The Sodium-Alanine Interaction in Rabbit Ileum

Effect of alanine on sodium fluxes

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ABSTRACT The interaction between Na transfer and alanine transfer across the mucosal border of rabbit ileum has been studied further by examining the effect of alanine on Na movement. Studies on strips of mucosa treated with ouabain showed that net Na movements against a Na concentration difference could be caused by a concentration difference of alanine. Na extrusion from mucosal cells was demonstrated when cellular alanine concentration exceeded that in the external medium. Conversely, the cells took up Na against a concentration difference when external alanine concentration was greater than cellular concentration. Unidirectional Na efflux from the cells toward the mucosal solution was increased by loading the cells with alanine. The relation between the increment in Na efflux and alanine efflux was approximately that predicted by the model of Curran et al. (reference 2) for the Na-alanine interaction at the mucosal border of the cells. The results offer further indication that the transport system is reversible and symmetrical.

Previous studies from this laboratory have examined certain aspects of the relation between Na fluxes and alanine fluxes across the mucosal border of rabbit ileum. Several points have been established: (a) Alanine influx from the mucosal solution into the cells depends on Na concentration in the external solution but not on Na concentration in the cells (1). (b) The presence of alanine in the mucosal solution causes an increase in Na influx and the increment in Na influx per unit alanine influx varies with Na concentration (2). (c) Alanine influx is not markedly altered by metabolic inhibitors or by ouabain (3). (d) Alanine efflux from the cells to the mucosal solution is influenced by cellular Na concentration (4). (e) The direction of net alanine transfer between the cells and the bathing medium is determined, at least in part, by the direction of the Na concentration difference (4). In mucosal strips treated with cyanide or ouabain, alanine is extruded from the cells against a concentration difference if cell Na concentration exceeds that in the external medium. Conversely if cell Na concentration is less than external concentration, alanine enters the cells against a concentration difference.

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These observations are all consistent with the model of the Na-alanine interaction proposed by Curran et al. (2) and with the "Na gradient hypothesis" (4, 5) as the mechanism of alanine transport. There remains at least one significant aspect of this system that has not yet been tested. The model predicts that efflux of Na from the cells to the mucosal solution should be affected by the presence of alanine in the cell. That is, there should be a coupling between the effluxes of Na and alanine just as there is a coupling between their influxes. The present experiments were undertaken to test this possibility by measuring net Na transfer into and out of mucosal cells and the unidirectional efflux of Na from the cells toward the mucosal solution.

METHODS

All experiments were carried out on the distal ileum of New Zealand white rabbits. The animals were killed by intravenous injection of sodium pentobarbital and the appropriate section of intestine was removed rapidly. It was cut open along the mesenteric border and placed in Ringer solution containing 140 mM NaCl, 10 mM KHCO$_3$, 0.2 mM KH$_2$PO$_4$, 1.2 mM K$_2$HPO$_4$, 1.2 mM MgCl$_2$, and 1.2 mM CaCl$_2$. Unless otherwise indicated, all solutions contained 10^{-4} M ouabain in order to inhibit the normal active Na transport system.

Net Na Transfer in Mucosal Strips

Small sheets of mucosa (50–100 mg wet weight) were prepared by scraping the tissue with a glass slide. As shown previously by Schultz et al. (1) these mucosal strips contain only epithelial cells and underlying connective tissue; they are completely devoid of muscle cells. The strips were incubated at 37°C in a large volume of solution and samples were removed at intervals for measurement of cell water and cellular Na concentration. All solutions contained inulin-^{3}H for estimation of extracellular space as described by Schultz et al. (1) and in most experiments ^{22}Na was also present. The pieces of tissue were removed from the bathing solution, blotted on filter paper, and weighed immediately on a torsion balance (wet weight). The tissues were then extracted for at least 2 hr in 0.1 N HNO$_3$. Aliquots of the extract and of the incubation media were taken to count ^{22}Na and inulin-^{3}H on a liquid scintillation spectrometer using Bray's solution (6). After extraction, the tissue was dried overnight at 80°C to determine the dry weight. Cell Na concentration was calculated from these data as previously described (1) using direct estimation of Na by flame photometry or from the ^{22}Na content of the extract and the assumption that the specific activity of the cell Na was equal to that in the medium. As discussed below, the two methods gave identical results.

