

# The Sodium-Alanine Interaction in Rabbit Ileum

## *Effect of sodium on alanine fluxes*

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**ABSTRACT** The model of the interaction between Na and alanine at the mucosal border of rabbit ileum has been tested further by examining the efflux of alanine from the cells toward the mucosal solution. Alanine efflux shows a tendency toward saturation as cellular alanine concentration increases and is influenced by cellular Na concentration. A decrease in cell Na concentration causes an increase in the apparent Michaelis constant with little change in maximal efflux rate. Studies on strips of mucosa treated with ouabain or cyanide showed that the direction of net alanine transfer between the cells and the medium is determined by the direction of the Na concentration difference. The cells extrude alanine against a concentration difference when cell  $[Na]$  exceeds medium  $[Na]$  and accumulate alanine when cell  $[Na]$  is less than medium  $[Na]$ . The observations are consistent with the model previously suggested involving a transport site that combines with and translocates both Na and alanine, and with the concept that the Na concentration difference between mucosal solution and cytoplasm provides at least part of the energy for active transport of alanine.

In a series of earlier studies, certain aspects of the interaction between Na and alanine transport in rabbit ileum (1-4) were examined in some detail. Those studies dealt mainly with the unidirectional influxes of Na and alanine across the brush border from the mucosal solution into the cells. They led to a model of the transport system involving a site that is capable of combining with both Na and alanine, and that can result in the translocation of both solutes across the brush border membrane. The influx of alanine via this mechanism is not markedly affected by metabolic inhibitors (4), suggesting that a direct link between metabolic energy and the transport system may not be involved. Further, alanine influx was not appreciably altered by ouabain, suggesting that the Na dependence of the process is not mediated by a Na-sensitive ATPase that is inhibited by ouabain. We felt that these ob-

servations were at least consistent with a mechanism for active transport of amino acid similar to the "Na gradient hypothesis" suggested by Crane (5) to account for sugar transport by intestine. According to this hypothesis, the energy required to transport the amino acid into the cell across the mucosal border against a concentration difference is provided by the Na concentration difference between the cytoplasm and the mucosal solution. This concentration difference is thought to be maintained by active extrusion of Na from the serosal side of the cell (6, 7). However, the previous experiments dealt only with the influx process and cannot, therefore, be considered to prove the Na gradient hypothesis even if they are consistent with it. Information on the process of efflux across the brush border from the cell to the mucosal solution is also required to test the hypothesis. The model proposed to explain alanine influx across the brush border can be used to make certain predictions regarding the efflux process if the Na gradient hypothesis is correct. The present experiments represent an attempt to test some of these predictions.

#### METHODS

Several types of experiments were carried out to measure unidirectional efflux of alanine from the cells to the mucosal solution and net efflux from the cells under certain conditions. In all studies, the distal ileum of New Zealand white rabbits was used. The animals were sacrificed by an intravenous injection of sodium pentobarbital, the abdomen was opened rapidly, and the appropriate section of intestine was resected. The tissue was washed free of intestinal contents and placed in Ringer solution. Subsequent treatment varied with the nature of the experiment as described below. The normal Ringer solution contained 140 mM NaCl, 10 mM KHCO<sub>3</sub>, 1.2 mM K<sub>2</sub>HPO<sub>4</sub>, 0.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgCl<sub>2</sub>, and 1.2 mM CaCl<sub>2</sub>. The pH was maintained at 7.2 by bubbling with 95% O<sub>2</sub>-5% CO<sub>2</sub>. In some experiments, all the NaCl in the Ringer solution was replaced by choline chloride or KCl; in others ouabain was added at a concentration of 10<sup>-4</sup> M. L-Alanine and <sup>14</sup>C-L-alanine (uniformly labeled, New England Nuclear Corp., Boston, Mass.) were added from stock solutions. All experiments were carried out in a warm room at 37°C.

##### *Direct Measurement of Alanine Efflux*

Alanine efflux was determined directly by loading the cells with <sup>14</sup>C-labeled alanine and following the appearance of radioactivity in unlabeled mucosal solution. The cells were loaded by incubating the whole tissue in a flask for 60 min in solution containing alanine-<sup>14</sup>C and the desired concentration of unlabeled alanine. At the end of the loading period, the tissue was dipped briefly into isotonic mannitol solution to remove excess surface activity and mounted as a flat sheet between Lucite half-chambers. Unlabeled solution was placed in the mucosal chamber (2.3 ml) and left for 1 min. The solution was removed by suction directly into a counting vial and the chamber was refilled with fresh solution. Bray's solution (8) was added to the vials and radioactivity determined in a liquid scintillation spectrometer. In most experiments, washout of <sup>14</sup>C was followed for eight to ten 1 min periods with bathing solution on the

mucosal side of the tissue only. Unless otherwise noted, the bathing solution contained alanine at the same concentration as the loading solution so that cellular alanine concentration may be considered constant during the washout period.

At the end of the experiment, the tissue was removed from the chamber and the exposed portion was cut out. The mucosa was separated from the muscle layer by scraping with a glass slide as previously described (1). The mucosa was then extracted for at least 2 hr in 0.1 N HNO<sub>3</sub> and the extract counted in order to estimate the amount of radioactivity remaining in the mucosa. The residue from the extraction was dried at 80°C for 12 hr to determine the dry weight of the mucosa. Since the tissue did not fragment during the extraction, this procedure provides an adequate estimate of dry weight. Cellular concentration of alanine was estimated as described below.

