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ALANINE TRANSPORT ACROSS ISOLATED RABBIT ILEUM

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SUMMARY

(1) When both surfaces of isolated segments of rabbit ileum are bathed with a Ringer's solution containing 140 mM Na^+ and 5 mM L-alanine, there is an average net flux of L-alanine from the mucosal to the serosal solution of 1.2 μmoles/h·cm^2. Under these conditions the unidirectional flux from mucosa to serosa is approximately ten times the flux in the opposite direction. The net flux of alanine is nearly equal to the previously observed increment in active Na^+ flux brought about by the addition of 5 mM alanine to the mucosal solution.

(2) Net transport of L-alanine is markedly inhibited if the Na^+ in the bathing solutions is replaced with choline, or if 10^{-4} M ouabain is added to the serosal solution in the presence of Na^+. In both instances, the inhibition of net alanine transport is the result of a decline in the unidirectional flux from mucosa to serosa. Addition of ouabain to the mucosal solution alone does not significantly inhibit transport of the amino acid.

(3) There is net transport of D-alanine across rabbit ileum in the absence of a concentration difference and the tissue accumulates D-alanine to relatively high concentrations. This accumulation is markedly inhibited by ouabain and by replacement of the Na^+ in the incubation medium with choline.

INTRODUCTION

Transport of neutral amino acids across small intestine from a low concentration in the mucosal solution to a higher concentration in the serosal solution requires Na^+ and is inhibted by digitalis glycosides[1-6]. In addition, a rapid and sustained increase in the rate of active Na^+ transport from mucosa to serosa[7] is observed when L-alanine is added to the solution perfusing the mucosal surface of rabbit ileum. The same effect, as measured by short-circuit current, is produced by several other amino acids, including D-alanine.

The present investigation explored further the interaction between Na^+ and neutral amino acid transport in small intestine. Undirectional fluxes of L-alanine

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across rabbit ileum have been measured in the same preparation *in vitro* used previously for studies of Na^+ transport[8] in order to estimate the quantitative relation between the net transmural fluxes of Na^+ and L-alanine. The effects of ouabain and Na^+ replacement have been determined in order to establish whether the decrease in net flux observed in sac preparations of small intestine is primarily the result of a decrease in mucosal to serosal flux or an increase in serosal to mucosal flux. Finally, unidirectional transmural fluxes and tissue accumulation of D-alanine have been measured to determine whether this amino acid, which appears to stimulate Na^+ transport, is itself actively transported. In view of previous reports that there is no transport of D-alanine into everted sacs of hamster intestine against a concentration difference[9], it seemed important to examine this process in rabbit ileum.

METHODS

New Zealand white rabbits, weighing 2.5–4.0 kg and maintained on normal food intake, were sacrificed by intravenous injection of pentobarbitol (50 mg/kg). A segment of distal ileum was quickly excised, opened along the mesenteric border, rinsed clean of luminal contents and clamped as a flat sheet between two identical lucite half-chambers. In this apparatus, both surfaces of the tissue are perfused and oxygenated by means of water-jacketed, gas-lift circulating systems which maintain the bathing solutions at 37°. The gas mixture employed was 95% O_2–5% CO_2. Unless otherwise indicated, the composition of the bathing solution was: NaCl, 140 mM; $KHCO_3$, 10 mM; K_2HPO_4, 1.2 mM; KH_2PO_4, 0.2 mM; $CaCl_2$, 1.2 mM, and $MgCl_2$, 1.2 mM. Apparatus and methods employed for the determination of the transmural electrical potential difference and the short-circuit current have been described previously[8].

