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AMINO ACID EFFLUX FROM RABBIT ILEAL ENTEROCYTES

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SUMMARY

1. A method is described for converting tissue concentrations of amino acid, determined autoradiographically using sections of rabbit distal ileum, into measurements of total uptake.

2. Using this method the quantity of amino acid recovered from the villus core following short-term incubation with tritiated amino acid was shown to be directly related to the intra-enterocyte concentration of amino acid determined at a site immediately adjacent to the basal membrane. No evidence was obtained for saturation of efflux across the basal membrane of the enterocyte.

3. Amino acid efflux from the mucosa to the villus core, calculated for a constant intra-enterocyte concentration of substrate, was found to be greater for methionine and leucine than for alanine, serine, lysine or arginine.

4. The distribution of amino acids within the core of the villus following efflux from the enterocyte could not be explained by diffusion alone.

5. It is suggested that quantitative autoradiography can be used as an alternative method to study mechanisms responsible for amino acid movement across the basal membranes of enterocytes. Advantages and limitations of the technique are discussed.

INTRODUCTION

The complete kinetic description of how amino acids cross the intestinal mucosa involves the measurement of fluxes across both brush border and basolateral membranes. Detailed characterization of amino acid entry has already been made through the introduction of new techniques of analysis (Schultz & Curran, 1970; Paterson, Sepúlveda & Smith, 1979, 1980*a*, *b*, 1981). A similar analysis of amino acid efflux has yet to be completed.

Use of compartmental analysis led, originally, to the conclusion that efflux was carrier mediated (Munck & Schultz, 1969*a*; Hajjar, Khuri & Curran, 1972; Danisi, Tai & Curran, 1976). The substrate specificity of this process has been assessed further by measuring the ability of different amino acids to affect the transport of each other during efflux (Munck & Schultz, 1969*b*; Cheeseman, 1981, 1982) and by measuring amino acid influx into vesicles prepared from basolateral membranes (Mircheff, van Os & Wright, 1980).

A means of studying amino acid flux across this membrane *in situ* has recently

become available through the use of quantitative autoradiography to measure cellular concentrations of transported amino acids (Paterson, Sepúlveda & Smith, 1982). Present work shows that it is possible to use these concentrations to obtain estimates of amino acid efflux across the basolateral membrane.

METHODS

General

The work described in the present paper was carried out on data obtained using animals and experimental techniques described previously (Paterson *et al.* 1982). Previous analysis of these results was used to describe the presence of concentration profiles for different amino acids across rabbit ileal mucosa. Present work was designed to convert these estimates of amino acid concentration into absolute quantities in an attempt to describe the characteristics of amino acid efflux from the intestinal mucosa into the core of the villus.

Converting tissue concentrations of amino acids into measurements of amino acid uptake into rabbit ileum

The autoradiographic measurements comprise ten consecutive 5 μm steps beginning at the brush border at 50 μm from the villus tip. The simplest convenient model for estimation of total uptake by a villus was a regular cylinder of 50 μm radius. The areas of concentric circles whose radii are multiples of 5 μm, are 1, 4, 9 ... times π (0·0005) cm^2. If concentration in the innermost circle is C_1, in the next 5 μm ring is C_2 etc. then the total quantity of amino acid taken up by this plane of section (Q_p) will be π (0·0005)^2 $[C_1+3C_2+\ldots(2n-1)Cn]$. Uptake into the villus core (Q_{vc}) can be calculated by applying the same formula to the five innermost estimates of amino acid concentration (values obtained beyond the epithelium). The units of measurement for Q_p and Q_{vc} are 10^{-11} mole cm^{-1}.

Previously it has been shown that amino acid uptake does not extend more than 0·0150 cm below the villus tip and that there is a nearly linear gradient in amino acid concentration along this region of the villus (King, Sepúlveda & Smith, 1981). The mean uptake for a single villus (Q_v) is 0·75 of that quantity of amino acid detected 50 μm from the tip (Q_p). Q_v then becomes equal to $0{\cdot}015 \times 0{\cdot}75\ Q_p = 0{\cdot}01125\ Q_p$ (10^{-11} mole).

