J. Physiol. (1982), **327**, *pp.* 53–64
With 8 text-figures
Printed in Great Britain

AMINO ACID INHIBITION AND STIMULATION OF 2-AMINOISOBUTYRIC ACID EXIT FROM ANURAN SMALL INTESTINE

BY C. A. R. BOYD AND VIVIEN S. PERRING*

From the Department of Physiology, University of Dundee, Dundee DD1 4HN and the Department of Human Anatomy, South Parks Road, Oxford OX1 3QX

(*Received 6 October 1981*)

SUMMARY

1. Using the vascularly perfused frog small intestine, the exit of the non-metabolized amino acid 2-aminoisobutyric acid (AIB) from the pre-loaded epithelium into the blood has been studied in winter animals.

2. Marked inhibition of the instantaneous rate constant for AIB exit into the vascular bed is observed when L-leucine, but not D-leucine, is added either to the intestinal lumen or to the vascular bed. The extent of the inhibition is related to the leucine concentration in an alinear fashion. The concentration of luminal L-leucine giving half maximal inhibition is 2·5 mM.

3. The instantaneous rate constant for AIB exit is similarly decreased by 10 mM-L-tryptophan and by L-phenylalanine added to the intestinal lumen and to a lesser extent by L-asparagine, L-valine, L-glutamine, L-isoleucine, and L-norleucine.

4. 10 mM-L-proline added to the lumen stimulates AIB exit from the pre-loaded epithelium into the blood. This stimulation is due to an increased rate constant for movement of AIB across the basolateral membrane.

5. No inhibition is found when the dipeptide L-leucyl-L-leucine (10 mM) is added to the intestinal lumen in the presence of 10 mM-L-leucine. When added to the vascular compartment this dipeptide has no effect upon AIB exit from the epithelium.

6. Possible mechanisms by which amino acids and peptides may influence AIB movement out of the epithelium into the blood are discussed and conclusions are drawn concerning AIB transport across the intestinal basolateral membrane of the intact epithelium.

INTRODUCTION

The different transport characteristics of the brush border and of the basolateral membrane impart polarity to the intestinal epithelium which underlies its absorptive function. However it has until relatively recently been easier to characterize experimentally the properties of the microvillous membrane, adjacent to the lumen, than to examine properties of the blood-facing membrane. Measurements of amino acid flux across the basolateral membrane in short-circuited intestinal sheets (Munck

* Present address: Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

& Schultz, 1969; Munck, 1980; Nassar, Khuri & Hajjar, 1980) and in membrane vesicles prepared by differential gradient centrifugation (Hopfer, Sigrist-Nelson, Ammann & Murer, 1976; Mircheff, van Os & Wright, 1980) have been made, yet the vascularly perfused intestine allows the exit step in absorption to be studied in the presence of vascular flow and therefore provides important additional information to that obtained under less physiological conditions. The present experiments complement such previous findings in demonstrating that in the intact epithelium of frog small intestine the basolateral membrane appears to possess a transport system for 2-aminoisobutyric acid (AIB) and for a specific group of other neutral amino acids. This group includes L-leucine whose transfer out of the epithelium recently has been described by Cheeseman (1979, 1981). This paper describes characteristics of this system and amplifies a previous preliminary report (Boyd & Perring, 1981).

METHODS

The vascularly perfused small intestine of the frog (*Rana ridibunda*) has been used as described by Boyd, Cheeseman & Parsons (1975). The epithelium is loaded for 45 min with the non-metabolized amino acid 2-aminoisobutyric acid (AIB) by perfusing [^{14}C] labelled AIB (1 mM) through the lumen until a steady-state rate of AIB transfer from lumen to vascular bed is achieved. The amino acid is abruptly removed from the lumen and its exit from the epithelium into the vascular bed studied during the subsequent washout. To test the specificity of the basolateral exit system(s) available to AIB, the intestinal lumen is pulsed briefly during AIB washout with a second amino acid (10 mM). In order to localize the site of action of any amino acid interacting with AIB during its washout, both luminal and portal venous effluents are collected allowing AIB appearance in lumen and vascular bed during washout to be monitored; this permits calculation of the instantaneous rate constants for AIB appearance in the luminal and vascular compartments.

All animals were housed in running water at ambient temperature and were allowed free access to food (crickets) twice a week. All experiments reported here were performed between December and February (i.e. using 'winter' animals; Boyd & Perring, 1980).

