Dicarboxylic Amino Acid Influx across Brush Border of Rabbit Ileum

Effects of amino acid charge on the sodium-amino acid interaction

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ABSTRACT Glutamate and aspartate influxes across the brush border of rabbit intestine are saturable processes that are subject to competitive inhibition and are markedly influenced by the Na concentration in the mucosal solution. Lowering the Na concentration increases the amino acid concentration needed to elicit a half-maximal influx but does not significantly affect the maximal influx. The interaction between Na and anionic amino acid influx can be described by the same kinetic model that has been applied to the influxes of neutral amino acids and lysine. Comparison of the kinetic parameters for anionic, neutral, and cationic amino acids suggests that amino acid charge influences (a) the stability of the binary (amino acid-site) complex and (b) the affinity of this binary complex for the subsequent binding of Na. A mechanistic interpretation of these interactions is proposed.

In his early studies of amino acid transport across everted sacs of rat small intestine, Wiseman (1) noted that the dicarboxylic (anionic) amino acids, aspartic and glutamic acids, were not transferred from the mucosal solution to the serosal solution against a concentration difference. Nevertheless, these amino acids were taken up from both the mucosal and serosal solutions and could not be recovered from the intestinal tissue. Subsequently, Wiseman and his coworkers (2, 3) discovered that aspartic and glutamic acids are rapidly transaminated by in vitro and in vivo preparations of small intestine. Everted sacs of hamster small intestine are also unable to transport anionic amino acids against a concentration difference (4, 5) and transamination of these
amino acids has been demonstrated for dog (3, 6), cat (6), rabbit (6), and rat (7) small intestines.

If a solute is metabolized rapidly, the results of studies of transmural transport and tissue accumulation are, at best, difficult to interpret. Certainly, failure to observe net transport of anionic amino acids against a concentration difference cannot be interpreted as conclusive evidence against the presence of a mechanism similar to those responsible for the active transport of neutral and basic amino acids since the function of such a system could be obscured by extensive metabolism. In the present study, we have attempted to obtain more definitive information on the transport of anionic amino acids by examining the characteristics of the unidirectional influxes of glutamic and aspartic acids across the brush border of rabbit intestine. The method employed is ideally suited for this study because the results are not influenced by tissue metabolism provided all the products are retained within the tissue. Studies on rabbit ileum were carried out in Pittsburgh and those on rabbit jejunum in New Haven. The results were quite similar in most respects and since the studies on the ileum are more extensive, we will report them in detail and summarize the results on the jejunum.

METHODS

Male and female white rabbits (2–4 kg), that had been maintained on normal food intake, were sacrificed by intravenous injection of pentobarbital. A section of distal ileum or proximal jejunum was excised, opened along the mesenteric border, rinsed free of intestinal contents, and mounted, mucosal surface up, in a chamber that has been described in detail previously (8). This apparatus permits exposure of eight defined areas (each 1.13 cm²) of the mucosal surface alone to solutions of desired composition; the serosal surface of the tissue rests on moistened filter paper. The procedure for determining the unidirectional influx of solutes from the mucosal solution across the brush border into the epithelium has been described in detail (8).

The mucosal solution contained (mM): NaCl, 120; KHCO₃, 10; K₂HPO₄, 1.2; KH₂PO₄, 0.2; CaCl₂, 1.2; and MgCl₂, 1.2. Glutamic and aspartic acids were added in the form of the potassium salts. In order to maintain constant osmolarity in studies of influx vs. amino acid concentration, the following procedure was employed: The K salt of the amino acid was added to obtain the desired final concentration and KCl was added to bring the total addition (K amino acid plus KCl) to 25 mM. Thus, the anionic amino acid replaced Cl in all solutions and the measured osmolarity was maintained at 300 ± 3 milliosmols. All experiments were performed at 37°C and the pH of the mucosal solution was maintained at 7.4 by bubbling with 95% O₂–5% CO₂ gas mixture. The Na concentration of the mucosal solution was varied by replacement of NaCl with choline chloride. A series of studies performed on the jejunum indicated that the inhibition of influx resulting from replacement of Na with choline, Tris, or Li, or replacement of NaCl with mannitol did not differ significantly.

L-Glutamic acid was obtained from Calbiochem (Los Angeles, Calif.) and the K salt was prepared by titration with KOH. Potassium aspartate was obtained directly...
from Mann Research Laboratories, Inc. (New York), $^{14}$C-L-Glutamate, $^{14}$C-L-aspartate, inulin-^3H, and $^{22}$Na were obtained from New England Nuclear Corp. (Boston, Mass.).

