J. Biosci., Vol. 1, Number 1, March 1979, pp. 1–12. © Printed in India.

Interaction of amino acids with glycyl-glycine transport in the mammalian intestine*

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MS received 27 October 1978; revised 1 January 1979

Abstract. In order to investigate a possible interaction between free amino acids and dipeptides during their mucosal uptake in man and monkey, perfusion studies *in vivo* and uptake studies *in vitro* using labelled and non-labelled dipeptides and amino acids have been carried out. In contrast to the observations of other workers, inhibition of glycyl-glycine uptake was observed with free leucine and methioninc but not with glycine, proline, hydroxyproline or alanine. Leucine and methionine caused inhibition of cytosol glycyl-glycine hydrolase activity, while glycine had no effect. The dipeptide uptake and dipeptide hydrolysis by cytosol enzyme was competitively inhibited by leucine. Although brush border glycyl-glycine hydrolase was also inhibited by leucine, the inhibition was noncompetitive. These data indicate that a few free amino acids can interact with dipeptides during uptake. This interaction might occur either at the transport step or at the stage of intracellular dipeptide hydrolysis.

Keywords. Intestine; peptide transport; peptidases; amino acid interaction.

Introduction

Dipeptides and amino acids use separate transport mechanisms for their entry into the enterocyte. Numerous experiments support this concept (for a review, see Das and Radhakrishnan, 1976; Matthews, 1975). The evidence includes (i) the lack of competition between amino acids and dipeptides during transport (Das and Radhakrishnan, 1974); (ii) increased rate of absorption of amino acids from peptides than from equivalent amount of free amino acids (Matthews *et al.,* 1968, 1969); iii) more rapid disappearance from the lumen of partial protein hydrolysates (obtained by enzymatic digestion) than that of the equivalent amino acid mixtures (Crampton *et al.,* 1971; Silk *et al.,* 1975); (iv) abolition of mutual inhibition of transport between amino acids when they are presented as dipeptides (Cheng *et al.,* 1971); and (v) the observations in amino acid transport defects such as

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Hartnup disease and cystinuria (Asatoor *et al.,* 1970; Hellier *et al.,* 1972). However, much remains to be understood about the mechanisms involved in the peptide transport and some studies have produced conflicting results suggesting that interaction may occur between amino acids and dipeptides during uptake (Adibi, 1971; Adibi and Soleimanpour, 1974; Caspary, 1973; Rubino *et al.,* 1971). There are also reports showing that amino acids can alter the rate of hydrolysis of dipeptides by mucosal homogenates (Cheeseman and Smyth, 1971; 1975). But the role of peptide hydrolases in the transport of peptides is still unclear. The aim of this study has, hence, been to investigate this problem by perfusion studies *in vivo* in man and tissue uptake studies *in vitro* in man and monkey.

Materials and methods

Unlabelled glycyl-glycine was obtained from Sigma Chemical Company, St Louis, MO., USA. Labelled [^{14}C] glycyl-glycine was purchased from the Radiochemical Centre, Amersham, Bucks., U.K. All amino acids were of the L-configuration and were either from Sigma Chemical Company, St. Louis, MO., USA or from the California Corporation for Biochemical Research, Richmond, CA., USA. Dioxan used in the scintillation mixture was obtained from British Drug House. Naphthalene was purchased from Eastman Organic Chemicals and 2,5-diphenyloxazole (PPO) and Cab-o-sil from Packard Instrument Company, USA. All other chemicals were of analytical reagent grade.

Perfusion studies

Perfusions were carried out in three normal Indian subjects who formed part of a control group which has been described previously (Hellier *et al.,* 1976). Subjects were admitted to a metabolic ward for assessment prior to the perfusion studies In both cases, the nature of the study was explained and informed consent obtained.

Perfusions were carried out using a modified double lumen tube incorporating a proximal occlusive balloon (Hellier *et al.,* 1970). This enabled direct measurements of absorption to be made. The tube was passed orally after an overnight fast and under radiological control, the infusion port was placed just distal to the duodeno-jejunal junction. The position of the tube was checked before the start and during the study.

The solutions which were studied are shown in table 1. Solutions were made up in 0·15 M saline such that the osmolality was approximately 300 mosm. and the pH adjusted to 7·0. Polyethyleneglycol, a non-absorbable marker (4 mg/ml), was added to correct for volume changes due to water movement.

