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Aminopeptidase activity in homogenates of various absorptive mucosae in the albino rabbit: implications in peptide delivery

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Summary

A methodology was developed to determine the type and activity of aminopeptidases in the conjunctival, nasal, buccal, rectal and vaginal homogenates, relative to duodenal and ileal homogenates, so as to define the aminopeptidase barrier to peptide absorption from non-oral routes. 4-Methoxy-2-naphthylamides of leucine, alanine, arginine and glutamic acid were used as substrates. Based on the pattern of substrate hydrolysis and the effect of activators and inhibitors on the rate of substrate hydrolysis, four to five aminopeptidases were estimated to be present in the mucosal homogenates studied. Aminopeptidase N was present in all these mucosae to the extent of 50–100% of ileal activity, whereas aminopeptidase A was present to the extent of 4–20% of ileal activity. Overall, the differences in aminopeptidase activity among the various non-oral routes were not large. This suggests that, before guidelines can be established to select a given route for optimal peptide delivery, further work is necessary to determine if the non-oral routes differ in non-aminopeptidase activity and in membrane permeability.

Introduction

As a result of rapid advances in knowledge on their diverse physiological functions, several biologically active peptides and proteins including somatostatin, interleukins, and tumor necrosis factor have been projected to be useful therapeutic agents. An important prerequisite in fulfilling this goal is an understanding of the nature as well as effectiveness of the transport and enzymatic barriers in restricting the systemic absorption of these substances. The enzymatic barrier to peptide absorption in the gastrointestinal tract is substantial and has been suggested to be principally responsible for the 0.1% or less of an orally administered dose of a peptide which is absorbed (Okada et al., 1982). Consequently, alternative routes which are presumed to be deficient in peptidase activity have been investigated to optimize peptide delivery. These include the nasal (Anik et al., 1984; Moses et al., 1983; Su et al., 1985), buccal (Ishida et al., 1981), rectal (Nishihata et al., 1983; Yoshioka et al., 1982), and vaginal (Okada et al., 1982) routes. However, there have been no reports on peptidase activity in these mucosal tissues and, in particular, on how it compares with the small intestine. As a result, guidelines on the selection of a given route for optimal peptide delivery are as yet unavailable.

We have initiated studies to characterize the enzymatic barrier to peptide absorption, thereby

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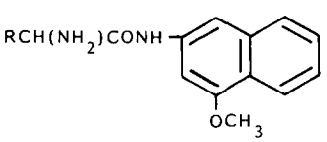
setting the stage for companion studies to define the intrinsic permeability characteristics of various routes of peptide delivery. We have chosen the aminopeptidases, a family of exopeptidases which cleave peptides and proteins at their N-terminus, from among other peptidases for these initial studies for 3 reasons: (1) the broad substrate specificities of these peptidases (McDonald and Schwabe, 1978); (2) their wide tissue distribution (Hiwada et al., 1977); and (3) their principal role in terminating the activity of several neuropeptides (Burbach et al., 1982; Palmieri et al., 1985; Schwartz et al., 1983). Results to date indicate that the corneal epithelium of the albino rabbit, which is anticipated to be deficient in peptidase activity, has up to 15% of the aminopeptidase activity in the ileum (Stratford and Lee, 1985). This appreciable aminopeptidase activity is principally responsible for the less than 0.1% intraocular bioavailability of enkephalins following topical ocular administration to the albino rabbit (Lee et al., 1985). It is possible that aminopeptidases in the mucosal tissues of the alternative routes are also as active and therefore may be responsible, at least in part, for the rather modest improvements in peptide bioavailability observed with these routes to date.

The objective of this study was to investigate if the homogenates of the conjunctival, buccal, nasal, rectal, vaginal, duodenal and ileal mucosae of the albino rabbit differed substantially in aminopeptidase activity, both in the presence and absence of aminopeptidase activators and inhibitors. Aminopeptidase activity was determined using a simple, continuous fluorescence assay which monitored the formation of a fluorescent leaving group (4-methoxy-2-naphthylamine) from one of four non-fluorescent aminopeptidase substrates, namely, 4-methoxy-2-naphthylamides of L-leucine (1), L-alanine (2), L-glutamic acid (3), and L-arginine (4).