The actual shape of villi in the rabbit distal ileum, viewed at a magnification of $\times$300 through a T.V. monitor using a technique for villus exposure described previously (James & Smith, 1981), can be seen to be leaflike with a major and minor axis at the base of approximately 200 and 100 μm respectively. Allowing for a spacing distance of about 20 μm it follows that each cm^2 of intestine will contain 3500–4000 villi. Amino acid uptake per cm^2 of ileum (Q_a) is then $0{\cdot}01125\ Q_p \times 3700 = 41{\cdot}6\ Q_p$ (10^{-11} mole cm^{-2}). Scaling to a 1 min uptake produces estimates of Q_a (n-mole cm^{-2} min^{-1}) suitable for direct comparison with previously published values for uptake, estimated directly using a double isotope method of analysis (Paterson *et al.* 1979, 1981).

Values of Q_a were, in practice, only used in order to establish that autoradiography could be used quantitatively to measure the absolute amount of amino acid taken up by the rabbit ileum. Subsequent estimates of the amount of amino acid entering the core of the villus from the intestinal mucosa have been calculated only for that plane of section encountered 50 μm from the villus tip (Q_{vc}).

RESULTS

Quantitative estimation of amino acid uptake using autoradiography

The concentration dependence of alanine, methionine and lysine uptake, calculated as described above from autoradiographic data presented previously (Figs. 5–7, Paterson *et al.* 1982), is shown in Fig. 1.

In each case there is clear evidence for the presence of a saturable process. A single hyperbola does not provide an adequate fit to the observed experimental points. If two entry processes are assumed to be present an improved fit is obtained, as shown in Fig. 1.

The calculated K_m values for amino acid entry on high affinity systems were $5{\cdot}4 \pm 3{\cdot}0$ (S.E.M.), $0{\cdot}9 \pm 0{\cdot}5$ and $1{\cdot}2 \pm 0{\cdot}4$ mM for alanine, methionine and lysine respectively. These values are very similar to previous estimates made using a dual isotope method of analysis (4·6, 0·4 and 1·0 mM for alanine, methionine and lysine respectively; Paterson *et al.* 1979, 1981). The uptake of alanine, methionine and lysine through their low affinity systems, poorly characterized with only two degrees of freedom, can be assumed to show an almost rectilinear concentration dependence with slopes of 0·00118, 0·00123 and 0·0056 cm min^{-1} respectively.

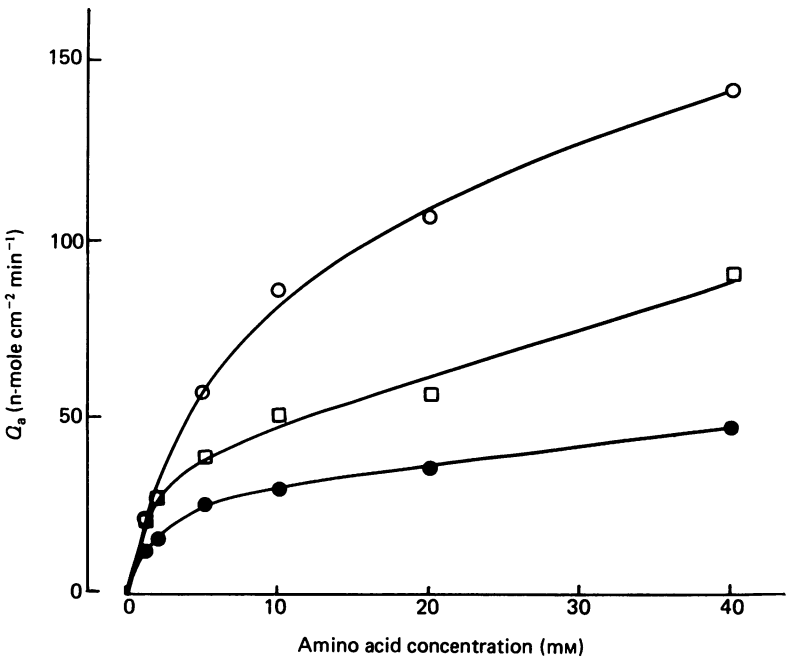

Fig. 1. Concentration dependence of amino acid uptake by rabbit ileum. Uptake of alanine (○), methionine (□) and lysine (●) into rabbit distal ileum was determined using quantitative microdensitometry of autoradiographs as described in the text. Each value of uptake (Q_a) gives the mean of estimates carried out on thirty-two villi. Curves fitting these points have constants described in the text.