All errors are expressed as standard errors of the mean.

**RESULTS**

**Kinetics of Influx**

A typical time course of L-glutamate uptake as a function of the duration of exposure of the mucosal surface of the ileum to a solution containing 5 mM glutamate and 120 mM Na is shown in Fig. 1. Studies of this type, using jejunum and ileum, indicate that glutamate uptake is a linear function of time for at least 1 min. As discussed previously (8), these findings indicate that the 1 min uptake is a valid measure of the unidirectional influx of glutamate across the brush border. It should be stressed that the fact that glutamate is transaminated by rabbit small intestine does not influence the interpretation of these data since none of the immediate products of this reaction is volatile (9). Thus, all the $^{14}$C that enters the tissue across the brush border is retained within the tissue for at least the 1 min duration of the influx measurement; any significant loss from the tissue would result in a deviation from linearity.

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**Figure 1.** Time course of glutamate uptake across brush border. The mucosal solution contained 5 mM glutamate and 120 mM Na. The slope corresponds to an influx of 2 μmoles/hr, cm².
Glutamate influx in the ileum was determined as a function of glutamate concentration in the presence of 120, 60, 30 mM Na, and a Na-free medium in which all the Na was replaced with choline. In all these experiments, the tissue was first incubated for 30 min in Na-free choline medium and influx was determined at several glutamate concentrations on tissue from the same animal. At 120 mM Na, influx was studied from solutions containing 2.5, 5, 10, and 25 mM glutamate and in each of three experiments duplicate determinations were obtained at each concentration. At 30 and 60 mM Na, influx was determined from solutions containing 5, 10, and 25 mM glutamate, and at 0 mM Na, influx was determined only from solutions containing 10 and 25 mM glutamate. The reason for this approach is that glutamate influx is markedly dependent on the Na concentration in the mucosal medium and at low Na concentrations (less than 30 mM) influx from solutions containing less than 10 mM glutamate is very low and subject to considerable experimental error.

The results of these studies are illustrated in Fig. 2. The values for $K_t$ and $J_o^{max}$ (maximal influx of glutamate) were estimated from Lineweaver-Burk, double reciprocal plots of influx vs. concentration. The maximal influxes at the four Na concentrations ranged between 6.0 and 7.5 μmoles/hr, cm² and do not differ significantly. In contrast, the $K_t$ increased significantly as the mucosal Na concentration decreased and averaged 9 mM at 120 mM Na, 17 mM at 60 mM Na, 30 mM at 30 mM Na and 90 mM in the absence of Na. The curves shown in Fig. 2 are the theoretical curves constructed using these parameters. The agreement between the experimental data and the theoretical curves indicates that glutamate influx is a saturable process that
conforms to Michaelis-Menten kinetics. Thus, glutamate influx \( J'_G \) at constant Na concentration can be described by means of the following equation:

\[
J'_G = \frac{J''_G[G]_m}{(K_t + [G]_m)}
\]

where \([G]_m\) is the glutamate concentration in the mucosal solution and \(K_t\) is the glutamate concentration required to elicit a half-maximal influx.

A similar series of experiments was carried out in New Haven to investigate the kinetics of glutamate influx in rabbit jejunum. The methods used were identical to those described above with two exceptions. First, solutions were prepared with Na glutamate rather than K glutamate and all solutions contained 13 mM K. Second, tissues were preincubated for 30 min in solutions having the same Na concentration as the test solutions rather than in Na-free solution as described above. However, control experiments showed that glutamate influx was unaffected by the Na concentration of the preincubation solution. Tissues were preincubated in either 140 mM Na or Na-free media and influx was measured from a mucosal solution containing 140 mM Na and 20 mM glutamate. Influxes were 0.80 ± 0.09 μmole/hr, cm² for preincubation in the presence of 140 mM Na and 0.76 ± 0.09 μmole/hr, cm² for preincubation in choline medium.