1, R = $CH_2CH(CH_3)_2$

2, R = CH_3

3, R = $(CH_2)_2COO^-$

4, R = $(CH_2)_3NHC(NH_2)=\overset{+}{N}H_2$

These substrates are not hydrolyzed by either serine or thiol proteinases (Sylven and Bois, 1962) and thus are relatively specific for aminopeptidases. Within limits, they are preferentially hydrolyzed by leucine aminopeptidase (EC 3.4.11.1), aminopeptidase N [1] (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), and aminopeptidase B (EC 3.4.11.6) and lysosomal aminopeptidase (no EC number), respectively.

Materials and Methods

Materials

The four synthetic aminopeptidase substrates (1–4) as well as two competitive aminopeptidase inhibitors, bestatin and puromycin, were obtained commercially (Sigma Chemicals, St. Louis, MO) and were used as received. Two aminopeptidase preparations, leucine aminopeptidase and porcine kidney microsomal aminopeptidase, were also obtained commercially (Sigma Chemicals, St. Louis, MO). Leucine aminopeptidase is a cytosolic enzyme while porcine kidney microsomal aminopeptidase is a membrane bound enzyme. These enzymes were used as received following appropriate dilutions of each in 0.05 M Tris maleate buffer, pH 7.4.

Collection of mucosal tissues

Two groups of six female albino rabbits (ABC Rabbitry, Pomona, CA), weighing approximately 2 kg, were used in this study. These two groups of rabbits were used 3 months apart in order to verify reproducibility of the results in aminopeptidase activity. Rabbits were fasted overnight and then killed by a lethal injection of sodium pentobarbital solution (Eutha-6, Western Medical Supply, Arcadia, CA) into a marginal ear vein. Mucosae were removed immediately in the following order: conjunctiva, buccal mucosa, nasal mucosa, duodenum, ileum, rectum and vagina. The approximate wet weight of each tissue specimen is listed in Table 1. Approximately 30 min

[1] This enzyme is also known as amino-oligopeptidase, aminopeptidase M, microsomal aminopeptidase, neutral arylamidase and particulate aminopeptidase.

were required to excise all these tissues from a single rabbit. The tissues were rinsed in 0.9% NaCl, pooled in boro-silicate glass vials by tissue type, and stored at −70°C. It was necessary to store these tissues at this temperature and in an unprocessed state since substantial loss in activity was observed upon storing them as their homogenates at either 4°C or −20°C for as brief as 24 h (unpublished data).

Bulbar and palpebral conjunctivae were excised from both eyes of each rabbit using a no. 11 surgical blade as previously described (Stratford and Lee, 1985). Buccal mucosa was excised from the lateral aspects of the oral cavity using a no. 11 surgical blade; sublingual mucosa was not excised. Nasal mucosa was obtained by making an incision along the length of the lateral wall of the nose on each side of the nasal septum followed by cutting the nasal septum using surgical scissors and lifting the nasal bone frontally to expose the nasal cavity fully. Mucosal tissue was then carefully freed and excised from the underlying cartilage and bone.

Small intestinal mucosae were obtained from the scrapings of 10 cm segments of the duodenum and ileum as previously described (Stratford and Lee, 1985). Rectal and vaginal mucosae were obtained by first exposing the respective luminal surface with a longitudinal incision followed by excising the mucosal tissues using a no. 11 surgical blade.

Preparation of tissue homogenates

Immediately before each experiment, a given tissue was thawed at room temperature for about 10 min, rinsed twice in 0.05 M Tris maleate buffer (pH 7.4), and then homogenized in 4–6 ml of the rinse buffer at 4°C using a Teflon–glass homogenizer. The homogenate was centrifuged at 3020 × *g* in a refrigerated (4°C) centrifuge (Sorvall RC-5B refrigerated superspeed centrifuge, Dupont Instruments, Newton, CT) for 10 min to remove cellular and nuclear debris. The resulting supernatant, which contained cytosol and relevant plasma and intracellular membrane fractions, was assayed immediately for aminopeptidase activity against each of the four substrates mentioned earlier. Its protein concentration was determined using a dye-binding assay (Bradford, 1976) with rabbit serum albumin as the standard. Table 1 lists the protein concentration in each tissue supernatant.