In order to determine the effect of cellular alanine on net Na movements, the tissues were first incubated for 1 hr in normal Na Ringer (140 mM Na) containing ouabain and 40 mM L-alanine. Some tissues were then transferred to a solution identical to the initial solution except that it contained no alanine. Control tissues remained in the initial medium. Tissues were removed from the control flask initially and at 15 min and from the experimental flask at 5, 10, and 15 min after transfer. To determine the ef-

fect of external alanine on Na movement, the tissues were initially incubated in Na medium plus ouabain (1 hr) and transferred to solution containing 40 mM alanine. Samples were taken as described above.

Na Efflux across the Mucosal Border

Experiments designed to estimate Na efflux were similar to those described by Hajjar et al. (4) for the determination of alanine efflux. The whole intestinal wall was incubated for 1 hr in Na medium containing ^{22}Na and ouabain. In each experiment paired adjacent tissues from the same animal were used. The incubation solution for the experimental tissue contained 40 mM L-alanine and for the control, no alanine. At the end of the loading period, the tissues were rinsed briefly in 0.3 M mannitol to remove excess surface activity and mounted as flat sheets between Lucite half-chambers. Nonradioactive solution (Na medium, no alanine) was added to the mucosal chamber and left for 1 min. The solution was removed by suction directly into a counting vial and fresh solution was added to the chamber. Efflux was followed for seven 1 min periods. No bathing solution was placed on the serosal side. At the end of the experiment, the exposed tissue was cut out and the mucosa scraped off with a glass slide. The mucosa was extracted in 0.1 N HNO$_3$ and the extract counted to determine the amount of ^{22}Na remaining in the mucosa. Dry weight of the mucosa was determined as described above.

RESULTS

The interpretation of the results of these experiments depends on the assumption that active extrusion of Na from the mucosal cells is completely or nearly completely inhibited by 10^{-4} M ouabain. Fig. 1 shows cell Na concentration in mucosal strips as a function of time after treatment with ouabain. After 60–75 min, the concentration approaches that in the external solution (140 mM) indicating that the Na extrusion system is virtually abolished. Control tissues show no significant gain in Na over 45 min and Schultz et al. (1) have found similar cell Na concentrations (45–55 mM) after 60–75 min incubation under identical conditions. When ouabain-poisoned tissues are incubated for 60–75 min in the presence of ^{22}Na, virtually all the cell Na exchanges with the tracer. Table I shows the results of estimation of cell Na concentration using both direct measurement (flame photometry) and the determination of tracer content on individual mucosal strips. The agreement is satisfactory.

The results of experiments testing the effect of net alanine efflux on the level of tissue Na are summarized in Fig. 2. Mucosal strips were incubated in Ringer solution containing ouabain and 40 mM alanine and then transferred to alanine-free medium. The external media contained 140 mM Na and the control tissues (which remained in the initial incubation medium) had a mean cell Na concentration of 133 ± 4 mM. This concentration did not vary significantly during the 15 min test period. The ratio of cell Na concentration

in test and control tissues was calculated for each test tissue using the mean control value observed in the individual experiment and the average value of the ratio is shown in Fig. 2. By 5 min after transfer of tissues to alanine-free medium, cell Na concentration has decreased to a level significantly below the concentration in the control tissues and in the external medium
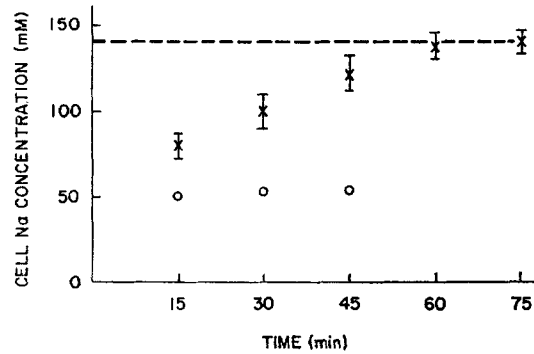

FIGURE 1. Effect of ouabain (10^{-4} M) on cell Na concentration of strips of mucosa. The incubation solution contained 140 mM Na; circles represent control tissues, crosses those ouabain-treated.

