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DISTRIBUTION OF TRANSPORTED AMINO ACID WITHIN RABBIT ILEAL MUCOSA

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SUMMARY

1. Pieces of rabbit distal ileum, incubated for short periods of time in solutions containing tritiated amino acids, have been processed for autoradiography and the profiles of amino acid concentration across the villi determined by microdensitometry.

2. The concentration profiles of a series of amino acids could be described in terms of two descending exponentials, one extending from the brush border to the basal membrane of the enterocyte and the other from the base of the epithelial layer to the centre of the villus.

3. Exponential coefficients describing the steepness of these gradients were highest for basic amino acids. Coefficients for short-chain amino acids were greater than for long-chain neutral amino acids. None of these values changed for times of incubation varying from 5 to 180 sec.

4. Enterocytes accumulated amino acids in the apical cytoplasm, against a concentration gradient, within the first few seconds of incubation. This step-up in concentration decreased as the external concentration was increased, in a manner dependent on the amino acid used.

5. It is suggested that amino acid concentration gradients within enterocytes arise by diffusion and that the amino acid specificity of this process originates from an ability of the more lipophilic amino acids to permeate structures acting as barriers to the more hydrophilic molecules.

INTRODUCTION

The use of autoradiography to determine which part of an intestinal villus is responsible for amino acid transport is now a well established technique (Kinter & Wilson, 1965; King, Sepúlveda & Smith, 1981, Smith & Syme, 1982). From this work it appears that amino acid absorption emerges as a late feature of enterocyte development. It is, however, also possible to use autoradiography on a more minute scale to study events taking place within and beyond the enterocyte during the early stages of amino acid absorption. The present work was started to determine the time and substrate dependency of amino acid distribution within the distal ileum of the rabbit. The possible origin and maintenance of amino acid gradients, found to exist within both the enterocyte and the core of the intestinal villus, are discussed.

METHODS

Animals

All work was carried out using 2·5–3·5 kg New Zealand White rabbits (Morton Commercial Rabbits, Stansted, Essex) deprived of food but allowed free access to water for 24 hr before experiment.

Experimental

Rapid uptake of ^{3}H-labelled amino acids was measured across the mucosal border of rabbit distal ileum after a 10 min pre-incubation in Na-free medium using a previously described method (Sepúlveda & Smith, 1978; Barker, Sepúlveda & Smith, 1980). Incubation for periods of time ranging from 5 to 180 sec using amino acids at concentrations varying from 1 to 40 mM was stopped by washing the surface of the intestine with a phosphate-buffered saline (Dulbecco 'A', Oxoid Ltd., Basingstoke, Hampshire) containing 4 % (v/v) glutaraldehyde and 2 % (w/v) sucrose. Pieces of fixed tissue were transferred to fresh glutaraldehyde-sucrose medium for a period of 1 hr, after which they were washed twice in phosphate-buffered saline and processed for autoradiography as described previously (King *et al.* 1981).

Estimation of the amount of transported amino acid becoming fixed to tissue protein following treatment with glutaraldehyde was carried out using a variety of amino acids at a concentration of 1 mM. Any effect of amino acid concentration on the efficiency of glutaraldehyde fixation was estimated by carrying out experiments with Ala and Lys at concentrations ranging from 1 to 40 mM. To determine percentage fixation of amino acid, paired pieces of tissue were exposed to ^{14}C-labelled amino acid for 45 sec in the presence of ^{3}H-labelled polyethylene glycol (PEG-900) as a marker of extracellular space. Uptake was stopped by rinsing the intestinal surface with isotonic mannitol or glutaraldehyde-sucrose buffer. Attempts were then made to extract the absorbed amino acid from the paired pieces of tissue using 0·1 N-HNO_3. The tissues were finally removed and the acid solution counted for radioactivity in the normal way. The amount of amino acid extracted following a mannitol wash, corrected for extracellular space, was taken to be equal to that entering the paired piece of tissue. The amount of amino acid extracted following glutaraldehyde fixation was subtracted from the total uptake to give the amount retained. The percentage of amino acid fixed at a concentration of 1 mM was: Ala, 59·8 ± 4·3; Ser, 85·3 ± 1·7; Met, 82·2 ± 1·5; Leu, 82·7 ± 4·4; Lys, 104·1 ± 3·2; Arg, 94·7 ± 4·0 %. The amount of Ala fixed at 1, 5, 20 and 40 mM was 59·8 ± 4·3; 63·6 ± 3·4; 68·0 ± 3·7 and 64·9 ± 5·4 % respectively. Corresponding values for Lys fixation were 104·1 ± 3·2; 95·8 ± 7·5; 108·4 ± 7·3 and 114·4 ± 9·6 % (all values give means ± S.E. of the mean of at least eight determinations). It is concluded that the concentration of amino acid does not determine the percentage fixation by glutaraldehyde. Mean estimates for percentage fixation were used subsequently to convert measurements of optical density into estimates of intracellular amino acid concentration.

