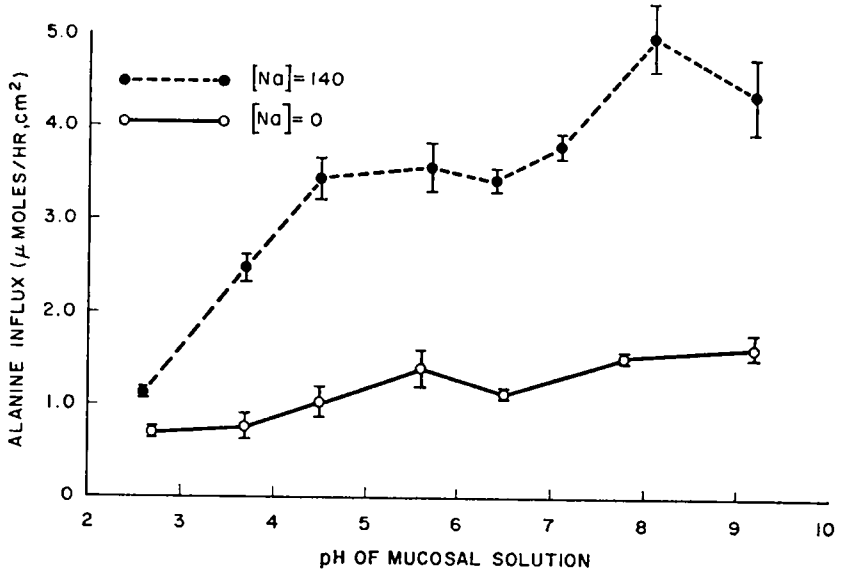


FIGURE 2. Alanine influx as a function of pH. Each point represents the average of at least four determinations.

the same as that of the preincubation solution. Alanine concentration in the test solution was 5 mM. Overlapping of the mean influx values in the pH range 6 to 7 indicates that the observed effects are due to the pH of the mucosal solution rather than to the buffer system employed. Alanine influx from a medium rendered Na-free by replacement of Na with choline declines slowly as pH decreases. However, although there appears to be a significant decrease in influx as pH is decreased from 5.6 to 4.5, the mean values over the range 2.5 to 4.5 do not differ significantly. Indeed, 23 influx determinations at pH 2.5 averaged 1.0 ± 0.1 μmoles/hr, cm^2 whereas 8 determinations at pH 5.0 averaged 1.3 ± 0.1 μmoles/hr, cm^2. In marked contrast, alanine influx from a medium containing 140 mM Na declines sharply as the pH is lowered from

4.5 to 2.5. At pH 4.5 alanine influx in the presence of Na is three to four times that observed in the absence of Na whereas at pH 2.5 alanine influx in the presence of Na is only 50% greater than that observed in the absence of Na. These data suggest that the stimulatory effect of Na on alanine influx is selectively and markedly suppressed at pH 2.5.

The effect of pH 2.5 illustrated in Fig. 2 was observed after 30 min exposure of the tissue to this pH. Qualitatively similar results are obtained if the tissue is preincubated for 30 min at pH 7.4 and influx is then determined using a test solution at pH 2.5. However, the degree of inhibition is generally not as

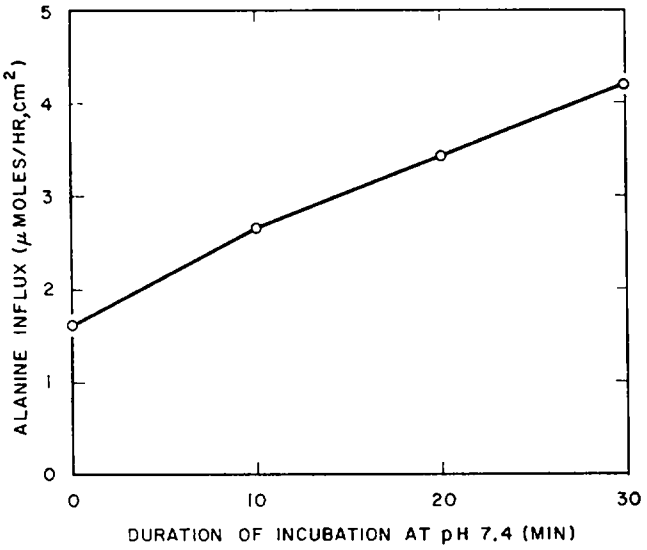


FIGURE 3. Reversal of the inhibition of alanine influx at pH 2.5 by subsequent incubation at pH 7.4.

marked. Exposure of the brush border to pH 2.5 for less than 1 min is sufficient to elicit approximately 80% of the effect observed after 30 min exposure (see below). Fig. 3 shows the results of a typical experiment designed to examine the reversibility of the inhibitory effect of pH 2.5. The tissue was exposed to a mucosal solution at pH 2.5 for 2 min. This solution was then withdrawn and replaced with a solution at pH 7.4 in which the tissue was allowed to incubate for varying lengths of time. Alanine influx was then determined from a mucosal test solution at pH 7.4. The figure shows alanine influx as a function of duration of exposure to pH 7.4 after a 2 min exposure to pH 2.5. The reversal of the inhibition is quite slow and normal influx values are observed only after approximately 30 min incubation at pH 7.4. However, the reversal of inhibition appears to be complete and studies performed after 30 min exposure to pH 7.4 disclose no history of a prior 2 min exposure to pH 2.5.

The slow reversal suggested an important modification in our experimental procedure. That is, we could elicit the pH 2.5 effect with a brief exposure of the mucosal surface to pH 2.5 and then determine influx from a test solution at pH 7.4. In this way the same test solution could be employed for control and experimental tissues, the only difference being the exposure of the latter to pH 2.5 immediately before the influx determination. The data illustrated in Fig. 4 indicate that a 2 min exposure to pH 2.5 is sufficient to obtain maximal inhibition even when influx is determined immediately thereafter from a solution at pH 7.4. Influxes determined immediately after a 2 min exposure to pH 2.5 do not differ significantly from those observed following a 30 min preincubation at pH 2.5.

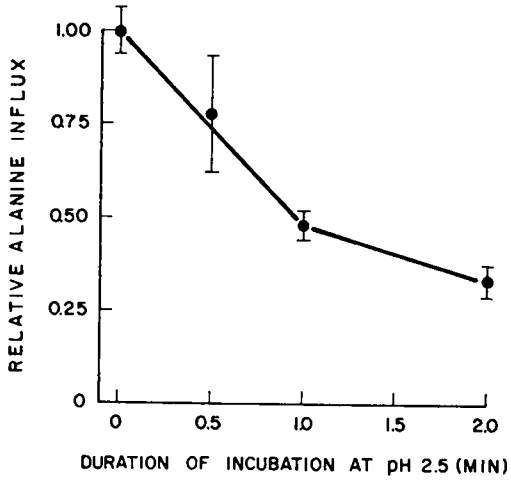


FIGURE 4. Alanine influx as a function of duration of preexposure to pH 2.5. Influx was determined using a test solution at pH 7.4 immediately after the exposure. Results are from one experiment and each point is the average of duplicate determinations on tissue from the same animal.