Materials

Frogs (*Rana ridibunda*) were supplied by Maved, Budapest, Hungary and Southern House Crickets (*Acheta domestica*) by Xenopus Ltd., Surrey. D- and L-amino acids, Sigma grade, were obtained from Sigma, London and 2-amino [1-^{14}C] isobutyric acid and 3-0-methyl-D-[1-^{3}H] glucose from Amersham International Ltd.

RESULTS

AIB washout from epithelium: effect of adding leucine to the lumen

Fig. 1 shows the washout of AIB into the portal venous effluent from the small intestinal epithelium which had been loaded previously from the lumen with 1 mM-amino acid. After the initial 25 min of washout, 10 mM L-leucine was added to the lumen for a 15 min period as a result of which AIB appearance in the portal venous effluent fell markedly (mean inhibition by 10 mM-L-leucine, $37 \pm 2\%$ ($n = 60$)). The experiment was then repeated following a further identical loading of the epithelium from the lumen with AIB; this time after 25 min of washout the lumen was pulsed with 10 mM-D-leucine which resulted in a very much smaller fall in vascular AIB appearance rate. This shows that the inhibitory effect of leucine upon AIB washout is stereospecific.

In order to find out with which face of the epithelium leucine was interacting to produce this inhibition of vascular AIB appearance the effluent flowing from both the portal vein and the intestinal lumen was studied during AIB washout. Knowing both the quantity of amino acid appearing in these solutions as a function of duration of washout and the quantity of amino acid remaining in the tissue at the end of the

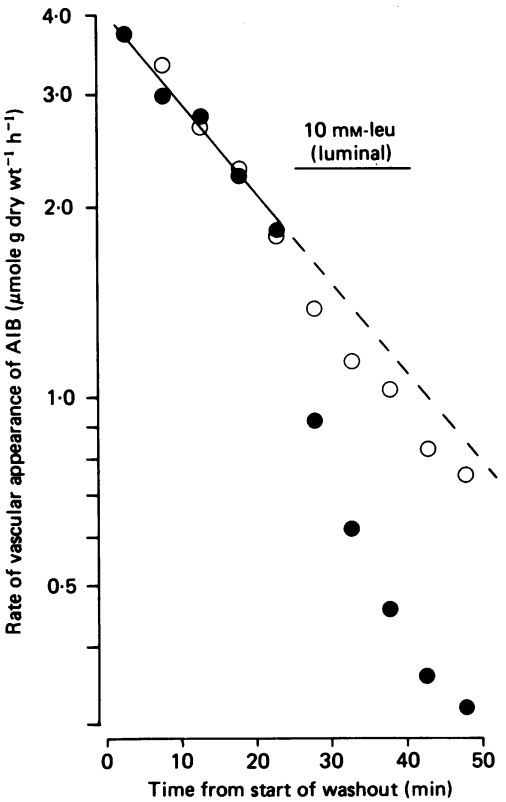

Fig. 1. Influence of leucine (leu; 10 mM) added to the intestinal lumen upon the washout of AIB from the pre-loaded small intestinal epithelium into the vascular bed. Two washouts were performed following identical loading with AIB and the results are superimposed. During the first (●) L-leucine and during the second (○) D-leucine was added to the lumen for the period indicated.

experiment, the instantaneous rate constants for AIB movement from the pre-loaded epithelium into the blood and into the lumen were calculated. Fig. 2 shows a plot of the mean normalized results from ten experiments. The finding that the rate constant for AIB vascular appearance is decreased markedly by adding leucine to the intestinal lumen whereas that for appearance in the lumen is virtually unaltered (Fig. 7 shows the appearance of the control curve for the AIB instantaneous rate constant) demonstrates that the inhibition shown in Fig. 1 is a consequence of a decrease in AIB exit from the epithelium into the blood and is not the result of increased backflux into the lumen. The extent of the inhibition of vascular AIB appearance by leucine added to the intestinal lumen is dependent upon the concentration of