TABLE 1

APPROXIMATE WET WEIGHTS OF VARIOUS MUCOSAL TISSUES IN INDIVIDUAL RABBITS AND PROTEIN CONCENTRATIONS IN THE SUPERNATANTS OF POOLED TISSUE HOMOGENATES

Tissue	mg tissue	Protein conc. (mg/ml)
Conjunctival	200	2.04
Nasal	25	0.58
Buccal	80	0.54
Duodenal	300	1.16
Ileal	300	0.85
Rectal	200	1.77
Vaginal	200	0.90

Preparation of stock solutions

Stock solutions of the four aminopeptidase substrates (1–4), each at 30 mM, were made in dimethylformamide, stored at −20°C, and used within 5 days of preparation. The final substrate concentration in an incubation mixture was 1.0 mM, which in preliminary experiments was shown to provide sufficient substrate for maximal hydrolytic velocity (V_{max}) measurements. This is necessary to provide meaningful comparisons of aminopeptidases content among the mucosal homogenates. The final concentration of dimethylformamide in an incubation mixture was 3.3%.

Assay of aminopeptidase

Aminopeptidase activity was determined using a modification of a previously reported assay (Stratford and Lee, 1985). Specifically, 100 μl of a tissue supernatant was preincubated in 2.8 ml of a 0.05 M Tris maleate buffer, pH 7.4, for 15 minutes in a fluorescence cuvet thermostated at 37°C. This pH was chosen not only because it represents physiological condition but also because it is at or close to the pH optima of most aminopeptidases, including lysosomal aminopeptidase (Mahadevan and Tappel, 1967). The reaction was initiated by adding 100 μl of a stock substrate solution to this mixture. The increase in fluorescence intensity was monitored at an excitation wavelength of 342 nm

and an emission wavelength of 426 nm for 5 min (Perkin-Elmer model 650-20S spectrofluorometer, Norwalk, CT). This was found to be linear for all the homogenates studied. Initial velocities were determined from plots of fluorescence intensity vs time and were expressed in terms of nmoles of substrate hydrolyzed per min per mg protein after correcting for chemical hydrolysis. All incubations were performed at least in duplicate.

The assays were repeated for each substrate in the presence of an aminopeptidase activator. The presence of leucine aminopeptidase, aminopeptidase N and aminopeptidase A was determined by studying the effect of 0.1 mM Mn^{2+}, 0.1 mM Co^{2+} and 10 mM Ca^{2+} on the hydrolytic rates of the leucine, alanine, and glutamate substrates, respectively (Emmelot and Visser, 1971; Feracci et al., 1981; Van Wart and Lin, 1981). The presence of aminopeptidase B and lysosomal aminopeptidase in a particular homogenate was determined by studying the effect of 0.1 M Cl^- and 0.1 mM dithiothreitol, respectively, on the hydrolysis of the arginine substrate, the preferred substrate for these two enzymes (Hopsu et al., 1966; Mahadevan and Tappel, 1967).

Aminopeptidase assays were also conducted for each substrate/activator combination in the presence of an aminopeptidase inhibitor, bestatin or puromycin. Bestatin has been shown to inhibit leucine aminopeptidase as well as aminopeptidases N and B (Suda et al., 1976), while puromycin has been shown to inhibit aminopeptidases N and B (McDonald and Schwabe, 1977) but not leucine aminopeptidase (McDonald et al., 1964). Neither inhibitor has an effect on aminopeptidase A (Auricchio et al., 1972; Suda et al, 1976). Bestatin and puromycin were used at a final concentration of 0.01 mM and 0.1 mM, respectively, the factor of 10 difference reflecting the greater potency of bestatin as an aminopeptidase inhibitor than puromycin (Schwartz et al., 1983). One-hundred μl of an inhibitor stock solution in buffer were added to 2.8 ml of tissue-buffer/activator preparation and incubated for 15 min at 37°C. Following incubation, a substrate solution was added to a final concentration of 1.0 mM and the production of fluorescence was monitored as described previously.