Solutions were perfused in randomised order at 15 ml/min over a 30 cm segment. For each solution, a half-hour equilibration period was used followed by the collection of three 10 min samples. All samples were collected on ice, acidified with HCl to pH 1·0 to reduce peptide hydrolysis and centrifuged to remove debris. Samples were stored frozen. Polyethyleneglycol was measured by the method of Hyden (1955). Amino acids were measured as copper-ninhydrin complexes using a quantitative paper chromatographic method developed in this laboratory using 75% ethanol as the eluent (Das and Radhakrishnan, 1973).

Table 1. Solutions used in human perfusion studies and the percentage inhibition of glycyl-glycine absorption.

Solution	Amino acid or dipeptide	Concentration	Inhibition of glycyl-glycine absorption (%)
1	Glycyl-glycine	50 mmol/l	
2	Glycyl-glycine + glycyl-leucine	50 mmol/l 30 mmol/l	38·4
3	Glycyl-glycine + glycine + leucine	50 mmol/l 30 mmol/l 30 mmol/l	31·2
4	Glycyl-glycine + glycine	50 mmol/l 30 mmol/l	0

Dipeptides were measured as cadmium-ninhydrin complexes by a similar chromatographic method described by Ganapathy and Radhakrishnan (1977).

Calculations and back diffusion

Details of the formula for calculating absorption have been described previously (Hellier *et al.*, 1970). During dipeptide absorption, free amino acids accumulate in the lumen. These free amino acids arise to a variable extent from brush border hydrolysis and back diffusion out of the cell after intact dipeptide uptake and hydrolysis inside the cell. For uniformity in calculating absorption, free amino acids are treated as though they resulted from hydrolysis of dipeptide at the brush border before dipeptide uptake and an appropriate correction was made to allow for this in calculating the dipeptide uptake rates.

Uptake studies

Uptake experiments were carried out as described by Das and Radhakrishnan (1975). Adult male monkeys (*Macaca radiata*) after an overnight fast were used. Since an earlier study by Das and Radhakrishnan (1975) has shown that maximal peptide uptake occurred at a distance of 35–40% of the total length of the intestine from pyloric to ileocaecal end, this region was used in this study. Small rectangular strips (whole wall) of the intestine weighing approximately 30–40mg were used. Experiments using five animals were performed in duplicate in each animal.

The surgical specimens from human intestine were resected from the jejunum of patients undergoing corrective surgery for complications following gastroenterostomy. The specimens were immediately transferred info ice-cold

oxygenated Krebs-Ringer bicarbonate buffer (Umbreit *et al.,* 1964) containing 5% glucose. Pieces of the mucosal layer weighing around 30–40 mg were carefully removed and were used for the uptake studies. The procedure was the same as for the monkey intestinal strips.

Each intestinal strip was incubated with an amino acid or dipeptide solution in 0·5 ml of Krebs-Ringer bicarbonate buffer containing 5% glucose. D-Mannitol was used to equalise the osmolality of the solutions. All incubations were at 37° C for 2 min except where otherwise mentioned. The uptake of glycyl-glycine was found to be linear over a 2 min incubation period. After incubation, the strip was gently blotted on filter paper and weighed. Experiments were done using both labelled and non-labelled glycyl-glycine. In the case of non-labelled glycyl-glycine, the strips were homogenised in 0·5 ml ethanol : water (4 :1,v/v). After centrifugation, the supernatant was used for the determination of glycyl-glycine.

In the case of labelled glycyl-glycine, the intestinal strip was digested with 0·5 ml NaOH (2M) in a scintillation vial at 75° C for 2 h. After digestion, 0·5 m of water and 10 ml of scintillation mixture were added. The scintillation mixture contained naphthalene (10%), 2,5–diphenyloxazole (0·5%) and Cab-o-sil (3%) in dioxan. The radioactivity was measured in a liquid scintillation spectrometer (Packard model).

For the determination of extracellular space, the tissue was incubated with [^{3}H]-inulin under the same conditions as the peptide solutions and the radioactivity determined as described above. The extracellular fluid for a 2 min incubation period was found to be 5% of the wet wt of the tissue. The total water content determined separately by drying the tissue at 110° C for 12 h was found to be 78% of the wet wt of the tissue.