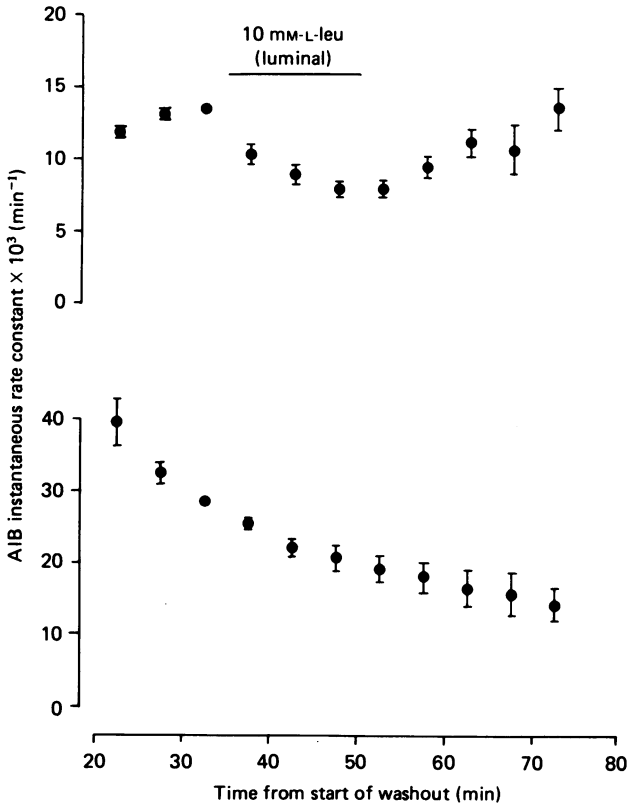

Fig. 2. Instantaneous rate constants for AIB washout from the pre-loaded intestine into the lumen (lower graph) and vascular (upper graph) compartments. 10 mM-L-leucine was added to the lumen for the period indicated. Mean (±S.E.M.) normalized results of ten different experiments are shown.

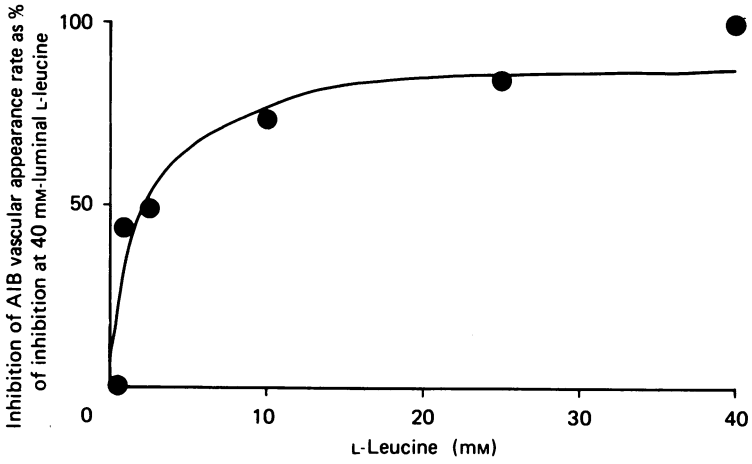

Fig. 3. Inhibition of AIB washout into the vascular bed as a function of luminal L-leucine concentration.

leucine used (Fig. 3). 0·1 mM-leucine gives no detectable inhibition whereas near maximal inhibition is found with 10 mM-leucine. The leucine concentration giving half-maximal effect is about 2·5 mM. Fig. 4 shows the result of an experiment designed to test the possibility that the saturation of the leucine inhibition of AIB efflux across the basolateral membrane (shown in Fig. 3) merely reflects the limited ability of the