Maximum Q_a values for alanine, methionine and lysine uptake of 111 ± 51, 31 ± 6 and 27 ± 4 n-mole cm^{-2} min^{-1} respectively (means ± S.E.M.) were higher than previous estimates for J_{max} obtained using the dual isotope method of analysis (52, 14 and 13 n-mole cm^{-2} min^{-1} for alanine, methionine and lysine respectively; Paterson *et al.* 1979, 1981). Part of this discrepancy could result from animal variation or assumptions made in calculating Q_a (Paterson *et al.* 1982). One of the assumptions, namely a villus density of about 4000 mm^{-2}, is about twice as high as published measurements for the human (Riecken, Zennek, Lay & Menge, 1979), rat (Robinson, Menge, Schroeder, Riecken & van Melle, 1980) and dog (Heneghan, Robinson, Menge & Winistörfer, 1981) small intestine. A similar villus density in the rabbit would halve the Q_a values quoted above, producing very good agreement between autoradiography and the conventional method to measure uptake.

It is concluded from these initial experiments that autoradiography can be used quantitatively to provide measurements of amino acid uptake. Subsequent work has been confined to a study of how such uptake partitions between epithelial and sub-epithelial compartments during different experimental circumstances.

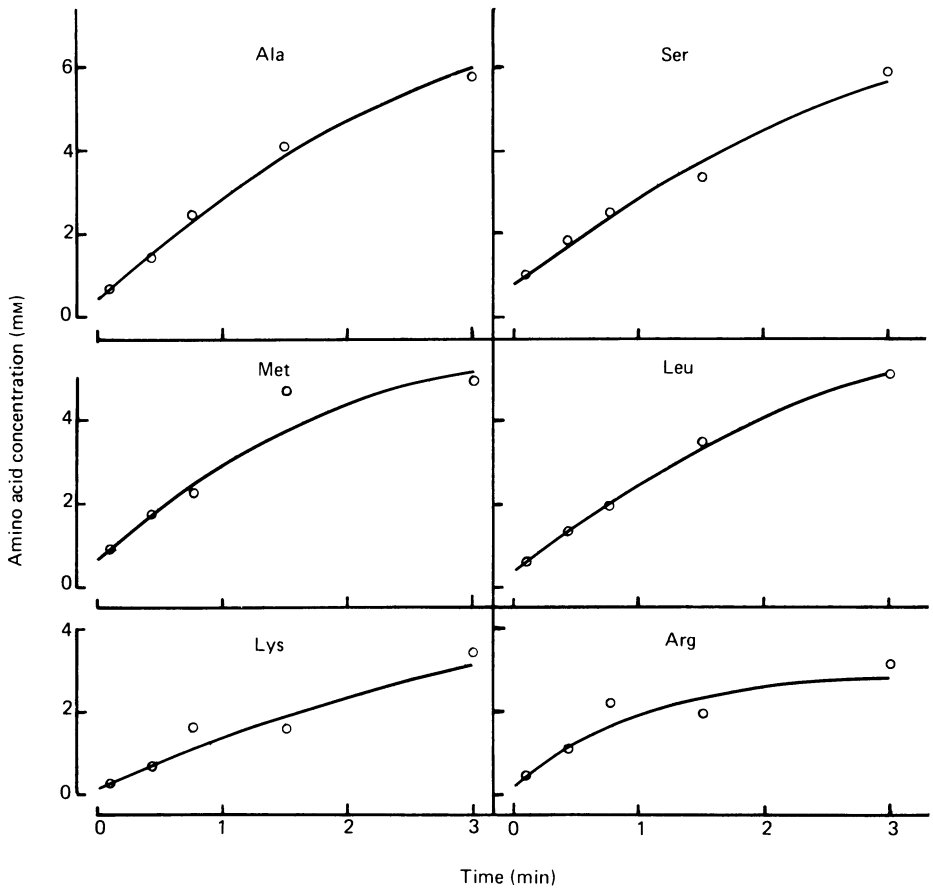

Fig. 2. Time dependence of changes in intra-enterocyte amino acid concentrations determined in rabbit ileal mucosa at a site immediately adjacent to the basal membrane. Values for the intra-enterocyte concentrations of alanine, serine, methionine, leucine, lysine and arginine, obtained after incubation of rabbit distal ileum with 1 mM-tritiated amino acid for 5–180 sec, were determined as described in the text. Curves to these points have been fitted (MLP) with increasing exponential functions $C_t = C_a + C_e\,(1 - e^{-kt})$.

Amino acid concentration at the base of enterocytes

Two difficulties immediately arise when considering how to analyse mechanisms responsible for amino acid efflux across the basal membranes of enterocytes. The first is to decide, in an unstained section, exactly where that membrane exists, and the second is to devise a way of relating the amount of amino acid entering the villus core to concentrations within the enterocyte which vary with time.

Previous work showed that at 25 μm from the brush-border membrane there was a change in exponential gradient of amino acid concentration and this was taken to

mark the position of the basal membrane (Paterson *et al.* 1982). Exponential functions describing these gradients are:

$$C_x = C_0 e^{-k_1 x} \; (x = 0\text{–}25\ \mu\text{m})$$
$$C_{x'} = C_a + C_{0'} e^{-k_2 x'} \; (x' + 25 = 25\text{–}50\ \mu\text{m})$$