Preparation of soluble and particulate fractions

After the monkeys were killed under nembutal anaesthesia, the entire small intestine was taken out. The intestine was washed with 0·154 M KCl and cut open longitudinally. The mucosa was scraped off and 20% homogenate prepared using 0·02 M sodium phosphate buffer, pH 7·0. The homogenate was filtered through nylon cloth (St. Martins Bolting Cloth 9N, Henry Simon Limited, Cheshire, UK), to remove unbroken cells. The filtrate was centrifuged at 105,000 g for 60 min (Beckman Spinco, Model L, rotor type 50) to obtain the soluble fraction. The pellet was washed twice by resuspending it in 0·02 M phosphate buffer, pH 7·0 and centrifuged at 105,000 g for 60 min. The washed pellet was designated as the particulate fraction.

Enzyme assay

The assay mixture contained glycyl-glycine (0·3–6 μmol), Tris-HCl bufer, pH 7·8 (5 μmol), cobalt chloride (0·005μmol) and the enzyme in a total volume of 0·05 ml. Cobalt ion has earlier been shown to activate glycyl-glycine hydrolase (Smith, 1948). Incubations were carried out at 37° C for a time sufficient to hydrolyse about 20% of the substrate and the enzyme was heat-inactivated. Glycine was estimated by the paper chromatographic method (Das and Radhakrishnan, 1973)·

When phenylalanyl-glycine and glycyl-phenylalanine were used as substrates, *p*-hydroxymercuribenzoate (0·1 mM) completely inhbits cytosol peptide hydrolases but does not affect the brush border peptide hydrolases (Heizer *et al.,* 1972). While 0·1 mM *p*-hydroxymercuribenzoate did not inhibit brush border glycyl-glycine hydrolase, it inhibited 92–100% of cytosol glycyl-glycine hydrolase. In the assay of the brush border glycyl-glycine hydrolase activity, *p*-hydroxymercuribenzoate (0·1 mM) was used.

Glycyl-glycine hydrolysis by both particulate and soluble fractions was inhibited markedly at high levels of the substrate when commercial glycyl-glycine (Sigma) was used. Therefore, glycyl-glycine, recrystallised from ethanol in the presence of 1 mM EDTA, was used n this work.

Results

Experiments using non-labelled glycyl-glycine

Perfusion studies : The percentage inhibition of glycyl-glycine absorption by glycine, leucine and glycyl-leucine are given in table 1. The numbers represent the mean of values from three subjects.

Glycyl-glycine absorption was inhibited by glycyl-leucine confirming the work of Adibi and Soleimanpour (1974). However, in contrast to their study, a mixture of glycine and leucine caused inhibition (31%) of glycyl-glycine absorption, though the inhibition was slightly less than that caused by glycyl-leucine (38%). But glycyl-glycine absorption was unaffected by glycine.

Uptake studies : Uptake of glycyl-glycine was inhibited by glycyl-leucine both in the monkey and in the human, the percentage of inhibition in both the cases being 25%. Methionine and leucine inhibited the uptake of glycyl-glycine, though the inhibition with leucine was higher than with methionine (37% and 24% respectively). Glycine had no effect on glycyl-glycine uptake. The control experiments with tissue in Krebs-Ringer bicarbonate buffer alone showed very high levels of glycine (6 μmol/gm wet tissue) but did not reveal free leucine or glycyl-glycine in the tissue. Because of the high concentration of glycine in the tissue, it was not possible to detect the differences in the concentrations of glycine in the tissues incubated with glycyl-glycine in the absence and presence of leucine. However, it was possible to measure the poorly hydrolysed glycyl-glycine inside the cell by the paper chromatographic procedure. The results are given in figure 1.

In the human, experiments were performed using surgical specimens from three subjects. Only the effect of leucine on glycyl-glycine uptake was studied. The results given in figure 2 show that leucine inhibits glycyl-glycine uptake by 32%, a value similar to that observed in the monkey.

Experiments using labelled glycyl-glycine

Time course of uptake: The time course of uptake of [^{14}C]-glycyl-glycine in monkey small intestine was studied and the uptake was shown to be linear upto 2 min (data not given). During this incubation period, the amount of glycine formed from glycyl-glycine hydrolysis was negligible.

