4. Protection of Bioactive Enkephalins from Enzymatic Degradation

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1. Introduction

Enkephalin, a natural opiate receptor agonist, has been identified by Hughes *et al.* (1975b), as a mixture of two pentapeptides; H-Tyr-Gly-Gly-Phe-Met-OH (methionine enkephalin, Met-Enk) and H-Tyr-Gly-Gly-Phe-Leu-OH (leucine enkephalin, Leu-Enk). Both Met- and Leu-Enks have potent agonist activity at opiate receptor sites in that they produce a dose-related inhibition of electrically evoked contractions of the mouse vas deferens and the quinea-pig ileum. These inhibitory effects can be completely antagonized by naloxone. Met-Enk is twenty times more active than morphine in the vas deferens. Leu-Enk has half the potency of that of Met-Enk in the vas deferens but in the guinea-pig ileum only one-fifth of that of Met-Enk (Hughes *et al.*, 1975b). On the basis of these and other evidence (Belluzzi *et al.*, 1976; Snyder and Childers, 1979), it has been proposed that Enk receptors may be sites at which morphine-like drugs exert their analgesic actions, and that the Enks are pentapeptides which produce as neurotransmitter or neuromodulator in peripheral and brain systems, pharmacological effects including analgesia, modulation of blood pressure, and alterations in gut motility.

It is particularly interesting that the Met-Enk sequence is present at residues 61-65 in beta-lipotropin isolated from pituitary glands of sheep, pig and man. Met-and Leu-Enks are distributed in both the central and peripheral nervous systems, plasma and olfactory of vertibrates (Beaumont and Hugher, 1979; Bogan *et al.*, 1982; Cupo *et al.*, 1988).

However, because of their enzymatic lability after systemic or intravenous administration, a number of analogs have been developed, which by virtue of containing a D-amino acid substitution, are more resistant to proteolysis (Coy *et al.*, 1876; Marks *et al.*, 1978; Hill and Pepper, 1978; Roemer and Pless, 1979). *In vitro* studies with brain homogenates, purified enzyme preparations and isolated brain capillaries have demonstrated that the Enks are subject to rapid enzymatic degradation, and suggested that Enk deactivating peptidases, enkephalinases, might play a role in physiological central nervous system enkephalin homeostasis (Gorenstein and Snyder, 1980; Pardridge and Meitus, 1981). In the light of these results, it is not surprising to find that, *in vitro*, the pharmacological action of natural Enks is very shor-tlived, even after intracerebroventricular (i.c.v.) injection, in comparison to that of the more potent D-Ala(2) analogs (Pert *et al.*, 1976; Schachter, 1981). Recently it was reported that the transport of Leu-Enk and D-Ala(2)-D-Leu-Enk across the blood brain barrier (BBE) was not facilitated and a low brain uptake combined with enzymatic hydrolysis of Leu-Enk would reduce the penetration of this peptide greatly through the blood-brain barrier. It was also considered that the resistance to hydrolysis of D-Ala(2)-D-Leu-Enk, together with a greater natural penetrance of this substance through the BBB, would make its uptake significantly greater than that of Leu-Enk (Zlokovic *et al.*, 1985).

Even though bioactive peptides have been the subject of extensive interest due to their natural roles in the regulations of biological activities, their rapid metabolism in the GI tract requires considering alternative routes of administration, such as mucosal (nasal, buccal, rectal, vaginal) and transdermal administration. Irrespective of which of these routes is considered, physicochemical properties of Enks, biophysical properties of mucosae, the propensity for route specific enzymatic metabolism, and enzyme inhibitors, should be elucidated prior to the formulation of a peptide drug.

2. Enzymatic Degradation of Enkephalins

The opioid peptides Met- and Leu-Enks are distributed in both the central and peripheral nervous systems. Current evidence indicates that they may participate in synaptic transmission related to the processing of pain information and the elaboration of emotional behaviour. The pharmacological action of the Enks is very short-lived owing to their rapid degradation by serum, brain, gut and mocosa enzymes.

It has been reported in a variety of systems that one of the major degradative pathways for Enks is the cleavage of the Tyr(1)-Gly(2) bond

Table I. Aminopeptidases which Hydrolyze Enkephalins

Reference	Traficante *et al.* 1980a and 1980b	Schnebli *et al.*, 1979 Barclay and Phillipps 1980b	Wagner and Dixon, 1981 Wagner *et al.*, 1981	Hershi *et al.*, 1980	Hayashi *et al.*, 1978a	Coletti-Previero *et al.*, 1981
Properties						
Molecular weight (subunit composition)	61,500	100,000 (48,000-50,000)	102,000	98,000	92,000	80,000-90,000
K_m	64 μM	30 μM	–	22 μM	167 μM	400 μM
pH optimum	7.4	7.0	6.5-7.0	–	7.0	8.0
Effect of Zn^{2+}	Inactivates	Activates	Activates	Activates	–	–
Alternative Substrates	γ-endorphin D-ala^2-met-enkephalin β-endorphin	β-napthylamide *p*-nitroanilides of neutral, basic and aromatic amino acids somatostatin and substance P	β-napthylamides of neutral or basic amino acids	di- and tri-peptide β-naphthylamides	di- and tri-peptides oliopeptides	Peptides with *N*-terminal aromatic amino acids other than Tyr-β-naphthylamide
Inhibitors	Bacitracin puromycin *o*-phenanthroline γ-endorphin	Puromycin *o*-phenanthroline *p*-chloromercuribenzoate EDTA amastatin, bestatin	Bestatin and analogues (K_i=0.8 - 5.0×10^{-7} M) bacitracin puromycin EDTA *o*-phenanthroline	Puromycin (K_i=60 nM) *p*-chloromercuri-benzoate EDTA	Bestatin (K_i=2.5×10^{-7} M) puromycin	
Activators	β-mercaptoethanol	–	–	–	thiol reagents	–

Source: Frederickson and Geary (1982).