Previous studies (1) indicated that cell water content increased as a result of uptake of alanine by the cells. In order to take account of this effect, cellular alanine concentration,  $[A]_c$ , was estimated from the following expression:

$$[A]_c = \frac{S_c}{V_c^0 + 0.0033S_c} \quad (1)$$

in which  $S_c$  is alanine content (micromoles per milligram dry weight) and  $V_c^0$  is cell volume (milliliters per milligram dry weight) in the absence of amino acid. (The method for determining  $S_c$  for washout experiments is described below.) The factor 0.0033 represents the change in cell volume per micromole of solute required to maintain isotonicity. As observed previously (1), the value of  $V_c^0$  depends on experimental conditions. For the present series,  $V_c^0$  was taken as 5.0  $\mu\text{l}/\text{mg}$  d.w. for incubation in normal Ringer, 5.9  $\mu\text{l}/\text{mg}$  for ouabain-treated tissue bathed in 140 mM Na, and 4.2  $\mu\text{l}/\text{mg}$  for choline medium. In order to evaluate the reliability of this estimate of  $[A]_c$ , a series of experiments was carried out on strips of mucosa in which  $[A]_c$  was estimated by equation 1 and also by conventional methods using inulin-<sup>3</sup>H as an extracellular space marker (see below, also reference 1). For tissues incubated in choline medium, equation 1 gave  $5.0 \pm 0.3$  mM for  $[A]_c$  while the direct estimate gave  $5.3 \pm 0.2$ . For tissues incubated in Na medium plus  $10^{-4}$ M ouabain the values for  $[A]_c$  were  $5.6 \pm 0.3$  for equation 1 and  $5.7 \pm 0.5$  for the direct estimate.

#### *Indirect Measurement of Alanine Efflux*

The method described by Curran, Herrera, and Flanigan (9) for Na movements in frog skin was used to obtain indirect estimates of alanine efflux. This method depends on observing the time course of the approach to a steady-state unidirectional flux after adding tracer to one of the bathing solutions. The tissue was mounted as a flat sheet separating mucosal and serosal bathing solutions in a chamber similar to that described by Schultz and Zalusky (6). At zero time, alanine-<sup>14</sup>C and unlabeled alanine were added to the mucosal solution and sampling of the serosal solution was begun immediately. 10 serosal samples were taken at intervals of 2–5 min depending on the nature of the experiment. Three additional samples were taken from the serosal solution at 10 min intervals to determine the steady-state mucosa-to-

serosa flux, and samples were taken from the mucosal solution to determine the specific activity. At the end of the experiment, the tissue was removed from the chamber and the mucosa scraped off and extracted as described above. Some experiments were carried out using the whole intestinal wall. In others, the method described by Parsons and Paterson (10) was used to remove most of the muscle before mounting the tissue in the chamber.

The details of the analysis of this type of experiment are given by Curran et al. (9). Briefly, we assume that the tissue can be described by a three-compartment series system consisting of the mucosal solution (compartment  $m$ ), the mucosal cells (compartment  $c$ ), and the serosal solution (compartment  $s$ ). When tracer is initially added to the mucosal side, the rate of appearance in the serosal solution ( $\dot{P}_s$ ) can be expressed as follows:

$$\ln \left[ 1 - \frac{\dot{P}_s}{\dot{P}_{s\infty}} \right] = -(k_{cs} + k_{cm})t \quad (2)$$

in which  $\dot{P}_{s\infty}$  is the steady-state rate of tracer appearance and  $k_{cs}$  and  $k_{cm}$  are rate coefficients for alanine flux from cell to serosa and cell to mucosa, respectively.<sup>1</sup> Also,

$$\dot{P}_{s\infty} = k_{cs} P_{c\infty} \quad (3)$$

in which  $P_{c\infty}$  is the steady-state amount of tracer in the cell compartment. From equations 2 and 3,  $k_{cm}$  and  $k_{cs}$  can then be evaluated separately ( $P_{c\infty}$  is determined by extraction of the mucosa at the end of the experiment as described above). Since there is no alanine in the serosal solution, the pool of alanine in the mucosa ( $S_c$ ) at the end of the experiment is given by

$$S_c = P_{c\infty}/p_m^*$$

where  $p_m^*$  is the specific activity (counts per minute per micromole) in the mucosal solution. The alanine fluxes from cell to mucosa ( $J_{cm}$ ) and cell to serosa ( $J_{cs}$ ) are given by  $k_{cm}S_c$  and  $k_{cs}S_c$ , respectively. Finally, since the measured mucosa-to-serosa flux ( $J_{ms}$ ) is also equal to the net flux under these conditions,

$$J_{ms} = J_{mc} - J_{cm} = J_{mc} - k_{cm} S_c$$

from which  $J_{mc}$  can be estimated.

#### *Measurement of Net Transfer in Strips of Mucosa*

These experiments were designed to examine the effect of cellular and extracellular Na concentrations on the direction of net transfer of alanine between the epithelial

<sup>1</sup> Derivation of equation 2 involves the assumption that the  $k$ 's are constant. As indicated by results presented below, this is not strictly true for  $k_{cm}$  because the cellular alanine concentration is changing with time. However, at the relatively low concentrations employed,  $k_{cm}$  does not change appreciably and the assumption appears reasonable.

cells and the surrounding medium. The method used was essentially that described by Schultz, Fuisz, and Curran (1). Strips of mucosa, obtained by scraping with a glass slide, were incubated in various solutions and changes in the intracellular concentration of alanine were determined. In one type of experiment, tissue was incubated for 30 min in normal Ringer solution containing  $10^{-4}$ M ouabain in order to elevate the cell Na concentration (1).  $^{14}$ C-labeled alanine was then added and incubation continued for an additional 30 min to allow alanine to enter the cells. Some of the pieces of tissue were then transferred to a flask containing Na-free Ringer (either choline or K substituted for Na) and  $^{14}$ C-labeled alanine at the same specific activity as the original solution. Both solutions also contained identical concentrations of inulin- $^3$ H for estimation of extracellular space. At various time intervals, samples of tissue were removed from both flasks, blotted, weighed, and extracted in 0.1 N  $\text{HNO}_3$ . The extracts and samples of the incubation medium were counted in the liquid scintillation spectrometer and the dry weight of the tissue determined as described above. These data were used to estimate extracellular space and intracellular alanine concentration as previously discussed (1). In another series of experiments, the initial incubation medium was Na-free choline medium containing 2 mM KCN and the second medium was normal Ringer solution, also containing 2 mM CN.