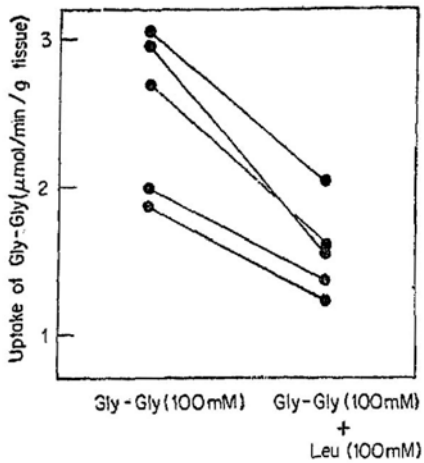

Figure 1. Effect of leucine on glycyl-glycine uptake in monkey.

The uptake of glycyl-glycine (100 mM) in the absence and presence of leucine (100 mM) was measured. The points represent the average of duplicate values from each of the five animals.

Effect of glycyl-glycine concentration on its uptake: The effect of glycyl-glycine concentration on its uptake is shown in figure 3. Measurements of tissue radioactivity after 2 min exposure of the mucosal cell to [^{14}C]-glycyl-glycine does not suggest the usual Michaelis-Menten kinetics for glycyl-glycine transport. Over the range of dipeptide concentration from 0 to 25 mM there seems to be two saturable transport process. But, as the concentration increases above 25 mM, there appears to be a sudden increase in the uptake which is not saturated even at the

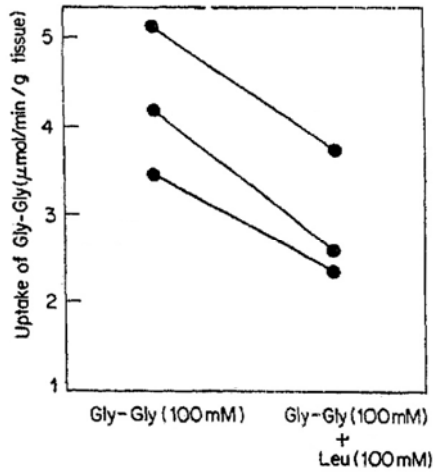

Figure 2. Effect of leucine on glycyl-glycine uptake in man.

The uptake of glycyl-glycine (100 mM) in the absence and presence of leucine (100 mM) was measured. The points represent the average of duplicate values from each of the three subjects.

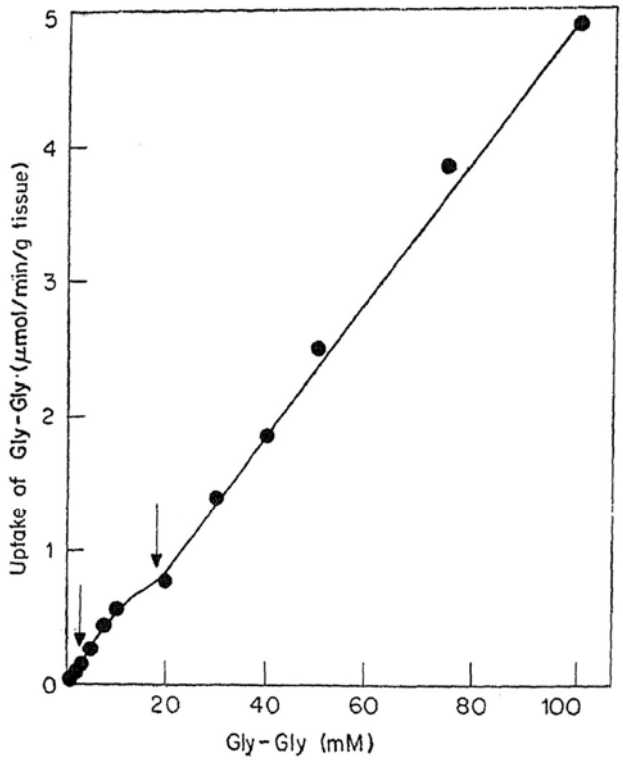

Figure 3. Uptake of [^{14}C]-glycyl-glycine (monkey intestinal strips) as a function of concentration of the dipeptide.
The arrows show the breaks in the curve corresponding to k_t values, 5 mM and 25 mM.

concentration as high as 100 mM. The fact that the uptake of glycyl-glycine at 100 mM was inhibited by leucine as revealed by the studies using non-labelled dipeptide suggests that this also might be a carrier-mediated process.

