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THE INTESTINAL ABSORPTION OF SOME DIPEPTIDES

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Fisher (1954) has recently discussed the form in which protein is absorbed from the intestine, and has concluded that the evidence for the classical view of absorption as amino acids is less satisfactory than has usually been supposed. He has also suggested that the idea of absorption of protein in the form of peptides deserves serious consideration. Very little information is available on absorption of peptides from the intestine, apart from a brief report by Agar, Hird & Sidhu (1953), who investigated a few peptides *in vitro*. It was therefore decided to carry out an investigation on the passage of peptides through the intestinal mucosa. The present work deals with four different dipeptides and a preliminary account has been given by Newey & Smyth (1957).

METHODS

The general principle was to study the passage of dipeptides across the intestinal mucosal barrier, and in particular to observe whether the peptides or the constituent amino acids appeared on the other side of the barrier. The dipeptides used were glycyl-glycine, glycyl-L-leucine, glycyl-L-tyrosine and DL-alanyl-DL-alanine. Both *in vitro* and *in vivo* procedures were used for studying absorption. In both types of experiment solutions containing the dipeptides were placed in contact with the mucosa. In the experiments *in vitro* dipeptides and amino acids were sought for in the solution bathing the serosal surface of the intestine, and in the experiments *in vivo* in the venous mesenteric blood draining the section of intestine containing the dipeptides.

In vitro. Three different techniques were used: (1) the sac of the everted small intestine as described by Wilson & Wiseman (1954); (2) the isolated intestinal preparation described by Wiseman (1953), as modified by Smyth & Whaler (1953); and (3) the preparation of isolated rat intestine suspended in air, as described by Smyth & Taylor (1954). In referring to these techniques, the term 'mucosal fluid' means the fluid in contact with the intestinal mucosa; that is, in the case of the everted sacs it is the fluid in which the sacs are suspended and in the other *in vitro* techniques it is the fluid which is circulated through the loop of the intestine. The term 'serosal fluid' refers to the fluid in contact with the serosal surface of the intestine; that is, in the case of the everted sacs it is the content of the sac, and in the other *in vitro* techniques it is the solution in which the outer surface of the intestine is bathed, or the solution which passes across the intestine and collects on the outer surface.

The sacs of everted intestine, approximately 12 cm long, were made from the jejunum of the rat. Each sac held 3 ml. Krebs–Ringer–phosphate solution (Krebs, 1933), containing 200 mg glucose/100 ml., and was suspended in 4 ml. of the same solution, to which the dipeptide was

added. The sacs were shaken in standard Warburg flasks for 60 min at 37° C and the mucosal and serosal fluids then collected and examined for dipeptides and amino acids as described in the subsequent section.

In the other *in vitro* methods the saline was circulated through isolated segments of intestine. One important difference between the two *in vitro* techniques must be remembered in interpreting the results. In one preparation (Wiseman, 1953) the isolated intestine is bathed in an outer serosal fluid so that fluid and solutes passing through the wall are diluted considerably. In the second (Smyth & Taylor, 1954) the intestine is suspended in air, and the solution passing through the intestinal wall is collected undiluted. In both cases the initial mucosal solutions were 25–50 ml. bicarbonate-saline (Krebs & Henseleit, 1932) containing 200 or 500 mg glucose /100 ml. and the dipeptide. In the preparation with the outer fluid, this consisted of 30 ml. bicarbonate-saline with the same concentration of glucose as the mucosal solution. The duration of the experiment was 60 min at 37° C.

In vivo. The *in vivo* method was that of Matthews & Smyth (1954), in which the vein draining a loop of the intestine is cannulated and the blood collected. A solution containing the dipeptide dissolved in NaCl solution 0·9% (w/v) was placed in the intestine, and the mesenteric blood draining this loop was subsequently analysed for dipeptides and amino acids. The details of the procedure were as follows. A dog was anaesthetized by intravenous injection of pentobarbitone sodium. The abdomen was opened and a loop of the small intestine was selected, about 20 cm long and drained by a vein suitable for cannulation. The selected loop was washed out with warm saline. After preparing the vein for cannulation the animal was heparinized and the vein cannulated. The cannula draining the vein from the loop was attached to a polythene tube which was brought out through a small hole in the abdominal wall. Immediately outside the abdomen it passed through ice-cold water, and the blood was then collected in a measuring cylinder. The cooling would reduce enzyme activity and perhaps also the rate of passage of amino acids or peptides into the red cells although, as Christensen, Cooper, Johnson & Lynch (1947) have shown, movement of glycine into erythrocytes is very slow. A sample of blood was collected from the vein for a period of 5–10 min. After the collection of this control sample the dipeptide was injected into the loop of the intestine. The collection of the second sample of blood was now commenced, and continued for a period of 10–25 min. The blood flow in the various experiments varied from 7–18 ml./min. The volume of blood collected was measured, and the haematocrit value was determined. The plasma was subsequently taken for analysis. At the conclusion of the experiment the intestinal loop was quickly removed, the contents were collected and the volume measured. The contents were heated to boiling-point to stop enzyme activity and were analysed for amino acids and dipeptides.