Results

Patterns of substrate hydrolysis

Table 2 is a summary of aminopeptidase activity in various mucosal homogenates, expressed as a percentage of ileal activity, based on each of the four substrates in the presence of activators but in the absence of inhibitors. Surprisingly, against the leucine, alanine and arginine substrates, aminopeptidase activities in non-intestinal tissue homogenates were comparable to the ileum. Quite unexpectedly, the conjunctival homogenate hydrolyzed the leucine and arginine substrates 3 and 2 times faster than the ileum, respectively. The ileal homogenate hydrolyzed the glutamic acid substrate at a substantially greater rate than the other homogenates. The nasal and duodenal homogenates hydrolyzed this substrate at 19.0 and 15.1% the rate in the ileal homogenate, respectively, while the remaining homogenates were 2.8–6.1% as active.

Fig. 1 shows the patterns of substrate hydrolytic rates in the various mucosal homogenates in the absence of activators. On the basis of similarities in the patterns of relative hydrolytic rates, the various mucosal tissues can be placed into 5 groups. These are nasal and duodenal, buccal and rectal, conjunctival and vaginal, and ileal. The unique hydrolytic pattern in the ileum may be attributed to aminopeptidase A. The appreciable activity against the alanine substrate in all the mucosal homogenates suggests that aminopeptidase N probably exists in all these tissues. In contrast, leucine aminopeptidase does not appear to be a dominant peptidase in the mucosal tissues studied, except the conjunctiva. This is inferred by comparing the patterns of substrate hydrolysis for mucosal homogenates in Fig. 1 with those for commercially purified leucine aminopeptidase in Fig. 2.

Effect of activators on aminopeptidase activity

Table 3 shows the effect of various activators on the hydrolytic rate of substrates, each of which was preferred by a certain aminopeptidase. Mn^{2+} significantly increased the hydrolysis of the leucine substrate in the duodenal homogenate only. Even then the extent of increase (19.0%) was 111 times less than that obtained with purified leucine

TABLE 2

SPECIFIC AMINOPEPTIDASE ACTIVITIES IN MUCOSAL TISSUE HOMOGENATES IN THE PRESENCE OF ACTIVATORS EXPRESSED AS PERCENTAGE OF ILEAL ACTIVITY [a]

Tissue	Substrate/activator combination [b]				
	Leu/Mn^{2+}	Ala/Co^{2+}	Glu/Ca^{2+}	Arg/Cl^-	Arg/DTT
Conjunctival	320.7	86.6	6.1	173.5	229.1
Nasal	108.7	96.1	19.0	65.9	71.4
Buccal	69.6	61.7	2.8	112.5	62.4
Duodenal	70.0	75.3	15.1	101.8	89.0
Rectal	87.1	96.6	5.3	138.0	103.3
Vaginal	102.7	51.6	3.0	82.0	83.5

[a] The ileal aminopeptidase activity in nmol/min/mg protein was 7.01 ± 0.26 for Leu/Mn^{2+}, 22.51 ± 0.73 for Ala/Co^{2+}, 25.73 ± 0.001 for Glu/Ca^{2+}, $4.40 + 0.34$ for Arg/Cl^-, and $2.99 + 0.33$ for Arg/DTT. For comparison, the cytosolic leucine aminopeptidase activity in nmol/min/mg protein was 44.72 ± 1.05 for Leu/Mn^{2+}, 3.21 ± 0.13 for Ala/Co^{2+}, 2.45 ± 0.11 for Glu/Ca^{2+}, 0.09 ± 0.10 for Arg/Cl^-, and 1.58 ± 0.10 for Arg/DTT. The porcine kidney microsomal aminopeptidase activity in μmol/min/mg protein was 2.60 ± 0.18 for Leu/Mn^{2+}, 10.6 ± 0.2 for Ala/Co^{2+}, 0.70 ± 0.002 for Glu/Ca^{2+}, 1.46 ± 0.015 for Arg/Cl^-, and 1.58 ± 0.015 for Arg/DTT.