## RESULTS

### *Direct Measurements of Efflux*

The results of a typical experiment on alanine efflux are shown in Fig. 1 in which log of the fraction of activity remaining in the mucosa is plotted against time of exposure to unlabeled solution. The curve can be described adequately in terms of two exponentials with half-times of 0.5 min and 22.4 min. Thus, the washout curve appears to be consistent with a system involving two compartments, a cellular compartment and a relatively small extracellular space in series with it (Fig. 2). The extracellular space is most likely an unstirred layer at the surface including the space between the microvilli of the brush border (see Passow (11) for a discussion of some aspects of the influence of this space). In such a system, the slopes and intercepts of the exponential curves are complex functions of rate coefficients and pool sizes and cannot be arbitrarily assigned to represent the properties of particular compartments (12). The individual rate coefficients and pool sizes in such a system can be estimated under certain conditions by an analysis similar to that discussed by Zadunaisky and Curran (13). However, one of the assumptions involved in that treatment is that there be no net flux between the two compartments. This condition is approximately met when the tissue is bathed in Na-free solution or treated with ouabain (14) but it is clearly not met under normal conditions (140 mM Na). Consequently, we have utilized a somewhat different approach to evaluate fluxes for these experiments.

First, the curves are fitted to two exponentials by a method of least squares

and the intercept of the slow component is taken as an estimate of the cellular pool of alanine- $^{14}\text{C}$ . As discussed by Huxley (15) this estimate will be in error, but under the present conditions when the fast exponent is 20–50 times larger than the slow, the error amounts to an overestimate of only 2–7%. In addition, in one experiment the extract of the mucosa taken at the end of a washout was analyzed on an amino acid analyzer. The cellular pool of alanine estimated from the intercept and the specific activity of the loading

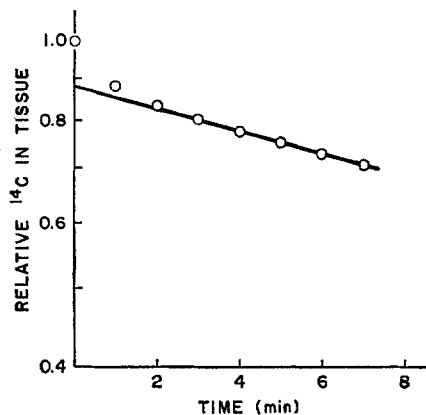


FIGURE 1. Washout of alanine- $^{14}\text{C}$  across the mucosal border of rabbit ileum. The bathing solution was normal Ringer with  $10^{-4}\text{ M}$  ouabain and 5 mM alanine.

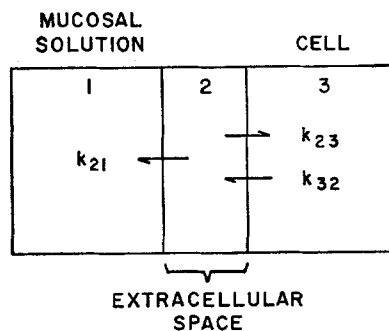


FIGURE 2. Three-compartment model for washout experiments. The  $k$ 's are rate coefficients.

solution was 2.36  $\mu\text{moles}$  and the directly measured alanine content was 2.21  $\mu\text{moles}$ . Thus, the intercept represents a reasonable estimate of the pool.

Second, we have made use of a simplification in the analysis arising if the coefficient  $k_{23}$  becomes negligible with respect to both  $k_{21}$  and  $k_{32}$ . Under these conditions, the slower exponential reduces to  $\exp[-k_{32}t]$ . Since the influx into the cells is a saturating process (3), the coefficient  $k_{23}$  must decrease as the external concentration of alanine increases and at some point should become negligible with respect to the other coefficients. In order to test this possibility, experiments were carried out in which the external alanine concentration was raised to 40 mM during the washout period. The results of two such experiments are shown in Fig. 3. In the ouabain-poisoned tissue, the

efflux of  $^{14}\text{C}$  increased, an effect consistent with a reduction in  $k_{23}$  or, in simpler terms, a reduction in the tendency to a recapture of  $^{14}\text{C}$  by the cells via the influx process.<sup>2</sup> In Na-free solution, there was virtually no effect of increasing external alanine concentration indicating that under these conditions  $k_{23}$  is already negligible. Since the influxes per unit concentration in ouabain-treated tissue at 40 mM alanine and in Na-free solution at 5 mM are approximately equal (3), 40 mM alanine should be sufficient to make  $k_{23}$  negligible. A series of such experiments carried out at different initial concentrations of alanine made possible evaluation of a correction factor at each concentration that was used to calculate a "true"  $k_{32}$ . The correction was simply the ratio of the flux immediately after elevation of alanine concentration to 40 mM to the flux immediately before. The fluxes were estimated

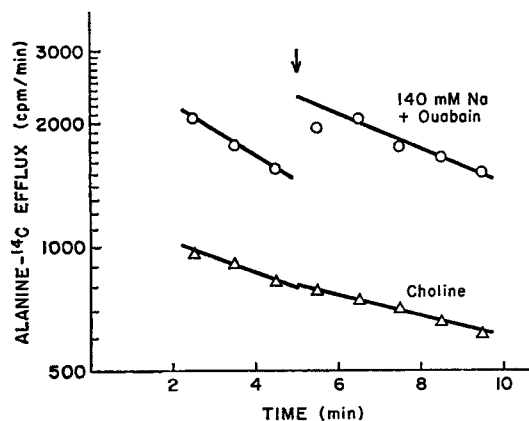


FIGURE 3. Effect of increasing alanine concentration in the mucosal solution on alanine efflux across the brush border. Washout was begun with solutions containing 5 mM alanine. At the arrow, alanine concentration was raised to 40 mM.

by extrapolating lines as shown in Fig. 3. Identical factors were obtained for ouabain-treated tissues and for tissues bathed in normal Na medium. The alanine efflux was then calculated as the product of the pool size and the corrected value of  $k_{32}$ .