The results of these studies are qualitatively similar to those obtained in the ileum. Glutamate influx measured at 2, 5, 10, and 20 mM could be described by equation 1 and Table I summarizes the results of three to four experiments at each of three Na concentrations. The maximal influx does not vary significantly with Na concentration while the \(K_t\) increases as Na concentration is decreased. The same kinetic dependence upon \([Na]_m\) has been observed for alanine, leucine, valine (10), lysine (11), glycine (12), phenylalanine and histidine (unpublished observations). It should be noted that although the \(K_t\)'s for glutamate influx in ileum and jejunum are very similar, the maximal influx in jejunum appears to be significantly lower than that in ileum. Although this could reflect differences between different sections of rabbit small intestine, it could also be the result of differences between rabbits obtained from local breeders in New Haven and Pittsburgh. Maximal influx is an extensive parameter that is directly dependent upon the total functional mucosal area. Thus, differences in distendibility, number of villi, number of 

### Table I

<table>
<thead>
<tr>
<th>[Na]ₘ</th>
<th>Kₜ</th>
<th>(J'<em>{G</em>{max}})</th>
</tr>
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<tbody>
<tr>
<td>mm</td>
<td>mM</td>
<td>μmole/hr, cm²</td>
</tr>
<tr>
<td>140</td>
<td>7.1±1.6</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>70</td>
<td>12.7±1.5</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>20</td>
<td>41.2±9.0</td>
<td>1.3±0.3</td>
</tr>
</tbody>
</table>
functional absorptive cells could result in large differences in this parameter. Additional studies are required to determine the factors that are responsible for the difference in maximal influxes. However, these studies are not relevant to the present investigation because all the subsequent discussion and conclusions are based exclusively on results obtained with ileum.

Fig. 3 shows a plot of $1/K_t$ for glutamate as a function of the Na concentration in the mucosal medium for both ileum and jejunum. A similar linear relation between $1/K_t$ and $[\text{Na}]_m$ was also found for alanine (10) and glycine (12) influx across the brush border of rabbit ileum and for alanine influx in rabbit jejunum (13). The extrapolated value of $K_t (100 \text{ mM})$ at $[\text{Na}]_m = 0$ is in good agreement with that estimated from influx measurements in the absence of Na.

The kinetics of aspartate influx into ileum in the presence of 120 mM Na were investigated in four experiments involving eight determinations of influx at each of four aspartate concentrations. The results of these studies are given in Fig. 4. The curve corresponds to a saturable process having a $K_t$ of 5 mM and a maximal influx of 3 μmoles/hr, cm² and appears to provide an adequate description of the experimental data. It is of interest that the maximal influx of aspartate is approximately one-half that calculated for glutamate. This cannot be attributed to differences between different animals. Studies of glutamate and aspartate influxes into tissue from the same animals indicate that aspartate influx is consistently lower than that of glutamate when the two amino acids are present at concentrations calculated to elicit near maximal influxes. Using paired tissues from the same animal glutamate influx from a solution containing 25 mM glutamate averaged $3.8 \pm 0.7$ μmoles/hr, cm² whereas influx from a solution containing 25 mM aspartate averaged $1.7 \pm 0.4$ μmoles/hr, cm² (four determinations). In terms of the model (10) for amino
acid influx in rabbit ileum, these differences in maximal influx could be attributed to (a) different amounts of membrane component capable of binding these amino acids; and/or (b) different translocation rates for the two amino acid-carrier complexes. Kinetic studies cannot distinguish between these possibilities so that further speculation on this point seems unproductive. Aspartate influx in the absence of Na averaged $0.32 \pm 0.04 \mu$mole/hr, cm$^2$ at 10 mM aspartate and $0.90 \pm 0.09 \mu$mole/hr, cm$^2$ at 30 mM aspartate (14 paired determinations at each concentration). Although these data are consistent with a linear relation between influx and concentration, they can also be very adequately described by a saturable process having a maximal influx of 3 $\mu$mole/hr, cm$^2$ and a $K_i$ of 75 mM. This process predicts influxes of 0.35 and 0.86 $\mu$mole/hr, cm$^2$ in the presence of 10 and 30 mM aspartate, respectively, values that are in good agreement with those observed. Because our estimates of the $K_i$'s for anionic amino acids in the absence of Na play an important role in the subsequent discussion, it is pertinent to consider the extent to which the nearly linear relation between influx and concentration can provide a minimal estimate of $K_i$. If influx is measured at two amino acid concentrations, $[A]_1$ and $[A]_2$, ($[A]_2 > [A]_1$), then $J_2/J_1 = \alpha [A]_2/[A]_1$, where $\alpha = 1$ for a strictly linear relation and $\alpha < 1$ for a saturable process. If the process conforms to Michaelis-Menten kinetics it follows that

$$(1 - \alpha)K_i = \alpha [A]_2 - [A]_1$$

Now, the standard error of our influx determinations is approximately 10% so that a deviation from linearity of 20% should be detectable; that is, a value
of $\alpha < 0.8$ should constitute a significant deviation from linearity. Using $\alpha = 0.8$ and $[A]_2 = 3[A]_1$, $K_t = 2.4 [A]_2$. Any value of $K_t$ less than $2.4 [A]_2$ would result in a readily detectable deviation from linearity. Thus, the minimal $K_t$ for aspartate is $(2.4) (30) = 72$ mm.