[b] The substrates were 4-methoxy-2-naphthylamides of leucine (Leu), alanine (Ala), glutamic acid (Glu), and arginine (Arg). DTT abbreviates for dithiothreitol.

aminopeptidase (2110.7%), indicating that this aminopeptidase was not a dominant peptidase in the mucosal tissues, as suggested earlier. Co^{2+} significantly enhanced the hydrolysis of the alanine substrate in all the mucosal homogenates. The extent of increase (29.4–78.7%) was comparable with that observed with purified aminopeptidase N (34.1%), suggesting the presence of this aminopeptidase in the mucosal homogenates, as mentioned earlier. The slightly greater increase observed in the mucosal homogenates was probably due to the presence of cytosolic leucine aminopeptidase, since Co^{2+} also enhanced the hydrolysis of the alanine substrate by this enzyme (399.4%). Unexpectedly, Ca^{2+} did not enhance the hydrolysis of the glutamic acid substrate in the nasal, duodenal, and ileal homogenates, as it should if aminopeptidase A were present. It is speculated that exogenous Ca^{2+} was sequestered by calcium binding proteins liberated during homogenization and therefore was unavailable for activating aminopeptidase A (Feracci et al., 1981).

Table 3 also shows that Cl^- significantly enhanced the hydrolysis of the arginine substrate by 19.5–80.7% in all the mucosal homogenates except the vaginal homogenate, suggesting the presence of aminopeptidase B. Interestingly, this anion inhibited the hydrolysis of the arginine substrate by

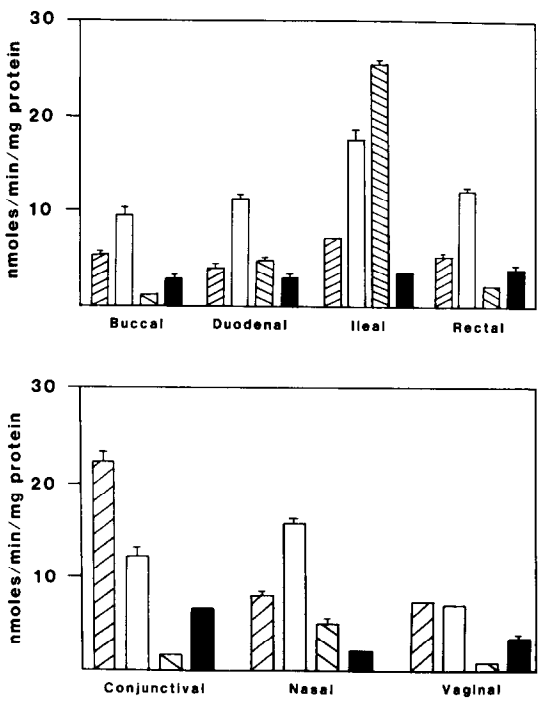


Fig. 1. Mucosal aminopeptidase activity in the albino rabbit against four L-amino acid-4-methoxy-2-naphthylamide substrates: L-leucine, ▨; L-alanine, □; L-glutamic acid, ▧; and L-arginine, ■. Activity was expressed in nmoles of substrate hydrolyzed per min per mg protein. The substrate concentration was 1 mM. Error bars represent standard deviations.

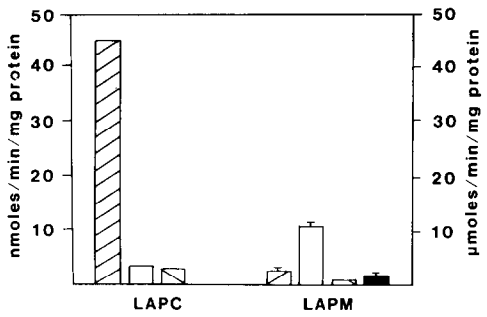


Fig. 2. Aminopeptidase activity of leucine aminopeptidase (LAPC) and porcine kidney microsomal aminopeptidase (LAPM) against four L-amino acid-4-methoxy-2-naphthylamide substrates: L-leucine, ▨; L-alanine, □; L-glutamic acid, ⊠; and L-arginine, ■. Activity was expressed as nmoles of substrate hydrolyzed per min per mg protein (LAPC) or μmoles of substrate hydrolyzed per min per mg protein (LAPM). The substrate concentration was 1 mM. Error bars represent standard deviations.

both commercially purified aminopeptidase, supporting its use to selectively screen for aminopeptidase B. The last activator, dithiothreitol, enhanced the hydrolysis of the arginine substrate in the conjunctival homogenate only, suggesting the presence of lysosomal aminopeptidase in this tissue (Mahadevan and Tappel, 1967).