TABLE I

COMPARISON OF DETERMINATION OF
CELL Na CONCENTRATION*

Na concentration		
(a) By ^{22}Na	(b) By flame photometer	Ratio (a/b)
_mM_		
130	131	0.99
112	113	0.99
117	112	1.04
72	70	1.03
105	103	1.02
121	118	1.03
120	120	1.00
94	92	1.02
Mean ± SEM		1.02 ± 0.01

* Concentration was determined by both methods on each piece of tissue. Extracellular space was estimated with inulin-^{3}H.

indicating an extrusion of Na against a concentration difference. At this time, the cell volume was near its original value. At later times, the cells tended to shrink and in the final 5 min period Na reentered so that the cellular concentration tended to approach the level in the control tissues. However, Na extrusion continued for 10 min and at the end of this time, cell Na concentration was still significantly below the control level.

The results of the converse experiment are summarized in Fig. 3. Tissues were incubated for 1 hr in normal Ringer plus 10^{-4} M ouabain and transferred

to solution containing 40 mm alanine. The mean concentration ratio was calculated for each test tissue as described above for the experiments shown in Fig. 2. For the first 10 min after transfer, there was a net entry of Na into the cells and the cellular Na concentration increased to a level significantly greater than that in the control tissues (143 ± 4 mm) and in the bathing solution (140 mm). At 15 min, there was a decrease in cellular Na content but the concentration did not fall because of a slight decrease in cell volume.
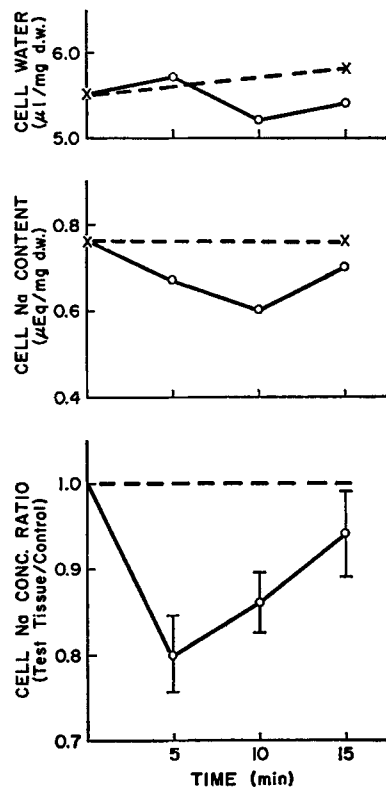

FIGURE 2. Extrusion of Na from mucosal cells i: the presence of an alanine concentration difference Mucosal strips were incubated in Ringer solutio: plus 10⁻⁴ m ouabain and 40 mm alanine. At zer time, they were transferred to alanine-free Ringe1 Control tissues (X) remained in the initial incuba tion solution. Mean cell Na concentration i: control tissues was 133 mm. Each point represent the average of 10 determinations and the bar show ± 1 SEM.

To test the possibility that the results shown in Figs. 2 and 3 were due to an artifact resulting from transfer of the mucosal strips from one solution to another, a single experiment was carried out in which the tissues were transferred between identical solutions. Changes in cell water and cell Na content were minimal when compared to the control tissue that remained in the original incubation medium.

Although the above results are consistent with predictions of the model for the Na-alanine interaction at the brush border proposed by Curran et al. (2), experiments with mucosal strips cannot distinguish between events at the mucosal and serosal sides of the cell. Thus, it is conceivable that in the

experiments shown in Fig. 2 the Na extrusion occurred at the serosal side and was unrelated to a transport system in the mucosal barrier. In order to test this possibility, intact sheets of intestine were loaded with ^{22}Na and washout across the mucosal surface was followed. The loading solutions were identical (Ringer plus 10^{-4} M ouabain) except that one contained 40 mM alanine in order to load the cells with amino acid. Fig. 4 shows the fraction of ^{22}Na remaining in the mucosa as a function of time for the paired tissues from a single experiment. ^{22}Na efflux can be described approximately by
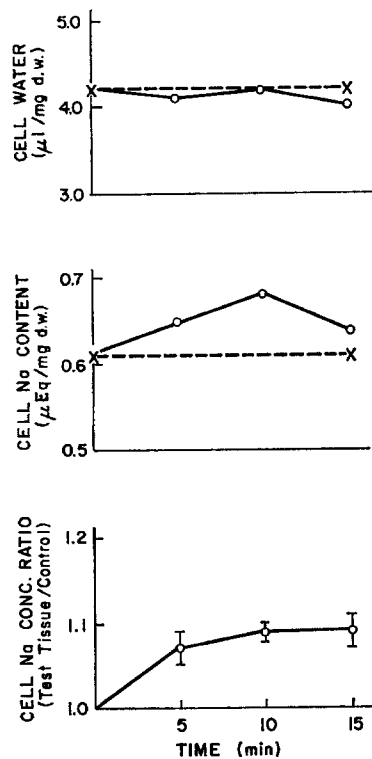
FIGURE 3. Uptake of Na by mucosal cells in the presence of an alanine concentration difference. Mucosal strips were incubated in Ringer solution plus 10^{-4} M ouabain. At zero time they were transferred to Ringer plus 40 mM alanine. Control tissues (×) remained in the initial incubation medium. Mean cell Na concentration in control tissues was 143 mM. Each point represents the average of 14 determinations and the bars show ± 1 SEM.