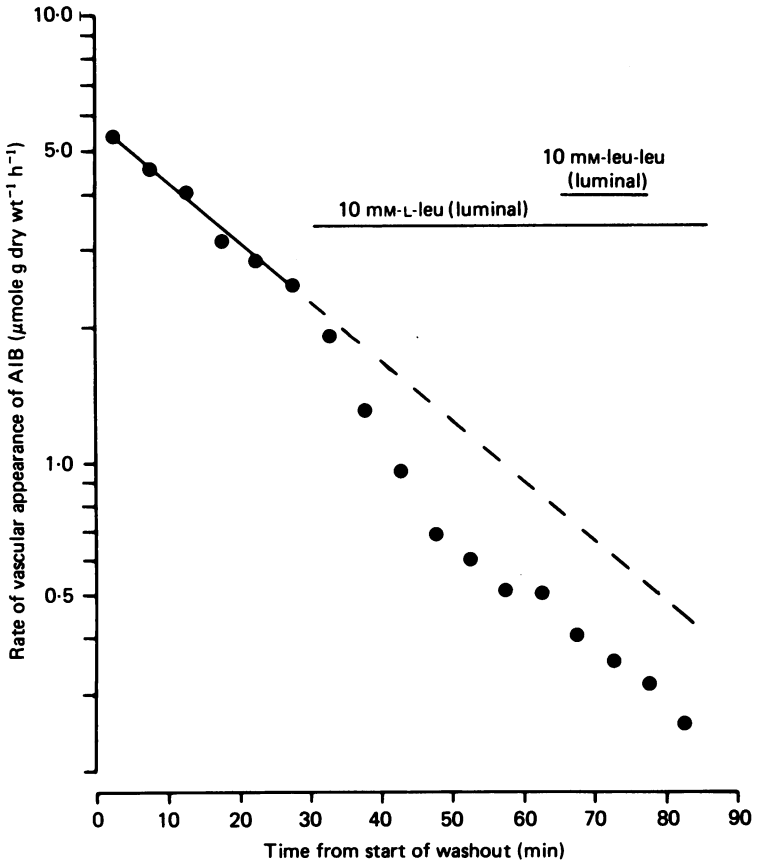

Fig. 4. Effect of 10 mM-luminal L-leucine and subsequently and additionally 10 mM-L-leucyl-L-leucine (leu-leu) upon AIB washout from the pre-loaded small intestine into the vascular bed.

inhibitor to gain access to the site of inhibition. Since leucine may separately enter the epithelium from the lumen in the form of intact dipeptide (Cheeseman & Parsons, 1974; Ward & Boyd, 1980) which is then hydrolysed intracellularly, luminal leucyl-leucine in the presence of a saturating concentration of monomer may be used to increase the intracellular concentration of amino acid. That no additional inhibition of AIB exit is seen under such circumstances (Fig. 4) strongly indicates that the maximal inhibitory effect observed is not a consequence of saturation of the brush border entry mechanism for leucine.

Steady-state rate of transepithelial AIB transfer: effect of adding leucine to the lumen

Fig. 5 illustrates an experiment the results of which enable washout experiments to be interpreted more easily. After an initial 85 min period during which the lumen was perfused with AIB, a steady-state rate of vascular AIB appearance is reached. On then adding 10 mM-L-leucine to the luminal perfusate a rapid decrease in the rate of transepithelial AIB transfer occurs, thus after 15 min exposure to leucine the rate

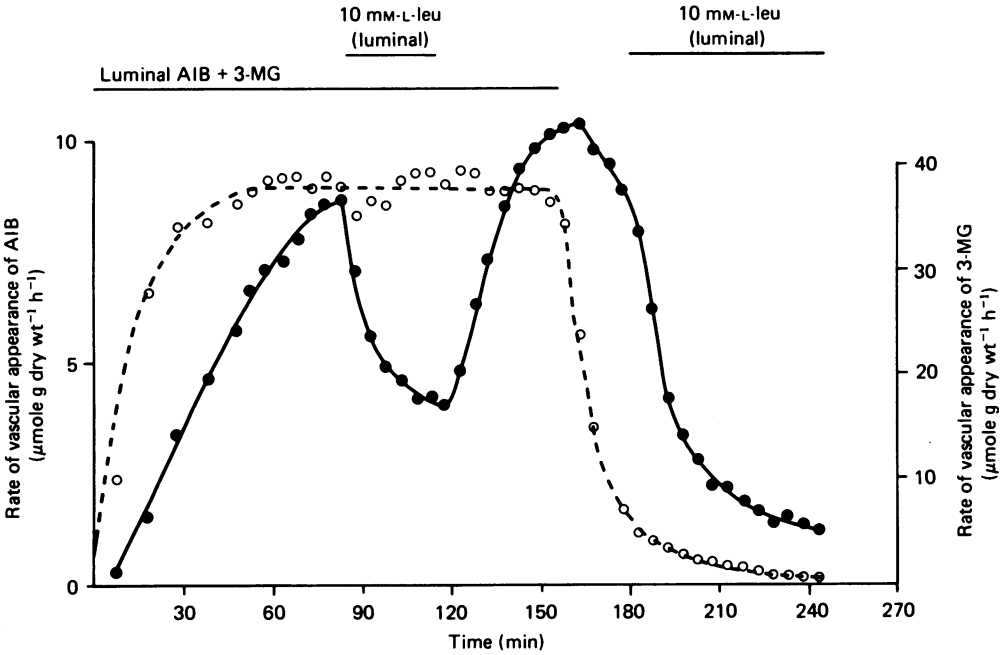

Fig. 5. The rate of vascular appearance of AIB (●) and 3-0-methyl glucose (3-MG) (○) following their addition (each 1 mM) to the lumen of the vascularly perfused small intestine of *R. ridibunda* for the period indicated. Note that the lumen was pulsed with L-leucine (10 mM) for two discrete periods beginning respectively at 85 and 180 min.