Two experiments were carried out at 5 mM alanine in ouabain-poisoned tissue to test the validity of this estimate. Washout samples were taken at 30 sec intervals to provide a better estimate of the fast exponential component and the "true value" of  $k_{32}$  was estimated by a procedure similar to that described by Zadunaisky and Curran (13). The value of  $k_{32}$  was found to be 1.4 times the slope of the slow component, in good agreement with the correction factor of 1.5 for 5 mM alanine determined from experiments such as that shown in Fig. 3.

The results of these experiments are summarized in Fig. 4 in which alanine

<sup>2</sup> The increase in efflux shown in Fig. 3 could also be due to a "transconcentration effect" mediated by the transport system. However, such an effect is unlikely in the present case because there are no transconcentration effects on alanine influx (3); influx is not affected by alanine in the cell.

efflux is shown as a function of cellular alanine concentration under three conditions, normal tissue, Na-loaded tissue (ouabain), and low Na tissue (choline). In normal and Na-loaded tissues, efflux,  $J_A^e$ , shows a tendency

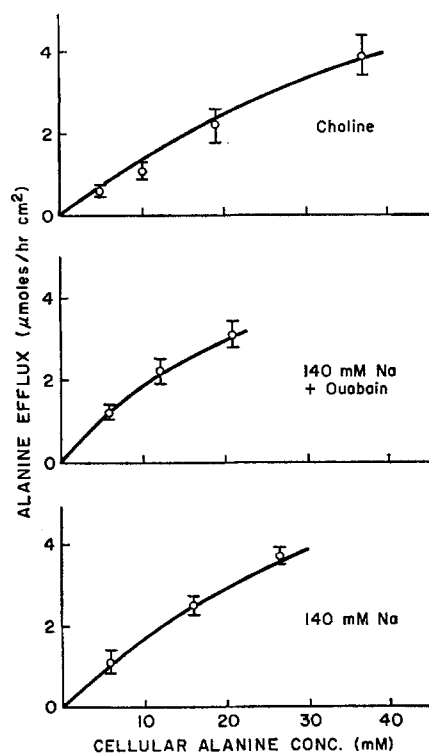


FIGURE 4. Relation between alanine efflux and cellular alanine concentration. Each point is the average of at least five determinations and the bars indicate  $\pm 1$  SEM. The lines are drawn from equation 4 using  $K_t$  and  $J_A^{e\max}$  from Table I.

toward saturation as concentration increases and can be described by the expression

$$J_A^e = \frac{J_A^{e\max}[A]_c}{K_t + [A]_c} \quad (4)$$

in which  $J_A^{e\max}$  is the maximal flux and  $K_t$  is the "apparent Michaelis constant." The values of  $J_A^{e\max}$  and  $K_t$  were determined by plotting  $[A]_c/J_A^e$  vs.  $[A]_c$  as shown in Fig. 5 and are given in Table I. The lines in Fig. 4 are determined by equation 4 using these values of maximal flux and  $K_t$ . In low Na tissues, the points do not differ significantly from a straight line. However, they can also be fitted within the limits of error by equation 4 using the values of  $J_A^{e\max}$  and  $K_t$  given in Table I.

At each level of cellular alanine concentration, efflux from Na-loaded tissue



(ouabain)<sup>3</sup> is greater than from low Na tissues (choline) indicating that efflux is influenced by cellular Na concentration. The data in Table I indicate that the effect of cell Na on alanine efflux is due primarily to an increase in  $K_t$  with decreasing Na concentration. The lower value of  $J_A^{e\max}$  in ouabain-treated tissue is perhaps not unexpected. Previous studies (4) showed that under the same conditions influx was reduced by about 40% from influx into

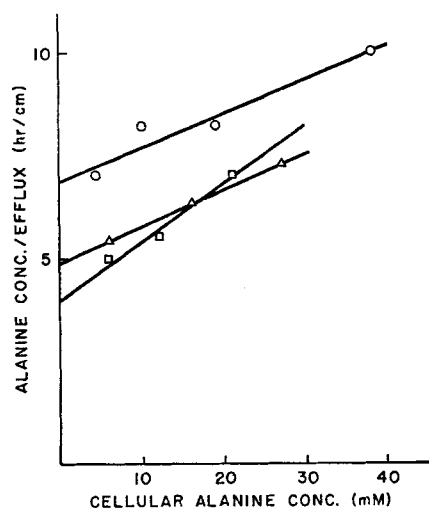


FIGURE 5. Relation between alanine concentration and concentration divided by efflux for 140 mM Na ( $\Delta$ ), 140 mM Na plus ouabain ( $\square$ ), and choline ( $\circ$ ). Data from Fig. 4.

TABLE I  
PROPERTIES OF THE ALANINE EFFLUX SYSTEM

Incubation medium	$K_t$	$J_A^{e\max}$
	mM	$\mu\text{moles/hr cm}^2$
Na medium	55	11.0
Na medium + ouabain	29	7.3
Na-free	80	12.0

normal tissue. The effect is apparently due to cell swelling and a similar effect might be expected for the efflux process.

#### *Indirect Measurement of Efflux*

The time course for the approach of transmural tracer flux to a steady-state value is shown for two conditions in Fig. 6. As indicated by equation 2, the plots of  $\ln [1 - \dot{P}_s/P_{s\infty}]$  vs. time yield straight lines with a slope  $-\lambda (=k_{cm} +$

<sup>3</sup> The effect is not dependent on the use of ouabain to attain a high cell Na concentration. Two experiments using 140 mM Na plus 2 mM KCN as incubation medium gave results identical with those obtained using ouabain.