For the determination of transmural unidirectional fluxes of L- or D-alanine, both sides of the tissue were bathed with 15 ml of solution containing equal concentrations of the amino acid. A tracer quantity of ^{14}C-labeled amino acid (New England Nuclear Corp.) was introduced into the solution bathing one side of the tissue, and, after a 35–40-min period (necessary for the achievement of a steady-state flux of tracer (see Fig. 1)) serial samples were withdrawn at 10-min intervals from the opposite bathing solution. The samples and suitably diluted aliquots of the initially labeled solution were assayed for ^{14}C using a liquid-scintillation spectrometer. Since L-alanine is not metabolized to any significant extent by rabbit ileum under these conditions[10], unidirectional fluxes may be calculated directly from the rate of appearance of ^{14}C on the unlabeled side, as described previously[8]. The amount of alanine transferred from mucosa to serosa during an experiment was less than 5% of the alanine content of the bathing solutions (see Table I) so that the concentrations of alanine remained essentially constant. In most experiments simultaneous flux measurements were made on two adjacent pieces of ileum from the same animal, thus permitting either a determination of both transmural unidirectional fluxes under the same condition, or a comparison of the unidirectional flux in one direction under two different conditions.

Accumulation of D-alanine by mucosal strips of rabbit ileum was determined as described previously[10]. Intracellular concentrations were calculated after correction for the D-alanine content of the extracellular space measured in each tissue with tritiated inulin.

Optical purity of D-*alanine*

D-Alanine, having a specific rotation, $[\alpha]_D$, of $-14.5°$, was obtained from the California Corporation for Biochemical Research (Calbiochem, Lot 68091). D-[1-^{14}C]-Alanine was obtained from the New England Nuclear Corporation (Lot 211-110-12) and was certified 99.7% optically pure by the manufacturers. The purity of the D-alanine (^{12}C and ^{14}C compounds) was checked in this laboratory by ascending paper chromatography using a butanol–acetic acid–water (25:4:10, v/v/v) mixture as solvent. The ^{12}C and ^{14}C compounds were chromatographically homogeneous and migrated together with L-alanine (Nutritional Biochem.). The optical purity of the D-[^{14}C]-alanine was tested using D-amino acid oxidase (crude preparation from hog kidney) obtained from Sigma Chemical Co. (Lot 26B-1820). The D-[^{14}C]alanine (0.29 μmole) was incubated with the enzyme (20 mg) at 37° in 3 ml of a pyrophosphate buffer (pH 8.3) under an atmosphere of 100% O_2. After 120 min only 2.1% of the original radioactivity migrated with D-alanine on paper chromatographs. To demonstrate that D-alanine is neither chemically altered nor racemized by rabbit ileum, tissue was incubated for 60 min in a Ringer's solution containing 5 mM D-[^{14}C]alanine. The incubation solution and the tissue extract were chromatographed before and after treatment with D-amino acid oxidase. Before treatment with the enzyme, 96% of the radioactivity migrated with D-alanine as a single peak. After 120 min of incubation with D-amino acid oxidase only 3.5% of the original radioactivity migrated with D-alanine. These results indicate that D-alanine is neither metabolized nor racemized to any significant extent by rabbit ileum. Thus, transport of D-alanine across the tissue and accumulation within the tissue may be determined from assays of ^{14}C as described above.

RESULTS

Unidirectional and net fluxes of L-*alanine*

The appearance of L-[^{14}C]alanine in the serosal bathing solution is plotted as a function of time in Fig. 1. A steady-state flux is not achieved until 35–40 min after addition of tracer to the mucosal solution. Two factors contribute to the delay: the time necessary for the mucosal cells to reach a steady-state concentration of L-[^{14}C]-alanine and the time necessary for L-[^{14}C]alanine to achieve a steady-state rate of

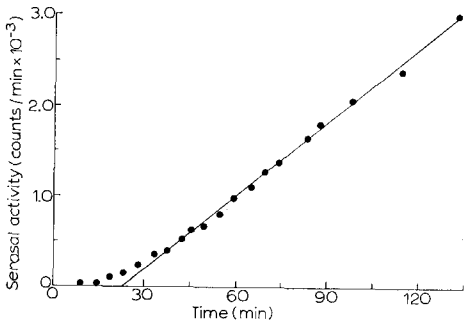

Fig. 1. L-[^{14}C]Alanine activity in the serosal solution as a function of time after the addition of the tracer to the mucosal solution. A steady-state flux of 1.4 μmoles/h·cm^2 (J_{ms}) is achieved after a delay of 37 min.

diffusion across the serosal tissues following exit from the mucosal cells. We are unable, at present, to estimate the relative contribution of each of these factors to the observed delay. MATTHEWS AND LASTER[11] have reported that a steady-state rate of net accumulation of amino acids in everted sacs of rat intestine is observed only after 10–15 min have elapsed. However, because serial sampling is difficult with the everted-sac technique, this lag has generally been ignored.