Because of the high apparent $K_t$'s for glutamate and aspartate influxes in the absence of Na, it is difficult to statistically exclude the possibility that influx is due to simple diffusion. In order to provide further evidence for carrier-mediated influx, the effect of aspartate on glutamate influx was in-

<table>
<thead>
<tr>
<th>TABLE II</th>
<th>COMPETITIVE INTERACTIONS BETWEEN GLUTAMATE AND ASPARTATE INFLUXES*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Na]$_{in}$ = 120 mm</td>
</tr>
<tr>
<td>Glutamate influx</td>
<td></td>
</tr>
<tr>
<td>5 mM Glu</td>
<td>1.90±0.25 (4)</td>
</tr>
<tr>
<td>5 mM Glu + 20 mM Asp</td>
<td>0.42±0.10 (4)</td>
</tr>
<tr>
<td>Observed $r$</td>
<td>0.22±0.06</td>
</tr>
<tr>
<td>Predicted $r$</td>
<td>0.28</td>
</tr>
<tr>
<td>Aspartate influx</td>
<td></td>
</tr>
<tr>
<td>5 mM Asp</td>
<td>1.13±0.11 (4)</td>
</tr>
<tr>
<td>5 mM Asp + 20 mM Glu</td>
<td>0.62±0.04 (4)</td>
</tr>
<tr>
<td>Observed $r$</td>
<td>0.53±0.07</td>
</tr>
<tr>
<td>Predicted $r$</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* Fluxes are in μmoles/hr, cm$^2$. The number of influx determinations is given in parentheses. $r$ is the ratio of the influx in the presence of inhibitor to that in the absence of inhibitor. The predicted $r$ is calculated from the values for the $K_t$'s of glutamate and aspartate in the presence and absence of Na assuming classic competitive inhibition (reference 10, page 1266). In every instance, influx in the presence of inhibitor was lower than influx in the paired adjacent control tissue. Inhibition in the absence of Na, though small, is statistically significant.

...investigated both in the presence and absence of Na. In experiments on ileum, the control medium contained 5 mM K glutamate and 20 mM KCl and the test medium contained 5 mM K glutamate plus 20 mM K aspartate; thus control and test media had equal osmolarities and K concentrations and the only difference was that 20 mM aspartate replaced 20 mM Cl in the test medium. Similar experiments were performed to examine the effect of glutamate on aspartate influx. The results of these experiments are given in Table II. Either anionic amino acid inhibits the influx of the other in the presence and absence of Na. Similar experiments were carried out on jejunum except that glutamate and aspartate were used at concentrations of 1.7 and 20 mM, respectively. At 140 mM Na, aspartate inhibited glutamate influx by 78% while in a Na-free solution the inhibition was 23%. These results are comparable to those obtained on ileum, and support the notion that glutamate influx in the presence and absence of Na is a carrier-mediated process subject...
to competitive inhibition by aspartate. Thus, in spite of the uncertainties in precisely evaluating $K_t$ in the absence of Na, the nearly linear relation between influx and concentration is almost certainly due to the fact that $K_t$ is at least two to three times the highest concentration employed.

**Coupling between Na and Glutamate influxes**

A characteristic of Na-dependent amino acid transport in a variety of animal cells (14) and Na-dependent sugar influx in rabbit ileum (15) is a positive coupling between the influxes of the amino acid or sugar and the influx of Na. Alanine and glycine influxes in rabbit ileum (10, 12) and alanine influx in rabbit jejunum (13) are associated with a concomitant increase in Na influx. The relation between Na and glutamate influxes in ileum was examined in 3 experiments involving 24 influx determinations. 12 determinations of Na influx in the absence of glutamate averaged $29 \pm 1.3 \mu$moles/hr, cm$^2$. 12 simultaneous determinations of Na and glutamate influxes, in the presence of 10 mM glutamate, yielded an average Na influx of $33 \pm 1.1 \mu$moles/hr, cm$^2$ and an average simultaneous glutamate influx of $3.5 \mu$moles/hr, cm$^2$. The increase in Na influx of $4 \pm 1.7 \mu$moles/hr, cm$^2$, associated with glutamate influx, is statistically significant ($p < 0.01$). The ratio between the increment in Na influx and the simultaneous glutamate influx averages $1.1 \pm 0.4$.