Chemical estimations. Glycine was determined before and after acid hydrolysis and the glycyl dipeptide calculated from the difference between free and total glycine contents. Glycine was determined on protein-free filtrates of plasma or intestinal fluids according to the method of Alexander, Landwehr & Seligman (1945) as modified by Christensen, Riggs & Ray (1951). Free glycine was determined directly on one sample and total glycine on a second sample after hydrolysis with 2 vol. concentrated HCl in a sealed tube at 105–110° C for 24 hr. The tube was then opened, the contents were washed out into a vacuum distillation apparatus and, after evaporating twice to dryness to remove hydrochloric acid, the residue was dissolved in a volume of water sufficient to give a concentration of glycine suitable for estimation. The recoveries of glycine added to saline solutions and to plasma varied from 94 to 106%. The recovery of dipeptide in saline varied from 94 to 110% and in plasma from 92 to 112%.

Chromatographic analysis of amino acids and dipeptides. Samples of 20–60 μl. were taken from the solution to be tested and with no preliminary treatment 'spotted' immediately on Whatman No. 1 Paper. In order to obtain an approximate estimate of the peptide and amino acid on the chromatograms, solutions containing known amounts of the substances were also run, and in some experiments known quantities of peptide were added to the experimental solutions. The solvents used were butanol–acetic acid–water (40:10:50) and propanol–water (80:20). The butanol–

acetic acid–water solvent was used for glycyl-leucine and glycyl-tyrosine, and propanol–water for glycylglycine and alanyl-alanine. The chromatograms were run for 24–72 hr at 18° C. They were dried at room temperature and sprayed with 0·1% ninhydrin in butanol.

RESULTS

In vitro experiments with sacs of everted small intestine

These were in the nature of preliminary experiments and were confined to chromatographic analysis of the solutions. Experiments were restricted to glycyl-L-tyrosine and glycyl-L-leucine. The peptide was initially present in the mucosal solution in a concentration of 500 mg./100 ml. The sacs were incubated for 1 hr and the serosal solutions were then examined. It was found that the final serosal solution contained no peptide but considerable amounts of the constituent amino acids. These preliminary experiments suggested that the peptide was transferred as amino acid.

In vitro experiments with isolated intestine suspended in air

Experiments with this preparation, followed by chromatographic analysis, confirmed the results of the experiments with everted sacs. Further experiments were then undertaken, in which the peptide was estimated quantitatively. Glycyl-L-leucine, glycyl-L-tyrosine, and glycyl-glycine were used and the results of the experiments are shown in Tables 1 and 2. In Table 1 the results from one experiment with glycyl-L-leucine are given in some detail to illustrate the method of calculation. It is seen that of the 125 mg of dipeptide originally present in the mucosal fluid, 44 mg was still present at the end of the experiment, and in addition 23 mg of glycine (equivalent to 58 mg dipeptide). There is thus at the end of the experiment a concentration of 215 mg glycyl-L-leucine/100 ml. In spite of this, no appreciable dipeptide appeared in the serosal fluid although it contained 6 mg of glycine (equivalent to 15 mg of glycyl-L-leucine). It thus appears that, even if a high concentration of dipeptide is present inside the intestine, it does not appear in the serosal fluid. Table 2 summarizes the results of a number of other experiments which were similar to those shown in Table 1. Of the dipeptides used, it is seen that only in the case of glycyl-glycine is any appreciable amount of dipeptide found in the serosal fluid, and even in this case the amount is small compared with the amount of glycine present.