$k_{es}$ ). As explained in the Methods section, this slope together with the steady-state transmural flux and the final content of radioactivity in the mucosa can be utilized to calculate fluxes across the mucosal and serosal borders of the epithelial cells. The results of a series of experiments under three conditions (normal Ringer, Na-free Ringer, and normal Ringer plus  $10^{-4}$  M ouabain) are summarized in Table IIA. The values obtained for efflux ( $J_{cm}$ ) in tissue with muscle removed are in reasonable agreement with those obtained by the

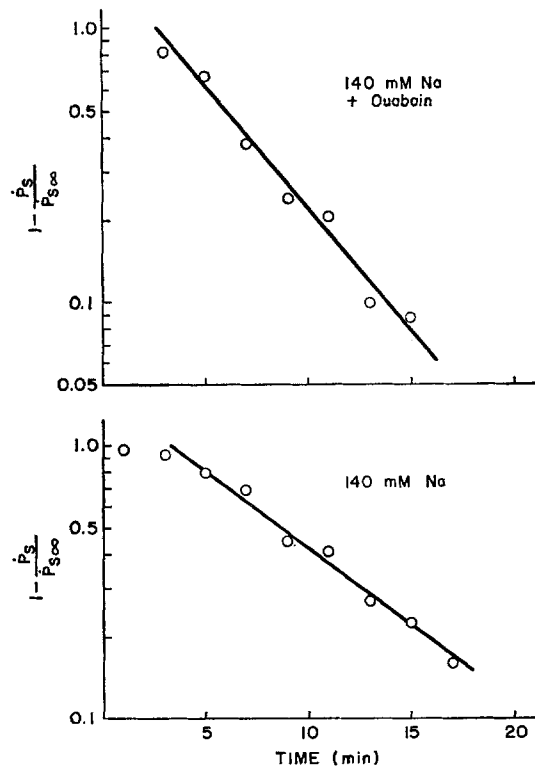


FIGURE 6. Approach of tracer flux to steady state. The two experiments were carried out on tissue from one animal. Alanine concentration in the mucosal solution was 5 mM.

direct method considered in the previous section. Again, the efflux observed in ouabain-poisoned tissue (high cell Na) is two to three times higher than the efflux observed in choline Ringer (low cell Na); the calculated cell alanine concentrations are approximately equal in the two conditions. The experiments in normal Ringer solution under the same conditions of 5 mM alanine in the external solution yield approximately the same efflux as the ouabain-treated tissues although the cellular alanine concentration was approximately four times higher. Thus, these experiments also indicate that alanine efflux from the cells is influenced by the cellular Na concentration. Included in Table IIA are directly measured values for alanine influx obtained in earlier experiments under similar conditions (4). The agreement between those direct

measurements and the present indirect estimates is reasonably good suggesting that the indirect method provides valid estimates of fluxes.

A few similar experiments were carried out on tissue with the muscle layer intact and rather different results were obtained. The slopes of the lines similar to those shown in Fig. 6 were appreciably smaller. As indicated in Table IIB the calculated unidirectional fluxes were considerably smaller than those obtained in tissues from which muscle had been removed. The estimated cellular alanine concentrations were higher, particularly for the case of normal Ringer solution. Although the relative values of the calculated efflux follow the pattern seen in other experiments for the three conditions, comparison of the flux values in Table IIA and IIB suggests that these studies with intact muscle

TABLE II  
UNIDIRECTIONAL ALANINE FLUXES BY THE INDIRECT METHOD

Bathing solution	[A] <sub>c</sub>	<i>J</i> <sub>cm</sub>	<i>J</i> <sub>mc</sub>
	mM		μmoles/hr cm <sup>2</sup>
A. Muscle removed			
Na medium	13.9	1.82 ± 0.68	3.40 ± 0.80 (2.86)*
Na medium + ouabain	3.2	1.60 ± 0.50	1.80 ± 0.51 (1.69)*
Choline	3.0	0.60 ± 0.30	0.78 ± 0.40 (0.60)*
B. Muscle intact			
Na medium	31.9	0.55	2.11
Na medium + ouabain	5.5	0.81	0.94
Choline	3.2	0.21	0.28

\* Values in parentheses are directly measured fluxes from mucosal solution to cell under the indicated conditions. They are taken from Tables II and IV of reference 4. Alanine concentration in the mucosal solution was 5 mM in all experiments.

produce incorrect flux values. Clearly the muscle layer provides a significant additional barrier to alanine movement that alters the rate of approach to steady-state flux. However, the steady-state flux from mucosa to serosa does not appear to be significantly altered by removal of the muscle layer. For example, for tissues bathed in normal Ringer with 5 mM alanine, the average mucosa-to-serosa flux was 1.52 μmoles/hr cm<sup>2</sup> with the muscle removed and 1.56 μmoles/hr cm<sup>2</sup> with intact muscle.

#### *Net Transfer of Alanine*

Strips of mucosa were used to examine the influence of the Na concentration difference between cells and medium on the direction of net alanine transfer. Two conditions were studied by preincubating mucosal strips in one solution and transferring them to another solution as described under Methods. These experiments are complicated by two factors: First, in the poisoned tissues

used, the initial difference in Na concentration is dissipated rather rapidly (15–30 min). Second, transferring the tissue from one type of bathing solution to another can lead to substantial changes in cell volume that could produce misleading results. We have overcome the first problem by taking samples at short time intervals and the second by measuring cell water content on each piece of tissue.