with the two functions being fitted independently using the maximum likelihood program MLP (C1980 Lawes Agricultural Trust). The concentrations of amino acids at the basal membrane (C_b) are given by $\frac{1}{2}(C_0 e^{-25k_1} + C_a + C_{0'})$.

The mucosal surface of rabbit ileum was exposed to 1 mM-alanine, serine, methionine, leucine, lysine and arginine for 5, 25, 45, 90 and 180 sec. The calculated concentrations at the basal membrane, shown in Fig. 2, can be seen to rise for all six substrates as the time of incubation increases from 5 to 180 sec. These observations were fitted to increasing exponential functions $C_t = C_i + C_e(1 - e^{-kt})$. The intercepts ($C_i$) on the ordinate range from 0·13 mM for lysine to 0·79 mM for serine. There appears to be an initial rapid influx of amino acid, which does not represent preferential binding to any particular cellular component since the profile of amino acid concentration across the whole tissue after 5 sec incubation is very similar to that seen at later times (Paterson *et al.* 1982). The half-times for increase in amino acid concentration at the base of the enterocyte were of the order of 1–3 min. Steady state concentrations of about 8 mM at the basal membrane would be attained after 5–15 min incubation.

Amino acid efflux from the rabbit ileal enterocyte

Calculation of the amount of amino acid in the villus core has been described in the Methods sections (Q_{vc} values). These values can be related to the concentrations at the basal membrane. It would be expected from the lack of variation in concentration gradients with time or concentration (Paterson *et al.* 1982) that there would be a close correlation between these two quantities and this is evident in Figs. 3 and 4.

Q_{vc} is related linearly to concentration at the basal membrane. In the case of alanine C_b attained 30 mM and even at this concentration there was no deviation from rectilinearity. If amino acids leave enterocytes by a carrier-mediated process, then its affinity for all six substrates must be very low.

The relative ease with which amino acids cross the basal membrane may be characterized by the slopes of these regressions which are given in Table 1. Methionine and leucine are clearly different from alanine, lysine, serine and arginine. The permeability to the hydrophobic amino acids is approximately 50% greater than for the others.

Concentration gradients in the villus core

The pattern of post-mucosal amino acid distribution in the villus core mentioned previously (Paterson *et al.* 1982) could arise from diffusion through extracellular space, from capture by cells in the connective tissue, or from a mixture of both effects. Attempts to distinguish these factors are important, since the possible rate of amino acid back-flux into the mucosa will depend on the amount of free amino acid present in the submucosal space.