Evidence for Mediated Influx at pH 2.5

A possible explanation for the observations illustrated in Figs. 2 and 4 is that the brush border mechanism for alanine influx is destroyed at pH 2.5 and that the observed influxes simply represent nonmediated diffusion through a damaged barrier. In order to explore this possibility, the effect of leucine on alanine influx was evaluated at pH 2.5 in the presence and absence of Na using paired tissues from the same animal. The tissue was preincubated at pH 7.4 for 30 min and the exposure to pH 2.5 was limited to the brief period (less than 1 min) during which the tissue was exposed to the test solution.[3] The results of these experiments are illustrated in Fig. 5. Influx from a test solution at pH 2.5 containing 140 mM Na is only 90% greater than influx from a Na-free test solution; at pH 7.4, influx in the presence of 140 mM Na is three to

[3] These experiments were performed prior to the discovery of the slow reversibility and the modification of the procedure for eliciting the pH 2.5 effect. Similar results are obtained following preexposure to pH 2.5.

four times that in the absence of Na. Previous studies have indicated that leucine inhibits alanine influx both in the presence and absence of Na; approximately 30 mM leucine is necessary to halve alanine influx from a Na-free solution containing 3.3 mM alanine (1). As seen in Fig. 5, leucine inhibits alanine influx at pH 2.5 both in the presence and absence of Na. In the absence of Na, 30 mM leucine brings about a 55% inhibition of alanine influx indicating that K_I of leucine in the absence of Na is approximately the same at pH 2.5 as it is at pH 7.4.[4] These results strongly suggest that alanine influx at pH 2.5 is a carrier-mediated process. Further, the good agreement between K_I for leucine at pH 7.4 and 2.5 in the absence of Na suggests that a brief exposure to pH 2.5 does not significantly affect the passive permeability of the brush border to alanine.

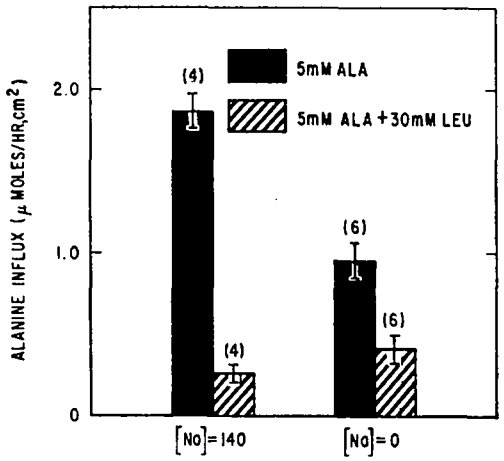


FIGURE 5. Inhibition of alanine influx by leucine at pH 2.5 in the presence and absence of Na.

Mannitol slowly crosses the brush border of rabbit ileum by simple diffusion (9, 10). Mannitol influx from a solution containing 5 mM mannitol was unaffected by preexposure of the tissue to pH 2.5 for 2 min and averaged 0.08 ± 0.01 μmole/cm², hr, a value identical to that observed in paired tissues exposed only to pH 7.4. Experiments were also performed to examine the effect of pH 2.5 on Na influx, a process that appears to be at least partially mediated (6). Na influx from a solution containing 140 mM Na at pH 7.4 averaged 24 ± 1 μmoles/hr,cm² and influx into tissue exposed to pH 2.5 for 2 min averaged 23 ± 1 μmoles/hr,cm² (12 paired determinations at each pH); these values do not differ significantly. These data corroborate the conclusion that passive permeability of the brush border is not affected by brief exposure to low pH.

[4] The K_I for leucine in the absence of Na is 31 mM (1). Using this value, and the previously published value for the K_t for alanine in the absence of Na (1), the predicted inhibition of alanine influx is 48%.

Cation Selectivity at pH 2.5

The following experiments were performed to determine whether the stimulatory effect of Na on alanine influx is assumed by other alkali metal cations at pH 2.5. The tissue was preincubated for 30 min in Na-free Ringer solution at pH 7.4 and then exposed to a Na-free solution at pH 2.5 for 2 min. Alanine influx was determined from a test solution containing 140 mM of the chloride salt of Na, Li, K, or choline, buffered at pH 7.4 with 10 mM Tris. Fig. 6 shows the reduced, but significant, stimulatory effect of Na following exposure to pH 2.5. The influxes of alanine from solutions containing Li or K as the major

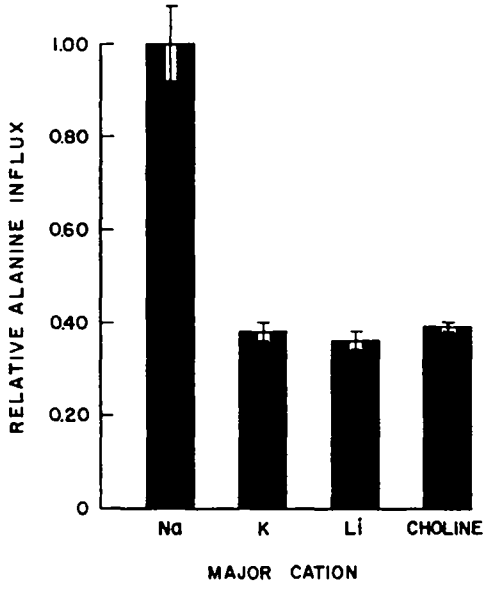


FIGURE 6. The effects of different substitutes for Na on alanine influx at pH 2.5. Each value is the average of four determinations.

cation do not differ from that observed when choline replaces Na. Thus, the specificity for Na is retained following exposure to pH 2.5.

Kinetics of Alanine Influx at pH 2.5

In order to examine the effect of pH 2.5 on the kinetics of alanine influx, the eight available tissue segments from the same animal were preincubated for 30 min using Na-free Ringer solution at pH 7.4. Four alternate segments of tissue were then exposed to pH 2.5 for 2 min. Alanine influx was determined using test solutions of 140 mM Na-Ringer containing 5, 10, 15, and 20 mM alanine at pH 7.4; at each concentration, influx was determined from the same test solution on control tissue (pH 7.4) and on adjacent areas of tissue that had been exposed to pH 2.5.

Alanine influx in the presence of 140 mM Na at pH 7.4 and 2.5 is plotted as

a function of alanine concentration according to the method of Lineweaver and Burk in Fig. 7. Alanine influx is a saturable process that conforms to Michaelis-Menten kinetics at pH 7.4 (as described previously [1]) and at pH 2.5. The data can be adequately described by two lines that have a common intercept on the ordinate corresponding to a common maximal influx of 12.5 μmoles/cm^2,hr. The slopes of the two lines differ markedly and indi-

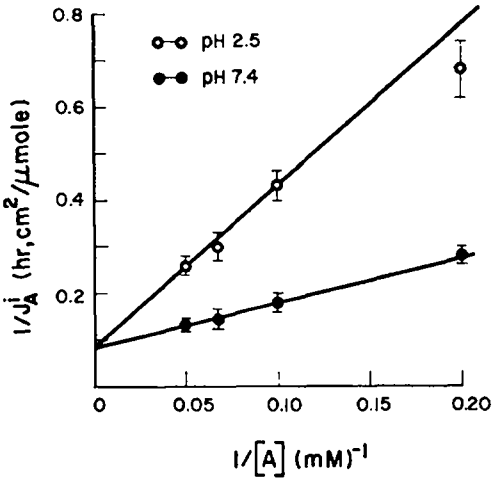


FIGURE 7. Lineweaver-Burk plots of alanine influx as a function of alanine concentration in the presence of 140 mM NaCl. Each point is the average of five determinations.

