Effect of Inhibitors on Alanine Transport in Isolated Rabbit Ileum

RONALD A. CHEZ, RICHARD R. PALMER,
STANLEY G. SCHULTZ, and PETER F. CURRAN

From the Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115.
Dr. Chez's present address is the Department of Obstetrics and Gynecology, University of
Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213. Dr. Schultz's present address
is the Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh,
Pennsylvania. Dr. Curran's present address is the Department of Physiology, Yale University
School of Medicine, New Haven, Connecticut 06510

ABSTRACT   The effects of metabolic inhibitors and ouabain on alanine trans-
port across rabbit ileum, in vitro, have been investigated. Net transport of
alanine and Na across short-circuited segments of ileum is virtually abolished by
cyanide, 2,4-dinitrophenol, iodoacetate, and ouabain. However, these inhibitors
do not markedly depress alanine influx from the mucosal solution, across the
brush border, into the intestinal epithelium, and they do not significantly affect
the Na dependence of this entry process. The results of this investigation indicate
that: (a) the Na dependence of alanine influx does not reflect a mechanism in
which the sole function of Na is to link metabolic energy directly to the influx
process; and (b) the inhibition of net alanine transport across intestine is, in part,
the result of an increased rate coefficient for alanine efflux out of the cell across
the brush border. Although these findings do not exclude a direct link between
metabolic energy and alanine efflux, the increased efflux may be the result of the
increased intracellular Na concentration in the presence of these inhibitors. The
results of these studies are qualitatively consistent with a model for alanine trans-
port across the brush border which does not include a direct link to metabolic
energy.

In vitro preparations of rabbit ileum are capable of accumulating L-alanine
within the epithelial cells (1) and of transporting it from the mucosal to the
serosal bathing solution against concentration differences (2). These transport
processes are inhibited by the cardiac glycoside ouabain and by depletion of
extracellular Na (1, 2). There is good evidence that the primary amino acid
transport mechanism is located at the mucosal border of the cells (3–5), and
methods for direct measurement of amino acid influx[1] across this membrane

---

[1] The term influx will refer to the unidirectional flux of alanine from the mucosal solution, across
the brush border, into the tissue. It should not be confused with the transmural flux from mucosa
to serosa.

2357

from mucosal solution to cell have been described (3, 4). Alanine influx was found to depend on Na concentration in the mucosal solution and to be accompanied by an influx of Na suggesting a direct coupling of the transfer of these two solutes. The model developed to explain these observations is consistent with the concept that the Na concentration difference between mucosal solution and cytoplasm provides the requisite driving force for alanine accumulation and transport. This idea has been suggested by a number of investigators to account for transport of both sugars (6, 7) and amino acids (8–10), but there is little direct, unequivocal evidence for such a mechanism. However, on the basis of our previous studies on alanine influx, a direct input of metabolic energy in this step of the over-all transport process could not be ruled out. The present experiments were undertaken to obtain evidence on this point by examining the effect of metabolic inhibitors on alanine influx across the brush border of rabbit ileum. In addition, effects of inhibitors on unidirectional transmural fluxes have been studied in order to obtain insight
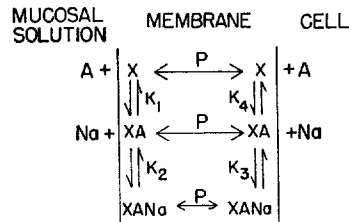

FIGURE 1. Model for alanine transport across mucosal border of rabbit ileum.

into the forces responsible for alanine transport against a chemical potential difference.

THEORETICAL CONSIDERATIONS

Previous studies (4) of the kinetics of alanine and Na influxes across the brush border of rabbit ileum have suggested the model shown in Fig. 1 for the interaction between Na and alanine influxes. According to this model, alanine (A) combines with a membrane component (X) to form a binary complex (XA); this reaction is assumed to be governed by the laws of mass action and characterized by a dissociation constant K_1. The complex XA may either cross the membrane or combine with Na to form a ternary complex $XANa$ that then crosses the membrane. The coefficients P describe the rates at which X, XA, and $XANa$ translocate across the membrane. They are assumed to be first order, equal in both directions, and equal for all three forms of the membrane component. The process of translocation is assumed to be much slower than the association-dissociation reactions, but the actual mechanism of translocation need not be further specified. With these assumptions, the unidirectional influxes of alanine, J_A^i, and Na, J_{Na}^i, are given by

$$J_A^i = \frac{J_A^{im}[A]_m}{\dfrac{K_1 K_2}{K_2 + [Na]_m} + [A]_m}$$ (1)

$$J_{Na}^i = \left[\frac{[Na]_m}{[Na]_m + K_2}\right] J_A^i + J_{Na}'$$ (2)

where J_A^{im} is the maximum influx of alanine in μmoles/hr cm^2, J_{Na}' is the alanine-independent Na influx in μeq/hr cm^2 and

$$K_1 = [A]_m[X]_m/[XA]_m = 70 \text{ mM}$$
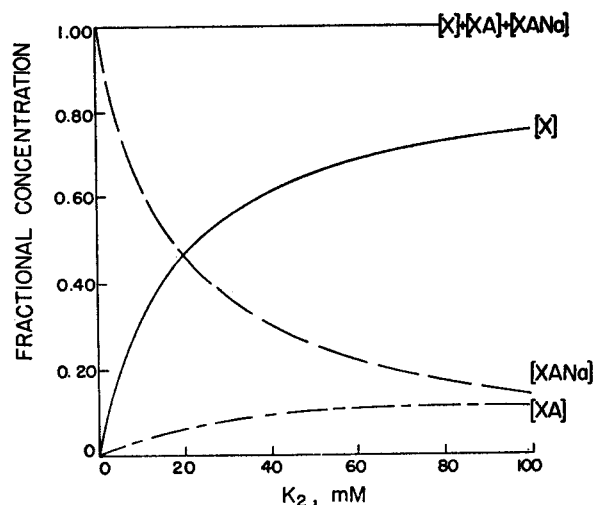
$$K_2 = [Na]_m[XA]_m/[XANa]_m = 17 \text{ mM}$$

FIGURE 2. Dependence of steady-state concentrations of X, XA, and XANa on K_2. All values are expressed relative to the total concentration of carrier in the membrane ($X + XA + XA$Na) and were calculated from the equations describing the model, given in Appendix A of reference 4, for the conditions $[A]_m = 10$ mM, $[Na]_m = 140$ mM, and $K_1 = 70$ mM.