The fact that very little peptide appears in the serosal fluid might be due to (1) the inability of peptide to pass through the intestinal barrier, (2) the hydrolysis of peptide while it is passing through the intestine, or (3) the hydrolysis of peptide in the serosal fluid. That this third possibility exists was shown by the fact that the serosal fluid was found to have a marked peptidase activity. It was therefore not possible to conclude from these experiments that peptides cannot pass through the intestinal barrier, but only that in these

particular conditions they were not found in the serosal fluid. There are various ways in which the effect of peptidase in the serosal fluid can be reduced. One method is to take drops of fluid from the surface of the intestine as soon as they appear, so that there is little time for peptidase activity to occur. Another is to chill the fluid dropping from the intestine by surrounding it with an ice jacket, while still maintaining the intestine at 37° C. Both these procedures were used but in no experiment was there any substantial amount of peptide in the serosal fluid. A third method of reducing peptidase activity is discussed in the next section.

TABLE 1. Transfer of glycyl-L-leucine by the *in vitro* intestinal preparation. The initial mucosal solution was 25 ml. Krebs bicarbonate-saline containing 200 mg glucose and 500 mg glycyl-L-leucine/100 ml. Initial amount of peptide 125 mg; duration of experiment 1 hr

	Final mucosal fluid	Final serosal fluid
Vol. (ml.)	20·5	3·4
Concn. of glycine before hydrolysis (mg/100 ml.)	112	184
Concn. of glycine after hydrolysis (mg/100 ml.)	198	185
Increase in glycine on hydrolysis (mg/100 ml.)	86	1
Equivalent concn. of peptide (mg/100 ml.)	215	2
Amount of glycine (mg)	23	6
Amount of peptide (mg)	44	0

TABLE 2. Transfer of dipeptides by the *in vitro* intestinal preparation. The initial mucosal solution in all cases was 25 ml. bicarbonate-saline containing 50 mg glucose (200 mg/100 ml.) and 125 mg peptide. Duration of experiment 1 hr

	Final mucosal solution		Final serosal solution	
Dipeptide	glycine (mg)	dipeptide (mg)	glycine (mg)	dipeptide (mg)
Glycyl-L-leucine	25·7	15·8	3·4	0·0
	31·5	42·4	6·1	0·0
	35·0	6·0	5·8	0·0
	23·0	44·0	6·3	0·0
Glycyl-L-tyrosine	21·0	8·5	4·7	0·0
	17·0	18·4	4·9	0·0
	18·8	42·5	5·7	0·0
	19·6	32·5	5·4	0·0
Glycyl-glycine	33·5	40·1	15·2	0·4
	36·0	42·3	16·5	0·4
	48·4	48·0	20·2	0·8
	36·7	66·8	7·9	0·7

In vitro experiments with intestine suspended in bicarbonate-saline

It seemed probable that the high peptidase activity of the serosal fluid might be reduced by using the form of preparation where the intestine is bathed in a serosal fluid, so that the peptidase will be greatly diluted. In these experiments the fluids were only tested chromatographically, but a rough quantitative estimate was made by running markers of known strength. The results were as follows. In four experiments with glycyl-L-leucine, 500 mg of peptide/100 ml. was initially present in the mucosal fluid, with no peptide in the serosal fluid. At the end of the experiment no peptide was found in the serosal fluid,

large amounts of glycine and leucine were present, and the peptidase activity of the serosal fluid was very low. In three experiments with glycyl-glycine, with 400 mg peptide/100 ml. initially present in the mucosal fluid, less than 10 mg peptide/100 ml. was present in the final serosal fluid, while more than 100 mg glycine/100 ml. was present. Again, there was a very low peptidase activity in the serosal fluid. Two similar experiments were also done with DL-alanyl-DL-alanine, which could not be estimated quantitatively by the method used for the other dipeptides. In these experiments the initial concentration of peptide was 400 mg/100 ml. in the mucosal fluid. At the end of the experiments less than 10 mg/100 ml. was in the serosal fluid and the concentration of alanine was about 100 mg/100 ml. No observable peptidase activity was present in the final serosal fluid. These experiments suggest that the hydrolysis of the dipeptides to amino acids does not take place after the dipeptides have appeared in the fluid, but rather that the amino acids, as such, are added to the serosal fluid by the intestine.