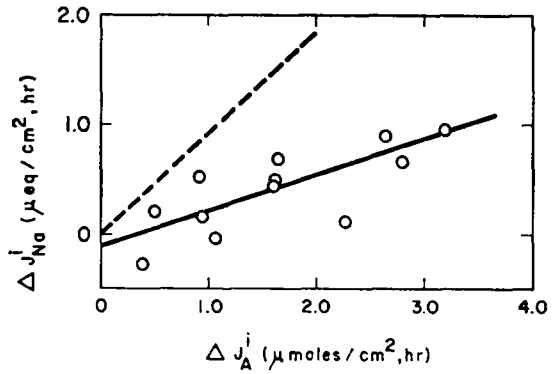


FIGURE 8. Coupling between Na and alanine influxes following exposure to pH 2.5. The dashed line indicates the relation observed at pH 7.4.

cate that the alanine concentration required to elicit a half-maximal influx (K_t) is much greater when the tissue has been exposed to pH 2.5 than when the tissue is only exposed to pH 7.4. The K_t for control tissues is 10 mM, in excellent agreement with the value reported previously (1). The K_t for tissues exposed to pH 2.5 is 50 mM.

Coupling Coefficient at pH 2.5

At pH 7.4, alanine influx is accompanied by an increase in Na influx (1). The following experiment was performed in order to evaluate the coupling coeffi-

cient between the unidirectional influxes of Na and alanine across the brush border after exposure to pH 2.5. After the standard preincubation, the tissue was exposed to pH 2.5 solution for 2 min and influx was then determined from a test solution at pH 7.4 containing 140 mM ^{22}Na and 5–20 mM alanine-^{14}C. Since the magnitude of the alanine-independent Na influx varies widely from animal to animal, the results were normalized by plotting the increment in Na influx (ΔJ^{i}_{Na}) associated with an increment in alanine influx (ΔJ^{i}_{A}); these

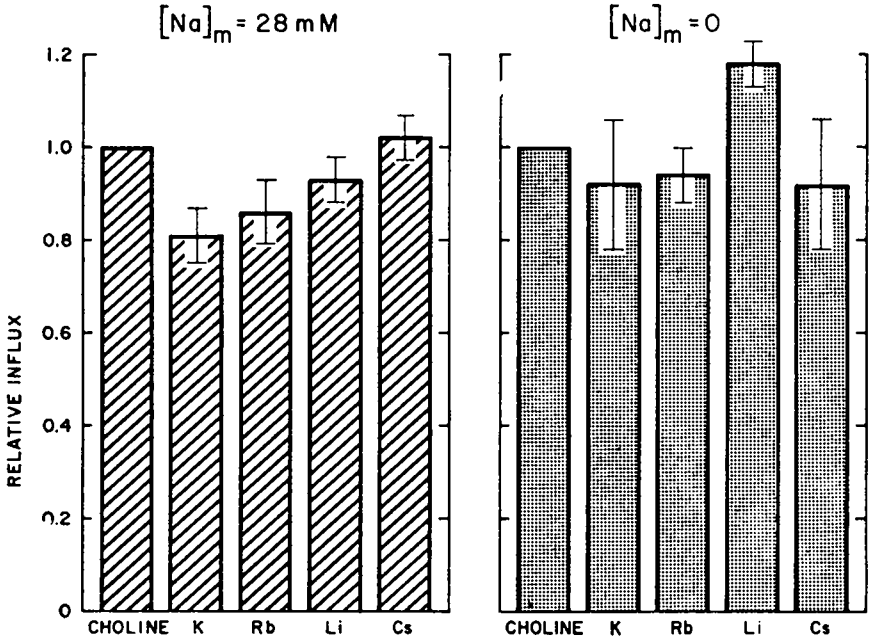


FIGURE 9. Comparison of the effects of different monovalent cations on alanine influx in the presence of 28 mM NaCl or choline Cl. The data are expressed relative to the influxes observed when choline Cl is used to replace 112 mM NaCl. These fluxes averaged 2.24 μmoles/hr, cm² in the presence of 28 mM NaCl and 0.96 μmole/hr, cm² when the Na was replaced with an additional 28 mM choline. These experiments were performed at pH 7.4 with 5 mM alanine in the test solution.

data are shown in Fig. 8. The coupling coefficient is given by the slope of the solid line, which by least squares analysis is 0.33 ± 0.08; the intercept of this line does not differ significantly from 0. From previous studies (1) at pH 7.4, the coupling coefficient in the presence of 140 mM Na is 0.90; this is indicated by the dashed line in Fig. 8. Thus, the coupling between Na and alanine influxes is substantially reduced by exposure to pH 2.5.

Effects of Other Monovalent Cations

Previous studies have shown that K and Li inhibit alanine influx in the presence of 22 mM Na (6). In view of the present observations that H is markedly

inhibitory in the presence of Na but does not affect influx in the absence of Na over the pH range 2.5 to 4.5, it was of interest to reevaluate the effects of other monovalent cations. Two types of experiments were performed. In the first, the effects of 112 mM K, Rb, Li, Cs, or choline were determined in the presence of 28 mM Na. In the second series the effects of these other cations were evaluated in the absence of Na with 28 mM choline serving as the substitute cation. Usually, the effects of several cations were determined on tissue from the same animal to facilitate quantitative comparisons. The results of these studies are illustrated in Fig. 9. In the absence of Na, neither K, Rb, nor Cs significantly inhibited alanine influx over and above the inhibition observed when choline was the sole substitute. On the other hand, alanine influx was significantly enhanced by Li($p < 0.05$). K and Rb significantly inhibited influx in the presence of 28 mM Na when compared to the inhibition observed when choline was the substitute cation. Neither Li nor Cs brought about a significant depression of influx, although influx in the presence of Li was consistently lower than that in the presence of Cs or choline.

The kinetics of K inhibition in the presence of Na are illustrated in Fig. 10.

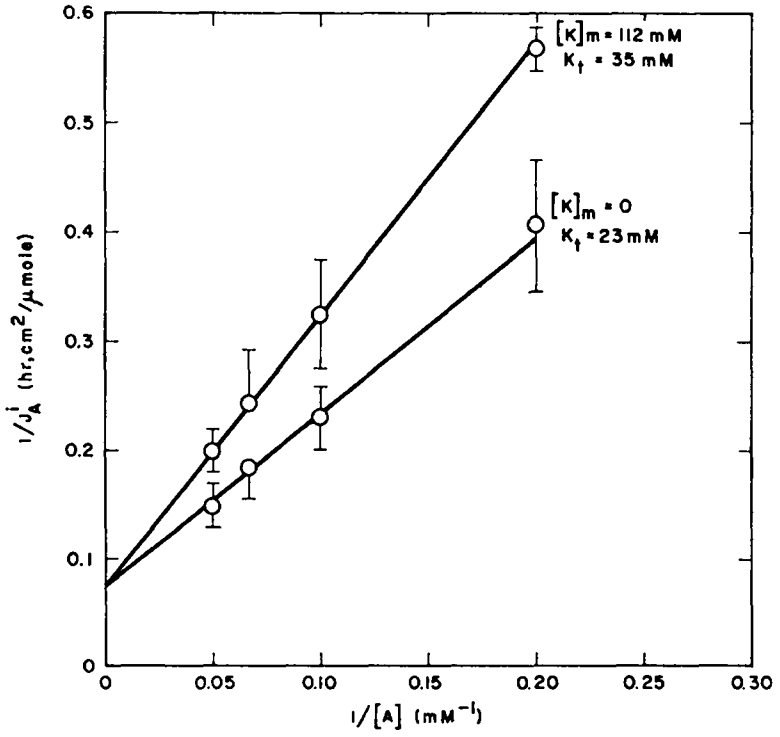


FIGURE 10. Lineweaver-Burk plot of alanine influx as a function of alanine concentration in the presence of 28 mM NaCl and either 112 mM choline Cl or KCl. Each point is the average of three determinations.