The subscript m denotes the mucosal bathing solution. The values of K_1 and K_2 for alanine were estimated from experimental observations (4).

Equations 1 and 2 have been shown to satisfy all available experimental observations on alanine and Na influxes in rabbit ileum (4). However, though this model has descriptive and predictive value, it gives little insight into the exact function of Na in this coupled influx process; it merely provides two kinetic parameters that account for the stimulation of alanine influx by Na in the mucosal medium. In terms of the model, Na enhances alanine influx because the ternary complex XANa is more stable than the binary complex XA as indicated by the relation $K_2 < K_1$. This effect is illustrated in Fig. 2 in which the relative distribution of the three forms of X is shown as a function

of K_2 using $[A]_m = 10$ mM, $[\text{Na}]_m = 140$ mM, and $K_1 = 70$ mM. As K_2 decreases (i.e. the stability of XANa increases) the total amount of free carrier (X) decreases and the influx of alanine (given by $P([XA]_m + [XA\text{Na}]_m)$) increases; as K_2 approaches zero, J^i_A approaches J^{im}_A, the influx that would be observed in the presence of an infinite alanine concentration.

Thus, within the framework of this model, the stimulatory effect of Na on alanine influx resides in the fact that $K_2 < K_1$. One possible explanation for the relatively low value of K_2 would be linkage of the formation of the XANa complex to an exergonic metabolic reaction, such as ATP hydrolysis. The role of Na in the coupled influx process could be to link energy from the hydrolysis of ATP to the amino acid transport mechanism. If, as suggested by Csáky, this process is mediated by a Na-K–activated ATPase that is inhibited by ouabain (11), the effects of Na, metabolic poisons, and ouabain on amino acid transport could be explained within the framework of the model. This
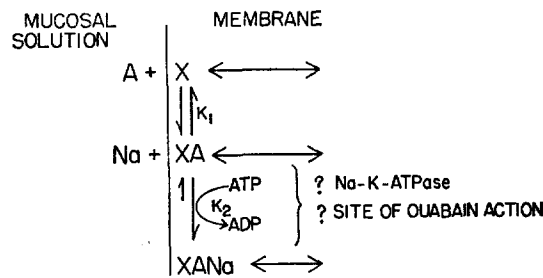

FIGURE 3. Possible coupling of ATP hydrolysis to model for alanine influx.

concept is illustrated in Fig. 3. A possible reaction sequence is

$$\text{Na}_m + XA_m + \text{ATP} \xrightleftharpoons{\text{ATPase}} XA\text{Na}_m + \text{ADP}$$

and the dissociation constant K_r for the reaction is

$$K_r = [\text{Na}]_m[XA]_m[\text{ATP}]/([XA\text{Na}]_m[\text{ADP}])$$

Thus, K_2 is given by

$$K_2 = \frac{[\text{Na}]_m[XA]_m}{[XA\text{Na}]_m} = \frac{K_r[\text{ADP}]}{[\text{ATP}]}$$

Clearly, if $[\text{ATP}] \to 0$ or if the reaction is completely inhibited with ouabain, $K_2 \to \infty$ and equation 1 reduces to

$$J^i_A = \frac{J^{im}_A[A]_m}{K_1 + [A]_m}$$

Thus complete inhibition of the reaction would eliminate the Na dependence of alanine influx. If the reaction were incompletely inhibited, or if the forma-

tion of the ternary complex were not tightly coupled to ATP hydrolysis, the Na dependence would not be completely abolished but the stimulatory effect of Na on alanine influx would be diminished. The primary purpose of the present experiments was to test this hypothesis for the role of metabolic energy in alanine transport.

METHODS

The technique used for determination of alanine and Na influxes across the mucosal border has been described in detail (3). Briefly, distal ileum from New Zealand white rabbits (killed by intravenous injection of pentobarbitol) was mounted in a chamber in which only the mucosal surface was bathed with Ringer's solution. The Ringer solution used contained 140 mM NaCl, 10 mM KHCO$_3$, 1.2 mM K$_2$HPO$_4$, 0.2 mM KH$_2$PO$_4$, 1.2 mM CaCl$_2$, and 1.2 mM MgCl$_2$, and had a pH of 7.2 when equilibrated with 5% CO$_2$-95% O$_2$. Na-free Ringer's was prepared by replacing all the NaCl by choline chloride. After a preincubation period of 30 min, the solution was removed from the chamber and a *test solution* containing L-alanine-^{14}C, inulin-^{3}H, and ^{22}Na was injected rapidly. This solution remained in contact with the mucosa for 60 sec and was then withdrawn. The chamber was rinsed rapidly with cold (4°C) isotonic mannitol solution, and the exposed tissue was immediately punched out and extracted in 0.1 N HNO$_3$. Aliquots of the extract and test solution were assayed for ^{14}C, ^{3}H, and ^{22}Na using a three-channel liquid scintillation spectrometer. The inulin-^{3}H in the extract was used to correct for contamination by labeled medium, and Na and alanine influxes were calculated from the tracer uptake by the tissue. Eight measurements were usually carried out on tissue from a single animal. Control experiments to test the suitability of this technique have been described previously (3).

In some experiments, tissues were preincubated in the Na-free choline medium, a procedure that has been shown to cause a marked reduction in intracellular Na concentration (3). The test solution from which influx was measured was either normal Ringer's solution or Na-free Ringer's. Inhibitors were added to the solutions to give the following concentrations: ouabain, 10^{-4} M; NaCN, 2 × 10^{-3} M; Na-iodoacetate (IAA), 1 × 10^{-3} M; 2,4-dinitrophenol (DNP), 10^{-4} M. The inhibitors were included in the preincubation solution bathing the mucosal surface, and the solutions containing cyanide were replaced every 10 min to minimize loss of cyanide by volatilization. In each experiment, influxes were measured under control and inhibited conditions on tissue from the same animal. Since ouabain has little effect on alanine and Na transport when present in the mucosal solution alone (2, 12), strips of intestine were incubated for 30 min in solution containing ouabain prior to mounting in the influx chamber. Influx was then determined after an additional 10 min preincubation. The control tissues for this series of experiments were treated in the same manner but without ouabain.