**Conclusions**

The present results provide, for the first time to our knowledge, compelling evidence that the movement of anionic amino acids from the intestinal lumen across the brush border into the absorptive epithelium is a carrier-mediated process. This conclusion is based on the findings that the influxes of both aspartate and glutamate are saturable processes that are subject to competitive inhibition and are markedly dependent on the Na concentration in the mucosal medium.

The implication of a carrier process in the intestinal absorption of anionic amino acids is not surprising in the light of previous observations that (a) glutamate and aspartate are rapidly absorbed in vivo and the rates of disappearance of these two amino acids from the lumen are comparable to the rates of absorption of neutral amino acids (16); (b) the L-stereoisomers of glutamic and aspartic acids are much more rapidly absorbed in vivo than are the D-stereoisomers (16); and (c) the addition of glutamic acid or aspartic acid to the solution bathing the mucosal surface of isolated rabbit ileum brings about a prompt increase in the short-circuit current (17). The earlier observations together with the present results indicate that the kinetic characteristics of the brush border transport mechanism for anionic amino acids closely resemble those for neutral and cationic amino acids. These
mechanisms for neutral and cationic amino acids appear to be responsible for transport from a lower concentration in the mucosal solution to a higher concentration within the cell (18), and evidence has been presented that the Na dependence of these systems together with the asymmetric distribution of Na across the brush border is, at least in part, responsible for this uphill transfer (14). In view of the marked Na dependence of glutamate and aspartate influxes, we might suggest that the brush border transport mechanisms for these anionic amino acids are capable of effecting transport against an electrochemical potential difference but that accumulation within the cell is prevented by rapid intracellular metabolism. Studies of anionic amino acid transport in the presence of specific inhibitors of transamination might establish this point conclusively. In any event, the present findings should once and for all dispel the notion that the failure to observe net transport of anionic amino acids against a concentration gradient using everted sacs or tissue segments implies that these amino acids do not interact with membrane components and can be treated as inert (19).

It is of interest to examine the recent findings of Hindmarsh et al. (20) in the light of the present results. These investigators have demonstrated that the transfer of sugars against concentration differences by everted sacs of hamster small intestine is inhibited by the addition of actively transported amino acids to the mucosal solution. However, neither glutamate nor aspartate affected sugar transport. If, as is quite likely, the hamster small intestine also possesses Na-dependent brush border transfer mechanisms for anionic amino acids, the observations of Hindmarsh et al. have important bearing on a current hypothesis for the mutual inhibitory interaction between sugar and amino acid transport in small intestine. Alvarado (21) has suggested that since the transport of all these compounds is Na-dependent they may "share a common polyfunctional carrier in which a series of separate binding sites, namely, one each for sugars, neutral amino acids, basic amino acids, and Na are joined together, as in a mosaic." One attractive feature of this hypothesis, as pointed out by Alvarado (21), is its ability to explain "in a unified and general way the activating effect of Na on a group of apparently different transport processes." Clearly, either hamster small intestine is qualitatively different from rabbit small intestine with respect to anionic amino acid transport or Na dependence per se cannot be the common denominator underlying these interactions. The anionic amino acids differ in two respects from neutral and cationic amino acids. They are not accumulated within the cell and, perhaps more important, transamination gives rise to readily metabolized intermediates (9, 22). Thus the failure of anionic amino acids to inhibit sugar transport could be consistent with the "energy limitation" notion suggested by Newey and Smyth (23) to explain these effects.