The effect of the dipeptide concentration in the medium on the accumulation of radioactivity inside the tissue is given in figure 4 as a Lineweaver-Burk plot The two transport processes observed below 25 mM have apparent k_t values of 5 mM and 25 mM. The effect of leucine (50 mM) on glycyl-glycine uptake when the dipeptide concentration ranged from 5 mM to 100 mM was studied. This study suggests that leucine inhibits the uptake process of glycyl-glycine (k_t=25 mM) competitively (figure 5). Though the transport prucess operating above 25 mM is also inhibited by leucine, kinetic analysis is difficult since the lines apparently pass through the origin.

Effect of leucine on glycyl-glycine hydrolysis

The effect of leucine on glycyl-glycine hydrolysis was checked using both the soluble and the particulate fractions of monkey intestinal mucosa. Soluble enzyme was more susceptible to leucine inhibition than the particulate enzyme. Significant inhibition (35%) of glycyl-glycine hydrolysis by the soluble fraction was

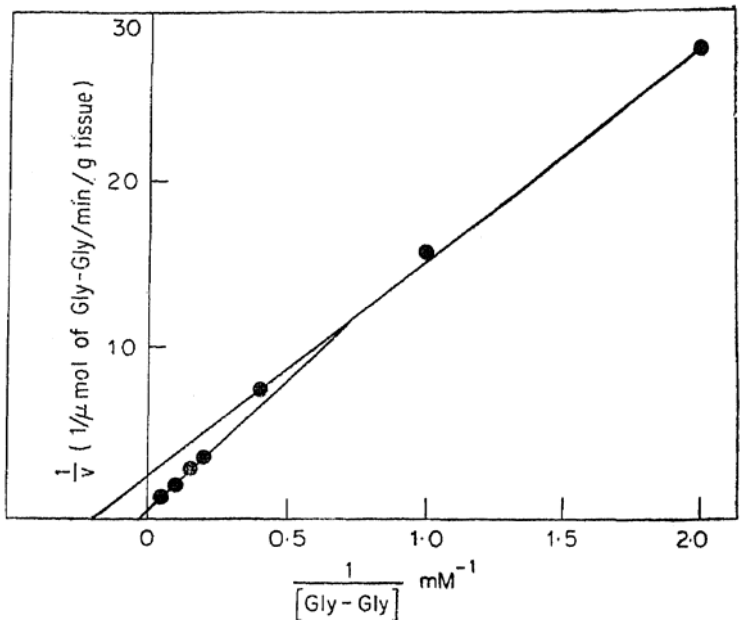

Figure 4. Lineweaver-Burk plot for the uptake of [^{14}C]-glycyl-glycine, Concentration used was 0·5 mM – 25 mM, with monkey intestinal strips k_t values : 5mM and 25 mM.

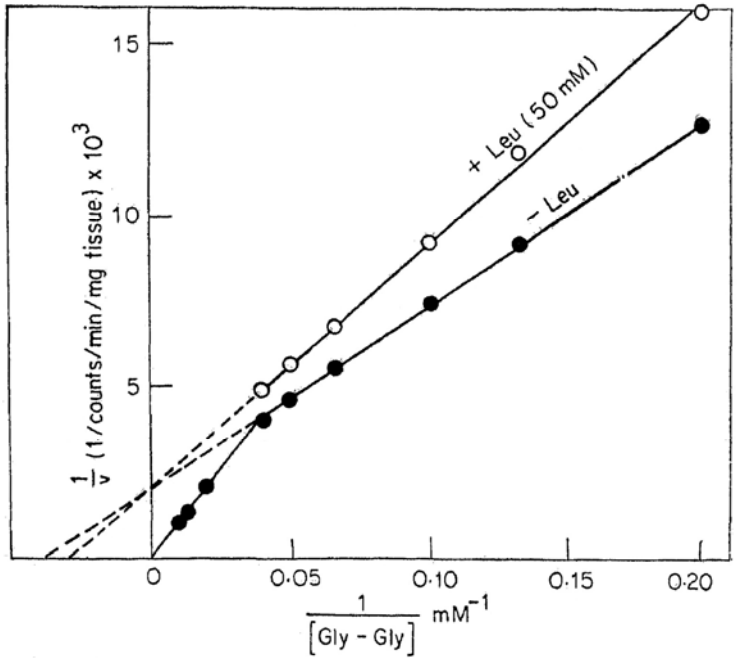

Figure 5. Lineweaver-Burk plot showing competitive inhibition of uptake of [^{14}C] glycyl-glycine by leucine, using monkey intestinal strips. Inhibitor concentration 50 mM.

noticed even when the concentration of leucine was only 1 mM and complete inhibition was observed at a concentration of 40 mM.