Transmural alanine fluxes were determined using the apparatus and techniques described by Schultz and Zalusky (12). A segment of ileum was mounted as a flat sheet between Lucite chambers and bathed on both sides by Ringer's solution bubbled continuously with 95% O$_2$-5%CO$_2$. L-Alanine-^{14}C was added to one bathing solution and its rate of appearance on the opposite side was determined. Inhibitors,

when used, and tracer were added immediately after mounting the tissue. Sampling for flux determinations was begun after a 30–40 min equilibration period. The unidirectional flux was calculated from the steady-state rate of tracer appearance on the "cold" side and the specific activity of the "hot" side. The tissue was kept short-circuited throughout the experiments.

RESULTS

Inhibition of Transmural Alanine Transport

The effects of metabolic inhibitors and of ouabain and Na-free conditions on mucosa-to-serosa (J_{ms}), serosa-to-mucosa (J_{sm}), and net (J_{net}) fluxes of alanine are shown in Table I. In each experiment, J_{ms} and J_{sm} were meas-

TABLE I
EFFECT OF INHIBITORS ON
TRANSMURAL ALANINE FLUXES*

	J_{ms}‡	J_{sm}	J_{net}	n§
	$\mu moles/hr\ cm^2$			
Control	1.30±0.10	0.13±0.02	1.17	16
CN(2 × 10^{-2}M)	0.27±0.02	0.16±0.01	0.11	8
DNP(10^{-4}M)	0.18±0.03	0.13±0.02	0.05	8
IAA(10^{-3}M)	0.32±0.04	0.18±0.01	0.14	8
Ouabain (10^{-4}M)	0.39±0.03	0.11±0.03	0.28	8
Na-free ¶	0.16±0.01	0.13±0.02	0.03	4

* Alanine was present in both mucosal and serosal solutions at a concentration of 5 mM in all experiments.

‡ J_{ms} is mucosa-to-serosa flux, J_{sm} is serosa-to-mucosa flux, and J_{net} is given by $J_{ms} - J_{sm}$.

§ No. of flux periods.

¶ All Na in the mucosal and serosal solutions was replaced by choline.

ured on two adjacent pieces of tissue from the same animal. The control data and those obtained in the presence of ouabain and in the absence of Na are from a previous study (2) and are included for comparison. In another series of experiments in the present study, J_{ms} alone was measured under control conditions and with ouabain: the average values obtained were 1.45 μmoles/ hr cm² and 0.44 μmole/hr cm² respectively, in good agreement with the data in Table I. All agents tested caused a marked decrease in net alanine flux from mucosa to serosa as a result of a decline in J_{ms} ; the unidirectional flux from serosa to mucosa is unaffected by these inhibitors and averaged 0.15 μmole/hr cm².

The short-circuit current across the ileum was also inhibited by all the agents listed in Table I. During the period in which alanine fluxes were determined, the short-circuit current averaged 5 μamp/cm² compared to an aver-
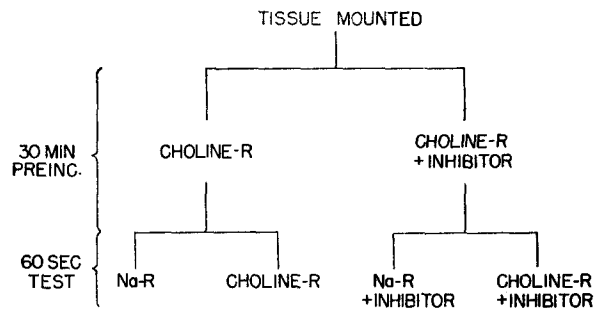
FIGURE 4. Flow chart describing procedure for experiments in which tissue was pre-incubated in Na-free, choline-Ringer's prior to influx determination.

TABLE II

EFFECT OF INHIBITORS ON ALANINE INFLUX.*
PREINCUBATION IN Na-FREE MEDIUM

| Inhibitor | 140 mM Na Alanine influx | | Δ‡ | Na-free Alanine influx | | Δ |
	Control	+ Inhibitor		Control	+ Inhibitor	
	$\mu moles/hr\ cm^2$			$\mu eq/hr\ cm^2$		
CN	2.46	2.49	0.03±0.03	0.66	0.67	0.01±0.02
DNP	2.16	1.77	−0.39±0.11§	0.52	0.41	−0.11±0.05
IAA	2.36	1.72	−0.64±0.13§	0.60	0.46	−0.14±0.03§
Ouabain	1.92	1.74	−0.18±0.10	0.36	0.33	−0.03±0.03

* All tissues preincubated in Na-free choline medium and influx measured from 140 mM Na or Na-free solutions. Alanine concentration 5 mM.

‡ Δ = treated minus control. The data were treated as paired by determining Δ for adjacent ports in the influx chamber. In all cases, control and inhibited tissues were alternated. No. of pairs, CN (8), IAA (12), DNP (12), ouabain (14).

§ Δ significantly different from zero, $p < 0.01$; other values $p > 0.05$.

TABLE III

EFFECT OF INHIBITORS ON Na INFLUX.*
PREINCUBATION IN Na-FREE MEDIUM

| Inhibitor | Na influx | | Δ‡ | n |
	Control	+ Inhibitor		
	$\mu eq/hr\ cm^2$			
CN	14.3	16.4	2.1±1.1	8
DNP	16.1	18.1	2.0±1.8	6
IAA	14.9	16.5	1.6±1.0	12
Ouabain	18.5	20.3	1.8±0.8	14

* Na concentration was 140 mM in test solution. All tissues preincubated in Na-free choline medium.