Effect of inhibitors on aminopeptidase activity

Table 4 shows the effect of bestatin and puromycin on substrate hydrolysis in both commercially purified aminopeptidase preparations and various mucosal homogenates. The hydrolysis of the leucine substrate by commercially purified leucine aminopeptidase was almost completely inhibited by bestatin but not inhibited appreciably by puromycin. This finding is consistent with literature reports (McDonald et al., 1964; Suda et al., 1976). However, this degree of inhibition of leucine substrate hydrolysis by bestatin was not observed in any of the mucosal homogenates, suggesting that leucine aminopeptidase may not be a dominant aminopeptidase in any of the mucosal tissues studied, as already noted.

The hydrolysis of the alanine substrate in mucosal homogenates was inhibited by bestatin from 62% to 85%, which was greater than the 49% inhibition determined for commercially purified aminopeptidase N. The greater inhibition in the homogenates could be due to inhibition of another bestatin-sensitive aminopeptidase, such as leucine aminopeptidase, which is also active against the alanine substrate (Fig. 1). Similarly, puromycin inhibited alanine substrate hydrolysis in the mucosal tissues from 21% to 62%, as compared with the 24% inhibition of substrate hydrolysis by commercially purified aminopeptidase N.

The hydrolysis of the glutamic acid substrate in the nasal, duodenal and ileal homogenates, which

TABLE 3

CHANGES IN MUCOSAL AMINOPEPTIDASE ACTIVITIES IN THE PRESENCE OF AMINOPEPTIDASE ACTIVATORS RELATIVE TO THE CONTROL BUFFER [a]

Tissue	% Change				
	Leu/Mn^{2}	Ala/Co^{2+}	Glu/Ca^{2+}	Arg/Cl^{-}	Arg/DTT
Conjunctival	+1.3	+60.4 [c]	−7.6	+19.5 [c]	+7.1 [c]
Nasal	−4.2	+38.0 [c]	−4.5	+39.8 [c]	+2.7
Buccal	−5.5	+46.8 [c]	−20.3 [c]	+80.7 [c]	−32.0
Duodenal	+19.0 [c]	+51.2 [c]	−19.5 [c]	+50.9 [c]	−10.5
Ileal	−3.6	+29.4 [c]	+1.4	+26.3 [c]	−14.3
Rectal	+14.1	+78.7 [c]	−36.3 [c]	+55.3 [c]	−21.0 [c]
Vaginal	+0.5	+67.2 [c]	+13.4	+10.1	−23.9 [c]
LAPC [b]	+2110.7 [c]	+399.4 [c]	+24.8 [c]	−88.9 [c]	+98.6 [c]
LAPM [b]	+14.4	+34.1 [c]	+27.2 [c]	−6.8 [c]	+0.9

[a] The substrates were 4-methoxy-2-naphthylamides of leucine (Leu), alanine (Ala), glutamic acid (Glu), and arginine (Arg). DTT abbreviates for dithiothreitol.

[b] LAPC and LAPM abbreviate for cytosolic leucine aminopeptidase and porcine kidney microsomal aminopeptidase, respectively.

[c] Experimental was significantly different from control at $P < 0.05$ by a Student's t-test.