Analytical

The general method used to scan autoradiographs was as described previously (King *et al.* 1981). In the present work scanning tracks were directed across the mucosa from the brush border to the centre of each villus. All measurements took place 50 μm from the villus tip. The diameter of the scanning spot was 5 μm. The total length of the scanning track used was 50 μm. This gave ten discrete readings of optical density. Mean optical densities decreased on moving from the brush border to the centre of the villus (see Figs. 1 and 2 in the Results section). In most cases the rate of change of amino acid concentration increased at 25 μm from the microvillar membrane. Between 25 and 50 μm, optical densities could be fitted by a mono-exponential function with non-zero asymptote; for conformity the optical densities for 0–25 μm have also been fitted by exponentials. Use of these smoothing functions aided the subsequent analysis of data. The functions $C(x) = C(0)e^{-k_1 x}$ (x = 0–25 μm) and $C(x) = C_{as} + C(25)e^{-k_2(x-25)}$ (x = 25–50 μm), where C equals the concentration of amino acid calculated from measurement of optical density, were fitted independently assuming lognormal distribution of residuals using the maximum likelihood program MLP (C1980, Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, Herts.). Areas under the histograms and exponentials were always in excellent agreement.

Readings of optical density were converted into intracellular concentrations of amino acid by comparison with a known arginine standard made up in gelatin and cut at the same section

thickness as that used for the tissue. The water content of mucosal scrapings taken from rabbit terminal ileum was found to be 80·3 ± 4·5 % (mean ± S.E. of the mean of nine determinations). Gelatin standards were made up as a 20 % (w/v) solution in order to correspond to this tissue water content. The specific activity of the amino acid presented to the tissue was identical to that of arginine used as standard (100 μc ml.$^{-1}$). Optical densities obtained from tissue and gelatin sections were each corrected for losses arising from incomplete retention during glutaraldehyde fixation. The percentage fixation of arginine to gelatin was 54·5 %.

Mathematical treatment of results

The observed optical densities (OD) showed inhomogeneity of variance. Standard deviations were proportional to mean values and the occurrence of many zero values for OD made necessary the use of ln (OD + c) as metameter. The origin c was estimated from the first three moments of the distribution of OD. Analysis of variance of the metameter showed that incubation time, substrate concentration, distance from the brush border and animal variation all produced effects on optical density. Animal variation was relatively small and has been ignored. In each of two rabbits the ileal mucosa was exposed to 1 mM-substrate for 5, 25, 45, 90 or 180 sec and in each of four rabbits the tissue was exposed for 45 sec to 1, 2, 5, 10, 20 or 40 mM-substrate. Eight replicate scans, involving ten measurements of optical density per scan, were made for each autoradiograph analysed. These designs have been treated as 5×10 ($n' = 16$) or 6×10 ($n' = 32$) two-way classifications, (n' = no. of animals × no. of replicates). The cell means from these tables were finally transformed back to optical density units and converted to tissue substrate concentrations as described in the previous section.

Materials

The following radioactive compounds were obtained from Amersham International Ltd., Amersham, Bucks.: L-[U-^{14}C] alanine (> 150 mc m-mole^{-1}); L-[2, 3-^{3}H]alanine (30–50 c m-mole^{-1}); L-[U-^{14}C]arginine monohydrochloride (> 300 mc m-mole^{-1}); L-[5(n)-^{3}H]arginine monohydrochloride (8–25 c m-mole^{-1}); L-[U-^{14}C]leucine (> 300 mc m-mole^{-1}); L-[4,5-^{3}H] leucine (130–190 c m-mole^{-1}); L-[U-^{14}C]lysine monohydrochloride (> 300 mc m-mole^{-1}); L-[4,5-^{3}H]lysine monohydrochloride (75–100 c m-mole^{-1}); L-[*methyl*-^{14}C] methionine (> 50 mc m-mole^{-1}); L-[*methyl*-^{3}H]methionine (70–85 c m-mole^{-1}); L-[U-^{14}C]serine (> 150 mc m-mole^{-1}); L-[3-^{3}H]serine (10– 20 c m-mole^{-1}). [1, 2-^{3}H] Polyethylene glycol, molecular weight 900 (6 mc g^{-1}) and L-[3-^{3}H(N)]ornithine (20–40 c m-mole^{-1}) were purchased from NEN Chemicals GmbH. D-6072 Dreieichenhain, F.R. Germany. All other reagents used were of AR grade.

RESULTS

Amino acid concentration profiles

The presence of a 'diffusion' gradient for methionine across the hamster jejunal enterocyte was reported originally by Kinter & Wilson (1965). Present experiments were designed to test whether similar gradients could be detected across the rabbit ileal enterocyte. Pieces of distal ileum were incubated for 45 sec in the presence of 1 mM-tritiated amino acid. The tissues were then fixed, processed and analysed as described previously. The resulting patterns of amino acid distribution, represented here as a percentage of total optical density to enable data from a number of preliminary experiments to be pooled, have been summarized in Fig. 1.