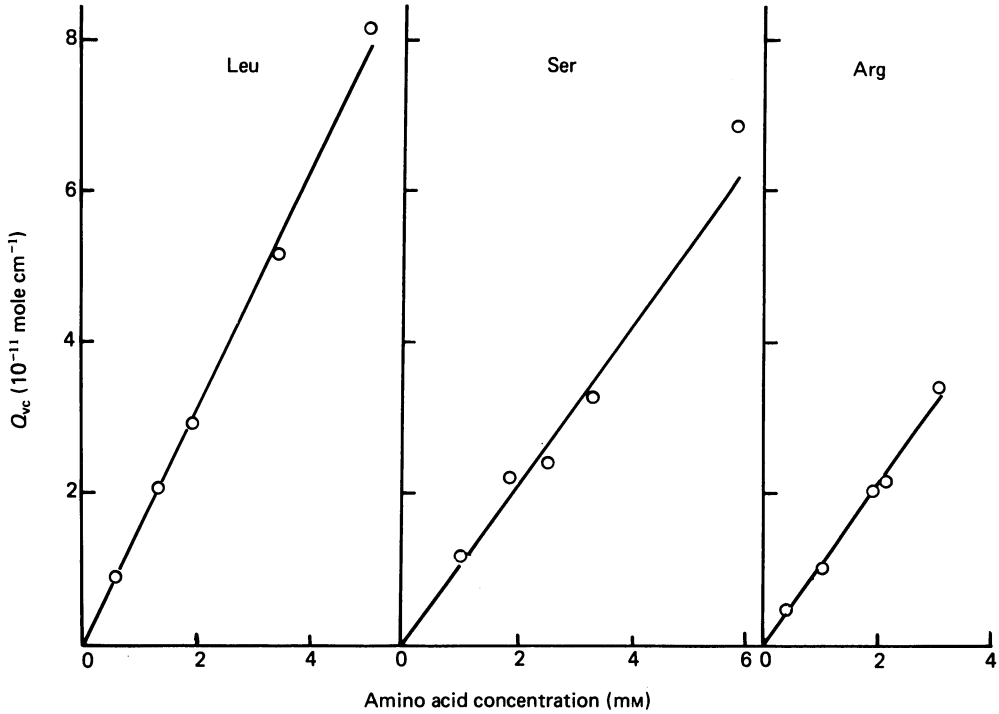

Fig. 3. Concentration dependence of amino acid efflux from rabbit ileal enterocytes. Values for the net efflux (Q_{vc}) of leucine, serine and arginine, obtained by integration of sub-epithelial concentration gradients, have been plotted against the concentration of amino acid at the basal membrane. Original data came from the paper of Paterson *et al.* (1982). Lines of best fit to these points have slopes given in Table 1.

TABLE 1. Concentration dependence of amino acid efflux across basal membranes of rabbit ileal enterocytes

Amino acid	Amino acid permeability (10^{-5} cm^2)
Methionine	1·64 ± 0·11
Leucine	1·57 ± 0·06
Alanine	1·22 ± 0·05
Lysine	1·21 ± 0·06
Serine	1·10 ± 0·09
Arginine	1·06 ± 0·05

Values for amino acid permeability give the slopes of the fitted regression lines ± S.E. of the mean of efflux concentration curves plotted in Figs. 3 and 4.

Exponential curves describing concentrations in the villus core have the form $C_{x'} = C_a + C_{o'}\, e^{-k_2 x'}$. Each set of concentrations $C_{x'}$, $x' = 2{\cdot}5$–$22{\cdot}5$ μm from the basal membrane was scaled by the $(C_a + C_{o'})$. In a two-factor analysis of variance of these scaled values only distance from the basal membrane gave significant variation. The standardized mean concentrations produced from this analysis are shown in Table 2.