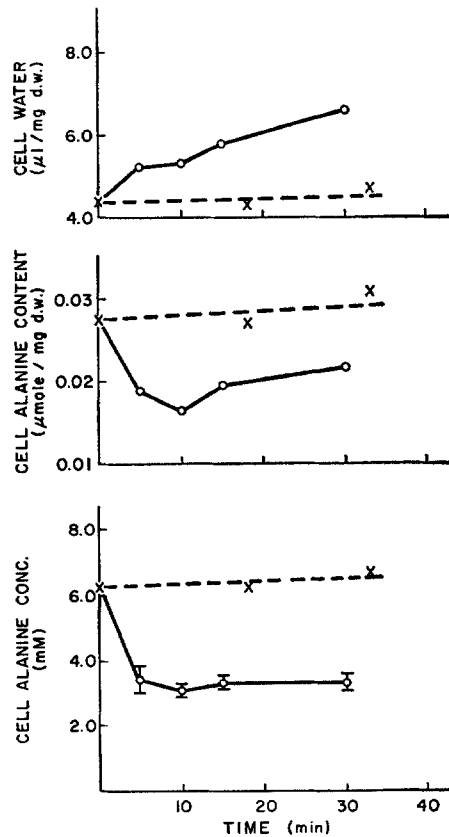


FIGURE 7. Extrusion of alanine from mucosal cells in the presence of a Na concentration difference. Mucosal strips were incubated in 140 mM Na plus ouabain and 5 mM alanine. At zero time they were transferred to Na-free KCl medium containing 5 mM alanine. Control tissues (X) remained in the initial incubation solution.

The results of experiments showing extrusion of alanine from the cell against a concentration difference are summarized in Fig. 7. The tissues were loaded with  $^{14}\text{C}$ -labeled alanine (5 mM) in 140 mM Na plus  $10^{-4}$  M ouabain and transferred to Na-free solution containing alanine at the same concentration and specific activity. When the mucosal strips were transferred to Na-free KCl medium, alanine was extruded from the cells and cellular alanine concentration fell to approximately one-half the level in control tissues and well below the medium concentration (5 mM). The cells swelled after transfer to KCl but in the first 10 min, swelling can account for only 30% of the decrease in cellular alanine concentration. Consequently, when Na concentration inside the

cell is greater than that outside, alanine is extruded from the cell against a concentration difference. Similar results were obtained when Na-loaded tissues were transferred to Na-free choline medium. In this case, the decrease in cellular alanine concentration is less dramatic because the cells shrink. However, alanine is extruded and at 5 min the cellular concentration falls significantly ( $p < 0.01$ ) below the medium level. In six observations, the ratio of

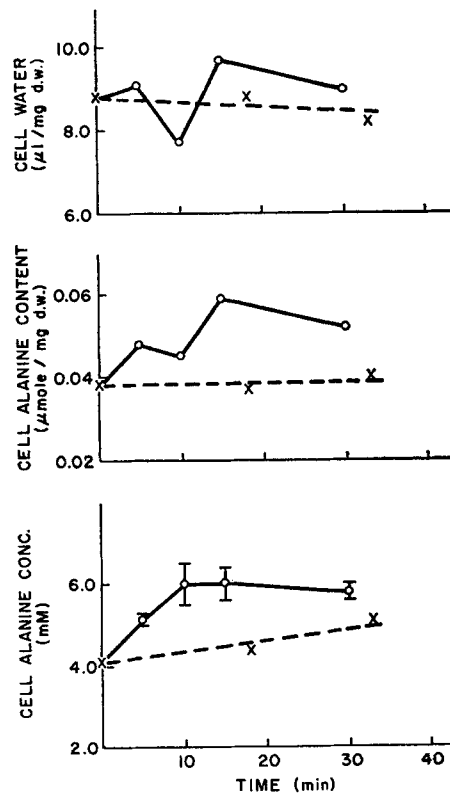


FIGURE 8. Uptake of alanine by CN-poisoned mucosal cells in the presence of a Na concentration difference. Mucosal strips were incubated in Na-free choline medium plus 2 mM KCN and 5 mM alanine. At zero time they were transferred to 140 mM Na plus 2 mM KCN and 4 mM alanine. Control tissues (x) remained in the initial incubation medium.

cellular alanine concentration in test tissues to that in control tissues at 5 min was  $0.77 \pm 0.04$  and at 10 min the ratio was  $0.87 \pm 0.04$ .

The converse experiment is illustrated in Fig. 8. The tissues were loaded with  $^{14}\text{C}$ -labeled alanine in Na-free choline medium containing 2 mM KCN to inhibit metabolism and then transferred to 140 mM Na (plus 2 mM KCN). Cellular alanine concentration increased above the level in the external solution (4 mM); cell volume did not change appreciably and there was clearly a net uptake of alanine. Thus in the absence of metabolic energy alanine can be transported into the cells against a concentration difference if the external Na concentration is greater than the cellular Na concentration. This transfer is probably more impressive than it appears to be at first sight because the tissue

is clearly in poor condition after 60 min in cyanide; it is very fragile and fragments easily and the cells have swollen quite markedly.

#### DISCUSSION

These experiments were undertaken to examine certain aspects of the model proposed by Curran et al. (3) for alanine transport at the mucosal border of rabbit small intestine. In particular, we were interested in the question of whether, within the context of the model, the Na gradient hypothesis (4, 5) is correct. The data presented offer considerable support for this hypothesis and for the detailed model of the transport system.

##### *The Na Gradient Hypothesis*

According to this hypothesis the efflux of alanine from the cell to the mucosal solution should depend on the Na concentration in the cell. Such a dependence has been observed (Fig. 4 and Table II) using two entirely different methods to estimate alanine efflux across the mucosal membrane. For both approaches, the efflux from Na-depleted cells is lower than from Na-loaded cells (ouabain-treated) by a factor of 2-3. Efflux from normal cells which have an intermediate Na concentration tends to fall between these two extremes. The data in Fig. 4 indicate that effluxes from normal and Na-loaded cells do not differ significantly. However, this observation is somewhat misleading in view of the depression of approximately 40% in maximal efflux rate in ouabain-treated tissues (Table I). As mentioned above, a similar depression was observed in alanine influx into poisoned tissues and has tentatively been attributed to cell swelling (4). If this factor is taken into account, the data show a relatively clear dependence of alanine efflux on cellular Na concentration. Such dependence is an essential feature of the Na gradient hypothesis.