The highest concentration of amino acid occurred at the intracellular face of the brush-border membrane. All amino acids showed a gradient of concentration across the enterocyte (0–25 μm). There was then a steeper gradient of amino acid concentration from the base of the epithelial layer to the centre of the villus (25–50 μm). Quantitative assessment of these different gradients was made by fitting exponential curves to results obtained within and beyond the enterocyte. These curves, which

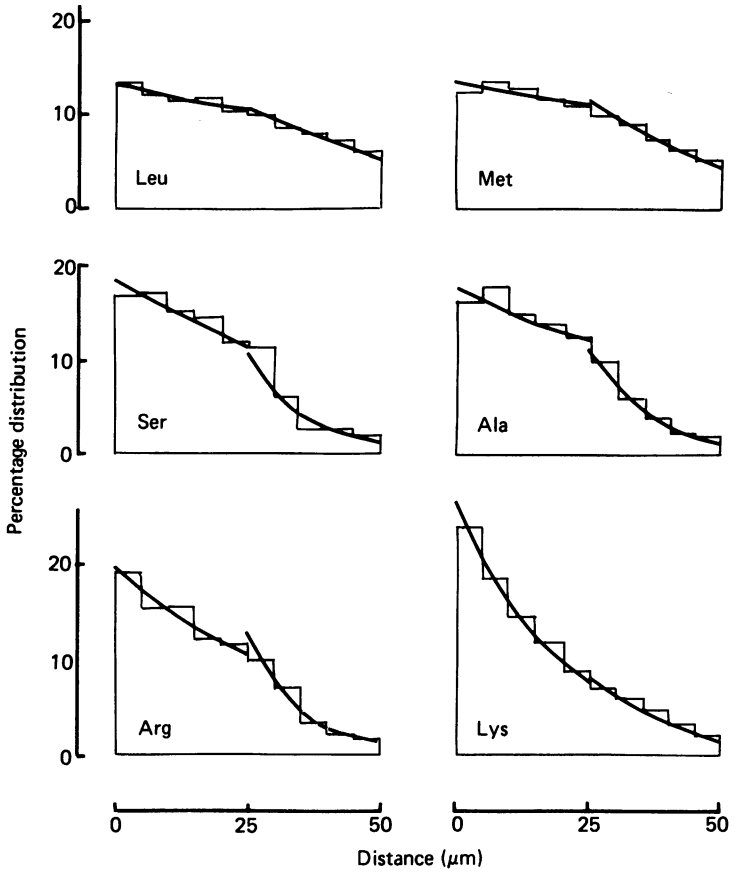

Fig. 1. Concentration gradients for different amino acids. Pieces of rabbit distal ileum, incubated for 45 sec in the presence of 1 mM-tritiated amino acid (100 μc ml.$^{-1}$) were fixed with glutaraldehyde and processed for autoradiography as described in the text. Optical densities, determined in 5 μm steps from the brush-border membrane to the back of the enterocyte (0–25 μm) and from there to the centre of the villus (25–50 μm), are shown as percentages of total optical density (0–50 μm). Values give the means of 32–40 determinations carried out on villi obtained from four to five rabbits. The constants of exponential curves fitted to these results are given in Table 1.

TABLE 1. Exponential coefficients used to describe trans-tissue amino acid gradients in rabbit terminal ileum

	Exponential coefficients	
Amino acid	Enterocyte k_1	Villus core k_2
Methionine	6·8 ± 4·1	35·8 ± 1·0
Leucine	9·8 ± 3·0	23·3 ± 2·5
Alanine	14·5 ± 4·3	82·2 ± 8·2
Serine	16·3 ± 3·8	85·8 ± 2·6
Arginine	24·4 ± 4·5	91·1 ± 10·8
Lysine	51·2 ± 0·5	58·0 ± 5·4

Experimental points, taken from Fig. 1, are fitted to decreasing exponential functions. Estimates ± S.E. of the mean of k(mm^{-1}) are given. The conditions of the experiments are as described for Fig. 1.

have been superimposed on the histograms shown in Fig. 1, provide good fits to the experimental data; their parameters have been summarized in Table 1.

Exponential slopes within enterocytes varied by a factor of 7 depending on the amino acid used. For neutral amino acids, the longer chain substrates methionine and leucine showed less gradient than the shorter chain amino acids alanine and serine. Steepest gradients were found for the basic amino acids lysine and arginine. Gradients over 25–50 μm from the brush border varied by a factor of 4, with methionine and leucine showing least slope.

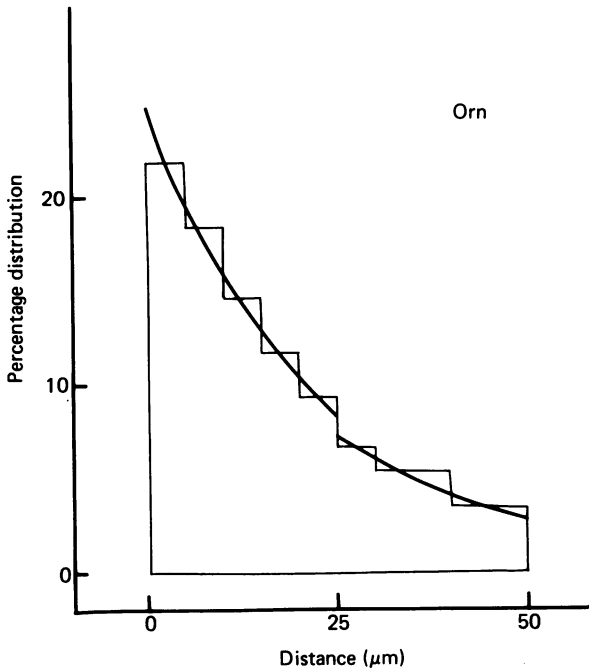

Fig. 2. Ornithine distribution across rabbit distal ileum. The experimental procedure and analysis of results was identical to that described for Fig. 1. Values give the means of sixteen determinations carried out on villi obtained from two rabbits.