of vascular AIB appearance is more than halved. Upon removal of leucine from the lumen the rate of AIB vascular appearance quickly recovers and reaches a value higher than that seen before the inhibitor was added. In this experiment neither of the two phenomena found for AIB, namely marked inhibition of transfer upon adding leucine to the lumen and the subsequent increased rate of transfer upon removal of luminal leucine, was seen for the sugar 3-0-methyl glucose. This excludes the possibility that the observed effects are non-specific. Moreover during washout of the pre-loaded epithelium at the end of the experiment the specificity of the inhibitory effect of the second pulse of luminal leucine on washout of the amino acid and not on that of the monosaccharide is shown clearly.

AIB exit from epithelium into vascular bed: effect of adding other inhibitory amino acids to the lumen

Experiments were carried out to see if other amino acids added to the lumen behaved similarly to leucine. In each animal the effect of the test inhibitor (10 mM)

amino acid was compared with that of 10 mM-L-leucine. As is shown in Table 1, eight amino acids behaved similarly to leucine in inhibiting AIB exit out of the epithelium into the vascular bed.

AIB exit from epithelium into vascular bed; stimulatory effect of L-proline added to the lumen

In contrast to all the other amino acids tested, L-proline (10 mM) when added to the lumen stimulated AIB appearance in the blood (Fig. 6). It is apparent that the effect, like the inhibitory effects, is due to alteration in AIB transport across the

TABLE 1. Amino acids inhibiting ($P < 0.05$) AIB washout from the pre-loaded small intestinal epithelium of *R. ridibunda* into the vascular bed.

Amino acid added to lumen (10 mM)	AIB exit: ratio of inhibition shown by amino acid to that shown by luminal L-leucine Mean ± S.E.M. (*n*)
leucine	1
tryptophan	1·66 ± 0·14 (9)
phenylalanine	1·35 ± 0·05 (9)
valine	0·45 ± 0·15 (12)
asparagine	0·36 ± 0·03 (3)
D-leucine	0·30 ± 0·05 (3)
norleucine	0·29 ± 0·09 (3)
glutamine	0·28 ± 0·05 (3)
isoleucine	0·17 ± 0·09 (6)

In ten animals the mean inhibition of AIB exit produced by luminal L-leucine (10 mM) is 37 ± 2 %.

basolateral membrane since the luminal washout is unaltered. However it is clear from the size of the standard errors that there was considerable variation in the magnitude of the stimulatory effect of L-proline upon AIB washout into the vascular bed.

Amino acids not inhibiting AIB exit into the vascular bed

When added to the intestinal lumen (10 mM) alanine, arginine, aspartic acid, cycloleucine, glutamic acid, glycine, histidine, lysine, methionine, serine, threonine and tyrosine were without effect on AIB washout.

AIB exit from the epithelium: effect of adding leucine or leucyl-leucine to the vascular bed

In addition to its effect from the luminal side of the epithelium, L-leucine (10 mM) added to the vascular bed acts as a potent inhibitor of AIB washout from the pre-loaded tissue. Fig. 7 shows the instantaneous rate constants for vascular and luminal AIB appearance in two washouts performed on the same animal. It is clear that neither vascular L-nor D-leucine affects AIB movement across the luminal membrane yet L-leucine, in a stereospecific manner, decreases the instantaneous rate constant for vascular AIB appearance by almost 50 %. Fig. 8 shows that vascular L-leucyl-L-leucine, unlike the monomer, has no effect on AIB washout into the vascular bed.

DISCUSSION

The site of interaction between AIB and other amino acids

In order to interpret the results of experiments in which AIB exit from the pre-loaded epithelium into the vascular bed is perturbed by the addition of another amino acid to either the luminal or vascular compartment of the intestine, we need to identify the site of action of the second amino acid. During washout, the rate of appearance