‡ Δ = treated minus control. Data from adjacent ports were considered paired in evaluating Δ and its standard error.

age initial short-circuit current of 104 μamp/cm². In the presence of alanine, the short-circuit current is a measure of the rate of active Na transport from mucosa to serosa (10). Thus, all the agents tested inhibited both net alanine transport and active transmural Na transport.

Effect of Inhibitors on Alanine Influx

We have previously demonstrated that the influx of alanine across the brush border from a medium containing 140 mM Na is independent of whether or not Na is present in the preincubation medium; the influx into tissue pre-incubated for 30 min in a Na-free, choline medium does not differ significantly from the influx into tissue preincubated in a medium containing 140 mM Na (3). For this reason, a series of experiments was performed in which the effects of inhibitors on alanine and Na influxes were determied on tissue preincubated in choline Ringer's. The test solution was either Na Ringer's or choline Ringer's. A flow chart of the experimental procedure is given in Fig. 4, and the results are summarized in Tables II and III. The data in Table II show that neither cyanide nor ouabain inhibits alanine in-
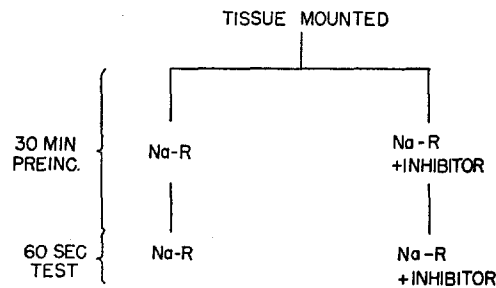
TISSUE MOUNTED

	Na-R	Na-R +INHIBITOR
30 MIN PREINC.		
60 SEC TEST	Na-R	Na-R +INHIBITOR

FIGURE 5. Flow chart describing procedure for experiments in which tissue was pre-incubated in Na-Ringer's prior to influx determination.

flux under these conditions. Both DNP and IAA bring about a small but statistically significant inhibition of alanine influx in the presence of Na. In the absence of Na, IAA also causes an inhibition and there appears to be an effect of DNP but it is not statistically significant. The fractional inhibitions caused by IAA and DNP do not differ for the two test conditions. The data in Table III show that Na influx across the brush border is not significantly altered by any of these agents that abolish active transepithelial transport of Na.

Somewhat different results were observed when influx of alanine was determined on tissue preincubated in Na Ringer's containing inhibitors. The experimental protocol is shown in Fig. 5 and the results are given in Table IV. All four inhibitors bring about significant decreases in alanine influx

under these conditions. Further, the fractional inhibitions are strikingly simi-
lar for the four agents, averaging approximately 40%. In contrast, Na influx
is slightly increased but the effect is statistically significant only for DNP. A
possible explanation for the reduced influx under these conditions is that
uptake of alanine-^{14}C is not a linear function of time over the 60 sec interval
during which the tissue is exposed to test solution. A significant back-flux of
alanine-^{14}C during this period would lead to spuriously low calculated in-
fluxes. In order to examine this possibility, the time course of alanine-^{14}C
uptake into tissue preincubated in Na Ringer's containing ouabain was in-
vestigated, as described previously (3). The results of a single experiment,
shown in Fig. 6, indicate that uptake is a linear function of time for at least

TABLE IV
EFFECT OF INHIBITORS ON ALANINE AND Na INFLUXES.*
PREINCUBATION IN Na RINGER'S

Inhibitor	Alanine influx		$\Delta\ddagger$	Na influx		Δ
	Control +	Inhibitor		Control +	Inhibitor	
	μmoles/hr cm²			*μeq/hr cm²*		
CN	2.92	1.77	-1.15 ± 0.21§	20.6	22.5	2.1 ± 1.8
DNP	2.87	1.63	-1.24 ± 0.23§	15.9	21.3	5.4 ± 1.3§
IAA	2.85	1.64	-1.21 ± 0.16§	18.8	20.4	1.6 ± 1.2
Ouabain	2.81	1.69	-1.12 ± 0.20§	18.5	19.5	1.0 ± 0.5

* All tissues preincubated in Na Ringer's and influx measured from solution containing 140 mM
Na.
‡ Δ = treated minus control. The data were treated as paired by determining Δ for adjacent ports
in the influx chamber. In all cases, control and inhibited tissues were alternated. No. of pairs, CN
(8), IAA (12), DNP (12), ouabain (14).
§ Δ significantly different from zero, $p < 0.01$; other values $p > 0.05$.

80 sec so that the rate of uptake is a valid measure of unidirectional influx. The
relatively marked difference between the average value for alanine influx into
control tissue in the ouabain series shown in Table II and that shown in
Table IV seems to be due to the necessity for mounting the tissue after pre-
incubation in the ouabain-containing medium (see Methods). Tissue pre-
incubated in this manner in choline medium contracts markedly and subse-
quent mounting is much more difficult than when the tissue is preincubated
in Na Ringer's. Since control and ouabain-treated tissues form a comparable
pair within each series of experiments, the differences arising from the
necessary variations in tissue handling seem relatively unimportant.

Analysis of Unidirectional Alanine Fluxes

The effects of metabolic inhibitors and ouabain on net transmural alanine
flux, summarized in Table I, were determined in the presence of Na. The

analogous experimental conditions for determination of alanine influx are those in which the tissue was preincubated in Na Ringer's with inhibitor for 30 min prior to the influx measurement (Table IV). Under these conditions, alanine influx is significantly depressed. Therefore, experiments were performed to test whether this decrease in influx is sufficient to account for the decrease in net transmural flux, or whether an increase in the unidirectional efflux of alanine out of the cell across the mucosal border must also occur. To examine this point, the unidirectional transmural flux of alanine from mucosa to serosa was measured simultaneously on two pieces of tissue from the same
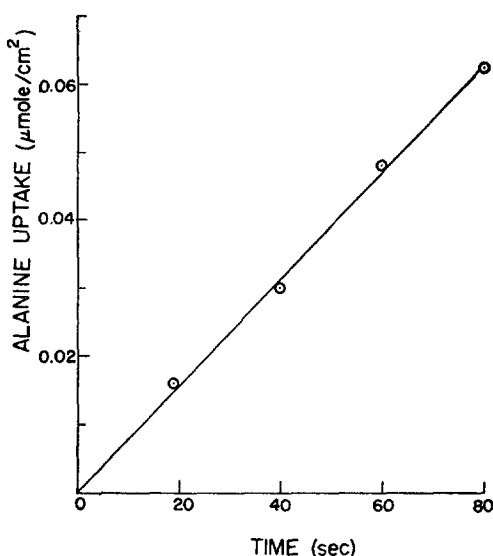