two exponentials. The more rapid one probably represents an "extracellular space" composed of an unstirred layer and the space between microvilli (see Hajjar et al. [4]), and the slower component is assumed to represent cellular Na. For the experiment shown, the rapid components are nearly identical (half-times of 0.5 and 0.6 min). The slower component has a smaller half-time for the tissue in which the cells contain alanine, suggesting that cellular alanine accelerates Na efflux. In order to estimate the actual Na efflux, the slope of the slow component was taken as an estimate of the rate coefficient for efflux. Since ^{22}Na exchanges completely with cell Na (Table I), the zero time intercept of the slow component was taken as an estimate of

the pool of cellular Na and the efflux was calculated as the product of the pool and the rate coefficient. In an effort to take into account the variations in the number of cells present in the chamber, efflux was calculated as microequivalents per hour per milligram dry weight of mucosa. The results of five experiments are shown in Table II. In each experiment, Na efflux was
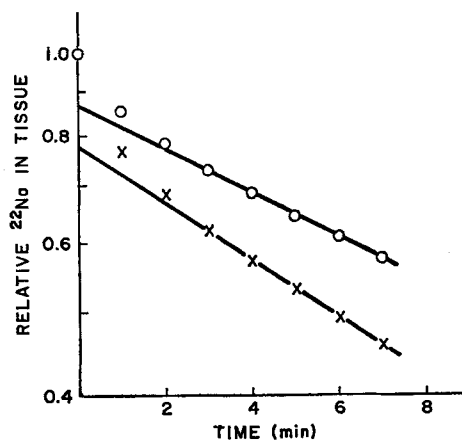

FIGURE 4. Washout of ^{22}Na across the mucosal border of rabbit ileum. Control tissue (O) was loaded with ^{22}Na in Ringer solution plus 10^{-4} M ouabain, and test tissue (×) in Ringer solution plus 10^{-4} M ouabain and 40 mM alanine.

TABLE II

EFFECT OF CELLULAR ALANINE ON Na
EFFLUX ACROSS THE MUCOSAL BORDER

| Na efflux | | |
(a) Control	(b) + Alanine	Ratio (a/b)
μeq/hr mg d.w.		
3.78	4.08	1.08
2.34	2.82	1.21
4.62	4.80	1.04
2.16	2.52	1.16
4.20	4.62	1.10
Mean ± SEM		1.11 ± 0.03

greater from the alanine-loaded tissue than from the paired control and the mean ratio of Na efflux with alanine in the cell to control efflux was 1.11 ± 0.03. In more conventional units, the average efflux under control conditions was 23.2 μeq/hr cm^2 so that the increment in Na efflux caused by alanine in the cell was 2.6 μeq/hr cm^2. This observation supports the concept that the Na extrusion shown in Fig. 2 is primarily the result of Na movement across the mucosal face of the cells.

DISCUSSION

These experiments were designed to examine certain aspects of the model suggested by Curran et al. (2) for the Na-alanine interaction at the mucosal border of rabbit intestine. According to this model, the membrane contains a transport site (X) that combines with alanine to form a complex (XA). This complex can be translocated across the membrane but can also combine with Na to form a ternary complex $(XA\text{Na})$ that can then be translocated. Studies on the influxes of Na and alanine (2) have indicated that the affinity of XA for Na is appreciably greater than that of X for A. Thus at high Na concentrations, most of the influx involves the ternary complex and a "co-transport" of Na and alanine. We have previously suggested that this transport system does not involve a direct interaction with metabolic energy-yielding processes and hence is readily reversible and symmetrical. That is, the transport system can also mediate the effluxes of Na and alanine from the cytoplasm to the mucosal solution, and the properties of the system with respect to association-dissociation reactions are the same on the cytoplasmic side of the membrane as they are on the side facing the mucosal solution. Evidence supporting this concept with respect to efflux of alanine has recently been presented by Hajjar et al. (4).