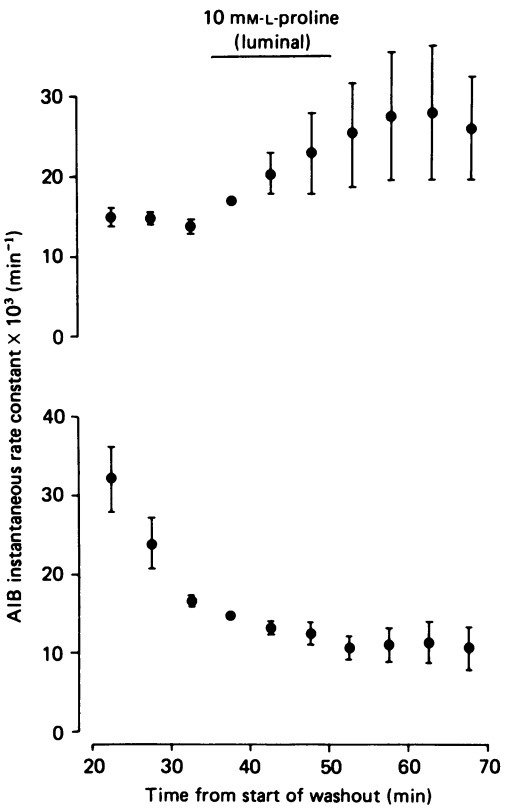

Fig. 6. Instantaneous rate constants for AIB washout from the pre-loaded small intestine into the lumen (lower) and vascular (upper) compartments. 10 mM-L-proline was added to the lumen for the period indicated. Mean (±S.E.M.) normalized results of five different experiments are shown.

of AIB in the blood, R, is normally well described by a mono-exponential function (Parsons & Sanderson, 1980). Thus $R = -KQ_t$ where K is the rate constant (min^{-1}) describing AIB exit into the vascular bed and Q_t is the quantity of AIB within the epithelium at time t after the start of washout. The rate of AIB vascular appearance may thus be altered by a change in the value of K and/or Q_t. If the intra-epithelial pool Q_t were to be decreased, for example as a result of increased AIB backflux into the intestinal lumen, then the rate of AIB appearance in the vascular bed would fall even in the absence of any effect on the exit rate constant, K. However, we have found that the amino acids which inhibit AIB exit from the epithelium do not alter the

instantaneous rate constant for AIB appearance in the lumen. Similarly the stimulatory effect of L-proline upon AIB appearance in the vascular bed cannot be explained by an increase in Q_t due to a decrease in the instantaneous rate constant for AIB appearance in the lumen. Non-specific causes for inhibitory and stimulatory effects on AIB movement can be ruled out for two reasons. Firstly, the effects

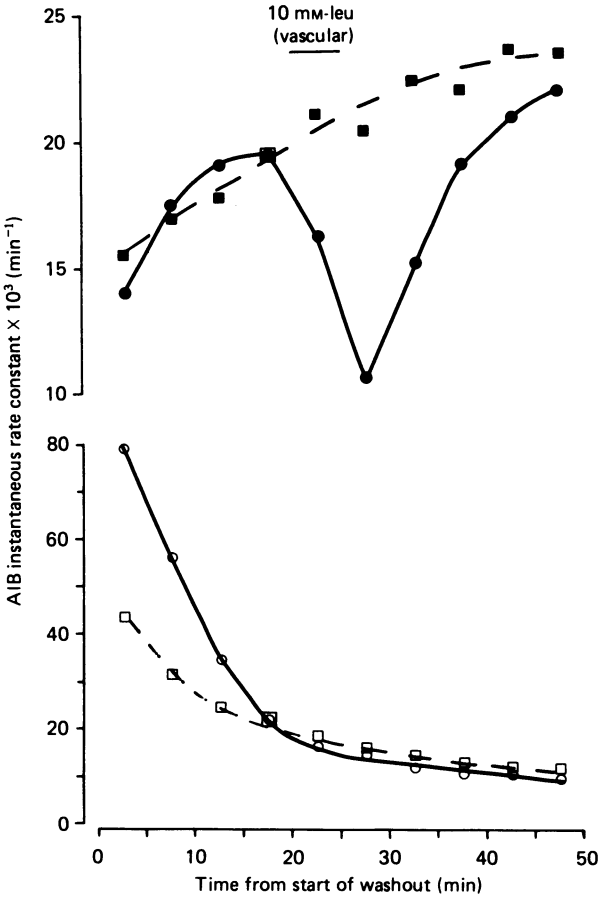

Fig. 7. Instantaneous rate constants (normalized) for AIB washout from the pre-loaded epithelium of the small intestine into the vascular (upper panel) and into the luminal (lower panel) compartments. Two washouts were performed on the same animal following identical loading with AIB, during the first (circles, continuous line) 10 mM-L-leucine, and during the second (squares, dashed line) 10 mM-D-leucine, was added to the vascular bed for the period indicated.

observed are limited to but a small group of structurally diverse amino acids and moreover show stereospecificity. Secondly, exit of the non-metabolized sugar 3-0-methyl glucose from the preloaded epithelium is unaffected by all amino acids including those which dramatically alter AIB exit (see for example Fig. 5). Furthermore, amino acid metabolism cannot be involved since L-norleucine (a non-metabolized amino acid) also inhibits AIB vascular appearance during washout.