Though 40 mM leucine completely inhibited the hydrolysis of glycyl-glycine, it was not possible to build up this concentration inside the enterocyte within the 2 min incubation period. But, the observation that 1 mM leucine inhibited soluble glycyl-glycine hydrolase significantly is very important because it has been shown that leucine concentration within the enterocyte can build upto 3 mM in 2min incubation period, leucine concentration in the medium being 100 mM Hence, leucine can be expected to have an inhibitory effect on the intracellular hydrolysis of glycyl-glycine under the conditions used here.

Kinetics of leucine inhibition of soluble and particulate glycyl-glycine hydrolases was also studied. Whereas soluble enzyme was competitively inhibited, the particulate enzyme was inhibited noncompetitively (figures 6 and 7). Though both the fractions of the enzyme were inhibited by leucine, the soluble enzyme was more susceptible than the particulate enzyme. Leucine concentration (2 mM) caused 50% inhibition of the soluble enzyme while 10 mM leucine was needed to cause the same amount of inhibition of the particulate enzyme. This difference in the degree of susceptibility to inhibition and also the kinetic pattern of the inhibition show that glycyl-glycine hydrolases in the soluble and the particulate fractions may be two different enzymes.

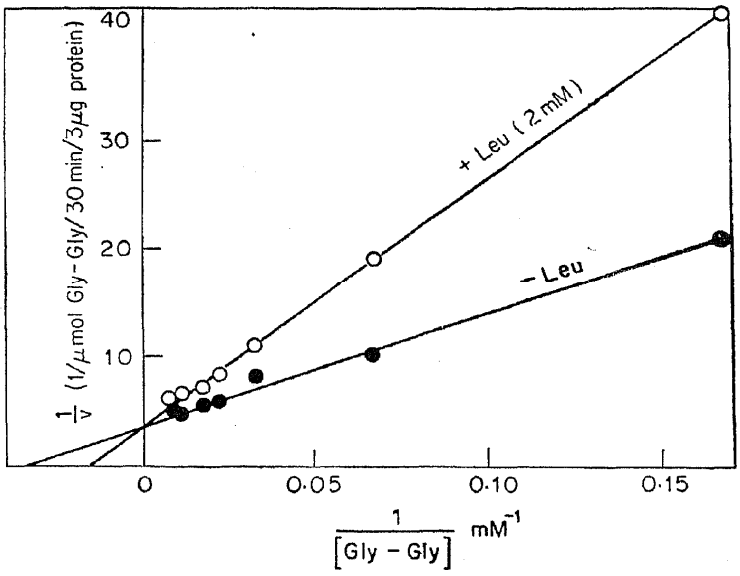

Figure 6. Lineweaver-Burk plot showing competitive inhibition of glycyl-glycine hydrolysis by leucine (soluble enzyme from monkey intestine).

Discussion

The data presented in this paper demonstrate beyond doubt that certain amino acids like leucine and methionine inhibit glycyl-glycine uptake. The precise