It is concluded that gradients of amino acid concentration exist within enterocytes and within the villus core during the early stages of amino acid transport and that the steepness of these gradients depends, to some extent, on the type of amino acid used in the experiment. Part of this anomalous distribution could result from metabolism, amino acids becoming bound to t-RNA or incorporated into protein during the time uptake is being measured. As a test for this it was decided to measure the distribution of ornithine across the rabbit mucosa, since this is an amino acid which is not coded for in eukaryotic systems. The results obtained using this amino acid are summarized in Fig. 2. The exponential coefficient for the gradient observed from 0–25 μm was $43{\cdot}1 \pm 1{\cdot}3$ mm^{-1}. This was similar to values obtained using other basic amino acids (Table 1).

Time-dependent changes in amino acid concentration

The following experiments were undertaken to determine the time dependence of amino acid accumulation within the rabbit ileal mucosa. The tissue was exposed to 1 mM-substrate (alanine, serine, methionine, leucine, arginine and lysine) for 5, 25, 45, 90 and 180 sec. The amino acid concentrations, within and beyond the enterocyte, calculated from optical densities of autoradiographs as described previously, are

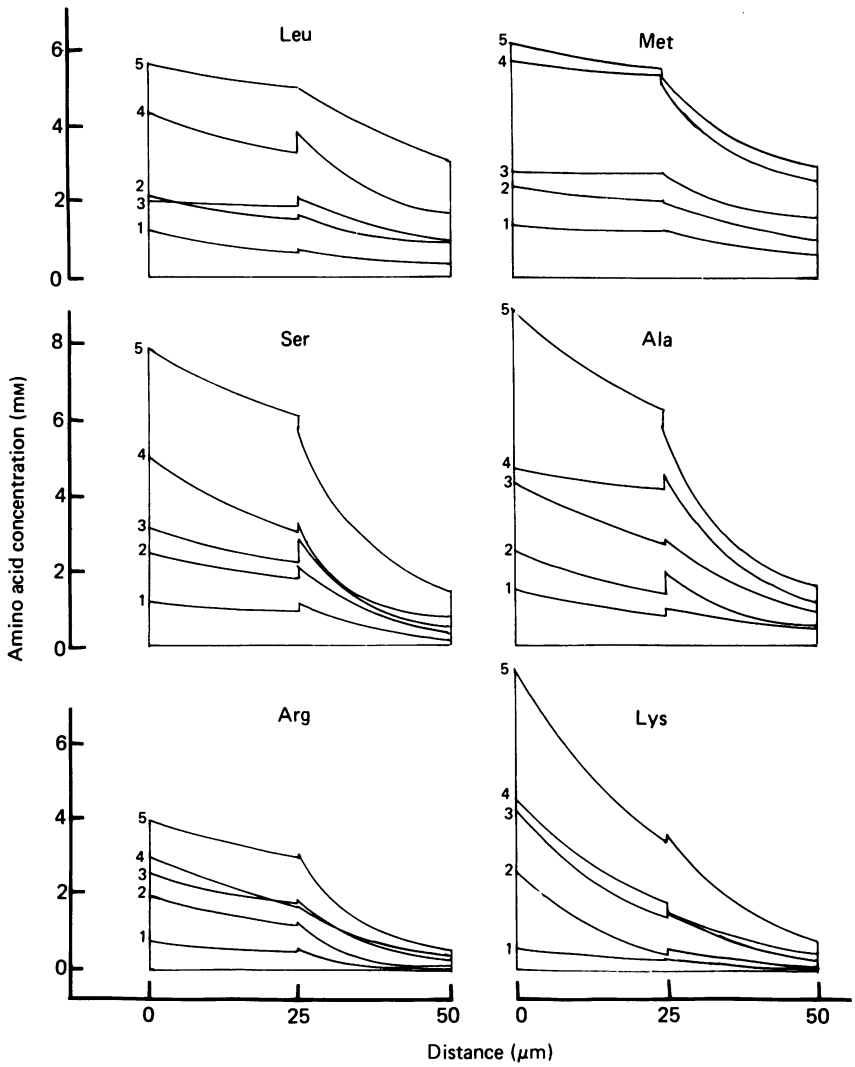

Fig. 3. Time-dependent changes in amino acid concentration occurring in rabbit distal ileum during incubation with 1 mM-tritiated amino acid. The experimental procedure was as described in Fig. 1. Exponential curves, fitted to results obtained after 5, 25, 45, 90 and 180 sec incubation (1, 2, 3, 4 and 5 respectively), are based on estimates of optical density carried out on 480 villi (six amino acids; sixteen villi per time point). Intracellular concentrations of amino acids were calculated by direct comparison with standards containing known amounts of radiactivity.

shown in Fig. 3. Within 5 sec the concentration immediately behind the brush border membrane was equal to or greater than 1 mM, except for the two basic amino acids. By 25 sec all six substrates showed a step-up in concentration over the microvillar membrane.