Since interactions at the brush border cannot account for the observed effects of

leucine or indeed of the other inhibitory amino acids on AIB washout it is of interest to study the effect of such inhibitors upon the steady-state transepithelial flux of AIB. There are two features apparent in the time course of leucine inhibition of steady-state AIB transepithelial transfer (Fig. 5) which support the findings made during washout experiments. Firstly, the rate of fall of AIB appearance in the vascular bed upon

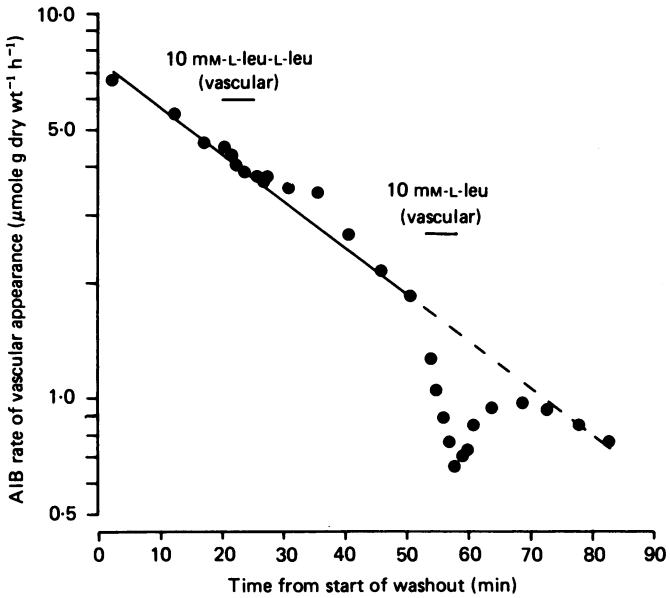

Fig. 8. AIB washout into the vascular bed of the pre-loaded frog small intestinal epithelium. For the periods indicated L-leucyl-L-leucine (10 mM) and L-leucine (10 mM) were added to the vascular infusate.

addition of leucine to the lumen is greater than that which is seen subsequently upon removal of AIB from the lumen. Secondly, upon removal of leucine from the lumen the rate of vascular AIB appearance rapidly increases and transiently exceeds the steady-state rate. This characteristic pattern of altered vascular appearance cannot occur as a result of changes in brush border transfer (Boyd, 1980).

Mechanism of interaction between AIB and other amino acids at the basolateral membrane

Classically, competition experiments have been used in order to characterize transport systems (Christensen, 1975) and the idea that a particular transport mechanism may not demonstrate absolute specificity towards a unique substrate underlies interpretation of the present experiments. The simplest explanation of our findings is that AIB is transported across the serosal face of the epithelium by a system which also accepts tryptophan, phenylalanine, leucine, asparagine, valine, glutamine, isoleucine and norleucine. Described here on the basis of its substrate specificity the basolateral AIB transport mechanism resembles the 'L' system, as does the sodium independent alanine pathway in rat intestinal basolateral membrane vesicles which Mircheff *et al.* (1980) suggest is responsible for efflux of neutral amino acids from the epithelium into the blood during absorption. Since one of the characteristics of the

'L' system is its ability to show trans-stimulation, we might expect vascular L-leucine to enhance AIB exit from the pre-loaded epithelium into the blood. However L-leucine added to the vascular bed inhibits AIB exit from the epithelium as strongly as when added to the intestinal lumen (Fig. 7 and 8). This suggests that the basolateral transport systems for AIB and leucine are highly asymmetric; under certain circumstances such an asymmetry could be important in supplying substrates to the epithelium from the blood.

The finding that vascular L-leucyl-L-leucine fails to inhibit AIB exit confirms that the basolateral membrane is, by the way of contrast to the brush border membrane, very impermeable to the dipeptide since L-leucyl-L-leucine is able to inhibit AIB exit when added alone to the lumen (data not shown). This confirms the view that leucine inhibition of AIB basolateral exit is due to an intracellular site of action.