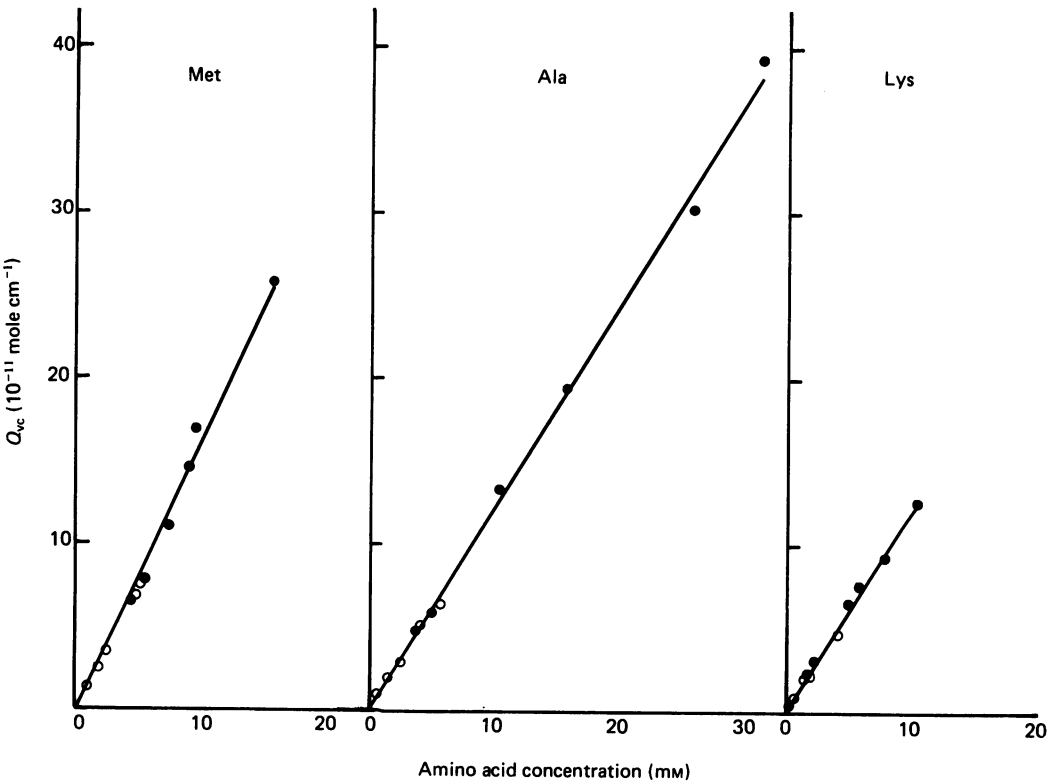

Fig. 4. Concentration dependence of amino acid efflux from rabbit ileal enterocytes. The net efflux (Q_{vc}) and intra-enterocyte concentrations of methionine, alanine and lysine were determined as described for Fig. 3. The original data comes from time course (–○–) and substrate concentration (–●–) curves plotted in a previous paper (Paterson *et al.* 1982). Lines of best fit to these points have slopes given in Table 1.

TABLE 2. Standardized concentrations of amino acids within the villus core of rabbit ileum calculated using methods described in the text

Distance from basolateral membrane	Standardized concentration of amino acid					
	Met	Leu	Ala	Ser	Lys	Arg
2·5	0·93±0·01	0·87±0·02	0·85±0·01	0·74±0·02	0·84±0·02	0·91±0·16
7·5	0·78±0·04	0·78±0·02	0·63±0·02	0·52±0·01	0·60±0·02	0·49±0·05
12·5	0·69±0·02	0·60±0·04	0·49±0·02	0·29±0·02	0·47±0·03	0·32±0·05
17·5	0·60±0·05	0·54±0·04	0·38±0·03	0·25±0·02	0·36±0·03	0·23±0·04
22·5	0·51±0·04	0·50±0·04	0·30±0·03	0·20±0·03	0·22±0·01	0·18±0·04

Values give means±S.E. of the mean of five (Ser, Leu, Arg) or eleven (Ala, Met, Lys) determinations.

There is a clear difference between methionine and leucine and the other four amino acids, which at the centre of the villus show concentrations about one-fifth of those present at the basal membrane, while for methionine and leucine the proportion is about one-half. A difference of this magnitude could not be accounted for solely by diffusion through extracellular space, since the diffusion coefficients are all very similar.