In the experiments just described the critical factor is the peptidase activity in the serosal solutions. Further experiments were therefore carried out in order to obtain more quantitative information about the peptidase activity. An experiment was carried out with glycyl-glycine initially present in the mucosal fluid only. After 60 min the serosal solution was removed and to this was added an amount of glycyl-glycine, which from previous experience was guessed to be necessary to produce the total glycine present in the serosal fluid. The serosal fluid was now incubated separately for a further 60 min and the total glycine again estimated. The increase in glycine during the second 60 min period was due to peptidase activity in the serosal fluid, and from this increase it could be judged whether the peptidase activity was capable of producing the amount of glycine actually found at the end of the first 60 min. In an experiment of this kind it might be argued that the peptidase activity was possibly inhibited by the high concentration of glycine present. Therefore a second experiment was done, similar to the first, except that no glycyl-glycine was present at the beginning of the first 60 min period, but this was added to the serosal fluid at the beginning of the second incubation period. The results of these experiments were as follows.

In the first experiment the intestinal preparation was incubated for 60 min with 400 mg glycyl-glycine/100 ml. and 200 mg glucose/100 ml. in the mucosal fluid, while the serosal fluid consisted of 30 ml. bicarbonate-saline with 200 mg glucose/100 ml. but no peptide. After 1 hr the serosal fluid was removed and the volume was found to be 35 ml. Of this, 5 ml. was used for glycine estimation; to the remaining 30 ml. was added 28 mg glycyl-glycine, and this was incubated for a further 60 min. At the end of this period the glycine was estimated. The glycine content of the total serosal fluid at the end of the first 60 min was 21 mg, and therefore the amount of glycine in 30 ml. was 18 mg. The

glycine content of 30 ml. serosal fluid at the end of the second 60 min was 20·6 mg. The increase in glycine during incubation of 30 ml. of serosal fluid during the second 60 min period was thus 2·6 mg. Since this was produced by the peptidase activity of 30 ml. serosal fluid, the total peptidase activity, i.e. in 35 ml., could have produced 3·0 mg. Since the amount of glycine present at the end of the first 60 min was 21 mg it is evident that only a small amount of this could have been produced by peptidase activity in the serosal fluid and it must therefore have been added to the serosal fluid as glycine.

In the second experiment, where no glycyl-glycine was present initially in the mucosal fluid, the final serosal fluid, when incubated with 100 mg peptide/100 ml. for a further period of 60 min, produced 2 mg glycine. This confirmed the very low activity of peptidase in the serosal fluid, and it is clear that this would be incapable of producing the amount of glycine present, when experiments were carried out with peptide initially present in the mucosal fluid.

In vivo experiments

In these experiments the blood was collected from the loop of the dog intestine before and after introduction of the peptide solution into the loop. Experiments were done with three different dipeptides, and the results are given in Table 3, which shows an analysis of the loop contents at the end of the experimental period, and an analysis of the plasma during the control period before introduction of peptide and also during the absorption of peptide. The term 'bound glycine' is used instead of peptide for the following reasons. The increase in glycine content on hydrolysis is the measure of the peptide present. The plasma sample taken in the pre-absorption period shows a small increase in glycine content on hydrolysis, and therefore contains a small amount of some substance which liberated glycine on hydrolysis. It is not known, however, what this particular substance was, and therefore the figures for glycine cannot be converted to peptide by multiplying by the appropriate factor. It is therefore given as 'bound' glycine. Since this value must be subtracted from the value obtained during the absorption period, this is also given as 'bound' glycine. The increase in 'bound' glycine in the plasma during the absorption period over the 'bound' glycine in the pre-absorption period gives the amount of 'bound' glycine absorbed, and this could be converted to peptide by multiplying by the appropriate factor. The necessity to do this does not arise, because the increase in the 'bound' glycine is in all cases very small. The largest amount of 'bound' glycine absorbed was obtained in an experiment with glycyl-glycine included in the table. The 'bound' glycine in the plasma during the absorption period was 12·0 mg/100 ml., an increase of 11·3 mg/100 ml. over the 'bound' glycine of plasma in the pre-absorption period. This corresponds to 10·0 mg glycyl-glycine/100 ml. The increase in free glycine in the absorption period over the pre-absorption period was

134 mg/100 ml. and hence even in this case it is clear that the greatest part of the peptide absorbed appears in the plasma in the form of amino acid. The difficulty in expressing the amount of peptide does not arise in the case of the content of the loop, and therefore it is given as peptide, and not as 'bound' glycine.