A kinetic model for amino acid influx across the brush border of rabbit
ileum has been proposed largely on the basis of detailed studies of the interaction between Na and alanine influx (10). According to this model, the amino acid \((A)\) first combines with a membrane component \((X)\) to form a binary complex \((XA)\). This complex may either cross the membrane, or it may combine with Na to form a ternary complex \((XANa)\) that then crosses the membrane. Assuming that the rate constants for the translocation of \(X\), \(XA\), and \(XANa\) across the membrane are equal and that translocation is rate limiting, amino acid influx is given by the following equation:

\[
J_A^i = J_A^m[A]_m/(K_t + [A]_m)
\]

where

\[
K_t = K_1K_2/(K_2 + [Na]_m)
\]

and \(K_1\) is the dissociation constant of the binary complex and \(K_2\) is the dissociation constant of the ternary complex (i.e., the equilibrium constant for the reaction \(XANa \rightleftharpoons XA + Na\)). According to this model, (a) the maximum influx is independent of \([Na]_m\) but the \(K_t\) increases as \([Na]_m\) decreases; (b) when \([Na]_m = 0\), amino acid influx is mediated by the binary complex and thus displays saturation kinetics, is subject to competitive inhibition, and \(K_t = K_1\); (c) a plot of \(1/K_t\) vs. \([Na]_m\) is linear, with the intercept equal to \(1/K_1\) and the slope equal to \(1/K_1K_2\). The results given in Tables I and II and Figs. 2–4 suggest that the influxes of glutamate and aspartate are consistent with this model. Further, according to the model, the increment in Na influx associated with a unit increment in amino acid influx (the coupling coefficient) is given by

\[
\Delta J_{Na}/J_A = [Na]_m/(K_2 + [Na]_m)
\]

For glutamate influx \((K_2 = 12 \text{ mM})\) in the presence of 120 mM Na, the predicted coupling coefficient is 0.91. The statistical uncertainty of \(\Delta J_{Na}\) in the present experiments is large because it represents the difference between two large numbers. Nevertheless, the observed coupling coefficient of 1.1 ± 0.4 does not differ significantly from the predicted value and is consistent with the model. In this respect it is pertinent to consider the extent to which the fact that glutamate is a monovalent anion places additional constraints on the coupling coefficient that are unnecessary for the case of neutral amino acids. The preservation of bulk electroneutrality requires that the net flow of glutamate across the brush border be accompanied by an equivalent net influx of cations, a net efflux of anions from the cell into the mucosal solution, or an equivalent combination of cation influx and anion efflux. It is important to stress that this constraint is concerned with net movements rather than with unidirectional fluxes. Thus there is no a priori need to require a coupling coefficient of unity for the unidirectional influx of glutamate across the brush
border, and no present need to modify the model. A definitive resolution of the manner by which electroneutrality is preserved would require a thorough accounting of the bidirectional flows of anions and cations across the brush border and an evaluation of the effect of glutamate influx on the electrical potential difference across this barrier.

The present results together with the results of detailed studies of glycine (12) influx and less detailed studies on the influxes of leucine, valine (10), phenylalanine (unpublished observations), and lysine (11) suggest that the model originally proposed for alanine influx may be generally applicable to

| TABLE III |
| KINETIC PARAMETERS FOR AMINO ACID INFUX* |
| Amino acid   | $K_1$ | $K_2$ |
| Glycine (12) | 125   | 25    |
| Alanine (10) | 70    | 17    |
| Valine (10)  | 32    | 23    |
| Leucine (10) | 29    | 24    |
| Phenylalanine† | 18 | 25 |
| Lysine (11)  | 35    | 56    |
| Glutamic acid | 90-100 | 12-13 |
| Aspartic acid | 75    | 9     |

* $K_1$ for glutamate is derived from the intercept of the lines shown in Fig. 3 (100 mM). The $K_1$ for aspartate is that consistent with the influx data in the absence of Na; as discussed above the uncertainty of this value is large, nevertheless it is unlikely to be much lower than 75 mM.† Unpublished observations.

amino acid influx across the brush border of rabbit small intestine. Values of $K_1$ and $K_2$ for the amino acids that have been investigated to date are given in Table III.