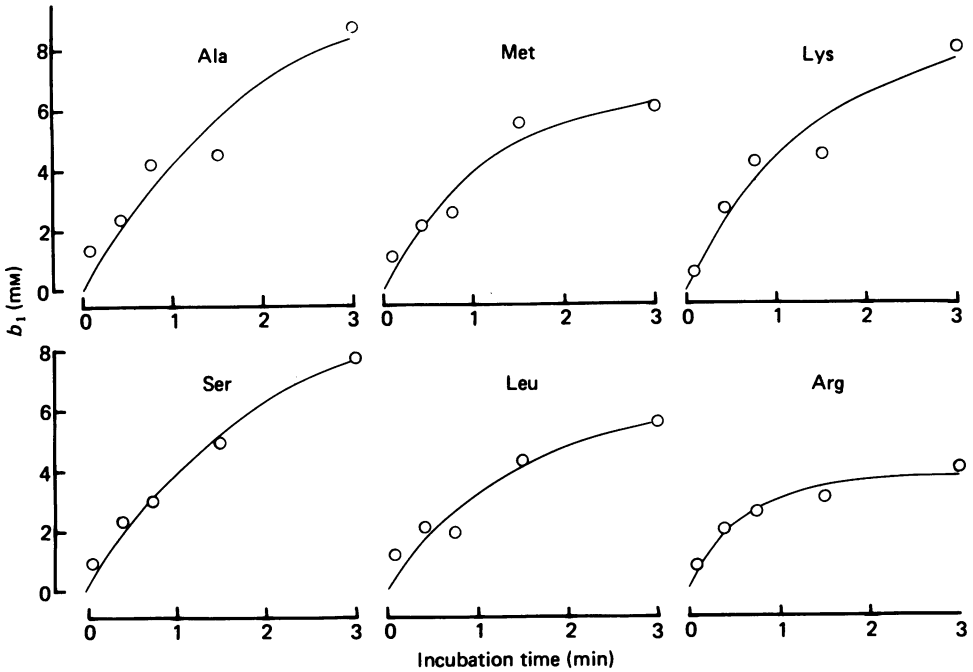

Fig. 4. Time-dependent increase in amino acid concentration in the apical cytoplasm of rabbit ileal enterocytes. Concentrations at 0 μm taken from Fig. 3(b_1) increase exponentially throughout incubation. The estimated parameters are given in Table 2.

TABLE 2. Time dependency of amino acid concentration within rabbit ileal enterocytes

Amino acid	k (min^{-1})	C_e (mM)	$t_{\frac{1}{2}}$ (min)
Alanine	0·50±0·33	10·7±4·0	1·4
Serine	0·52±0·16	9·6±1·6	1·3
Methionine	0·88±0·29	6·7±1·0	0·8
Leucine	0·68±0·33	6·4±1·5	1·0
Lysine	0·70±0·34	8·6±2·0	1·0
Arginine	1·56±0·44	3·8±0·4	0·4

Pieces of rabbit distal ileum were incubated with tritiated amino acid, as described in the text, for 5–180 sec. Amino acid concentrations at 0 μm, taken from Fig. 3, were fitted to exponential curves $C(t) = C_e\ (1-e^{-kt})$. The time for each concentration to reach half-maximal value is also given ($t_{\frac{1}{2}}$). Estimates±S.E. of the mean are given for the parameters.

The amino acid concentrations behind the brush-border membrane have been plotted against time of incubation in Fig. 4. These data have been fitted by increasing exponentials and the parameters for these are given in Table 2. The estimated equilibrium concentration for different amino acids behind the brush border (C_e) was

4–11 mM, the short-chain neutral amino acids being accumulated more, but with a longer time course, than the long-chain amino acids. There was no consistency between results obtained for arginine and lysine. Arginine reached an estimated intracellular concentration of 3·8 mM with a half-time of 0·4 min, values which are half those found for lysine. 97 % of the equilibrium concentration would be attained in times ranging from 2 min for arginine to 7 min for alanine. The corresponding time needed to achieve steady-state conditions for transport across stripped preparations of rabbit ileum is about 30 min (Schultz, Fuisz & Curran, 1966; Munck & Schultz, 1969; Hajjar, Lamont & Curran, 1970).

TABLE 3. Accumulation of amino acids within rabbit ileal enterocytes

Incubation medium	Amino acid concentration (mM) Within the enterocyte		
	Alanine	Methionine	Lysine
1	8·0±0·3	5·9±0·2	6·5±0·3
2	9·3±0·1	6·7±0·1	6·8±0·2
5	21·3±1·0	11·3±0·4	9·1±0·6
10	32·2±1·2	16·6±0·4	10·0±0·3
20	31·4±2·7	16·2±1·7	10·8±1·5
40	40·7±5·2	28·9±2·8	15·4±1·3

Pieces of rabbit distal ileum were incubated with tritiated amino acid for 45 sec as described in the text. Mean values±S.E. of the mean are shown for the concentrations of different amino acids immediately behind the brush border membrane, $C(O)$, obtained from the exponential curves in Figs. 5–7.