According to the analysis of the model proposed by Curran et al. (2), the net Na flux, J_{Na}, across the mucosal membrane via this specific system should be given by

$$J_{\text{Na}} = J_{\text{Na}}^i - J_{\text{Na}}^e = \left(\frac{[\text{Na}]_m}{K_2 + [\text{Na}]_m}\right) J_A^i - \left(\frac{[\text{Na}]_c}{K_3 + [\text{Na}]_c}\right) J_A^e \qquad (1)$$

in which K_2 is the dissociation constant for the reaction $\text{Na} + XA \rightleftharpoons XA\text{Na}$ at the mucosal solution side of the membrane and K_3 is the dissociation constant for the same reaction at the cytoplasmic side of the membrane. The subscripts, m and c, denote mucosal solution and cell interior and J_A^i and J_A^e are the unidirectional influx and efflux of alanine, respectively. (For the present purpose, the explicit expressions for J_A^i and J_A^e are unnecessary.) If the transport system is symmetrical, $K_2 = K_3$.

The present experiments can be considered in terms of the above expression, ignoring for the moment the effect of other paths for Na movement between the cells and the external medium. If the transport system is reversible and symmetrical, the effluxes of Na and alanine should be coupled in a predictable manner just as are the influxes of Na and alanine (2). The expected relations between the unidirectional Na and alanine fluxes are apparent from equation 1. In addition, the model predicts that a net efflux of alanine across the brush border should be accompanied by a net Na efflux

and a net influx of alanine should be accompanied by an increase in net Na influx. In particular, if $[Na]_m = [Na]_c$ and $K_2 = K_3$, equation 1 becomes

$$J_{Na} = \frac{[Na]}{K_2 + [Na]} (J_A^i - J_A^s) \tag{2}$$

Thus, under the conditions of the present experiments in which cellular and extracellular Na concentrations are approximately equal initially, the direction of net Na flux should be determined by the direction of net alanine flux $(J_A^i - J_A^s)$.

Both these effects have been demonstrated. The increase in Na efflux across the brush border caused by loading the cells with alanine (Table II) is consistent with a direct coupling or cotransport of Na and alanine from the inside of the cell toward the mucosal solution. Further, the increment in Na efflux appears to be quantitatively consistent with the concept of a symmetrical system. Although alanine efflux was not measured in the present experiments, the average efflux observed by Hajjar et al. (4) under exactly identical conditions was 3.1 μmoles/hr cm². Since the increment in Na efflux was 2.6 μeq/hr cm², the ratio, $\Delta J_{Na}^s/J_A^s$, is 0.84 at a cell Na concentration of approximately 140 mM. The predicted value for this ratio assuming that $K_2 = K_3$ and using the value of K_2 obtained from influx measurements (2), is 0.89. In other terms, the value of K_3 calculated from these data and equation 1 is 27 mM while the value of K_2 estimated previously is 17 mM. This agreement between observed and predicted values seems quite satisfactory in view of the uncertainties in the present experiments.

The net Na extrusion from mucosal strips accompanying net alanine efflux (Fig. 2) is entirely consistent with the predictions of equation 2. The studies on Na efflux suggest that this transfer occurs primarily at the mucosal side of the cell, and the net Na transfer can be accounted for reasonably well in terms of unidirectional Na fluxes across the mucosal border. From Fig. 2, the net Na efflux during the initial 10 min period takes place at a rate of approximately 1 μeq/hr mg dry weight. The unidirectional Na efflux under these conditions was found to be 25.8 μeq/hr cm². The unidirectional Na influx into ouabain-treated tissue with 140 mM Na and no alanine in the mucosal solution is approximately 18.0 μeq/hr cm² (3) so that the expected initial net Na efflux would be 7.8 μeq/hr cm². Since there are 6–10 mg dry weight of mucosa per cm², the observed net efflux is within the predicted range.[1]