DISCUSSION

The present work sought to establish that it was possible to use autoradiography to measure the net uptake of amino acids into rabbit distal ileum and to partition that uptake between epithelial and sub-epithelial compartments. Preliminary work, plotting the concentration dependence of amino acid uptake assessed autoradiographically, produced results in reasonably good agreement with those published previously but this does not mean that autoradiography should now become the method of choice to analyse mechanisms for amino acid transport across membranes. This is mainly because of the labour involved in obtaining results for even one set of experimental circumstances. Defining the kinetic properties of neutral amino acid entry into rabbit ileum measured by dual isotope methods, for instance, originally involved collecting results for eighty-three data points, each of which was eventually based on an average of eight separate determinations (Paterson *et al.* 1979). It would be quite impossible to carry out this amount of work using autoradiography. It is therefore recommended that this technique be reserved for situations which are difficult to analyse by any other means. Describing the concentration and substrate dependency of amino acid efflux across the basal membranes of enterocytes would be one example where such effort might seem justified.

Before discussing these results, however, it is worthwhile considering the efforts of earlier workers to define amino acid efflux mechanisms in intestine. It is, for instance, theoretically possible to provide a complete description of bidirectional amino acid movements across both brush border and basal membranes of the intestinal mucosa using compartmental flux analysis (Hajjar *et al.* 1972; Danisi *et al.* 1976). Adopting this technique it was demonstrated that alanine was unable to saturate its efflux mechanism for enterocyte concentrations rising up to 30 mM, a result in agreement with that obtained in the present work. What makes such agreement spurious is the knowledge that only a small fraction of mucosal cells actually transport amino acids (Kinter & Wilson, 1965; King *et al.* 1981), a fact not allowed for in the original calculation of tissue alanine concentrations, and the present observation that cells within the lamina propria also appear capable of taking up amino acids. Both observations cast doubt on the assumption that amino acids enter a single homogeneous cellular compartment in this tissue (Hajjar *et al.* 1972; Danisi *et al.* 1976).

Attempts to define the mechanisms of amino acid efflux through the use of basolateral membrane vesicles will likewise prove inconclusive until it can be proved that initial kinetics are being measured, that the preparation contains no trace of brush border membrane and that the basolateral membranes are not changed by the procedure used to prepare them (Mircheff *et al.* 1980). The availability of a non-invasive technique of analysis, of the type described in the present paper, avoids many of the disadvantages listed above.

Having criticized previous methods of analysis it must be admitted that present results tend to raise more questions than they answer. One of these questions concerns the anatomical location of amino acids once they have left the epithelial layer. Explaining the profile of amino acid concentration across the lamina propria in terms of diffusion involves postulating a diffusion coefficient of about 10^{-8} cm^2 sec^{-1}, a value two orders of magnitude slower than that found in free solution, suggesting that at least part of amino acid movement is across cells. It is not known whether any appreciable back-flux of amino acid takes place from villus core to mucosa under our experimental conditions. One might imagine that such a back-flux, if it did occur, would take place on an L-type carrier as suggested by Mircheff *et al.* (1980), making the values for methionine and leucine efflux minimal estimates and the difference between these two amino acids and the rest even greater than appears from comparison of values shown in Table 1, but there is no definitive evidence to prove that this is the case. The only safe conclusion to make is that the net movement of methionine and leucine from the enterocyte to the villus core is considerably greater than for alanine, serine, lysine and arginine and that movement of all these amino acids across the basal membrane shows no saturation by high intracellular concentrations of substrate.

Further consideration of this point is interesting for it is a consistent finding to observe the unidirectional mucosa to serosa flux of basic amino acids like lysine to be considerably less than for neutral amino acids such as alanine. Present results, however, suggest that the reduced rate of lysine appearance at the serosal surface might be due to a relative failure of this amino acid to reach the base of the enterocyte in high enough concentration to maintain a rapid exit. It can be seen in Fig. 4 and Table 1 that alanine and lysine have almost identical slopes relating Q_{vc} to C_b, but alanine attains concentrations at the basal membrane three times greater than for lysine estimated under identical conditions. It is only through the use of autoradiography that such a possibility can be appreciated. Further experiments utilising this technique of analysis might also help to explain the numerous interactions seen to take place between neutral and basic amino acids during efflux to the serosal surface (Munck & Schultz, 1969*b*; Cheeseman, 1981, 1982).

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