Mucosa-to-serosa (J_{ms}), serosa-to-mucosa (J_{sm}) and net fluxes (J_{net}) of L-alanine, when both surfaces of the tissue were bathed with the standard Ringer's solution containing 5 mM L-alanine, are given in Table I. Under these conditions, J_{ms} was

TABLE I

TRANSMURAL FLUXES OF L- AND D-ALANINE

Number of experiments in parentheses. Each experiment involved at least four sampling periods.

Amino acid	*Medium*	J_{ms} *(μmoles/h · cm²)*	J_{sm} *(μmole/h · cm²)*	J_{net} *(μmoles/h · cm²)*
L-Alanine 5 mM	Normal Ringer's $[Na^+]$ = 140 mM	1.3 ± 0.1 (19)	0.13 ± 0.02 (4)	1.2
	Choline Ringer's $[Na^+]$ = 0	0.16 ± 0.01 (4)	0.13 ± 0.02 (3)	0.03
	Normal Ringer's *plus* 10^{-4} M ouabain in serosal solution*	0.39 ± 0.03 (2)	0.11 ± 0.03 (2)	0.28
D-Alanine 20 mM	Normal Ringer's	1.8 ± 0.3 (4)	0.29 ± 0.07 (4)	1.5

* Fluxes were determined at least 60 min after addition of ouabain.

approx. ten times greater than J_{sm} and a net alanine flux from mucosa to serosa of 1.2 μmoles/h · cm² was observed. Flux measurements on open-circuited and short-circuited preparations have been combined in the table because transmural electrical potential differences between 0 and 10 mV do not significantly effect L-alanine fluxes. In four paired experiments J_{ms} was 1.3±0.3 μmoles/h · cm² when the tissue was short-circuited and 1.2±0.6 μmoles/h · cm² when the transmural electrical potential difference was clamped at 10 mV, serosa positive.

Unidirectional L-alanine fluxes across intestine perfused on both sides with medium in which all sodium was replaced by choline were determined on paired tissue from the same animal (see Table I). Net L-alanine transport disappeared, confirming the observations of others on the Na^+ dependence of amino acid transport[1,3–6]. The inhibition of net flux is entirely the result of a marked decline in J_{ms}.

The effect of 10^{-4} M ouabain in the serosal solution on unidirectional L-alanine fluxes is also shown in Table I. J_{ms} and the net flux were decreased by 80%, while J_{sm} remained unchanged. This concentration of ouabain also abolished the transmural electrical potential difference within 30 min and has previously been shown to inhibit active sodium transport completely[8]. In four experiments, J_{ms} was determined on paired tissues from the same animal under control conditions and in the presence of $2 \cdot 10^{-4}$ M ouabain in the mucosal solution. The average values of J_{ms} were 1.1±0.3 μmoles/h · cm² in control tissues and 1.0±0.2 μmole/h · cm² in the presence of ouabain. The average decrease in the transmural potential difference after 1 h exposure to

ouabain in the mucosal solution was only 28% compared to a 7% spontaneous decrease for the control tissue.