In these experiments the mucosal test solution contained 28 mM Na and either 112 mM K or 112 mM choline. Clearly, the maximal influx is the same when either K or choline is employed as a substitute cation (14 μmoles/hr, cm^2). However, the K_t in the presence of choline (23 mM) is significantly lower than that in the presence of K (35 mM). Thus, the effect of K resembles that of H and both are kinetically indistinguishable from the effect of lowering the Na concentration in the mucosal solution.

Experiments were performed to determine whether 112 mM K inhibits influx in the presence of 28 mM Na at pH 2.5. In order to facilitate comparisons, paired experiments at pH 7.4 and 2.5 were performed on tissue from the same animal. The results, given in Table I, indicate that whereas alanine influx in the presence of Na is inhibited by K at pH 7.4, this inhibitory effect is abolished at pH 2.5.

TABLE I

EFFECT OF K AT pH 7.4 AND pH 2.5

Test solution*	Alanine influx, *μmoles/hr, cm²*	
	pH 7.4	pH 2.5
112 mM choline + 28 mM Na	3.4 ± 0.2	0.88 ± 0.04
112 mM K + 28 mM Na	2.0 ± 0.1	0.83 ± 0.01
	$p < 0.01$	$p > 0.05$

* The alanine concentration in the test solution was 5 mM. There are four determinations in each case.

Effects of Heavy Metals and Group-Reactive Agents

In contrast to the effects of monovalent cations which specifically inhibit the Na-dependent fraction of alanine influx, several agents appear to act more nonspecifically. The effects of those agents which have been examined in the presence and absence of Na are given in Table II. The agent was included in the preincubation solution only and the influxes are expressed relative to control. Of the agents tested, Hg^{++}, mersalyl, Ag^{+}, Cu^{++}, UO_2^{++}, and La^{+++} inhibit alanine influx. All but La^{+++} inhibit both the Na-dependent and Na-independent fractions; La^{+++} inhibits influx in the presence of Na but not in its absence. Silver ion and UO_2^{++} at 10^{-4}–10^{-5} M inhibited the Na-dependent fraction of alanine influx only, but at higher concentrations also affected the Na-independent influx. The inhibitory effects of Hg^{++} and mersalyl suggest the involvement of SH groups (11). The failure of NEM to inhibit does not invalidate this conclusion inasmuch as many SH groups may be inaccessible to this agent. Rothstein (12) has reported that 7% of SH groups in the erythrocyte membrane are attacked by NEM but that 77% react with Hg^{++}.

TABLE II

EFFECT OF HEAVY METALS AND GROUP-REACTIVE AGENTS ON ALANINE INFLUX*

Agent	Relative influx	
	$[Na]_m$ = 140 mM	$[Na]_m$ = 0
Hg^{++} (10^{-3} M)	0.22 ± 0.04 (4)	0.56 ± 0.02 (2)
Hg^{++} (10^{-4} M)	0.38 ± 0.04 (4)	0.69 ± 0.02 (4)
Hg^{++} (10^{-5} M)	0.92 ± 0.08 (4)	1.03 ± 0.09 (4)
Hg^{++} (10^{-6} M)	1.03 ± 0.08 (6)	1.11 ± 0.13 (6)
Ag^{+} (10^{-3} M)	0.43 ± 0.06 (8)	0.84 ± 0.07 (6)
Ag^{+} (10^{-4} M)	0.70 ± 0.06 (8)	0.94 ± 0.12 (8)
Ag^{+} (10^{-5} M)	0.77 ± 0.10 (4)	1.03 ± 0.10 (4)
$(UO_2)^{++}$ (10^{-3} M) pH 5.0	0.41 ± 0.04 (4)	0.55 ± 0.04 (4)
$(UO_2)^{++}$ (10^{-4} M) pH 5.0	0.70 ± 0.03 (2)	0.98 ± 0.06 (2)
$(UO_2)^{++}$ (10^{-5} M) pH 5.0	0.89 ± 0.03 (2)	0.95 ± 0.08 (2)
La^{+++} (10^{-3} M)	0.60 ± 0.07 (4)	0.93 ± 0.06 (4)
Cu^{++} (10^{-3} M)	0.31 ± 0.03 (3)	0.49 ± 0.04 (3)
Pb^{++} (10^{-3} M)	1.08 ± 0.02 (2)	0.92 ± 0.18 (2)
Ni^{++} (10^{-3} M)	1.00 ± 0.22 (2)	0.96 ± 0.29 (2)
NEM (10^{-3} M)‡	1.00 ± 0.02 (2)	1.11 ± 0.16 (2)
Mersalyl (10^{-3} M)	0.33 ± 0.10 (2)	
Zn^{++} (10^{-3} M)	1.03 ± 0.31 (2)	

* The number of determinations is given in parentheses. Experiments with uranyl were performed at pH 5.0 to reduce complex formation with water which occurs at neutral pH.
‡ *N*-Ethylmaleimide.

The effect of UO_2^{++} and La^{+++} strongly implicate carboxylate or phosphoryl groups (13) and will be discussed further below.

CONCLUSIONS

These results indicate that alanine influx across the brush border of rabbit ileum in the presence of Na is rapidly and markedly inhibited by lowering the pH of the mucosal solution from pH 4.5 to pH 2.5; this effect is slowly but completely reversed by subsequent exposure to pH 7.4. In contrast, alanine influx observed in the absence of Na is only slightly, if at all, affected by lowering the pH of the test solution in this range. Thus, the effect of H on alanine influx in the pH range 2.5 to 4.5 appears to be largely restricted to the Na-dependent fraction. One question that must be considered is: To what extent is the inhibitory effect on alanine influx attributable to the effect of pH on the

charge distribution of this dipolar amino acid? Thus, it is conceivable that the effect of pH 2.5 is due, in part, to a direct effect on the brush border transport mechanism and, in part, to the fact that approximately 40% of the alanine is present in the form of a monovalent cation at pH 2.5. The data illustrated in Fig. 4 indicate that most of the inhibitory effect must be attributed to a direct effect on the brush border since alanine influx from a test solution at pH 7.4 is inhibited by exposing the tissue to pH 2.5 for 1–2 min immediately prior to the determination of influx. Under these conditions, influx is determined from a solution in which virtually all the alanine is present in the neutral zwitterionic form. Since the subsequent discussion will be based on results obtained using a test solution at pH 7.4, the possible complications arising from the effect of pH on the ionic distribution of alanine can be ignored[5]. An interesting feature of the pH effect is the finding that whereas the maximum inhibitory effect of pH 2.5 is observed within 1–2 min, reversal of this inhibition is quite slow; much too slow to be attributed to the presence of a residual unstirred layer at low pH. This hysteresis may be interpreted as evidence for the interposition of an energy barrier in the transition pathway between the protonated and deprotonated states (14), a barrier that does not hinder the transition from the deprotonated state to the protonated state. Protonated carboxylate groups and protonated phosphate groups can participate in hydrogen bonding. In addition, protonation of carboxylate groups could result in a lipophilic moiety that could engage in hydrophobic bonding. These new bonds are dependent upon protonation and must be broken before deprotonation. Their bond energies could be responsible for the transitional hysteresis. Similar phenomena have been observed in the titration of carbonylhemoglobin (15), membranes isolated from *Halobacterium halobium* (16), and other macromolecules (17, 18). In this regard it is appropriate to consider whether the observed behavior of the alanine transport mechanism, either in the presence of low pH or when tested immediately after exposure to low pH, can be attributed directly to the protonation of certain groups. Alternatively, it could be argued that our results are attributable to the processes responsible for the slow reversibility, e.g. conformational changes, and thus are only indirectly related to protonation. This possibility cannot be dismissed unequivocally. However, as will be shown below, all our observations can be accounted for, quantitatively, with the assumption that they are directly attributable to protonation. The secondary effects of protonation appear to perpetuate or preserve the effect on alanine influx but need not be invoked to explain the effect. For this reason, in the subsequent discussion

[5] The good agreement between influxes determined from a test solution at pH 2.5 and those determined from a test solution at pH 7.4 after exposure to pH 2.5, suggests that the ionic distribution of alanine does not significantly influence influx. However, further study is necessary to test this point explicitly.

we will use the phrases "at low pH" and "after exposure to low pH" interchangeably.

