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HYDROLYSIS AND ABSORPTION OF PROLINE DIPEPTIDES ACROSS THE WALL OF SACS PREPARED FROM EVERTED RAT INTESTINE

LEO J. SAIDEL and IDA EDELSTEIN

Department of Biochemistry, The Chicago Medical School, Chicago, Ill. 60612 (U.S.A.)

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SUMMARY

1. Transfer of intact glycyl-L-proline, L-prolylglycine, L-prolyl-L-proline, L-leucyl-L-proline, L-prolyl-L-hydroxyproline and *N*-formyl-L-leucine to the interior of sacs prepared from 5 cm segments of everted rat intestine was observed. The amounts varied from 0.24 ± 0.15 (S.E.) μmole per 30 min for leucylproline to 2.54 ± 0.23 μmole per 30 min for prolylproline. Of the two dipeptides containing glycine and proline there was significantly more transfer of prolylglycine than of glycylproline.

2. For most of the compounds examined, an inverse relationship was found between the intact dipeptide or *N*-formylleucine transferred in 30 min and the total hydrolysis in the same period.

INTRODUCTION

It is generally accepted that very little intact peptide passes through the intestinal lining to enter the circulatory system [1]. Yet the work of Ziff et al. [2], Prockop et al. [3], Meilman et al. [4] and Hueckel and Rogers [5] demonstrating the excretion of large amounts of bound hydroxyproline or proline after ingestion of gelatin indicated that proline peptides might be absorbed into the circulatory system intact. These investigations were all done with intact animals where there might be some question about further action on the amino acids within the body after absorption. The use of everted intestinal sacs reduces this possibility greatly although it has other obvious disadvantages in being less physiological. There were a number of other in vitro studies demonstrating intact peptide transfer across the intestinal lining for a few peptides [6–9], but at the time that this work was started there were no such studies with proline or hydroxyproline. Since then Hueckel and Rogers [10] have published their work demonstrating intestinal transfer of L-prolyl-L-hydroxyproline (Pro–Hyp) using hamster gut segments.

Using ninhydrin amino acid and peptide assay methods principally, we were able to demonstrate that glycyl-L-proline (Gly–Pro), L-prolylglycine (Pro–Gly), L-prolyl-L-proline (Pro–Pro), L-leucyl-L-proline (Leu–Pro) and Pro–Hyp all crossed the intestinal wall. Moreover, most of the dipeptides collected in the serosal compartment in amounts inversely related to the hydrolytic capacity of the system for the corresponding dipeptide.

EXPERIMENTAL

Everted intestine procedure

Male Sprague–Dawley rats weighing about 250 g and fed on pellets of Rockland rat stock diet up until the time of killing were used. The animals were stunned, decapitated and the intestines removed. The intestines were separated from mesenteric tissue and rinsed with the buffer to be used during incubation. The everted intestine procedure of Wilson and Wiseman [11] was followed using 5-cm segments, each about 0.5 g wet wt, beginning 16 cm from the pyloric sphincter. All segments were filled with 1.00 ml Krebs–Henseleit bicarbonate buffer [12] at pH 7.4 containing 0.2 % (w/v) glucose and saturated with O_2–CO_2 (95 : 5, v/v). Experimental segments were placed in 5.00 ml of the same solution containing 10 mM dipeptide or *N*-formylleucine in 25-ml glass stoppered Erlenmeyer flasks. Control segments contained no dipeptides. The sacs were incubated for 30 min in an atmosphere of O_2–CO_2 (95 : 5, v/v) oscillating 130 times per min (7-mm amplitude) in a bath at 37 °C. This rate of oscillation is somewhat high but when we varied the rate from 60 to 130 oscillations per min, we observed no significant change in the protein content of the mucosal fluid (0.8 mg/ml) nor in the microscopic appearance of the tissues. After 30 min incubation the flasks were chilled and the sacs were rinsed, blotted and opened. The serosal solution inside and the mucosal solution outside the sacs were treated immediately with 2 μl of 37 % (w/w) HCl per ml of solution and centrifuged in preparation for dipeptide and amino acid analysis.