Effect of temperature on L-*alanine transport*

Studies of the unidirectional influx of L-alanine across the brush border of rabbit ileum[22] have indicated that this process has an unusually large temperature dependence ($Q_{10} = 4.5$). Furthermore, the increase in short-circuit current across rabbit ileum elicited by actively transported sugars or amino acids is markedly depressed at room temperature (22°). The Q_{10} of this process, for 10 mM glucose, is 4.8 (ref. 12). In view of these observations, it was of interest to examine the effect of temperature on transmural L-alanine flux. In four experiments, J_{ms} for 5 mM L-alanine was determined at 22° and at 37° on paired tissues. J_{ms} was 1.2 ± 0.2 μmoles/h·cm^2 at 37° and 0.18 ± 0.03 μmole/h·cm^2 at 22°; the average Q_{10} was 3.7.

Transport and accumulation of D-*alanine*

A time-course of D-alanine uptake by mucosal strips incubated at 37° in standard solution containing 5 mM D-alanine is shown in Fig. 2. D-Alanine was rapidly accumulated by the tissue and after 3 min the intracellular concentration exceeded that in the incubation medium. The intracellular concentration after 30 min was approx. 10 times the external solution concentration. The time-course shown in Fig. 2 closely

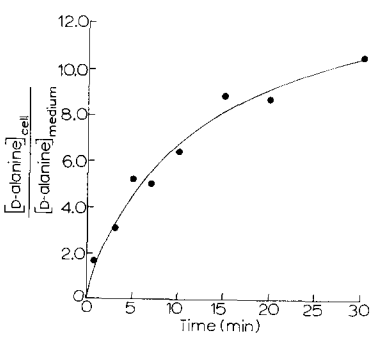

Fig. 2. Time-course of D-alanine uptake. All tissues were from the same animal and were preincubated for 30 min in the standard buffer before addition of 5 mM D-alanine.

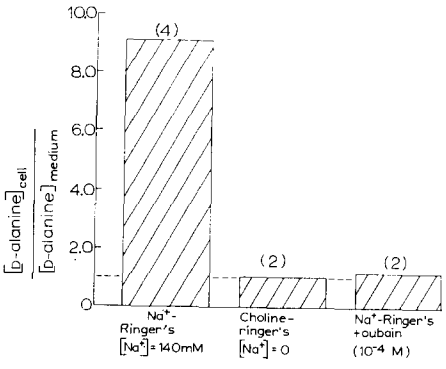

Fig. 3. D-Alanine concentration ratios under different conditions. All tissues were preincubated for 30 min under the conditions indicated and then for an additional 30 min in the presence of 5 mM D-alanine. The numbers of experiments are given in parentheses.

resembles that previously reported for L-alanine uptake by mucosal strips[10]. The effects of Na^+-free media and ouabain on the ratio of intracellular to extracellular D-alanine concentrations obtained after 30 min incubation at 37° in the presence of 5 mM D-alanine are shown in Fig. 3. D-Alanine accumulation was markedly inhibited in both instances. A similar effect of Na^+-free medium and ouabain on L-alanine accumulation was previously reported[10].

Unidirectional fluxes of D-alanine were determined when the concentration of this amino acid in both the mucosal and serosal bathing solutions was 20 mM. In four

experiments, J_{ms} and J_{sm} were determined simultaneously on adjacent pieces of tissue from the same animal. The results, shown in Table I, indicate a net flux of 1.5 μmoles/h·cm^2 under these conditions.

DISCUSSION

L-*Alanine transport*

Addition of L-alanine to the solution bathing the mucosal surface of short-circuited rabbit ileum elicits an immediate increase in the rate of active Na^+ transport from mucosa to serosa[7]. One purpose of the present investigation was to determine the rate of L-alanine transport across this tissue using methods identical to those employed for the Na^+ studies. The rate of net L-alanine transport was 1.2 μmoles/h·cm^2 at 5 mM alanine and the increase in active Na^+ transport in the presence of 5 mM L-alanine was 1.3 μmoles/h·cm^2 (Table I and Fig. 2 of ref. 7). Although these rates of L-alanine and Na^+ transport were not determined simultaneously, both studies were carried out on approximately the same region of distal ileum from the same species and size of rabbit, utilizing the same techniques so that the results should be roughly comparable. Thus the rate of net L-alanine transport and the increase in the rate of active Na^+ transport induced by the alanine appear to be nearly equivalent. Recent studies[22] have also indicated that the unidirectional influx of L-alanine across the mucosal border of rabbit ileum in the presence of 140 mM Na^+ is accompanied by an increase in Na^+ influx which is approximately equal to the alanine influx.