Further, the hypothesis postulates that the amino acid transport system is reversible and that the direction of net amino acid transfer is determined in part by the direction of the Na concentration difference between cytoplasm and mucosal solution. Experiments testing this feature of the hypothesis have yielded positive results. The data in Fig. 7 show that alanine can be extruded from the mucosal cells against a concentration difference if the cell Na concentration is greater than the external Na concentration. The data in Fig. 8 show the converse effect. Under normal conditions, treatment of mucosal strips with cyanide prevents cellular accumulation of alanine (4). However, under conditions in which external Na concentration was greater than cellular concentration, alanine could be made to enter the cells against a concentration difference. This observation is consistent with the Na gradient hypothesis and indicates that "active" uptake of alanine by the cells can occur in the absence of metabolism. The maximum concentration ratio (cell/medium) attained in these experiments was much smaller than the maximum observed in normal

tissue (1). However, the rapid dissipation of the Na concentration difference between cell and environment precludes examination of the maximum degree of alanine accumulation that can be reached for a given difference in Na concentration.

Although experiments on mucosal strips do not permit a distinction between transfer across the mucosal and serosal sides of the cell, the net alanine transfer observed in these experiments appears to be due primarily to the amino acid transfer mechanism located in the mucosal or brush border membrane. Both influx (1) and efflux (Fig. 4 and Table II) across this membrane depend on Na concentration in a manner that would provide an adequate explanation for the net changes. In addition, there is no evidence at present suggesting that alanine fluxes across the serosal border of the cell are Na-dependent. In fact the present experiments utilizing the indirect method for estimating fluxes provide an indication that alanine efflux from the cell to the serosal solution ( $J_{es}$ ) is independent of cell Na concentration. For the experiments summarized in Table II,  $J_{es}$  was  $0.22 \pm 0.02 \mu\text{mole/hr cm}^2$  for Na-loaded tissues (ouabain) and  $0.19 \pm 0.03 \mu\text{mole/hr cm}^2$  for low Na tissues (choline). The cellular amino acid concentrations were approximately equal in the two sets of experiments. Finally, the net extrusion of alanine shown in Fig. 7 is in reasonable agreement with the known unidirectional fluxes across the brush border membrane. In the initial 5 min period after transfer of the mucosal strips to KCl medium,  $0.0065 \mu\text{mole}$  of alanine was extruded from the cell per mg dry weight so that the initial rate of net extrusion was  $0.08 \mu\text{mole/hr mg d.w.}$  Unidirectional fluxes across the mucosal border can be converted to the same units since in the present experiments we have found an average mucosal dry weight of  $10 \text{ mg/cm}^2$ . From Fig. 4, alanine efflux from cells with high Na concentration and an alanine concentration of  $5 \text{ mM}$  would be  $0.12 \mu\text{mole/hr mg d.w.}$  From previous studies (2), alanine influx into cells bathed on the mucosal side by Na-free KCl medium would be  $0.05 \mu\text{mole/hr mg d.w.}$  at an external alanine concentration of  $5 \text{ mM}$ . Thus, on the basis of unidirectional fluxes across the brush border, a net alanine extrusion of  $0.07 \mu\text{mole/hr mg d.w.}$  would be expected for conditions in which the cells contain  $5 \text{ mM}$  alanine and  $140 \text{ mM}$  Na and the mucosal solution contains  $5 \text{ mM}$  alanine and no Na. This prediction is in good agreement with the observed rate of alanine extrusion from mucosal strips under similar conditions and lends support to the concept that the extrusion occurs primarily at the mucosal side of the cells. Similar arguments can be made regarding the experiments on alanine accumulation shown in Fig. 8. The observed net alanine uptake rate is  $0.12 \mu\text{mole/hr mg d.w.}$ ; the calculated value from the appropriate unidirectional influxes (efflux from Fig. 4, influx with  $140 \text{ mM}$  Na in the mucosal solution from Table II, reference 4) is  $0.11 \mu\text{mole/hr d.w.}$

These results are all consistent with the concept that at least part of the

energy required for transport of alanine against a concentration difference by mucosal cells is provided by the Na concentration difference. They also indicate that the alanine transport system is essentially reversible since the amino acid can be "actively" taken up or extruded by the cells under appropriate conditions. In this respect, the present results are similar to those obtained by Vidaver (16) and Eddy (17) for glycine transport by pigeon erythrocytes and Erlich ascites cells, respectively. In both these cases, the direction of net glycine transport was shown to depend on the direction of the Na concentration difference between the interior and exterior of the cells. Crane (18) has given a brief report of a similar experiment in which 6-deoxyglucose was shown to be extruded from villus cells of hamster intestine when cellular Na concentration exceeded that in the medium. The present results do not, however, show that the Na gradient is the sole source of energy for alanine transport. Proof of this for the mucosal cells would be extremely difficult because of the impossibility of maintaining artificial Na gradients for substantial periods of time in the presence of metabolic inhibitors. Thus, it is impossible at present to demonstrate that inhibited cells can transport alanine entirely normally if the normal Na gradient is maintained.

#### *Model for Alanine Transport*

The present results also provide additional support for and insight into the specific model of the alanine transport system proposed by Curran et al. (Fig. 4 of reference 3). In particular, they suggest that the transport system is reversible and does not involve a direct input of metabolic energy. If this prediction is correct, relations between alanine efflux and the cellular concentrations of alanine and Na should be the same as the relations between alanine influx and concentrations of alanine and Na in the mucosal solution (for convenience, the flux equations derived from the model are given in the Appendix). The data in Table I indicate that the relations between alanine efflux and cellular concentrations conform at least qualitatively to the predictions of the model. First, alanine efflux as a function of cellular alanine concentration can be described by Michaelis-Menten kinetics (equation 1). Second, a decrease in cell Na concentration results in an increase in the apparent Michaelis constant,  $K_t$ , with little change in maximal efflux (the probable cause of the decreased maximal efflux in ouabain-treated tissue has already been discussed). These are two of the important characteristics of the alanine influx process (3).