Ninhydrin analysis for dipeptide and amino acids

For the analysis of dipeptide and component amino acids in a mixture of all three, such as would result from partial hydrolysis, an automated procedure was developed based on the Matheson and Tattrie [13] modification of the Yemm and Cocking [14] ninhydrin method. This method was chosen because it maximizes the difference in color yields between dipeptide and component amino acids. By measuring color yields at two wavelengths, viz., 440 and 570 nm both before and after acid hydrolysis in N_2 filled borosilicate tubes [15] and comparing them with standard values, it was possible theoretically to calculate the concentration of most proline dipeptides and the component amino acids in any mixture of the three. Such an analysis is possible only because of the great difference in color yields between proline and other amino acids at both wavelengths. Thus it could not be applied to Pro–Hyp successfully unless it was assumed that proline and hydroxyproline were present in equal concentrations.

Paper chromatography

Aliquots of serosal solutions and standard dipeptide solutions were spotted 1 cm from the center of 15 cm Whatman No. 1 filter paper disks and developed radially with butanol-1, acetic acid, water solvent on adjacent segments. The peptides were located on the paper by spraying it with ninhydrin or NaOCl and starch iodide [16] and the positions of the resulting colored rings were compared.

Column cation-exchange chromatography

In some experiments dipeptide and amino acid concentrations were measured

by cation-exchange chromatography applied to samples from both compartments and from the sac tissue after treatment with $Ba(OH)_2$ and $ZnSO_4$ solutions to precipitate proteins. Each sac was rinsed, blotted and immediately homogenized at high speed for 2 min with 1.5 ml of protein precipitant in a Potter–Elvehjem tissue grinder using a glass pestle with fine clearance all submerged in crushed ice. After the precipitates were centrifuged, suitable aliquots of the supernatant solutions were analyzed for dipeptides and amino acids on the Technicon amino acid analyzer packed with Chromobeads A. For sacs the concentrations of peptides are based on the wet weights of the sacs and the analysis of 83 % water content.

Materials

The peptides and amino acids were research grade and were used without further purification. Gly–Pro, Pro–Gly and Leu–Leu were obtained from Nutritional Biochemicals; Leu–Pro hydrochloride, Pro–Pro and Pro–Hyp were obtained from Cyclo Chemical. The amino acids were all obtained from Calbiochem. *N*-Formyl-L-leucine was synthesized and characterized in our laboratory. The ninhydrin color yields at 440 nm of the peptides after acid hydrolysis agreed with that of molar equivalents of amino acids ±5 %.

RESULTS AND DISCUSSION

Transfer of intact dipeptides and N-formylleucine to serosal compartment

As indicated in Table I, after 30 min varying amounts of all of the proline dipeptides tested, as well as *N*-formylleucine were found in the serosal compartment. These findings were confirmed for the dipeptides by paper chromatography and cation-exchange chromatography procedures outlined in Experimental. In contrast no

TABLE I

DIGESTION AND TRANSFER OF DIPEPTIDES WITH EVERTED RAT INTESTINAL SACS

All results except those indicated were averages ±S.E. obtained aerobically with at least 4 sets of sacs from at least 2 animals using the ninhydrin-acid-hydrolysis analysis. Although there were small differences in the initial substrate concentration (usually 10.0±0.5 mM) in the 5.00 ml solution outside the sac (mucosal), all results here are normalized to 10.0 mM of dipeptide. Values are expressed in μmoles in sac (serosal) at 30 min based on 1.0 ml initial vol.