D-*Alanine transport*

The early studies of WISEMAN and his collaborators indicated that amino acid transport mechanisms in small intestine show a marked preference for the L-stereoisomer[13]. Subsequent investigations have shown, however, that this optical specificity is not absolute. JERVIS AND SMYTH[14] demonstrated that D-methionine is transported against a concentration gradient across the small intestine of rat, and CHRISTENSEN[15] reported that the rates of L- and D-valine transport by isolated rat intestine are nearly the same. Furthermore, several investigators reported that the transport of some L-amino acids is inhibited by the presence of high concentration of D-amino acids[16,17], and, conversely, that the rate of D-amino acid transport is markedly inhibited in the presence of the L-stereoisomer[16,18]. Although these findings suggest that D- and L-amino acids may share the same carrier mechanisms and that only their affinities for these mechanisms differ markedly, there are reports that several D-amino acids, including D-alanine, are neither accumulated by intestinal tissue nor transported against a concentration difference to a significant extent[9,18]. The observation that D-alanine elicits a rapid increase in the short-circuit current across rabbit ileum *in vitro* prompted an investigation of transport of D-alanine by this preparation; if net transport of D-alanine by rabbit ileum in the absence of a concentration gradient could not be demonstrated, our present concepts regarding the interaction between Na^+ transport and active transport of non-electrolytes would require major revision. The present data demonstrate that the mucosal cells of rabbit ileum accumulate D-alanine against a concentration difference and that transmural transport of D-alanine occurs in the absence of a concentration difference. The transport processes for the stereoisomers of alanine appear to be qualitatively similar. Since the degree of optical

asymmetry of alanine is relatively small compared with other amino acids (with the exception of glycine) our findings with respect to D-alanine cannot be generalized to the transport of other D-amino acids.

Effect of Na^+ and ouabain

In the absence of sodium, or when ouabain is added to the serosal solution, net L-alanine transport is almost completely abolished. This is entirely the result of a marked inhibition of the flux from mucosa to serosa. A previous investigation from this laboratory[19] demonstrated that the unidirectional influx of L-alanine from the mucosal solution across the brush border into the cell, which results in transport from a lower to a higher L-alanine concentration, is markedly inhibited if the Na^+ in the mucosal solution is replaced by a variety of charged or uncharged solutes. This study and those of other investigators[20], provide evidence that the mechanism responsible for the transport of L-alanine against a concentration difference is located on or near the brush border of the mucosal cell. However, ouabain causes a marked inhibition of net L-alanine transport only when present in the serosal solution. Ouabain in the mucosal solution has little, if any, effect. CSAKY AND HARA[21] have made a similar observation with respect to the inhibitory effect of ouabain on 3-*O*-methylglucose transport across bullfrog small intestine and have further demonstrated that little or no ouabain enters the cell water or the mucosal solution when this agent is added to the serosal solution. The present results, and the observation of CZAKY and HARA, suggest that ouabain does not act directly on the Na^+-dependent L-alanine transport mechanism at the brush border but that it exerts its inhibitory action on a process located on or near the serosal border of the cell. In a previous study, evidence was presented that the process responsible for the transmural transport of Na^+ against its electrochemical potential gradient is located on or near the serosal border[10] and it has also been shown that the inhibitory effect of ouabain on active transmural Na^+ transport is most marked when the ouabain is added to the serosal bathing solution[8]. These observations support the hypothesis[7] that ouabain inhibits sugar and amino acid transport indirectly through its inhibitory action on the sodium transport mechanism; no direct action on non-electrolyte transport mechanisms need be postulated. The extrusion of Na^+ from the cell and the resulting Na^+ concentration difference across the mucosal border appear to be indispensable prerequisites for net amino acid transport.

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