TABLE 4

PERCENT INHIBITION OF MUCOSAL AMINOPEPTIDASE ACTIVITIES BY 0.01 mM BESTATIN AND 0.1 mM PUROMYCIN

Tissue	Bestatin				Puromycin			
	Leu [a]	Ala	Glu	Arg	Leu	Ala	Glu	Arg
Conjunctival	28.1	73.2	25.5	46.8	1.8 [c]	28.4	5.9	2.3 [c]
Nasal	38.1	71.5	1.4 [c]	52.3	2.2 [c]	16.2	3.4 [c]	7.5 [c]
Buccal	32.1	84.6	16.0	64.6	4.1 [c]	51.3	17.9	40.9
Duodenal	36.1	71.5	−5.1 [c]	56.4	3.0 [c]	47.4	0.8 [c]	21.2
Ileal	46.0	62.1	−1.5 [c]	45.8	0.7 [c]	20.5	0.5 [c]	4.5 [c]
Rectal	34.2	79.3	39.3	64.4	1.0 [c]	62.3	14.6 [c]	18.9
Vaginal	33.5	74.2	20.8	48.7	2.5 [c]	50.2	9.2	11.5 [c]
LAPC [b]	98.6	69.7	−7.0 [c]	0 [c]	3.9 [c]	13.7	−7.0 [c]	0 [c]
LAPM [b]	39.8	49.0	3.5	17.6	22.8	23.7	5.3	4.6

[a] The substrates were 4-methoxy-2-naphthylamide of leucine (Leu), alanine (Ala), glutamic acid (Glu), and arginine (Arg).
[b] LAPC and LAPM abbreviate for cytosolic leucine aminopeptidase and porcine kidney microsomal aminopeptidase, respectively.
[c] Experimental was *not* significantly different from control at $P < 0.05$ by a Student's *t*-test.

we postulated to be richer in aminopeptidase A than its companion aminopeptidases, was virtually unaffected by either inhibitor. This lack of inhibition is consistent with the presence of aminopeptidase A (Auricchio et al., 1972; Suda et al., 1976). Finally, the hydrolysis of the arginine substrate in all the homogenates in the presence of Cl^- activation, conditions which optimized aminopeptidase B activity, was inhibited from 46% to 64% by bestatin. This finding is consistent with the inhibitory effect of bestatin towards aminopeptidase B (Suda et al., 1976). Puromycin, which also inhibits aminopeptidase B (Ellis and Perry, 1966), significantly inhibited the hydrolysis of the arginine substrate in the buccal (41%), duodenal (21%), and rectal (19%) homogenates only.

Discussion

The fraction of peptide absorbed intact into the systemic circulation from its site of administration depends on the ability of a peptide to cross the mucosal barrier and to resist degradation by peptidases and proteases present at both the site of administration and the mucosal barrier. To date, the relative contribution of these transport and enzymatic barriers to the bioavailability of a peptide has not been systematically studied. The enzymatic barrier is generally thought to be very effective in degrading orally administered peptides thus leading to poor oral peptide bioavailability. At the same time, this barrier is assumed to be moderate in effectiveness at routes of administration other than oral, thereby favoring peptide bioavailability. Indeed, the delivery of leuprolide, one of the more widely studied peptides, via the nasal, rectal and vaginal routes was demonstrated to improve its bioavailability by a factor of 3, 35 and 112 times over the oral route, respectively (Okada et al., 1982). However, the fraction of peptide absorbed systemically from the respective routes was only 0.1%, 1.2% and 3.8% of the applied dose (Okada et al., 1982). Since subcutaneous administration allowed over 65% of a leuprolide dose to be absorbed systemically (Okada et al., 1982), it is possible that the enzymatic barrier at the alternative routes of peptide administration is more substantial than has been anticipated.

This hypothesis of substantial aminopeptidase activity in alternative routes of peptide delivery is supported by our findings that, against 3 of the 4 substrates studied, aminopeptidase activity in the nasal, buccal, rectal and vaginal mucosal homogenates was, on the average, 87.1% ± 23.0% (n = 16) of the ileal activity. Nonetheless, modest im-

provement in bioavailability with these alternative routes over the oral route has been observed. This improvement may be attributed to 4 possibilities, as follows.