Peptide or amino acid used	Dipeptide	N-terminal amino acid		C-terminal amino acid
Gly–Pro	0.28±0.17	3.95±0.34		4.07±0.28
Pro–Gly	1.65±0.20	1.80±0.15		1.90±0.26
Pro–Pro	2.54±0.23		1.46±0.22	
Leu–Pro	0.24±0.15	1.99±0.21		2.65±0.46
Pro–Hyp	1.27±0.26		0.70±0.16	
Leu–Leu*	0		4.04±0.37	
N-Formylleucine*	2.01±0.13			0.08±0.08
Gly–Pro**	0.11±0.06	3.61±0.61		4.09±0.58

* Results obtained with 2 sets of sacs from a single animal.

** Results obtained with 3 sets of sacs from a single animal using cation-exchange chromatography.

Leu–Leu was found in this compartment by any of the procedures used. Of the two dipeptides containing glycine and proline, significantly ($P < 0.005$) more-Pro–Gly than Gly–Pro was found in the serosal compartment after 30 min.

Relationship between rate of transfer and rate of hydrolysis

It was anticipated that the amount of dipeptide found in the serosal compartment might depend on the rate of dipeptide hydrolysis with only the more slowly hydrolyzed dipeptides surviving passage through the intestinal wall. Different relative measures of dipeptide hydrolysis were obtained, such as the decrease in dipeptide concentration or the increase in component amino acid concentrations. In Fig. 1, the total amount of dipeptide or *N*-formylleucine found within each individual sac (serosal compartment) at 30 min is plotted as ordinate against one-half the total increase in component amino acids found both inside and outside each sac at 30 min as abscissa. The inverse relationship evident here was observed no matter which of the possible measures of hydrolysis was used. The values for five of the dipeptides and *N*-formylleucine scatter fairly well around the drawn line with slope of −0.060 and ordinate intercept of 2.65 ($r = -0.911$; $r^2 = 0.830$). The values for Pro–Hyp deviate considerably and were not included in the calculations. (If included, then $r = -0.786$; $r^2 = 0.618$). Of course any rapidly hydrolyzed dipeptide as Leu–Leu which does not appear intact in the serosal compartment would give results falling on the line at the abscissa because of the fixed amount of substrate used in all of the experiments (50 μmoles).

Although it has been evident for some time that most peptide bonds are hydro-

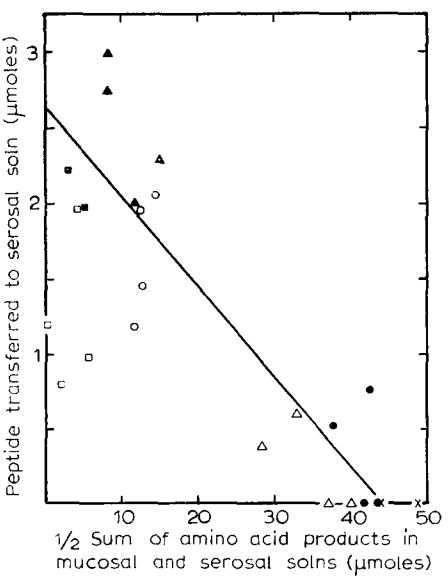

Fig. 1. Transfer of peptide vs hydrolysis of peptide in each individual 5 cm everted rat intestinal sac. Initial charge was 50 μmoles peptide in 5 ml mucosal solution under aerobic conditions. Serosal solution was 1 ml buffer only. The correlation coefficient for the line of regression, omitting only the points for Pro–Hyp, is $r = -0.911$; $r^2 = 0.830$. △, Leu–Pro; ▲, Pro–Pro; □, Pro–Hyp; ×, Leu–Leu; ●, Gly–Pro; ○, Pro–Gly; ■, *N*-formylleucine.

lyzed before or during the process of absorption of protein breakdown products, there has also been increasing evidence that many intact peptides are able to cross the intestinal wall to a limited extent. The data presented here show that all of the proline dipeptides examined are able to do so and that the extent to which they get through the wall is inversely related to the rate of hydrolysis. That the points in Fig. 1 appear to cluster around a straight line with the above regression constants is largely attributable to the particular conditions of concentration and time selected but the possibility remains that some common mechanism of absorption of certain dipeptides is indicated. As might be expected, there is some indication in Table I that the free amino acids derived from the dipeptides which are more slowly hydrolyzed tend to appear in lesser amounts in the serosal compartment.