The Effect of Amino Acid Charge on the Affinity of the Binary Complex for Na

Recent studies on the effect of pH on alanine influx have suggested that fixed anionic groups having a pK_a between 3 and 4 are involved in the binding of Na to the binary complex, $XA$ (24, 25). Protonation of these groups markedly inhibits the stimulatory effect of Na on alanine influx and kinetic data are consistent with a competitive interaction between Na and H for these anionic groups. These observations suggest that the affinity of the binary complex for Na may be explicable in terms of the anionic field strength hypothesis developed by Eisenman and his collaborators (26). Examination of the values of $K_2$ for neutral, cationic, and anionic amino acids suggests that the charge of the bound amino acid also influences the affinity of the binary complex for
Na. The values of $K_2$ for five neutral amino acids do not differ significantly (Table III). On the other hand, $K_2$ for lysine is approximately twice that of the neutral amino acids and the values of $K_2$ for anionic amino acids do not differ from each other but are approximately one-half those of the neutral amino acids. Thus, the binding of lysine to its site produces a binary complex that has relatively low affinity for Na whereas the binding of aspartate or glutamate to their sites yields binary complexes with relatively high affinities for Na. These differences in $K_2$ are consistent with previous observations on the degree to which the influxes of different amino acids are dependent upon Na (25, 27). The removal of Na from the mucosal solution has the largest effect on the influxes of anionic amino acids, the smallest effect on the influxes of lysine and arginine, and an intermediate effect on the influxes of five neutral amino acids. Further, within any group the fractional inhibitions resulting from removal of Na are very similar. According to the model, the ratio of $K_t$ at $[Na]_m = 0$ to $K_t$ at $[Na]_m = 140 \text{ mM}$ is

$$\frac{K_t(0)}{K_t(140)} = \frac{(K_2 + 140)}{K_2}$$

Thus, as $K_2$ increases, the effect of Na on $K_t$ decreases. For neutral amino acids, the average $K_2$ is 23 mM so that $K_t(0)/K_t(140) = 7$; for lysine this ratio is 3.5 and for the anionic amino acids it is 15. It should be noted that although $K_t(0) = K_1$, the decrease in $K_t$ resulting from the presence of Na is dependent only on $K_2$.

The relation between the standard free energy change and equilibrium constants ($\Delta F^o = RT \ln K$) indicates that the binary complex involving lysine has an affinity for Na that is smaller by approximately 400 cal/mole than the binary complex involving a neutral amino acid. On the other hand, the binary complex with an anionic amino acid has an affinity for Na larger by about 400 cal/mole than the complex with a neutral amino acid. One explanation for this behavior would involve a coulombic interaction between the charged group of the amino acid and the anionic field responsible for the binding of Na. That is, we might assume that the Na binding sites involved in the transport of neutral, anionic, and cationic amino acids are identical in structure and that their different affinities for Na are entirely attributable to the net charge of the amino acid. Alternatively, we must keep in mind the possibility that the Na sites involved in transport of the three classes of amino acids are structurally different as an explanation of the differing affinities for Na. If the former possibility (coulombic interaction) is correct, these data may provide some insight into the distance between the amino acid binding site and the Na binding site. The potential energy of an ion-ion interaction is given by (28):

$$F = 332 \frac{z_1 z_2}{Dd}$$
where \( z_1 \) and \( z_2 \) are the valences of the two ions, \( D \) is the dielectric constant, and \( d \) is the distance between the ions. When \( d \) is expressed in Å units, \( F \) has units of kcal/mole. Assuming that this interaction is influenced predominantly by the bulk dielectric constant of water (i.e., that it is truly interfacial), the relation between dielectric constant and interionic distance may be approximated by the equation (28):

\[
D = 6d - 7
\]

Thus,

\[
F = 332 \frac{z_1 z_2}{d(6d - 7)}
\]

For \( F = 400 \) cal/mole, the distance between the charged end group of lysine, glutamate, or aspartate and the Na site is \( \sim 12 \) Å assuming that, to a first approximation, the charged amino acid may be treated as a monopole. Thus a relatively short separation between the Na binding site and the amino acid binding site appears sufficient to account for the observed interaction. This separation is certainly consistent with the notion implicit in our model that the binding sites for Na and the amino acid are located on the same macromolecule.

The data in Table III also suggest that cationic amino acids form more stable binary complexes than anionic amino acids (\( K_1 \) is smaller for lysine than for glutamate and aspartate). Again, this difference could be due to structural differences in the transport sites for the two types of compounds but it could also be due, in part, to the charge on the side chain of the amino acids. This notion would be consistent with the hypothesis that a negatively charged group (or groups) is involved in conformational changes that confer stability on the amino acid–Na–membrane complex and that the function of Na is to promote the stable conformation through electrostatic interaction with these groups. The binding of a cationic amino acid could, through interaction with these anionic groups, mimic the action of Na and bring about stabilizing conformational changes and, at the same time, reduce the affinity for the subsequent binding of Na. Conversely, the binding of an anionic amino acid could, through electrostatic interaction with these anionic groups, result in an unstable binary complex with a high affinity for the subsequent binding of Na. This inverse relation between \( K_1 \) and \( K_2 \) for anionic and cationic amino acids was suggested several years ago by the observation that the influxes of lysine and arginine are relatively high in the absence of Na and only moderately stimulated by the presence of Na. Conversely, the influxes of aspartic and glutamic acids are extremely low in the absence of Na and are increased severalfold by the presence of 140 mM Na (27).