FIGURE 6. Time course of alanine-^{14}C uptake by tissue preincubated in Na-Ringer's containing 0.1 mM ouabain. The test solution contained 140 mM Na and 5 mM alanine. All values were obtained on tissue from the same animal and each point represents the average of duplicate determinations. The slope represents an alanine influx of 2.9 μmoles/hr cm².

animal under control and inhibited conditions. The tissues were bathed on both sides with Na Ringer's containing 5 mM alanine and inhibitor was added to the solutions bathing one of the tissues. At the same time, unidirectional influx of alanine, J_{mc}, was determined on tissue from the same animal according to the protocol given in Fig. 5 except that 5 mM alanine was included in the preincubation solution.[2] The measurements of J_{ms} and J_{mc} were carried out under as nearly identical conditions as the experimental procedures permitted; the same solutions were used and fluxes were determined over the same time interval after identical preincubation periods.

The data from these experiments were utilized to estimate individual unidirectional alanine fluxes across the mucosal and serosal boundaries of the tissue. Under steady-state conditions, these fluxes are related to the trans-

[2] Previous studies (3) using paired tissues from the same animal have shown that preincubation in the presence of alanine does not alter alanine influx. There is no "transconcentration effect" of intracellular alanine on alanine influx in rabbit ileum.

mural unidirectional fluxes as follows (13):

$$J_{ms} = J_{mc}J_{cs}/(J_{cm} + J_{cs}) \tag{3}$$

$$J_{sm} = J_{sc}J_{cm}/(J_{cm} + J_{cs}) \tag{4}$$

$$J_{ms} - J_{sm} = J_{mc} - J_{cm} = J_{cs} - J_{sc} = J_{net} \tag{5}$$

in which the subscripts m, c, and s refer to the mucosal medium, the tissue, and the serosal medium respectively and the subscript sequence ij denotes the
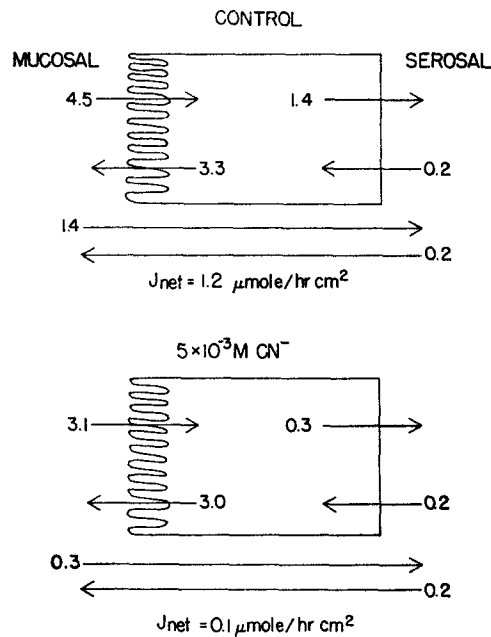

Figure 7. Effect of CN on unidirectional alanine fluxes across rabbit ileum. Above, average values for control tissue. Below, average values obtained on tissues from the same animals treated with 2 mM NaCN. The mucosal and serosal solutions contained 140 mM Na and 5 mM alanine. Fluxes are in μmoles/hr cm^2.

flux from compartment i to compartment j. Thus, knowledge of J_{mc}, J_{ms}, and J_{sm} permits calculation of the remaining three fluxes. Since, as shown in Table I, the value of J_{sm} is relatively invariant from tissue to tissue and is not affected by any of the inhibitors, an average value of 0.15 μmole/hr cm^2 was used for the calculations.

The unidirectional fluxes across control tissue and tissue poisoned with 2 mM cyanide are given in Fig. 7 (J_{mc} is the average of twelve determinations for each condition, and J_{ms} is the average of nine determinations). Data comparing fluxes under control conditions and in the presence of ouabain are given in Fig. 8 (J_{mc} is the average of eight determinations and J_{ms} is the average of six determinations). The influxes, J_{mc}, in the presence of ouabain and cyanide are approximately 30% less than their control values in agreement with the data in Table IV. These decreases in influx account for most of the

observed decreases in the steady-state transmural net flux, so that the efflux of alanine out of the tissue across the mucosal border, J_{cm}, is only slightly different in control and treated tissues. In contrast, both cyanide and ouabain bring about a marked decline in the unidirectional efflux of alanine out of the tissue across the serosal border, J_{cs}, which accounts for almost all the change in the net flux across that boundary.

Comparison of the data of Figs. 7 and 8 with those of Table IV indicates that the fractional inhibition of alanine influx by CN or ouabain is independent of whether or not the tissue was preloaded with alanine. Thus the effect of these inhibitors does not depend upon whether alanine influx is directed
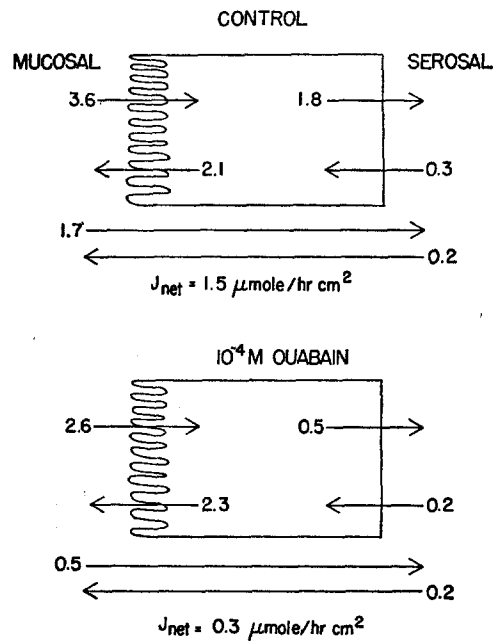

FIGURE 8. Effect of ouabain on unidirectional alanine fluxes across rabbit ileum. Above, average values for control tissues. Below, average values for tissues from the same animal treated with 0.1 mM ouabain. The mucosal and serosal solutions contained 140 mM Na and 5 mM alanine. Fluxes are in μmoles/hr cm².

from a high concentration to a low concentration (tissue preincubated in the absence of alanine, Table IV) or from a low concentration to a higher concentration (tissue preincubated in the presence of alanine, Figs. 7 and 8).