The inhibitory effect of leucine on alanine influx at pH 2.5 and the observation that this process conforms to saturation kinetics strongly suggest that influx at pH 2.5 is carrier-mediated. Indeed, the observation that the maximal influxes at pH 7.4 and 2.5 do not differ significantly suggests that the same carrier mechanism is involved at these two pH's and that prior exposure to pH 2.5 does not detectably reduce the capacity of this mechanism. Any decrease in the amount of carrier due to irreversible denaturation or desquamation of cells in the presence of pH 2.5 should result in a decrease in the maximal influx. In addition, significant tissue damage should be reflected by alterations in mannitol and Na influxes; no such changes were observed. In this respect rabbit ileum resembles rat small intestine (19) and rabbit gallbladder (20) in being relatively resistant to brief exposure to pH's as low as 2.5.

It has been demonstrated that Na affects the K_t of the alanine influx process but does not significantly affect the maximal influx; lowering the Na concentration in the mucosal solution brings about an increase in the K_t(1). The results illustrated in Fig. 7 indicate that the effect of pH 2.5 on alanine influx in the presence of Na is to increase the K_t without affecting the maximal influx. *Thus, the effect of increased H concentration on the kinetics of alanine influx in the presence of Na is indistinguishable from the effect of lowering the Na concentration of the mucosal solution at pH 7.4.*

A Kinetic Model

On the basis of these observations we have extended the previously proposed model to account, to a first approximation, for the effect of H on the influx process. This model is illustrated in Fig. 11.

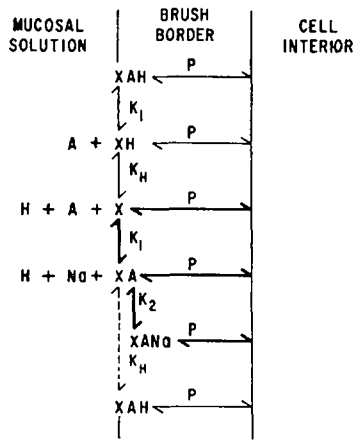


FIGURE 11. Kinetic model for the effect of H on alanine influx across the mucosal border.

According to this model, alanine (A) combines with a membrane component (X) to form a binary complex (XA). Na may then combine with XA to form the ternary complex (XANa). These steps are identical with those proposed previously and are indicated by the bold lines in Fig. 11. In addition, we postulate that H can combine with X to form XH and A can combine with XH to form XAH. The binding of H does not significantly affect the subsequent binding of A. However, XA can combine with either H or Na but not with both. We further assume that all forms of the carrier can translocate across the membrane. The translocation mechanism is not specified providing its rate is first order with respect to the carrier concentrations, the rate constants (P) are equal in both directions, and the rates of translocations are rate-limiting. These assumptions were employed in the derivation of the earlier model.

Under these conditions, the association-dissociation reactions at the interface may be considered at equilibrium and are governed by the following dissociation constants:

$$K_H = [H]\,[X]/[X\text{H}] = [H]\,[XA]/[XA\text{H}]$$
$$K_1 = [A]\,[X]/[XA] = [A]\,[X\text{H}]/[XA\text{H}]$$
$$K_2 = [\text{Na}]\,[XA]/[XA\text{Na}]$$

The solution of this model for alanine influx (J_A^i) (see Appendix A) is:

$$J_A^i = \frac{J_A^{im}[A]}{\frac{K_1K_2}{\alpha[\text{Na}] + K_2} + [A]} \tag{1}$$

where J_A^{im} is the maximal influx and is equal to PX_t (X_t is the total concentration of binding sites at the interface between the brush border and the mucosal solution) and

$$\alpha = K_H/(K_H + [H])$$

Equation 1 possesses the Michaelis-Menten form, where

$$K_t = K_1K_2/(\alpha\,[\text{Na}] + K_2) \tag{2}$$

Clearly, increasing H in the presence of Na increases K_t, however, J_A^{im} is independent of pH. According to this model, the pH of the mucosal medium will have no effect on alanine influx in the absence of Na. As discussed previously, this is strictly true only in the pH range 2.5 to 5, the range over which the inhibitory effect of H on influx in the presence of Na is observed. The model does not account for the effect of H on influx in the pH range above 5 because only a single protonation reaction characterized by a single K_H is included. Above pH 5 other reactions involving H occur and a vastly more

complex model would be necessary to account for the effect of H on influx over the entire pH range studied. In essence, the effect of H (through the fraction α) is to modify the "effective" Na concentration, *and an increase in H can be precisely simulated by a decrease in Na.*

Previous studies have indicated that K_1 and K_2 are equal to 70 mM and 17 mM, respectively (1). Using these values (a procedure that is justified by the excellent agreement between the K_t observed in the present investigation and that reported previously) and the observation that K_t = 50 mM when [H] = 3.2×10^{-8} M (Fig. 7), a value for K_H of 1.5×10^{-4} M can be calculated using equation 2. Thus, at pH 7.4 [H] $\ll K_H$ so that $\alpha \backeq 1$ and equation 1 reduces to the expression for alanine influx proposed previously. Furthermore, using these values for K_1, K_2, and K_H alanine influx from a solution containing 140 mM Na and 5 mM alanine can be calculated as a function of pH by means of equation 1. The calculated and observed alanine influxes, relative to the influxes at pH 6 (where $\alpha \backeq 1$), are plotted as functions of pH in Fig. 12 and are clearly in good agreement.

The coupling coefficient predicted by the model is given by

$$\frac{\Delta J^i_{Na}}{\Delta J^i_A} = \frac{[Na]_m}{K_2 + [Na]_m + K_2[H]/K_H}$$

At pH 2.5 the predicted coupling coefficient is 0.28, in good agreement with the observed value of 0.33. As discussed previously, the most stringent test of these models is their abilities to correctly predict the coupling coefficient between Na and amino acid influxes; this prediction is independent of the constraints employed in constructing the model.[6] The agreement between observed and predicted coupling coefficients and the data illustrated in Fig. 12 constitutes compelling evidence for the adequacy of this model.

The Nature of the Anionic Sites

Inspection of the data in Fig. 2 as well as the formal kinetic analysis indicates that the pK_a of the group(s) responsible for the effect of H on the Na-alanine interaction is approximately 4. This essentially implicates carboxylate groups although the data do not permit a distinction between end (pK_a = 3) or side chain (pK_a = 4) COO^- groups (21). Another prevalent functional group that could be involved is phosphoryl residues but these are generally assigned either a lower pK_a (2 for primary phosphates) or a higher pK_a (6 for secondary phosphates) (22). Although we cannot distinguish between these possibilities with certainty, present evidence favors carboxylate groups. This conclusion is supported by the effects of heavy metals and group-reactive agents

[6] The model is constructed to account for the effect of H on alanine influx, and the coupling coefficient emerges as a *de novo* prediction.