This, admittedly speculative, hypothesis can be visualized as illustrated in Fig. 5a. This mechanistic illustration is based on the "flexible active site"
hypothesis suggested by Koshland (29) to account for enzyme activation and regulation. In essence the sequence depicted consists of (A) the binding of an amino acid which results in (B) conformational changes that permit the sub-

sequent binding of Na (C). This sequence is demanded by our kinetic data. The interaction of Na with the anionic groups confers stability upon the entire complex. This interpretation is consistent with the effect of Na on the $K_t$ of the influx process and, as noted above, anionic groups having a pK of 3–4 have been directly implicated in the mechanism by which Na stimulates amino
acid influx. The essential feature suggested by the data in Table III is that a charged amino acid can interact with the anionic groups either to favor (cationic amino acid) or oppose (anionic amino acids) a stable binary con-
formation as illustrated in Fig. 5b. Thus, the binding of a cation partially fulfills the role of Na and at the same time reduces the affinity for the subsequent binding of Na whereas the opposite is true if an anionic amino acid is bound. It should be stressed that the essential elements of this hypothesis do not rest with the details of the illustration but with the interactions it depicts.

We must stress again that current evidence makes it very unlikely that all classes of amino acids are handled by the same transport system in intestine. In fact, there is reasonable kinetic evidence suggesting at least four systems for (a) neutral, (b) basic and (c) acidic amino acids, and (d) imino acids (30). The identification of distinct genetic defects that result in selective malabsorption of neutral or basic amino acids in man (31) supports these deductions. The degree to which the specificities of these systems overlap is not clear and further careful studies on this point are necessary. However, the data available on rabbit small intestine indicate that systems a-c have very similar kinetic properties so that considerations of possible specific effects of amino acid charge per se on these properties seem reasonable. Further studies may make possible a distinction between such effects and ones due to specific structural differences among the transport sites themselves.

Several observations on other cell systems are consistent with this line of thought. Fox et al. (32) have shown that lysine accumulation by rat kidney cortex slices is not as markedly affected by removal of Na from the incubation medium as is the accumulation of neutral amino acids. These authors have stated: “The ability of the cationic amino acid lysine to be actively accumu-
lated by the kidney cortex slice in the absence of Na in the medium raised the possibility that lysine may satisfy for itself the transport requirement for a positively charged moiety, as Na satisfies the requirement for the neutral amino acids.” Further, lysine transport appears to be unaffected by Na in toad bladder epithelial cells (33) and human leukocytes (34), and lysine influx into Ehrlich ascites cells (35) and rabbit reticulocytes (36) is only slightly, if at all, influenced by Na. Recently, Christensen and coworkers (37-39) have demonstrated that neutral amino acids can inhibit cationic amino acid uptake “provided Na or another suitable cation is present to take the position normally occupied by the distal amino group of the diamino acid.” One important difference between our findings and those reported by Christensen concerns the extent to which the terminal amino group of lysine can substitute for Na. In the Ehrlich cell and rabbit reticulocyte, lysine influx appears to be entirely independent of Na. Thus the notion that the terminal amino group of lysine physically occupies the position that is assumed by Na in the presence of certain neutral amino acids is reasonable. In rabbit ileum, lysine influx is
significantly enhanced by Na (11). Thus, although the terminal amino group appears partially to fulfill the function of Na it cannot occupy the Na site when Na is present. It is possible that the ε-NH$_3^+$ occupies the Na site when Na is absent but is displaced in the presence of Na. However, the interpretation that the ε-NH$_3^+$ and Na occupy separate sites and that the former exerts an electrostatic influence on the latter is certainly more direct.

Finally, we should stress that although the mechanism illustrated by Fig. 5 is consistent with all our data, it is not offered as a final explanation but rather as a working hypothesis that suggests directions for further investigation. Studies employing charged amino acid analogues and an investigation of the effects of pH on the kinetics of cationic and anionic amino acid influx will constitute important tests of this hypothesis.

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