DISCUSSION

Several conclusions, having important bearing on the role of Na in amino acid transport across small intestine, emerge from this study. The Na dependence of transmural amino acid transport and amino acid accumulation by the tissue does not reflect a mechanism in which the sole function of Na is to link metabolic energy directly to the influx mechanism for amino acids at the

brush border of the cell. The results obtained with tissue preincubated in a Na-free medium indicate that neither cyanide nor ouabain significantly affects the Na-dependent alanine influx (Table II), yet these agents markedly inhibit transmural transport (Table I) and tissue accumulation (1). Although DNP and IAA inhibit alanine influx slightly under these conditions, both the Na-dependent and Na-independent fractions are inhibited to the same extent. In view of the results obtained with cyanide and ouabain, it is not unreasonable to ascribe these small effects of DNP and IAA to some nonspecific action unrelated to their roles as metabolic inhibitors. The effects of DNP and IAA on Na-dependent alanine influx are clearly small compared to their effects on transmural net transport.

We have previously demonstrated that mucosal strips of rabbit ileum incubated for 30 min in a Na-free choline medium are not irreversibly damaged since they will accumulate alanine following resuspension in Na Ringer's (1). Also, alanine influx into tissue preincubated in Na-free choline medium does not differ significantly from influx into tissue preincubated in the normal Na Ringer (3). The failure of metabolic inhibitors to affect alanine influx significantly under these conditions unequivocally rules out a direct link between metabolic energy and the influx process. In terms of the model proposed in Fig. 1, these results exclude any direct intervention of metabolic energy in the steps described by K_1, K_2, and P, so that the model suggested to account for the stabilizing effect of Na (Fig. 3) must be rejected. Moreover, any other hypothesis involving a direct link between ATP hydrolysis and alanine influx seems untenable.

Although the present experiments do not provide an explanation for the fact that $K_2 < K_1$, they do narrow the field of possibilities by excluding a direct link with metabolic energy. The question that is as yet unanswered may be rephrased as follows: what is the mechanism by which combination of the carrier–amino acid complex (XA) with Na confers increased stability on the bond between the carrier site and the amino acid? An attractive explanation emerges from recent work on the role of allosteric transitions in enzyme action (14). Thus, it is quite possible that combination of XA with Na results in conformational changes that stabilize the carrier–amino acid bond. If, in addition, X alone has little affinity for Na but combination of X with A markedly increases the affinity of the complex for Na, the requirement that the amino acid combine with the carrier first (discussed in detail in reference 4) can be satisfied. Speculations that carrier mechanisms (15) and, in particular, the role of Na in intestinal sugar transport (16) may be mediated by allosteric transitions have been raised, but direct kinetic evidence for such a mechanism has not been presented.

The results obtained with tissue preincubated in Na Ringer's differ from

those obtained following preincubation in choline Ringer's in that all agents tested inhibited alanine influx by approximately 40%. Nonetheless, influx in the presence of these inhibitors is still markedly enhanced by Na so that Na-dependent influx has not been abolished. Thus, these results support the conclusion that the Na dependence of alanine influx cannot be completely attributed to an obligatory requirement of Na for the direct coupling of metabolic energy to the influx process. The observation that these inhibitors reduce influx into tissue preincubated in Na medium is puzzling. At least two explanations are possible. First, when tissue is incubated in Na Ringer's in the presence of a metabolic inhibitor or ouabain, the intracellular Na concentration rises rapidly; in the presence of ouabain, the intracellular Na concentration of mucosal strips becomes equal to that of the medium within 30 min (1). Although reduction of intracellular Na does not affect alanine influx (3), raising the intracellular Na concentration well above the normal level may cause inhibition. Second, in the presence of inhibitors, the gain of Na and Cl by the cells exceeds the loss of K so that the cell water content increases (1) and cell swelling may lead to depression of alanine influx. Since choline does not appear to penetrate intestinal cells readily (1), marked swelling would not be expected in a tissue incubated in choline Ringer's containing metabolic inhibitors. The possibility that tissue swelling may be responsible for the inhibition of alanine influx is supported by two additional observations. First, tissue preincubated in a medium containing high K concentration exhibits a marked increase in water content (1) and a decreased alanine influx (3). Second, several experiments have been carried out to test the effect of artificially induced swelling on alanine influx. Influxes into tissues bathed in isotonic and hypotonic (tonicity 50% of normal) media were compared; both media contained the same Na and K concentrations and 5 mM alanine. Influx of alanine into tissue swollen by exposure to the hypotonic medium was inhibited by approximately 28% and averaged 2.6 μmoles/hr cm² compared with the control value of 3.6 μmoles/hr cm². Thus, the increased intracellular Na concentration and/or the swelling that characterize tissue preincubated in Na Ringer's with inhibitor might be responsible for the differences noted between preincubation in Na and Na-free solutions. However, since we are unable to devise appropriate experiments to test these possibilities explicitly under well-defined conditions, the suggestions must remain speculative.

Na influx across the mucosal border is not significantly affected (if anything, it is slightly increased) by metabolic inhibitors and ouabain which abolish active transmural transport of Na. Thus, in the steady state, the depression of net transport across the mucosal border must be due to an increase in the efflux out of the cell across that boundary. This increase may be entirely due to an increase in the intracellular Na concentration resulting from inhibition

of an active extrusion mechanism for Na at the serosal or lateral membranes. Although there is evidence that Na transport across the mucosal border is not entirely attributable to simple diffusion (3, 4), all our results (2, 10, 12) are compatible with a single metabolically dependent, ouabain-sensitive active transport mechanism for Na at the serosal border of the cell.