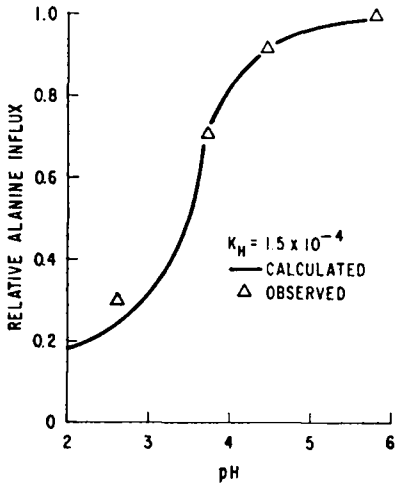


FIGURE 12. Alanine influx as a function of pH of the mucosal solution. Points are the experimental values calculated from the data shown in Fig. 2. The solid curve was calculated from equations 1 and 2.

reported in Table II. According to the original model, there are three parameters that can be affected by an inhibitor. If the inhibitor impeded the translocation process or irreversibly reduced the total number of functional sites, the maximal influx would be reduced but the fractional inhibition would be the same in the presence and absence of Na. If an inhibitor only affects the binding of amino acid to its site (increases K_1), the K_t of the process would increase but the fractional inhibition in the absence of Na would be greater than or equal to that in the presence of Na. For an inhibitor to bring about a greater degree of inhibition in the presence of Na than in the absence of Na, it must interfere with the binding of Na or itself bind with the Na site. Of the inhibitors tested La^{+++}, Ag^{+}, and UO_2^{++} inhibited influx to a significantly greater extent in the presence of Na than in its absence. Although Ag^{+} does complex with carboxylate groups this action is not specific and it also forms stable complexes with thiol and imidazole groups (22). Uranyl ion has a very high affinity for carboxylate and phosphate groups (13) and its marked effect on the Na-dependent fraction of alanine influx is consistent with the involvement of either of these residues. Indeed, failure of UO_2^{++} to affect Na-dependent influx would be difficult to reconcile with the presence of either of these reactive groups unless steric restrictions that prevent access of UO_2^{++} to the anionic site were invoked.

The effect of La^{+++} is restricted almost entirely to the Na-dependent fraction of influx. This metal ion has a high affinity for carboxylate and phosphate groups and has been shown to affect cation selectivity of artificial (23–25) and biological membranes. Van Breemen has demonstrated that porous phospholipid-cholesterol membranes that are normally permselective for cations become permselective for anions after exposure to La^{+++} (23). Similar findings have been reported by Wright and Diamond (20) for the

case of rabbit gallbladder. In both instances the effect is attributed to binding of the trivalent cation to carboxylate and/or phosphate groups resulting in the conversion of fixed negative charges to fixed positive charges. The possibility that charge reversal occurs in this system is currently under investigation.

All the other agents examined either did not affect influx or affected Na-dependent and Na-independent influx to similar extents. The observations reported in Table II also suggest the involvement of sulfhydryl and imidazole groups but further study is necessary to define the identities and roles of reactive groups involved in alanine binding and translocation.

Effects of Other Monovalent Cations

The data given in Figs. 9 and 10 indicate that K and Rb resemble the action of H in that they significantly inhibit influx in the presence of Na but not in its absence. Further, the effect of high K concentration is kinetically indistinguishable from the effect of high H concentration. Although influx at 28 mM Na in the presence of Li was consistently lower than that in the presence of choline the difference is not statistically significant. However, Li clearly enhances influx in the absence of Na. Thus, the inhibitory effect in the presence of Na is, in all likelihood, underestimated by the ability of Li to partially mimic the stimulatory action of Na. This dual action of Li will be discussed in greater detail below. The effect of Cs does not differ significantly from that of choline in the presence or absence of Na.

The observation that K inhibits influx in the presence of Na at pH 7.4 but has no effect at pH 2.5 supports the notion that K and H compete for a common site that has a much greater affinity for H. These data suggest that the model illustrated in Fig. 11, derived to account for the effect of low pH, can also account for the effects of K, Rb, and Li. Using equation 1 and the data given in Fig. 9, the mean values of K_K and K_{Rb} are 207 and 324 mM, respectively. The calculated value of K_{Li} is 715 mM but this is likely to be an overestimate due to the dual action of Li. The value of K_K derived from the kinetics illustrated in Fig. 10 is 100 mM and is probably a better estimate than the value of 207 mM derived at a single alanine concentration.[7] These data indicate that the affinity of X for H is three orders of magnitude greater than that for K, Rb, or Li.

Mechanistic Interpretations

The essential feature of the model illustrated in Fig. 11 is that the alanine carrier can bind either H or Na but not both. The simplest interpretation of

[7] The ranges of the dissociation constants calculated from the variance in Fig. 9 are: K_K, 133–351 mM; K_{Rb}, 180–736 mM; and K_{Li}, 388–3000 mM.

this model is that H and Na compete for the same anionic sites and that protonation of these sites abolishes Na binding. Unfortunately this simple interpretation has a major shortcoming. It implies that the conformational change, brought about by the binding of alanine, which permits the subsequent binding of Na, has no detectable effect on K_H. Further, if the same mechanism is responsible for the inhibitory effects of H, K, and Rb this simple interpretation becomes physically unrealistic. It would then imply that a conformational change that results in at least a 50-fold increase in the affinity for Na has no effect on the affinities for K or Rb. It should be stressed that our dissatisfaction with this interpretation is based entirely on a strict quantitative analysis of the model. As discussed in Appendix A, several major variants of this model can be excluded. However, the extent to which the affinities of H, K, and Rb for X could differ from those for XA and still remain compatible with our data is uncertain. For this reason, although we cannot exclude the above interpretation, we have sought alternatives. Two alternative interpretations that are based on simplifications of the general model (see Appendix A) will be discussed in detail below. In this discussion, we will assume that the effects of other monovalent cations and the effect of H reflect the same underlying interaction, with quantitative differences attributed solely to differences in dissociation constants (binding affinities).

A. *Two-Site Model*

A two-site model is illustrated in Fig. 13 b. C^+ designates either H, or other alkali metal ions (Li, K, Rb, Cs, Na) and K_c is the dissociation constant of the appropriate ion-site complex. According to this model the binding of A to X brings about a conformational change that creates a site that is highly

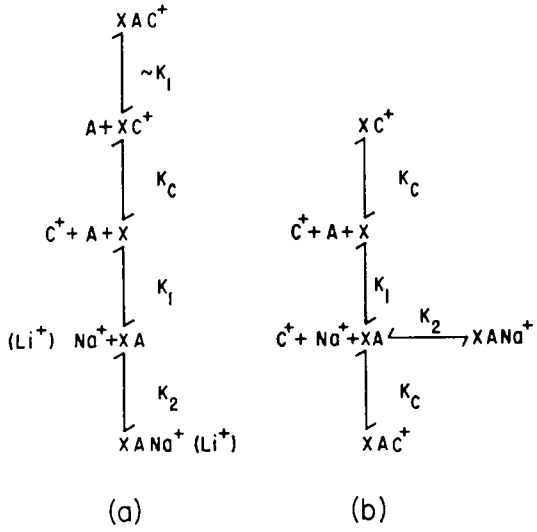
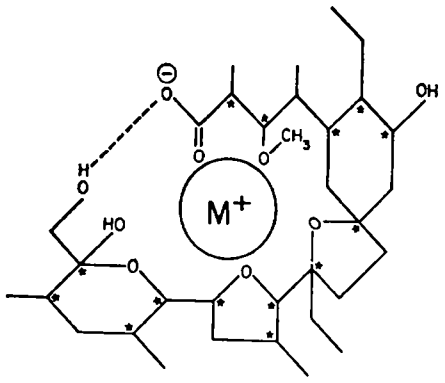


FIGURE 13. Mechanistic models of cation effects on alanine influx; (a) one-site model, (b) two-site model.