Concentration-dependent changes in amino acid accumulation

Previous predictions as to how the steady-state concentration of amino acid might depend on the external concentration of substrate have been based on measurements of alanine flux into and out of stripped preparations of intestinal mucosa (Curran, 1973). The following experiments use quantitative autoradiography to measure this directly within a single enterocyte.

Rabbit ileal mucosa was exposed for 45 sec to 1, 2, 5, 10, 20 and 40 mM-alanine, methionine or lysine. The concentration profiles calculated from optical densities of autoradiographs from these experiments, together with smoothing exponential functions, have been plotted in Figs. 5–7. The general features are similar to those already described showing concentration gradients to exist between the brush border and basal membranes of the enterocyte with steeper gradients describing the fall-off in concentration from the basal membrane to the centre of the villus.

Even in histogram form these results show evidence for the saturability of the entry process for amino acids. The detailed relation between enterocyte and medium concentration for these three substrates has been summarized in Table 3. In the case of alanine there is a marked step-up in concentration at the brushborder membrane for external medium concentrations less than 40 mM. In the case of methionine the step-up in concentration can be seen using 10 but not 20 mM-substrate while the ability of the enterocyte to concentrate lysine ceases using external concentrations greater than 5 mM (all results for 45 sec incubation only). A similar pattern is seen at 25 μm from the brush border where methionine and lysine show concentrations

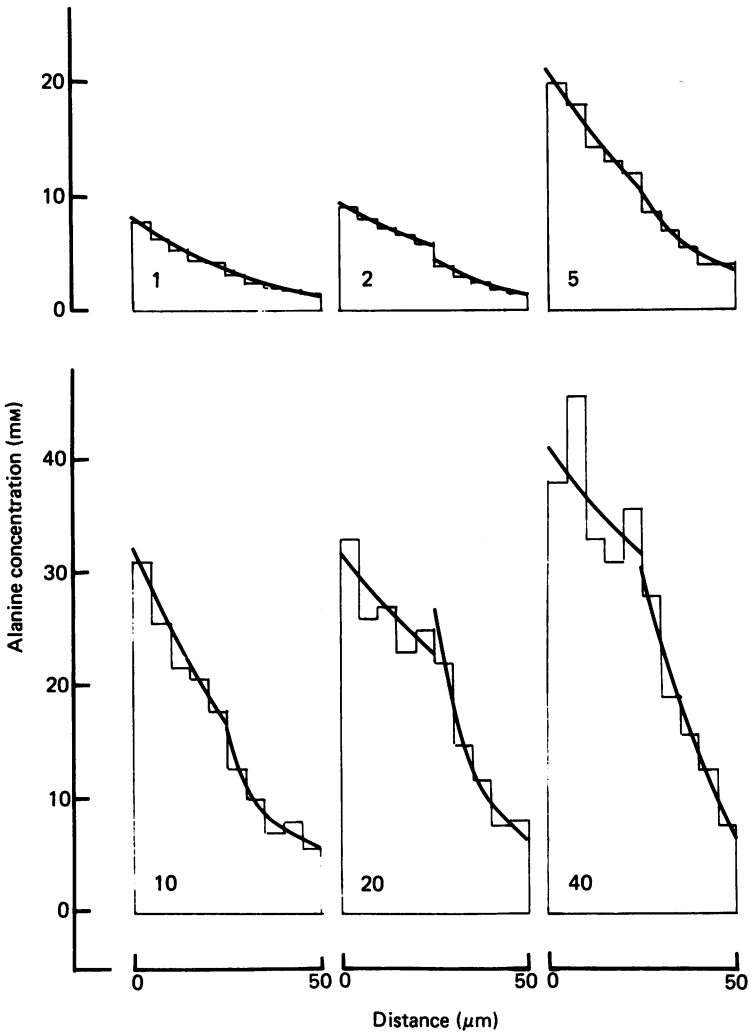

Fig. 5. Concentration-dependent changes in tissue alanine distribution following 45 sec contact of rabbit distal ileum with tritiated amino acid. The numbers 1, 2, 5, 10, 20 and 40 refer to the mM concentrations of alanine in the external medium. Experimental procedure and analysis of results was as described in the legends to earlier Figures. Exponential curves fitted to these results are based on estimates of optical density carried out on 192 villi (thirty-two villi for each concentration of alanine).