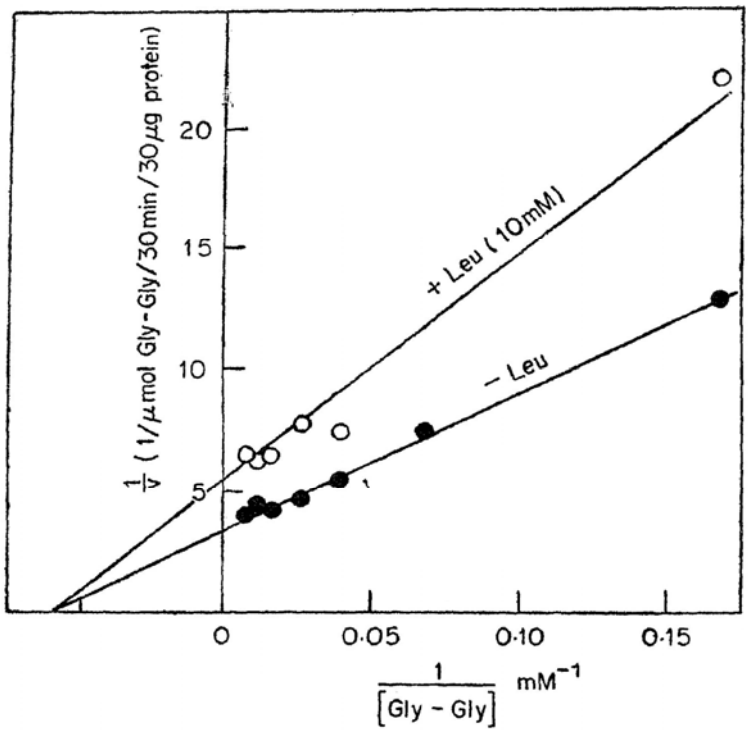

Figure 7. Lineweaver-Burk plot showing non-competitive inhibition of glycyl-glycine hydrolysis by leucine (particulate enzyme from monkey intestine).

interactions responsible for this inhibition has not been clearly defined. There are three possible sites of interaction : the transport step at the brush border, brush border,dipeptide hydrolysis and cytosol dipeptide hydrolysis. For several reasons, interactions with brush border hydrolase can be discounted in the case of glycyl-glycine. First, the inhibition of glycyl-glycine uptake by leucine is competitive while leucine inhibits brush border glycyl-glycine hydrolase non-competitively. Secondly, if the brush border hydrolysis of glycyl-glycine is an integral part of glycyl-glycine uptake process, then glycyl-glycine would not be detected intracellularly. The presence of glycyl-glycine intracellularly rules out hydrolysis prior to uptake.

The evidence presented in this paper is suggestive of an interaction of leucine at the stage of cytosol dipeptide hydrolysis. The main evidence for this is the observation that leucine inhibits both glycyl-glycine uptake and cytosol glycyl-glycine hydrolase competitively. Glycine has no effect on glycyl-glycine uptake or on the cytosol glycyl-glycine hydrolase. Under the conditions of this study the intracellular concentration of leucine was approximately 3 mM, and it has been shown that 1 mM leucine caused significant inhibition of the cytosol glycyl-glycine hydrolase. This suggests that inhibition of intracellular hydrolysis of glycyl-glycine could have occurred and may have been responsible for the inhibition of uptake (figures 1 and 2). However, it is also possible that there is a direct interaction between leucine and the dipeptide transport at the brush border, independent of the observed effect on cytosol hydrolysis.

In the kinetic studies reported here, since the radioactivity present inside the tissue was measured, it might be argued that leucine might have its inhibitory effect on the uptake of radioactive glycine in the medium which might have resulted either from the brush border hydrolysis or from the efflux of the amino acid from the inside of the cell or both. However, this argument is not tenable since glycine concentration in the medium after 2 min incubation with the tissue was negligible. When the concentration of the dipeptide in the medium was 50 mM, after 2 min incubation with a 40 mg piece of the intestinal tissue, the concentration of glycine was only 0·45 mM which is less than one-hundredth of the dipeptide concentration. This concentration of free glycine in the medium is negligible and hence the uptake of free glycine cannot contribute to the accumulation of radioactivity inside the tissue to any significant extent. Also, Rubino *et al.,* (1971) have shown that some amino acids inhibit the transport of glycyl-proline in the rabbit ileum. This dipeptide is taken up intact into the enterocyte because of the lack of glycyl-proline hydrolysing activity in the rabbit brush border (Rubino and Guandalini, 1977). The absence of the enzyme has been confirmed also in the case of monkey intestinal brush border (unpublished data). The results presented in this paper would thus support the idea that the amino acids have a definite effect on the transport of the dipeptide, glycyl-glycine. The degree of inhibition by glycyl-leucine of glycyl-glycine absorption compared to the inhibition by an. equimolar concentration of glycine and leucine suggests that the mode of inhibition by the dipeptide and the free amino acids is different.

Competition between dipeptides during transport across the mucosal border has been clearly demonstrated by many workers (for a review, see Matthews, 1975; Matthews and Adibi, 1976; Das and Radhakrishnan, 1976). It was argued that the interacting dipeptides compete for the same transport system. In addition, the evidence presented in this paper for the interaction of free amino acids with dipeptides during transport indicate that the inhibition of glycyl-glycine uptake glycyl-leucine is the combined effect of the dipeptide glycyl-leucine and leucine which could have accumulated inside the cell by the mucosal hydrolysis of the dipeptide. This would probably explain our preliminary findings that the inhibition of glycyl-glycine uptake by glycyl-leucine is greater than the inhibition of glycyl-leucine uptake by glycyl-glycine.