selective for Na. C^+ may combine with both X and XA at a site *other than* the Na site on XA, both binding processes being characterized by the same or very similar dissociation constants. The binding of C^+ (at this second site) prevents the formation of, or disrupts, the Na site. A good example of this type of interaction is afforded by monensin, a macrocyclic antibiotic, whose structure is shown in Fig. 14. In its cyclic form, monensin binds monovalent cations, M^+, with the following order of preference: Na > K > Rb > Cs (26). The cyclic configuration is dependent upon hydrogen bonding between a hydroxyl group and a dissociated carboxylate group (27). Protonation of the carboxylate group disrupts the cyclic structure and abolishes cation-binding affinity (26). If the binding of M^+ assists in stabilizing the cyclic structure, H would behave kinetically as a competitive inhibitor of M^+



Monensin Complex

FIGURE 14. Structure of the macrocyclic antibiotic, monensin. *Figure reprinted by permission from Federation Proceedings, 1968, 27:* 1283.

binding in spite of the fact that two distinct sites are involved. Further, if the anionic group involved in cyclization can form salts with other monovalent cations (as is, theoretically, always possible), then C^+ in general could behave as competitive inhibitors of M^+ binding.

B. *Single-Site Model*

A single-site model in which the same anionic group(s) are involved in the binding of C^+ and Na^+ is illustrated in Fig. 13 a. According to this model, the anionic site can undergo a conformational change that alters its cation selectivity. C^+ may combine with X to form XC^+. Further, alanine can combine with XC^+ to form XAC^+ with a dissociation constant that does not differ markedly from K_1. However, if A combines with X first, a conformational change takes place that makes the anionic site highly selective for Na and, to

a far lesser degree, Li.[8] In essence, the binding of alanine shifts the same site from a H-preferring to a Na-preferring conformation. This model may be considered in terms of the "induced fit" model proposed by Koshland (28) in that the binding site may be viewed as existing in two possible states or conformations, a H-preferring state and a Na-preferring state. The transition from the former to the latter is occasioned by the binding of alanine. The presence of C^+ stabilizes more of the carrier in the H-preferring conformation; the reason influx is inhibited is the pooling of carrier in the XC^+ form. Increasing A or Na increases the amount of carrier that contains A and thus contributes to influx (XA or XANa).

Although these two alternatives differ with respect to underlying mechanism, they are kinetically indistinguishable and have several common features.

1. According to both models, Na can only bind to the carrier, to an appreciable extent, after the binding of amino acid. Any significant binding of Na to X would lead to predicted coupling coefficients that differ significantly from those observed (1). Further, the H-preferring site (or conformation) cannot have a significant affinity for Na otherwise (i) $1/K_t$ would no longer be a linear function of $[Na]_m$ as has been observed for alanine (1, 29), glycine (30), histidine (Chez, Schultz, and Curran, unpublished observations), and glutamate (3), and (ii) the predicted coupling coefficient would differ from that observed.

2. A two-site model or a single-site that shifts affinities is consistent with the observation that Li slightly inhibits influx in the presence of Na but stimulates influx in the absence of Na. As pointed out in Appendix A (model II), if Li were solely competing for the Na site it should enhance influx in the presence and absence of Na. According to the present interpretation the inhibitory effect of Li in the presence of Na results from its interaction with the H-preferring site. In the absence of Na, the small affinity of Li for the Na site enhances influx.

3. The sequence of cation affinities for the H-preferring site or conformation is: H > K > Rb > Li > Cs, Na. The relative selectivities for H, K, and Rb are approximately $1:1 \times 10^3:2 \times 10^3$, respectively. This sequence corresponds closely to sequence IV of the 11 possible sequences predicted

[8] Because the binding of A to X is presumed to bring about a conformational change that does not take place when A binds to XC^+ the dissociation constants of these two reactions cannot be identical. However, K_1 is very large (70 mM) so that a change in this parameter is not readily detectable. For example, if the dissociation constant of the reaction $A + XC^+ \rightleftharpoons XAC^+$ were 1.5 K_1, influx from a Na-free solution containing 5 mM alanine would be 30% lower at pH 2.5 than at pH 6. There is a slight decrease in influx in the absence of Na as the pH is decreased from 6 to 2.5. However, this effect is clearly of second-order importance so that the assumption that similar dissociation constants govern the binding of A to X or to XC^+ would appear to be a valid first approximation.

by the anionic field strength theory developed by Eisenman (31) (see also [32]).

4. The Na-preferring site or conformation is highly specific for Na, has a discernible affinity for Li but no detectable affinity for H or other alkali metal ions. According to the anionic field strength theory, this high preference for Na can be attributed to restrictions on the amount of water permitted in the vicinity of the Na site (32). The preference sequence Na > Li > other cations has been encountered several times in studies of transport processes and, in particular, Na-coupled processes; examples will be discussed below.

Finally, it should be stressed that these mechanistic models represent strict interpretations of the simplest kinetic model that provides a satisfactory description of our data. Although these interpretations are speculative, they are consistent with current concepts of enzyme regulation and ion selectivity and they represent an attempt to employ kinetic studies to elucidate possible underlying mechanisms; kinetic studies are otherwise of limited usefulness. The success of this approach depends upon the extent to which the kinetic model, with all its assumptions and oversimplifications, provides a relatively undistorted reflection of reality; this question can only be answered by further investigation. These speculations should not obscure the experimental observations on the effects of H and K on alanine influx, and the possible physiological significance of these effects.

Physiological Implications

The present observations have important implications regarding two, as yet incompletely resolved, problems. The first is concerned with the energetics of amino acid accumulation and the second with the mechanism of amino acid transport across the brush border.

A. THE "NA-GRADIENT HYPOTHESIS" Abundant evidence, both direct and indirect, suggests that the energy necessary for the intracellular accumulation of amino acids by a wide variety of animal cells and the transport of sugars by small intestine and kidney is derived, at least in part, from the asymmetric distribution of Na across the cell membrane (4). It has been previously pointed out that the experimentally observed Na distribution ratio across the brush border of rabbit ileum is insufficient to account for the steady-state intracellular concentration of alanine, which may achieve levels 10 times that in the surrounding medium (1, 8). However, uncertainties regarding the activity and distribution of intracellular Na precluded any conclusive statement on the adequacy of the "Na gradient" (1). Recently, careful studies by Eddy at al. (33) and by Jacquez and Schafer (34) on amino acid accumulation by Ehrlich ascites cells have provided strong evidence that the Na-gradient alone is insufficient to account for accumulation but that a combined

Na-K gradient could suffice. Thus, the effects of a low intracellular Na together with the inhibition of efflux by a high intracellular K concentration may combine synergistically to enhance accumulation. A similar synergism has been suggested by Crane et al. (35) on the basis of the observation that extracellular K competitively inhibits the action of Na on sugar accumulation by hamster small intestine. The present results support the notion that the Na and K distribution ratios may combine synergistically to provide the energy for amino acid accumulation by small intestine. The intracellular K concentration in rabbit ileum is approximately 140 mM (8). Assuming that the inhibition of influx by K can be accounted for by the model illustrated in Fig. 11 and that influx and efflux are kinetically symmetrical, α_K for the efflux process is approximately 0.5; that is, the high intracellular K inhibits efflux to the same extent as would be obtained by halving the intracellular Na concentration. The present results also suggest that H gradients could contribute to the energy required for amino acid accumulation. Although it is generally believed that large H gradients between the intracellular and extracellular compartments do not exist, local gradients generated by metabolic processes adjacent to the brush border cannot be ruled out.