TABLE 4. Ranking sequence for different amino acids

Parameter	Ranking	Ref.
Enterocyte gradient	Lys > Orn > Arg > Ser = Ala > Leu > Met	1
1/RF (collidine)	Lys = Orn > Arg > Ala > Ser > Met > Leu	2
H_2O solubility	(Lys; Orn; Arg) > Ala > Ser > Met > Leu	3
1/liposome permeability	Ser > Ala > Met > Leu	4
	Ala > Met	5

Sequenes obtained from (1) material presented in the present paper; (2) Dent (1948); (3) The Merck Index; ninth edition; (4) Wilson & Wheeler (1973); (5) Klein, Moore & Smith (1971).

greater than those in the external medium up to 5 mM and in the case of alanine up to 20 mM. The coefficients of the exponentials used to describe these concentration gradients in enterocytes also show some dependence on the concentration of substrate used. This is most noticeable for lysine where the k value falls from 52·6 to 17·9 mm^{-1} as the external medium concentration is increased from 1 to 40 mM. Methionine and alanine showed variable intra-enterocyte exponential coefficients but with no pronounced trend. There was no such dependency of exponential gradient on concentration, between 25 and 50 μm from the brush border.

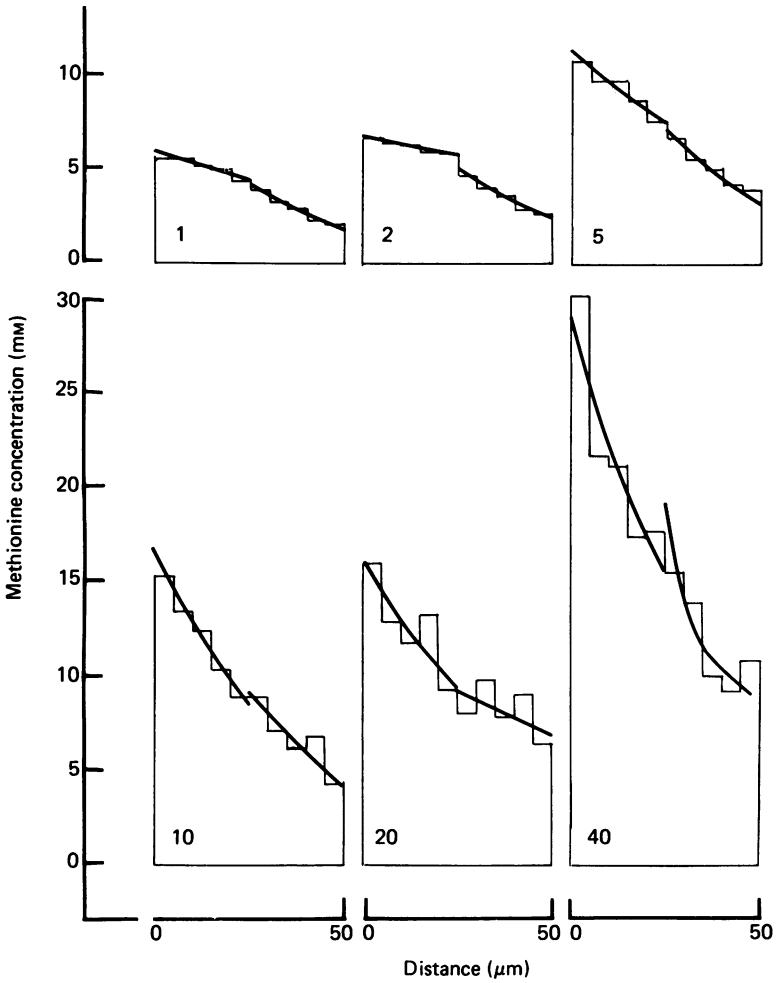

Fig. 6. Concentration-dependent changes in tissue methionine distribution; details as for alanine in Fig. 5.

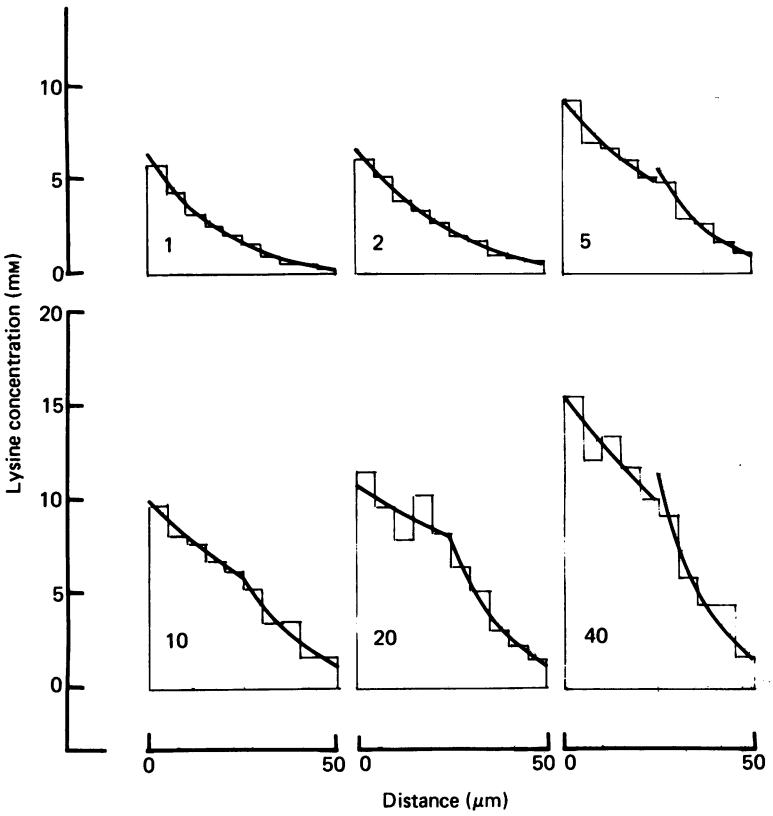

Fig. 7. Concentration-dependent changes in tissue lysine distribution; details as for alanine in Fig. 5.