TABLE 3. Absorption of dipeptides from loops of intestine of the dog. The figures give the concentrations of glycine and 'bound' glycine present in the mesenteric plasma draining the loop before and during absorption of the peptide, and also the absolute values for the amounts of glycine and dipeptide present in the loop at the beginning and end of the absorptive period

	Mesenteric plasma					Contents of loop			
	Control period		Absorption period			Initial		Final	
Peptide	Glycine (mg/ 100 ml.)	'Bound' glycine (mg/ 100 ml.)	Glycine (mg/ 100 ml.)	'Bound' glycine (mg/ 100 ml.)	Duration (min)	Glycine (mg)	Peptide (mg)	Glycine (mg)	Peptide (mg)
Glycyl-L-leucine	7·6	0	25·7	0·0	16	0	120	10	0
	3·8	0·4	16·7	1·4	13	0	150	27	2
	6·5	0·3	38·3	0·0	20	0	200	29	27
Glycyl-L-tyrosine	4·7	0·6	25·3	0	20	0	200	20	53
Glycyl-glycine	3·3	0·7	137	12·0	20	0	200	26	58
	5·7	0·8	94	0·6	20	0	110	7	34

DISCUSSION

The results show that when solutions containing dipeptides are in contact with the intestinal mucosa, most of the dipeptide appears on the other side of the mucosal barrier in the form of amino acids. Only in the case of glycyl-glycine was there evidence of appreciable amounts of peptide in the blood or serosal fluid, and even in this case the peptide found accounted for only a small part of that disappearing from the mucosal side. The original problem was the form in which proteins were absorbed into the blood stream, but it now seems essential to re-state this in more precise terms. There are in fact two problems: (1) What is the form in which protein enters the mucosal cells from the intestinal lumen? (2) What is the form in which it enters the blood stream from the mucosal cells? The first question is difficult to answer, since it is uncertain in the present experiments how much hydrolysis takes place in the lumen of the intestine. The products of hydrolysis may be removed very rapidly so that the final composition of the mucosal fluid does not give any information about the amount of hydrolysis which has occurred. It is indeed possible that unchanged peptide can enter the mucosal cells from the intestinal lumen.

The second question is one to which a more definite answer can be given, and from the present results it can be concluded that dipeptides from the intestinal lumen appear in the blood stream chiefly in the form of amino acids.

Since our preliminary communication was published (Newey & Smyth, 1957), a brief report has appeared by Johnston & Wiggans (1958) in which very similar results have been obtained with L-alanyl-L-phenylalanine and DL-alanyl-DL-phenylalanine.

Fisher (1954) has suggested that the time required for production of amino acids by digestion is too great to allow complete break-down of proteins to amino acids in the intestine before absorption. It is possible, however, that peptides may enter the mucosal cells and be hydrolysed intracellularly into amino acids.

In discussing the form in which proteins are absorbed, consideration must be given to the size of molecule which can pass through the intestinal mucosa. It has been frequently pointed out that protein molecules can pass through the intestinal barrier, as is shown by immunological or anaphylactic reactions. It appears to us that evidence of this kind is not very relevant to the problem. The question is whether substances pass through the intestinal barrier at a rate sufficiently rapid to account for absorption of the amount of protein required by the animal, and the amount of protein required to demonstrate an immunological reaction and that required to keep an animal in protein balance are of a different order of magnitude. An anaphylactic reaction has been produced in guinea-pigs sensitized by 0·05 μg ovalbumin after injection of 1 μg antigen (Coulson, Stevens & Shimp, 1949), and immunological reactions are also caused by very minute amounts of protein.

The results of the present investigation indicate that, for the particular peptides used, at most only a very small fraction of the amount introduced into the intestinal lumen can cross the intestinal barrier as such, and most of it appears in the blood stream in the form of amino acids. The work is being extended to polypeptides.

SUMMARY

1. A study was made of the transfer of a number of dipeptides by the intestine both *in vivo* and *in vitro*.

2. In both methods the dipeptides disappeared from the mucosal side, but only very small amounts appeared on the serosal side.

3. Most of the dipeptides transferred appeared on the serosal side as the constituent amino acids.

4. The relation of these findings to absorption of proteins is discussed.

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