B. THE BRUSH BORDER TRANSPORT MECHANISM Amino acid absorption is associated with an increase in the rate of active Na transport from mucosa to serosa (36) and evidence has been presented that the increase in net Na absorption can be accounted for by the coupled movements of alanine and Na across the brush border (1). The preservation of bulk electroneutrality requires that the net transfer of Na across the brush border (coupled to alanine transfer) be accompanied by an equivalent net influx of anions, a net efflux of cations (other than Na), or a combination of these movements. The model illustrated in Fig. 11 is consistent with a mechanism by which Na influx can be coupled to an efflux of K and, possibly, H. Studies designed to test this hypothesis directly are in progress. In this respect, it is of interest that several investigators have noted reciprocal movement of Na and K associated with amino acid uptake by Ehrlich ascites cells (37, 38) and nucleated erythrocytes (39).

Other Studies

Studies on Na-dependent transport processes in other epithelial and nonepithelial cells have yielded data that, in many respects, resemble the present results.

Oxender and Christensen (40) (see also [41]), have suggested that sensitivity to low pH is one of the characteristics of Na-dependent transport agencies in Ehrlich cells, pigeon erythrocytes, and rabbit reticulocytes.

Numerous investigators have reported that high K concentrations inhibit Na-dependent transport processes in epithelial and nonepithelial cells (4).

Holt (42) demonstrated that K and Li inhibit bile salt accumulation by slices of rat ileum in the presence of Na but have no inhibitory effect in the absence of Na. Kipnis (43) has reported that K antagonizes the action of Na on AIB influx into intact rat diaphragm; the effect of K conformed to the kinetics of a competitive inhibitor. Crane et al. (35) have reported that K is a competitive inhibitor of sugar accumulation by hamster small intestine and that it interacts with the Na-binding site on the carrier and results in a "greatly decreased affinity for the substrate." Similarly, Eddy et al. (33, 44) have shown that K is a competitive inhibitor of the action of Na on glycine influx in the presence of Na. These authors suggest that K competes with Na for the same negative site on the carrier, that the resulting complex can cross the membrane, and that this mechanism may be responsible for the reciprocal net movements of Na and K observed during glycine accumulation.

Bosackova and Crane (45) have reported that Li, K, Rb, Cs, and NH_4 inhibit sugar accumulation by hamster intestine *in the presence of Na* whereas Bihler and Adamic (46) have demonstrated that Li slightly stimulates sugar accumulation by the same preparation *in the absence of Na*. The latter authors concluded that Li may activate the carrier when alone but that it antagonizes the activation produced by Na when both ions are present. The ability of Li to partially substitute for Na has been demonstrated for several transport processes. For the case of Na-dependent transport, in addition to the findings of Bihler and Adamic, Li has been found to partially mimic the stimulatory effect of Na on the rate of sugar uptake by rat small intestine (47). Allfrey et al. (48) have shown that Na-dependent alanine uptake by isolated thymus nuclei is four times greater in the presence of Li than in the presence of K; high concentrations of K, Rb, or Cs were inhibitory. Eddy and Hogg (44) have recently reported that glycine influx into Ehrlich cells is stimulated by Li. In the virtual absence of Na, Li was about 15 times less effective than Na. In the presence of 16 mM Na, Li was still stimulatory but less so than in the absence of Na. Finally, Christensen et al. (49) have reported that Li partially mimics the action of Na in amino acid uptake by Ehrlich cells and rabbit reticulocytes.

The common features that emerge from these studies and the present data are:

1. In all instances, K (and Rb) inhibit Na-dependent transport and, wherever studied, the inhibition appears to be of the competitive type. Further, the effect of K is observed only in the presence of Na; no compelling evidence for inhibition of residual uptake in the absence of Na has been reported.

2. Li appears to have a dual effect. In many instances Li is inhibitory in the presence of Na but has either no effect or is slightly stimulatory in the absence of Na.

It is of interest that the two cation selectivity sequences implicated in these

studies are consistent with the anionic field strength theory developed by Eisenman and his collaborators (31) and are encountered very frequently in biological transport processes.

Sequence IV (H > K > Rb > Li, Cs, Na) appears to describe the relative affinities for cation transport in yeast (50); exchange rates in frog sartorius muscle (51); the ability of external cations to stimulate Na efflux in striated muscle (52) and squid axon (53); and the ability of different monovalent cations to mimic the action of K in the maintenance of the frog skin potential (54) and the transepithelial electrical potential across bullfrog urinary bladder (55).

Lithium is capable of partially mimicking Na in the generation of action potentials by cat cardiac muscle (56), cation extrusion by yeast cells (57) and the maintenance of electrical potential difference (54), and short-circuit current (58) in frog skin. (Na > Li > other cations is consistent with Eisenman's sequence X.)

Finally, the mechanism implicit in the single-site model illustrated by Fig. 13 a resembles in some respects current notions on the behavior of Na-K-ATPase. The cation site on the dephosphorylated ATPase is highly specific for Na (59). Phosphorylation of a glutamic acid carboxylate group (60) appears to initiate a conformational change (61) that alters the selectivity of the cation site to K, Rb, NH_4 > Cs > Li > Na (59, 61). Dephosphorylation of the L-glutamyl-phosphate residue restores Na specificity. In our single-site model, the binding of alanine is analogous to this dephosphorylation step.

APPENDIX A

In arriving at the kinetic model illustrated in Fig. 11, several less complex alternatives were examined; all were rejected because they did not satisfy our experimental observations. Because of the mechanistic implications of the model it seems relevant to outline these alternatives and the reasons for their rejection:

The solution of these models follows the general approach discussed by Hearon (62) and has been described in detail (reference 1, Appendix A).

Model I (i) A combines with X to form XA; (ii) Na or H can combine with XA to form XANa or XAH; (iii) XAH *cannot* translocate across the membrane ($P_{XAH} = 0$).

This model predicts that (i) J^{im} decreases with increasing [H]; and (ii) the coupling coefficient is unaffected by pH.

Model II (i) and (ii) same as above, but (iii) XAH *can* translocate across the membrane.

This model predicts that K_t will decrease with increasing [H] at constant [Na]. In essence, H acts as an acceptable substitute for Na, and stimulates rather than inhibits influx.

Model III (i) H combines with X to form XH; (ii) A combines with X to form XA; (iii) Na combines with XA to form XANa; (iv) A cannot combine with XH; and (v) all forms of X can translocate. This model differs from the one-site model illustrated in Fig. 13 a only in that A is prohibited from binding to XH.

This model predicts that (i) K_t increases with increasing [H] but the inhibitory effect of H is equally marked in the presence and absence of Na; and (ii) the coupling coefficient is unaffected by pH.

Thus, these three modifications of the original model satisfy neither the experimentally observed kinetics nor the effect of pH on the coupling coefficient. The model illustrated in Fig. 11 differs from model III, above, by one step and appears to be the simplest model that satisfies all our observations.

It should be noted that the complete model illustrated in Fig. 11 includes five association-dissociation reactions and thus five equations of the form

$$K[XY] - [X][Y] = 0$$

However, because K_1 and K_H are independent of the order of binding of A and H, respectively, only four of the five equations are independent. That is, one equation is redundant and can be derived from the other four. Thus, one step in the model can be omitted without affecting the final solution and the same solution applies to (i) the complete model as illustrated; (ii) a model in which the step $A + XH \leftrightarrow XAH$ is omitted (Fig. 13 a); and, (iii) a model in which the step $H + XA \leftrightarrow XAH$ (the dashed reaction in Fig. 11) is omitted (Fig. 13 b). In essence Fig. 11 contains one unnecessary reaction and, the two simplest models consistent with our data are illustrated in Fig. 13.

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