Analysis of Unidirectional Fluxes

Further insight into the effects of inhibitors on Na-dependent alanine transport is derived from analysis of the unidirectional fluxes across the mucosal border, J_{mc} and J_{cm}. Direct measurement indicates that the decrease in J_{net} (Figs. 7 and 8) can be largely accounted for by a decrease in J_{mc} but there must also be marked change in the mechanism responsible for the alanine flux from cell to mucosal solution, J_{cm}. The intracellular alanine concentration of mucosal strips of rabbit ileum incubated in the presence of 5 mM alanine averages 40–50 mM under control conditions, and only 7 mM in the presence of ouabain (1). However, in spite of this marked decline in intracellular alanine concentration, the data in Fig. 8 show that J_{cm} is somewhat greater in the presence of ouabain than in its absence. This observation indicates that the rate coefficient for alanine efflux out of the cell across the mucosal border[3] is increased by ouabain. A similar argument applies to the data obtained using cyanide as inhibitor. Although these observations could also be attributed to a Na-independent efflux mechanism that is saturated at intracellular alanine concentrations greater than 5 mM, this alternative is rendered unlikely by previous data on alanine fluxes in a Na-depleted system given in Fig. 6 of reference 3. The effect of Na depletion in the absence of inhibitors on the steady-state intracellular alanine concentration in mucosal strips is very similar to that of ouabain; the final intracellular alanine concentration does not significantly exceed that in the incubation medium. However, J_{cm} is reduced from a control value of 0.9 μmole/hr cm^2 to 0.5 μmole/hr cm^2 by removal of Na and is, thus, much lower than the values observed with ouabain and cyanide. Consequently, the efflux process does not appear to be saturated at low cellular alanine concentrations.

Although many kinetic treatments could undoubtedly be used to explain these findings, the model we have previously proposed (Fig. 1) to account for the Na-dependent alanine influx agrees qualitatively with all our observations on net flux across the mucosal border. According to this model

$$J_{cm} = \frac{J_A^{im}[A]_c}{\dfrac{K_3 K_4}{K_3 + [\text{Na}]_c} + [A]_c}$$

[3] This coefficient is defined by the ratio of efflux to intracellular concentration, $J_{cm}/[A]_c$.

and, using equations 1 and 5

$$J_{\text{net}} = J_{mc} - J_{cm} = J_A^{im} \left[\frac{[A]_m}{\dfrac{K_1 K_2}{K_2 + [\text{Na}]_m} + [A]_m} - \frac{[A]_c}{\dfrac{K_3 K_4}{K_3 + [\text{Na}]_c} + [A]_c} \right]$$

When the tissue is incubated in a Na-free medium ($[\text{Na}]_m = 0$), $[\text{Na}]_c$ falls rapidly and both J_{mc} and J_{cm} should decline from values under control conditions of $[\text{Na}]_m = 140$ and $[\text{Na}]_c \approx 50$ mM. J_{net} decreases because the decline in J_{mc} is greater than the decline in J_{cm}. If the model shown in Fig. 1 is sym-
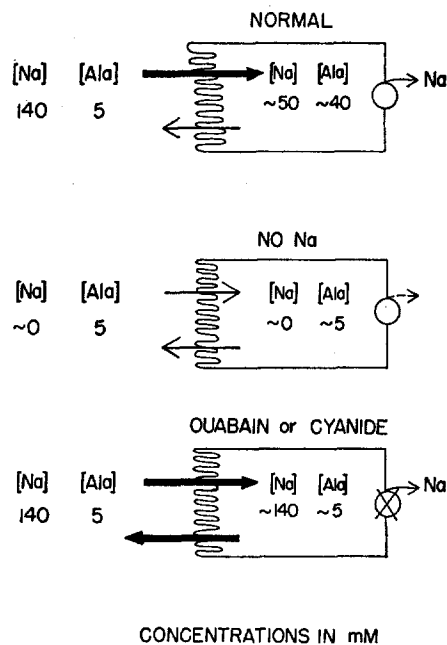

FIGURE 9. Schematic summary of alanine fluxes across mucosal border of rabbit ileum under control conditions (top) in the absence of Na (middle), and in the presence of either metabolic inhibitors or ouabain (bottom). Heavy arrows indicate relatively high flux, light arrows relatively low flux.

metrical so that $K_1 = K_4$ and $K_2 = K_3$, $J_{\text{net}} = 0$ when $[\text{Na}]_m = [\text{Na}]_c$ and $[A]_m = [A]_c$. When the tissue is incubated in a medium containing 140 mM Na plus ouabain the intracellular Na concentration rises and the rate coefficient for alanine efflux ($J_{cm}/[A]_c$) increases. If the model is symmetrical, $J_{\text{net}} = 0$ when $[\text{Na}]_c = [\text{Na}]_m$ and $[A]_c = [A]_m$. Thus, both Na depletion and ouabain decrease the rate of net alanine uptake by mucosal strips of rabbit ileum and abolish accumulation and transmural transport against concentration differences. However, as shown schematically in Fig. 9 quite different mechanisms are involved in spite of the gross similarities between the over-all effects. In the absence of Na, both J_{mc} and J_{cm} are markedly reduced, while in the presence of ouabain the bidirectional fluxes across the mucosal border are still quite high and the inhibitory action is primarily the result of a

six- to eightfold increase in the efflux constant. As pointed out previously (1) these differences in the modes of action of Na depletion and ouabain could not be discerned by studies of net uptake alone, since in both instances the rates of net uptake are reduced.