Stimulation of AIB exit

The mechanism by which luminal proline is able to stimulate AIB exit from the epithelium into the vascular bed remains unclear. There may be an intracellular interaction involving the two amino acids at the internal face of the basolateral membrane; such an interaction might be indirect, for example involving changes in the driving forces available or might be directly on the same 'carrier' protein. Scriver & Mohyuddin, (1968), have described an analogous effect in the kidney, namely that external proline stimulates AIB uptake into cortical slices, and have suggested that this may be due to a co-operative binding of proline, in a similar way to sodium to a site on the AIB carrier. This explanation is similar to that proposed by Jacquez (1963) to explain methionine stimulation of tryptophan transport into Ehrlich cells, and such interactions may explain both our results and those of Munck & Schultz (Munck & Schultz, 1969; Munck, 1980) who describe a stimulation by intracellular leucine of lysine efflux across the basolateral membrane of rabbit ileum. Krupka & Deves (1978) have described how binding of a competitive inhibitor to a carrier may produce such effects.

V.S.P. thanks the M.R.C. for a Research Studentship.

REFERENCES

BOYD, C. A. R. (1980). Intestinal absorption studied by vascular perfusion. In *Adv. Physiol. Sci.* vol. 12, ed. GATI, T., SZOLLAR, L. G. and UNGVARY, G., pp. 413–417. Oxford: Pergamon Press.

BOYD, C. A. R., CHEESEMAN, C. I. & PARSONS, D. S. (1975). Amino acid movements across the wall of *Anuran* small intestine perfused through the vascular bed. *J. Physiol.* **250**, 409–429.

BOYD, C. A. R. & PERRING, V. S. (1980). Factors influencing amino acid exit from the vascularly perfused intestinal epithelium. *J. Physiol.* **301**, 71–72*P*.

BOYD, C. A. R. & PERRING, V. S. (1981). Routes available for the exit of an A system amino acid from the small intestinal epithelium into the blood. *J. Physiol.* **316**, 55–56*P*.

CHEESEMAN, C. I. (1979). Factors affecting the movement of amino acids and small peptides across the vascularly perfused *Anuran* small intestine. *J. Physiol.* **293**, 457–468.

CHEESEMAN, C. I. (1981). The mechanism of transfer for L-leucine into the vascular bed of the *Anuran* small intestine. *J. Physiol.* **317**, 91–102.

CHEESEMAN, C. I. & PARSONS, D. S. (1974). Intestinal absorption of peptides. Peptide uptake by small intestine of *Rana pipiens*. *Biochim. biophys. Acta* **373**, 523–526.

CHRISTENSEN, H. N. (1975). *Biological Transport*, 2nd edn. Reading, MA: W. A. Benjamin.

HOPFER, U., SIGRIST-NELSON, K., AMMANN, E. & MURER, H. (1976). Differences in neutral amino acid and glucose transport between brush border and basolateral plasma membrane of intestinal epithelial cells. *J. cell Physiol.* **89**, 805–810.

JACQUEZ, J. A. (1963). Carrier-amino acid stoichiometry in amino acid transport in Ehrlich ascites cells. *Biochim. biophys. Acta* **71**, 15–33.

KRUPKA, R. M. & DEVES, R. (1978). The membrane valve: a consequence of asymmetrical inhibition of membrane carriers. I. Equilibrating transport systems. *Biochim. biophys. Acta* **550**, 77–91.

MIRCHEFF, A. K., VAN OS, C. H. & WRIGHT, E. M. (1980). Pathways for alanine transport in intestinal basal lateral membrane vesicles. *J. memb. Biol.* **52**, 83–92.

MUNCK, B. G. (1980). Lysine transport across the small intestine. Stimulating and inhibitory effects of neutral amino acids. *J. memb. Biol.* **53**, 45–53.

MUNCK, B. G. & SCHULTZ, S. G. (1969). Interactions between leucine and lysine transport in rabbit ileum. *Biochim. biophys. Acta* **183**, 182–193.

NASSAR, C. F., KHURI, R. N. & HAJJAR, J. J. (1980). Alanine influx across serosal border of *Pestudo graeca* intestine. *Biochim. biophys. Acta* **595**, 121–125.

PARSONS, D. S. & SANDERSON, I. R. (1980). Influence of vascular flow on amino acid transport across frog small intestine. *J. Physiol.* **309**, 447–460.

SCRIVER, C. R. & MOHYUDDIN, F. (1968). Amino acid transport in kidney. Heterogeneity of α-aminoisobutyric acid uptake. *J. biol. Chem.* **243**, 3207–3213.

WARD, M. R. & BOYD, C. A. R. (1980). Intracellular study of ionic events underlying intestinal membrane transport of oligopeptides. *Nature, Lond.* **287**, 157–158.