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[1] Table IV of reference 3 gives Na influx into ouabain-treated tissue from 140 mM Na as 19.5 μeq/hr cm² when alanine influx is 1.7 μmoles/hr cm². At 140 mM Na, alanine-dependent Na influx is 0.89 times alanine influx so that the calculated Na influx in the absence of alanine is 18.0 μeq/hr cm².
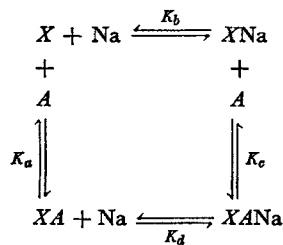
The net Na efflux takes place against a concentration difference since cell Na concentration decreases to 106 mM compared to an external concentration of 140 mM. Such an effect could in principle be caused by an activation of the normal Na extrusion mechanism or by a change in electrical potential difference (PD) across the mucosal membrane. The former seems highly unlikely since active Na extrusion has been completely inhibited in all experiments by the use of a relatively high concentration of ouabain. While there is no direct evidence available regarding changes in PD, this explanation for the net efflux also seems rather unlikely. The unidirectional flux data discussed above indicate that a minimum PD of approximately 9 mv, cell interior positive, would be required to account for the observed flux ratio if the fluxes were due to simple diffusion. This PD, which is reversed in orientation from normal (7, 8), would have to be caused by the presence of a concentration difference of alanine between the interior and exterior of the cell. Since in ouabain-treated tissue, the cellular Na concentration is approximately equal to that in the bathing media, there is probably little PD between the cells and the medium initially. Further, microelectrode studies in Greek tortoise (7, 8) intestine have shown that concentration differences of sugars and amino acids across the brush border membrane do not cause changes in PD across that membrane. These considerations argue against a change in PD as the cause of the Na extrusion shown in Fig. 2. Thus, this Na transfer probably occurs against an electrochemical potential difference; it has the characteristics of an "active" transport but the energy is supplied by a concentration difference of alanine. Such behavior is predicted by the model for Na-alanine interactions.

The reversible nature of the transport system with respect to Na movements is shown by the data in Fig. 3. A net entry of Na into mucosal strips takes place against a concentration difference when there is net entry of alanine as predicted by equation 2. The observed Na entry also appears to be consistent with information on unidirectional fluxes at the brush border. The net Na influx occurs at a rate of approximately 0.4 μeq/hr mg dry weight. From previous studies (2) the alanine influx across the mucosal membrane from a solution containing 140 mM Na and 40 mM alanine can be calculated to be approximately 5 μmoles/hr cm². Since under these conditions the increment in Na efflux is 0.89 times the alanine influx, the expected increase in Na influx is 4.4 μeq/hr cm². This value should also represent the net entry of Na and with a dry weight of 6–10 mg/cm², it is in reasonable agreement with the observed value.

The present experiments appear to offer considerable additional support for the specific model of the relation between alanine and Na transport at the brush border of rabbit small intestine. In particular, they show the existence of coupling of the effluxes of Na and alanine across the mucosal

membrane and provide additional evidence that the transport system is reversible and symmetrical, at least under conditions in which the tissue has been treated with ouabain. These results and those of Hajjar et al. (4) show clearly that the direction of net transfer via this system is determined by the directions of the Na and alanine concentration differences across the brush border membrane.

The experimental results have been discussed in relation to the model proposed by Curran et al. (2) because that model seems best able to account for the known properties of the alanine transport system in rabbit ileal mucosa. It is worth pointing out, however, that the prediction that an alanine gradient will drive Na ions uphill does not depend on the assumption that the transport site can combine with Na ions only after first combining with the amino acid. For if there is no preferred order of reaction we may write

$$
\begin{array}{ccc}
X + \mathrm{Na} & \xrightleftharpoons{K_b} & X\mathrm{Na} \\
+ & & + \\
A & & A \\
K_a \updownarrow & & \updownarrow K_c \\
XA + \mathrm{Na} & \xrightleftharpoons[K_d]{} & XA\mathrm{Na}
\end{array}
$$

For each reaction in the cycle, the free energy change, $\Delta G = \Delta G^0$, plus a concentration term, and summing round the cycle the concentration terms cancel leaving $\Sigma \Delta G^0 = 0$. It follows that $K_a \cdot K_b \cdot K_c \cdot K_d = 1$, where each equilibrium constant is defined as the ratio of the clockwise to the anti-clockwise velocity constant. If combination with Na increases the affinity of the carrier for alanine, $K_a \cdot K_c$ is greater than 1, so that $K_b \cdot K_d$ must be less than 1; that is, combination with alanine increases the affinity for Na. More generally, if any carrier combines with two ligands, A and B, then if combination with A increases (or decreases) the affinity for B, combination with B must increase (or decrease) the affinity for A. It follows that any system which employs an effect of this kind to couple the downhill movement of one molecular species to the uphill movement of another should show reciprocal transport effects. Such effects can, of course, also have other explanations.

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