The results are also in reasonable quantitative agreement with the predictions of the model. If the system is reversible the maximal fluxes in the two directions across the brush border should be equal. The values of maximal efflux, 7.3–12.0  $\mu\text{moles/hr cm}^2$  fall within the range (6.1–13.7  $\mu\text{moles/hr cm}^2$ ) reported previously (3) for maximal influx. If the transport system is reversible



and symmetrical, the values of  $K_t$  for influx and efflux should be the same at the same Na concentrations. As indicated by Table III, this does not appear to be the case; at similar Na concentrations,  $K_t$  for influx is appreciably lower than for efflux. However, evaluation of the  $K_t$  for efflux is complicated by the presence of amino acids other than alanine in the cytoplasm. In the one case in which the extract of mucosal cells was subjected to amino acid analysis, significant amounts of several other neutral amino acids were found.<sup>4</sup> Since alanine efflux appears to be a mediated process, these amino acids should compete with alanine and the competitive effect will increase the estimated value of  $K_t$  for alanine. The extent of this effect cannot be determined accurately because sufficient information is not available. However, an estimate of the

TABLE III  
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[Na]	Influx $K_t$ *	Observed efflux $K_t$	Corrected efflux $K_t$ †
<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
140	9	29	10
40	22	55	25
0	70	80	62

\* From Curran et al. (3). The value at 40 mM is calculated from the relation between  $K_t$  and [Na].

† Corrected for the effect of other amino acids in the cell using the relation

$$\text{Corrected } K_t = \frac{\text{Observed } K_t}{1 + [I]/K_I}$$

where [I] is the concentration of other amino acids and  $K_I$  is their effective Michaelis constant.

effect can be made by assuming that competing amino acids are present in the cell at a concentration of 20 mM and that their effective  $K_t$  for the transport system is 10 mM at 140 mM Na and varies with Na concentration in the same way that the  $K_t$  for alanine influx varies with Na concentration. The results of calculating the "true  $K_t$ " for alanine efflux with these assumptions are given in Table III. The values are in good agreement with the  $K_t$  for alanine influx. This calculation simply indicates that the correct  $K_t$  for efflux is probably similar to the value for influx. Proof that the values are identical would require considerable additional study, probably with methods other than those used here.

However, in discussing these quantitative aspects of efflux an important reservation must be kept in mind. The intracellular Na and amino acid concen-

<sup>4</sup> The principal amino acids present that should compete with alanine were glycine (estimated concentration 8.6 mM,  $K_t$  for influx at 140 mM Na = 22 mM), valine (2.3 mM,  $K_t$  = 5.0 mM), leucine (2.3 mM,  $K_t$  = 4.2 mM), and serine (6.0 mM,  $K_t$  not known).

trations are calculated on a volume average basis (cell content/cell water) assuming a uniform distribution of the solute throughout the cell water. This assumption is certainly difficult to justify on the basis of current information regarding the behavior of cell organelles such as nuclei and mitochondria. Consequently, the calculated cellular concentrations may not represent those at the inner surface of the brush border membrane and the latter are the important concentrations with respect to the model. Nonetheless, the fact that several aspects of the data agree reasonably well with predictions of the model suggests that the agreement is not entirely fortuitous. For example, the fact that net fluxes of alanine can be predicted satisfactorily from influx and efflux values suggests that the error in efflux cannot be too large. Since the efflux calculation assumes no compartmentalization of alanine, the agreement offers some justification for the assumption that the volume average concentration provides a fairly reasonable estimate of the concentration at the membrane. Those aspects of the data relating to the Na gradient hypothesis are more dependent on qualitative changes in concentration than on the actual values of the concentration so that the reservation is less important in this case.

In summary, the present studies provide several lines of evidence supporting the concept that at least a part of the energy required for active alanine transport by rabbit ileum is supplied by the Na concentration difference between the mucosal solution and the cytoplasm of the epithelial cells. The question of whether this Na gradient is the sole source of energy remains unanswered and will require additional study. The results have also provided additional support for the specific model of the Na-alanine interaction proposed previously. The efflux of alanine from the cell toward the mucosal solution is in qualitative, and to a reasonable extent, quantitative agreement with the predictions of the model on the assumption that the transport system is reversible, completely symmetrical, and does not involve a direct coupling to energy-yielding metabolic processes.

#### APPENDIX

The model of alanine transport across the brush border membrane assumes the existence of a transport site,  $X$ , confined to the membrane phase that combines with Na and alanine as follows:



in which the subscript  $m$  denotes the mucosal solution side of the membrane. Similar reactions are postulated to occur at the cytoplasmic side of the membrane. The forms  $X$ ,  $XA$ , and  $X\text{ANa}$  can all be translocated across the membrane. The rate coefficients for translocation are assumed to be equal in both directions, equal for all three forms and the rate-limiting steps. As discussed previously, the unidirectional

influx ( $J_A^i$ ) and efflux ( $J_A^e$ ) of alanine predicted by this model will be

$$J_A^i = \frac{X_t P [A]_m}{\frac{K_1 K_2}{K_2 + [Na]_m} + [A]_m}$$

and

$$J_A^e = \frac{X_t P [A]_c}{\frac{K_3 K_4}{K_3 + [Na]_c} + [A]_c}$$

in which  $X_t$  is the total concentration of transport site,  $P$  is the rate coefficient for translocation,  $K_1$  and  $K_2$  are dissociated constants for reactions A-1 and A-2,  $K_4$  and  $K_3$  are dissociation constants for the reactions at the cytoplasmic side of the membrane, and the subscripts  $m$  and  $c$  denote mucosal solution and cytoplasm. If the transport system is entirely symmetrical,  $K_1 = K_4$  and  $K_2 = K_3$ .

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