The present results are thus *qualitatively* consistent with the model presented in Fig. 1 and support the concept that the net flux of alanine across the mucosal border is determined primarily by the concentrations of alanine and Na on the two sides of this barrier. The data indicate that metabolic energy is not coupled directly to alanine influx. However, the results do not rule out a direct link between metabolic energy and efflux across the brush border. That is, metabolic energy could be utilized to alter X and to favor dissociation of XA or XANa at the cytoplasmic side of the membrane. Since, in the present model, influx is not dependent on events at this side of the barrier, (equation 1, see also Appendix A, reference 4), we cannot assume that measurements of influx provide any insight into these events. Nonetheless, a direct link between efflux and metabolism does not, at present, appear necessary, and the effects of metabolic inhibitors may be due simply to the increased intracellular Na concentrations resulting from the inhibition of the mechanism responsible for active extrusion of Na from the cell. A more definitive statement awaits an explanation for the action of inhibitors on influx into tissue preincubated in the presence of Na, and a detailed study of alanine efflux as a function of intracellular Na concentration. The latter is hampered by the difficulties involved in determining intracellular Na concentrations, the uncertainties regarding extracellular vs. intracellular compartments, and the possible lack of homogeneity in the distribution of intracellular Na.

Finally, it is of interest to consider the effects of inhibitors on the bidirectional fluxes across the serosal membrane, J_{cs} and J_{sc}. These fluxes are entirely calculated from equations 3–5 and are subject to uncertainties that do not affect the analysis of steady-state fluxes across the mucosal border. Equations 3 and 4 are derived assuming that the tissue is a single, well-mixed compartment, and they do not take into account the role of the serosal tissues as a barrier to diffusion of alanine into the serosal solution after it has left the epithelial cells. During net transport, there must be a concentration gradient for alanine in the serosal tissues and the difference between the concentration in the submucosal space immediately adjacent to the epithelial layer and that in the serosal solution must depend on the rate of alanine transport and the permeability of the serosal barrier. An explicit evaluation of the effect of this diffusion barrier is difficult, but the observation that marked reduction in the rate of net transport does not significantly influence the calculated J_{sc} (Figs. 7 and 8, and also Fig. 6 of reference 3) suggests that the diffusion barrier does not seriously affect the compartmental analysis.

Constancy of J_{sc} would be expected if this flux were entirely determined by

the alanine concentration in the serosal solution, and, like J_{mc} (3), were not subject to transconcentration effects. Similarly, J_{cs} behaves as if it were directly determined by the intracellular alanine concentration since it declines markedly in the presence of ouabain and CN. As pointed out previously (3), the alanine flux ratio across the serosal membrane does not appear to differ significantly from the expected alanine concentration ratio. For example, the data given in Fig. 8 are consistent with a serosal concentration of 5 mM, a control intracellular concentration of 35 mM, and an intracellular concentration of 7.5 mM in the CN-poisoned tissue; in the light of previous studies, these values for intracellular concentrations are not unreasonable (1). The above considerations suggest that under these conditions alanine transport across the serosal membrane is a passive process, but they do not permit distinction between simple diffusion and carrier-mediated transfer.

In summary, the present results suggest that the effects of metabolic inhibitors and ouabain on alanine accumulation and transmural transport are due primarily to a decrease in the net flux of alanine across the mucosal membrane. This decrease is accounted for, in part, by a decrease in influx and, in part, by a marked increase in the rate coefficient for alanine efflux across the mucosal border. The latter effect may be attributed to an increase in intracellular Na resulting from inhibition of active Na extrusion from the cell. No compelling evidence for a direct link between metabolic energy and alanine transport has been demonstrated, nor can the role of Na in alanine influx be solely that of coupling metabolic energy to this transport process. The possibility that the stabilizing effect of Na on the carrier—amino acid complex is due to an allosteric transition brought about by combination with Na has been raised. Alanine transport across the serosal membranes appears to respond passively to changes in the steady-state intracellular alanine concentration caused by events at the brush border.

BIBLIOGRAPHY

1. Schultz, S. G., R. E. Fuisz, and P. F. Curran. 1966. Amino acid and sugar transport in rabbit ileum. *J. Gen. Physiol.* 49:849.
2. Field, M., S. G. Schultz, and P. F. Curran. 1967. Alanine transport across isolated rabbit ileum. *Biochim. Biophys. Acta.* 135:236.

3. Schultz, S. G., P. F. Curran, R. A. Chez, and R. E. Fuisz. 1967. Alanine and sodium fluxes across mucosal border of rabbit ileum. *J. Gen. Physiol.* **50**:1241.

4. Curran, P. F., S. G. Schultz, R. A. Chez, and R. E. Fuisz. 1967. Kinetic relations of the Na-amino acid interaction at the mucosal border of intestine. *J. Gen. Physiol.* **50**:1261.

5. Kinter, W. B., and T. H. Wilson. 1965. Autoradiographic study of sugar and amino acid absorption by everted sacs of hamster intestine. *J. Cell Biol.* **25** (2, Pt. 2): 19.

6. Crane, R. K. 1962. Hypothesis for mechanism of intestinal active transport of sugars. *Federation Proc.* **21**:891.

7. Schultz, S. G., and R. Zalusky. 1964. Ion transport in isolated rabbit ileum. II. The interaction between active sodium and active sugar transport. *J. Gen. Physiol.* **47**:1043.

8. Riggs, T. R., L. M. Walker, and H. N. Christensen. 1958. Potassium migration and amino acid transport. *J. Biol. Chem.* **233**:1479.

9. Vidaver, G. A. 1964. Glycine transport by hemolyzed and restored pigeon red cells. *Biochemistry.* **3**:795.

10. Schultz, S. G., and R. Zalusky. 1965. Interactions between active sodium transport and active amino acid transport in isolated rabbit ileum. *Nature.* **205**:292.

11. Csáky, T. Z. 1963. A possible link between active transport of electrolytes and non-electrolytes. *Federation Proc.* **22**:3.

12. Schultz, S. G., and R. Zalusky. 1964. Ion transport in isolated rabbit ileum. I. Short-circuit current and Na fluxes. *J. Gen. Physiol.* **47**:567.

13. Ussing, H. H., and K. Zerahn. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23**: 110.

14. Monod, J., J. Wyman, and J. Changeux. 1965. On the nature of allosteric transitions: A plausible model. *J. Mol. Biol.* **12**:88.

15. Jardetzky, O. 1966. Simple allosteric model for membrane pumps. *Nature.* **211**: 969.

16. Crane, R. K. 1965. Na-dependent transport in the intestine and other animal tissues. *Federation Proc.* **24**:1000.