Acknowledgements

M.D.H was generously supported by a research grant from the Endowment Fund, St. Thomas' Hospital, London. We wish to thank Dr Anne Gammon for her help in the perfusion studies and Mr. R. Jacob and Miss K. M. Roshini for their technical help. We are grateful to Prof. S. J. Baker for his helpful discussion and to Prof. V I. Mathan whose patients were studied.

References

Adibi, S. A. (1971) *J. Clin. Invest.,* **50,** 2266.

Adibi, S. A. and Soleimanpour, M. R. (1974) *J. Clin. Invest.,* **53,** 1368.

Asatoor, A. M., Cheng B., Edwards, K. D. G., Lant, A. F., Matthews, D. M., Milne, M. D., Navab. F. and Richards, A. J. (1970) *Gut,* **11,** 380.

Caspary, W. F. (1973) in *Biochemical and clinical aspects of peptide and amino acid absorption* eds, K. Rommel, and H. Goebell, (Stuttgart, Germany : Schattauer) p. 29.
Cheeseman, C. I. and Smyth, D. H. (1971) *J. Physiol.* (*London*), **218,** 39.
Cheeseman, C. I. and Smyth, D. H. (1975) *Proc. R. Soc.* (*London*), **B190,**149
Cheng, B., Navab, F., Lis, M. T., Miller, T. N, and Matthews, D. M. (1971) *Clin. Sci.,* **40,** 247.
Crampton, R. F., Gangolli, S. D., Simson, P. and Matthews, D. M. (1971) *Clin. Sci.,* **41,** 409
Das, M. and Radhakrishnan, A. N. (1973) *Biochem. J.,* **135,** 609.
Das, M. and Radhakrishnan, A. N. (1974) *Indian J. Biochem. Biophys.,* **11,** 12
Das, M. and Radhakrishnan, A. N. (1975) *Biochem. J.,* **146,** 133.
Das, M. and Radhakrishnan, A. N. (1976) *World Rev. Nutr. Diet.,* **24,** 58.
Ganapathy, V. and Radhakrishnan, A. N. (1977) *Indian J. Biochem. Biophys.,* **14,** 83
Heizer, W. D., Kerley, R. L. and Isselbacher, K. J. (1972) *Biochim. Biophys. Acta,* **264,** 450
Hellier, M. D., Perrett, D. and Holdsworth, C. D. (1970) *Br. Med. J.,* **4,** 782.
Hellier, M. D., Holdsworth, C.D., Perrett, D. and Thirumalai, C., (1972) *Clin. Sci.,* **43,** 659.
Hellier, M. D., Radhakrishnan, A. N., Ganapathy, V., Gammon, A. and Baker, S. J. (1976) *Br. Med. J.,* **24,** 186.
Hyden, S. (1955) *Ann. Agri. Fenn.,* **22,** 139.
Matthews, D. M., Craft, I. L., Geddes, D. M., Wise, I. J. and Hyde, C. W. (1968) *Clin. Sci.,* **35,** 415.
Matthews, D. M., Lis, M. T., Cheng, B. and Crampton, R. F., (1969) *Clin. Sci.,* **37,** 751.
Matthews, D. M., (1975) *Physiol. Rev.,* **55,** 537.
Matthews, D. M., and Adibi, S. A. (1976) *Gastroenterology,* **71,** 151.
Rubino, A., Field, M. and Schwachman, H. (1971) *J. Biol. Chem.,* **246,** 3542.
Rubino, A., and Guandalini, S. (1977) in *Peptide transport and hydrolysis,* (CIBA Found Symp. 50) (North-Holland : Elsevier, Excerpta Medica).
Silk, D. B. A., Clark, M. L., Marrs, T. C., Addison, J. M., Burston, D., Matthews, D. M. and Clegg, K. M. (1975) *Br. J. Nutr.,* **33,** 95.
Smith, E. L. (1948) *J. Biol. Chem.,* **173,** 571.
Umbriet, W. W., Burris, R. H. and Stauffer, J. F. (1964) in *Manometric techniques and tissue metabolism* 4th ed. (Minneapolis : Burgess Pub. Co.), p. 132.