First, non-oral mucosae may be deficient in proteinases such as pepsin, trypsin and chymotrypsin which are present in gastric and intestinal secretions and are expected to contribute to peptide hydrolysis. Second, similar to small drug molecules, peptides administered via the alternative routes possibly are much less susceptible to first pass metabolism in the liver, if at all, than from oral administration. Third, it is possible that the epithelial tight junctions in the alternative routes are leakier than those in the small intestinal epithelia (Hayashi et al., 1985) thereby favoring paracellular transport of a peptide and thus minimizing its contact with luminal and epithelial membrane peptidases. Fourth, the surface area to which a peptide would be exposed in a given alternative route such as the nasal cavity (0.016 m^2) would be considerably smaller when compared with the small intestine (550 m^2). Moreover, considering the substantial resident volume of fluid in the gastrointestinal tract, a given amount of peptide administered orally would be diluted much more than when administered via any of the alternative routes. This would reduce not only the driving force for peptide absorption across the plasma membrane but also the ratio of peptide to cellular aminopeptidase, thus increasing peptide hydrolysis. The implication of these possibilities is that oral peptide delivery would be feasible provided peptide release is confined to the vicinity of the mucosal surface in a region of the small intestine that favors peptide uptake into the lymphatic circulation. This possibility needs to be investigated.

In selecting a route for peptide delivery, it would be helpful to have information on the type of peptidase responsible for peptide hydrolysis in that route in addition to information on the overall peptidase activity present. Since none of the substrates we used was absolutely specific for a given type of aminopeptidase, we relied on 3 lines of evidence to determine whether the same or different types of aminopeptidases were present in various mucosal tissues. These were pattern of substrate hydrolysis (Fig. 1), the effect of aminopeptidase activators on the rate of substrate hydrolysis (Table 3), and the effect of inhibitors on the rate of substrate hydrolysis (Table 4). On this basis, 4–5 aminopeptidases are estimated to be present in the mucosal homogenates studied. Aminopeptidase N, a plasma membrane bound peptidase (Vannier et al., 1976), is present in all the mucosae. Aminopeptidase A, another plasma membrane-bound peptidase (Feracci et al., 1981), appears to be most abundant in the ileum, 20% as abundant in the nasal and duodenal mucosae, and 10% or less as abundant in the conjunctival, buccal, rectal, and vaginal mucosae. Aminopeptidase B, a cytosolic enzyme (Hopsu et al., 1966), is present in all the mucosae studied except the vaginal mucosa, whereas leucine and lysosomal aminopeptidases are present primarily in the conjunctival mucosa. Overall, the various mucosae do not appear to differ substantially in the range of aminopeptidases present. Nevertheless, they may differ in the relative proportion of aminopeptidases thereby affecting the extent of hydrolysis of peptides applied. This possibility needs to be investigated in order to understand the nature of the aminopeptidase barrier to peptide absorption.

Although our assay revealed that leucine aminopeptidase as well as aminopeptidases N and B were present in non-oral mucosal homogenates at levels comparable to the ileum, the ability of the 4 substrates to screen for all types of aminopeptidases must be considered. Indeed, Lafferty et al. (1984) reported that only 2 of the 6 aminopeptidases present in soluble extracts of bovine and human lenses were capable of hydrolyzing the substrates used in this study. Thus, it is conceivable that aminopeptidases which are inert towards these substrates are also present in the non-oral absorptive mucosae but in quantities significantly less than the small intestine. Similarly, other types of peptidases known to exist in the small intestine, such as carboxypeptidases, di-aminopeptidases, and various endopeptidases, may not be present in the other mucosae at comparable levels. These possibilities need to be explored by expanding the present methodology to include a determination of the role of temperature and pH on aminopeptidase activity and to include other fluoromet-

ric substrates capable of detecting non-aminopeptidases.

In summary, essentially the same types of aminopeptidases (except aminopeptidase A) as found in the duodenum and ileum are present in the conjunctival, buccal, nasal, rectal and vaginal mucosae. The magnitude of aminopeptidase activity in mucosal homogenates of these non-oral routes does not appear to differ markedly from the oral route. Clearly, further work is required to establish whether the subcellular organization of these aminopeptidases, which was disrupted using our methodology, varies substantially among the mucosae to cause significant differences in peptide bioavailability from the various routes.

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