DISCUSSION

The present work demonstrates that amino acids distribute themselves asymmetrically across enterocytes during the initial stages of intestinal transport. Such asymmetry could arise as an artifact. It could also represent a diffusion gradient or a gradient produced through the preferential adsorption, transport or metabolism of amino acids within the apical portion of the absorbing enterocyte.

Artificial production of intracellular gradients

Any process which causes the preferential binding of amino acids to the apical or removal of amino acids from the basal part of an enterocyte will itself produce an intracellular gradient of amino acid concentration. The use of glutaraldehyde to wash as well as fix the tissue could produce this effect, either by cross-linking extracellular amino acids to the microvillar surface or by allowing amino acids to leave the enterocyte before becoming fixed. Also, intercellular spaces are known to become wider towards the base of the epithelium. Any scanning track passing over these spaces might be expected to record lower optical densities than when passing over adjacent tissue.

However, similar intracellular gradients have been seen using frozen sections of

hamster and rat intestine (Kinter & Wilson, 1965; Johnson & Bronk, 1980). This eliminates the possibility that glutaraldehyde could, by itself, produce the gradients of amino concentration recorded in the present work. The increase in intercellular space towards the base of the cells should be independent of the amino acid used; the wide range of gradients observed with different substrates suggests that this effect of tissue geometry must be small. Our routine technique of directing scanning tracks across the densest part of the enterocyte should, in any case, have minimized this source of error.

A variant of the artifact hypothesis is to suggest that the observed gradients are real, but that they indicate the location of binding sites for amino acids rather than transcellular diffusion profiles. The fact that ornithine distributes itself asymmetrically across enterocytes rules out the possibility that such binding could be associated with protein synthesis. Kinter & Wilson (1965) recognized the difficulty in disproving that concentration gradients could result from intracellular binding. In the case of monosaccharides which show similar concentration profiles, they showed that rapid thawing and re-freezing of sections eliminated the gradients. They concluded from this that substrate remained essentially free within the cytoplasm. Johnson & Bronk (1980) showed that the transcellular gradient of galactose could be similarly dissipated by further incubation in substrate-free medium, suggesting that the sugar was available to continue diffusion and movement beyond the enterocyte.

Intracellular gradients and amino acid diffusion

The use of autoradiography has provided clear evidence of the magnitude of the concentration step-up over the microvillar membrane, and it would be expected that amino acids would diffuse towards the base of the enterocyte, driven by the gradient in concentration. Diffusion alone, however, does not explain why amino acids of similar molecular size should produce gradients differing in steepness, and it does not explain why these gradients should persist for relatively long periods of time (for at least 3 min in the present work; up to 20 min in the case of Kinter & Wilson, 1965). To reconcile these differences it is necessary to modify the concept of diffusion along the enterocyte by assuming that there may be barriers which have finite permeability to the more lipophilic amino acids. Some credence can be given to this view by the recent description of cytoplasmic ground substance using high-voltage electron microscopy. This can now be seen to consist of a microtrabecular lattice interconnecting throughout the cell with both membranous and non-membranous organelles (Wolosewick & Porter, 1980). Amino acids migrating from the brush border to the basal membrane must circumnavigate such structures as well as the endoplasmic reticulum, mitochondria and nucleus. The effect of this will be to lengthen the actual diffusion path length and this could help to explain the constancy of gradient with time.

An assumption that some intracellular structures might be permeated more readily by certain amino acids could explain the observed differences in gradient between substrates. In Table 4 we have ranked these in order of steepness of intra-enterocyte gradient, and have noted that this order is the same as that given by other measurements of amino acid hydrophobicity.

The absolute concentration of amino acid reached within the rabbit ileal enterocyte and the time course leading to its full equilibration in the cell are both similar to

previous values reported for hamster intestine using a less quantitative method of analysis (Kinter & Wilson, 1965). Both speed of entry and intra-enterocyte equilibration take place much faster than can be assumed from measurements carried out on whole pieces of intestinal tissue, even when that tissue has been previously stripped of its muscle layers (Schultz *et al.* 1966; Munck & Schultz, 1969; Hajjar *et al.* 1970). The use of autoradiography, though still time consuming, has many advantages over previous methods of analysis. Not only does it enable one to study events taking place in the absorbing enterocyte directly but it also allows one to follow the movement of that amino acid with time as it crosses the enterocyte into the submucosal tissue. Such an analysis of amino acid efflux from rabbit ileal enterocytes forms the subject of the following paper (Paterson, Sepúlveda & Smith, 1982).

We would like to thank Mr I. S. King, who was responsible for preparing all the autoradiographs analysed in this work.

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