After this paper was first submitted for publication, Boullin et al. [17] reported that intact Gly–Pro and Pro–Gly as well as carnosine, glycylglycine, glycyl-L-phenylalanine and glycyl-D-phenylalanine could be detected in the superior mesenteric vein after intraluminal injection of dipeptide into anaesthetized rats and that there was an apparent inverse relationship between rate of hydrolysis and detection of intact peptide. The general agreement of these in vivo results with our in vitro results indicates that permeability through the unstirred layer recently discussed by Winne [18] is not rate limiting for these dipeptides which can be compared.

Dipeptides in the sac tissue

An attempt was made to estimate the dipeptide concentration in sac tissue after 30 min incubation with dipeptide in order that the concentration gradient from mucosal to serosal compartments might be studied. Hueckel and Rogers [10] had observed a large decrease in serosal concentration of Pro–Hyp compared to the tissue concentrations of this dipeptide in hamster intestine segments. Our direct cation exchange analysis of individual deproteinized rinsed sacs, gave the following concentrations of dipeptides in μmole/ml of sac tissue water after incubation with these respective dipeptides: Gly–Pro, 0.10, 0.26; Pro–Gly, 0.41, 0.97; and Pro–Pro, 0.48, 0.86. These values are all less than the corresponding concentrations in the serosal solutions listed in Table I or those measured in this experiment in the serosal solutions within the corresponding sacs. However, to conclude that these results demonstrate active transport of these dipeptides in this system would not be warranted at this time. Further investigation of the variables is necessary.

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REFERENCES

1 Smyth, D. H. (1972) in Ciba Found. Symp., Peptide Transport in Bacteria and Mammalian Gut (Elliott, K. and O'Connor, A., eds) pp. 59–70, Elsevier, Amsterdam
2 Ziff, M., Kibrick, A., Dresner, E. and Gribetz, H. J. (1956) J. Clin. Invest. 35, 579–587
3 Prockop, D. J., Keiser, H. P. and Sjoerdsma, A. (1962) Lancet 2, 527–528
4 Meilman, E., Urivetzky, M. M. and Rapaport, C. M. (1963) J. Clin. Invest. 42, 40–50

5 Hueckel, H. J. and Rogers, Q. R. (1970) Comp. Biochem. Physiol. 32, 7–16
6 Agar, W. T., Hird, F. J. R. and Sidhu, G. S. (1953) J. Physiol. London 121, 255–263
7 Newey, H. and Smyth, D. H. (1959) J. Physiol. London 145, 48–56
8 Wiggans, D. S. and Johnston, J. M. (1959) Biochim. Biophys. Acta 32, 69–73
9 Ciba Foundation Symposium (1972) Peptide Transport in Bacteria and Mammalian Gut (Elliott, K. and O'Connor, M., eds), Elsevier, Amsterdam
10 Hueckel, H. J. and Rogers, Q. R. (1972) Can. J. Biochem. 50, 782–790
11 Wilson, T. H. and Wiseman, G. (1954) J. Physiol. London 123, 116–125
12 Krebs, H. A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–36
13 Matheson, A. T. and Tattrie, B. L. (1964) Can. J. Biochem. 42, 95–103
14 Yemm, E. W. and Cocking, E. C. (1955) Analyst 80, 209–213
15 Roach, D. and Gehrke, C. W. (1970) J. Chromatogr. 52, 393–404
16 Groskopf, W. R., Holleman, J. W., Margoliash, E. and Klotz, I. M. (1966) Biochemistry 5, 3783–3796
17 Boullin, D. J., Crampton, R. F., Heading, C. E. and Pelling, D. (1973) Clin. Sci. Mol. Med. 45, 849–858
18 Winne, D. (1973) Biochim. Biophys. Acta 298, 27–31