(Marks *et al.*, 1977; Craves *et al.*, 1978; Craviso and 1978). In addition, a brain membrane-bound aminopeptidase also appears to catalyze cleavage of the Tyr(1)-Gly(2) bond of Enk while a membrane-bound dipeptidyl carboxypeptidase cleaves the Gly(3)-Phe(4) bond of Enk (Malfroy *et al.*, 1978).

The Enks are vulnerable to peptidases at a number of sites. Among the enzymes which can digest Enks are aminopeptidases, dipeptidyl carboxypeptidases (enkephalinase and angiotensin-converting enzyme; ACE) and carboxypeptidases A. *In vitro* tissue preparations or homogenized preparations expose Enks to a host of enzymes of varing specificity which may never be encountered *in vivo* during physiological action. The enzymes described below represent current thinking about the catabolism of Enks (Schwartz *et al.*, 1981; Frederickson and Geary, 1982; Giros *et al.*, 1986).

2-1. Aminopeptidases.

Within the brain, at least 5 aminopeptidases have been described. Another enzyme from human plasma has also been characterized (Coletti-Priviero et al., 1981). This enzyme cleaves the N-terminal tyrosine from Enk and from short peptides provided their first amino acid is aromatic. The properties of these enzymes are summarized in Table I. All of these enzymes are soluble enzymes. The five enzymes isolated from brain tissue are metalloenzymes which can be inhibited by chelating agents such as *o*-phenanthroline and EDTA. The substrate specificities of the enzymes differ although they all have a high affinity for the Enks. Overall, the distribution of aminopeptidase activity does not parallel that of the opioid receptor; however, the individual contribution to Enk degradation of each of these five brain aminopeptidases is obscured by the others, plus the large number of other types of peptidases.

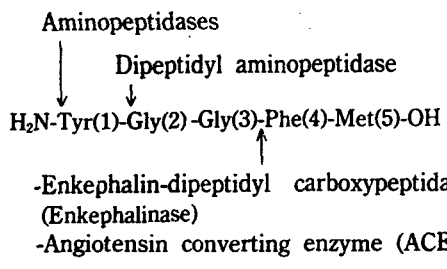
Soluble aminopeptidases cleaving the Tyr(1)-Gly(2) bond of Enk have been purified from monkey brain (Hayashi and Oshima, 1975; Hayashi, 1978), rat brain (Schnebli *et al.*, 1979), and bovine brain (Hersh and McKelvy, 1981), and have been identified as arylamidases. All three enzymes were inhibited by *p*-chloromercuribenzoate and puromycin, but there were both structural and kinetic differences amongst the three enzymes. The purified enzyme obtained from bovine brain hydrolyzed the Tyr(1)-Gly(2) bond of Met-Enk, exhibiting a K_m of 22 μM and a V_{max} of 28 μmol Tyr liberation per min per mg of protein. The activity of the enzyme towards a number of Enk analogs is shown in Table II (Hersh and McKelvy, 1981). As anticipated, substitution of Gly in position 2 of Enk by D-Ala or 2-methylalanine virtually abolishes enzyme activity. Puromycin was found to be a potent inhibitor of Enk hydrolysis and the calculated K_i for puromycin inhibition was 60 nM.

Table II. Comparison of the Reactivity of Various Enkephalin Analogues

Enkephalin or analogue (0.1 mM)	% Activity
Methionine^5-enkephalin	(100)
Leucine^5-enkephalin	94.0
Alanine^2-methionine^5-enkephalin	47.2
D-Alanine^2-methionine^5-enkephalin	0.9
D-Alanine^2-methionine^5-enkephalinamide	0.6
D-Alanine^2-D-leucine^5-enkephalin	0.5
2-Methylalanine^2-methionine^5-enkephalinamide	<0.1

Enzyme activity was measured by following tyrosine liberation with the coupled L-amino acid oxidase-horseradish peroxidase assay system described in Methods. The specific activity with met-enkephalin as substrate was 28.2 μmol/min/mg enzyme. Soure: Hersh and McKelvy (1981)

In human cerebrospinal fluid, aminopeptidases, dipeptidyl aminopeptidase, dipeptidyl carboxypeptidase, and carboxypeptidase which were capable of hydrolyzing Enks were detected (Hazato *et al.*, 1985). Among these enzymes, two distinct aminopeptidases, designated C-AP1 and C-AP2, were partially purified. Although these enzyms were not purified thoroughly, the characteristics of C-AP2 were similar to those of an aminopeptidase purified from monkey brain. The inhibitory activity of amastatin on C-AP2 was stronger, but that of substance P was negligible. On the other hand, characteristics of C-AP1 was extremely different from those of C-AP2 or an aminopeptidases puri-



Scheme 1—Modes of breakdown of enkephalins and enzyme activities involved

fied from monkey brain. C-AP1 had an optimum pH in the more acidic range (the highest at pH 6.0) and was not inhibited by any of the protease inhibitor tested including bestatin and amastatin.

2-2. Dipeptidyl carboxypeptidase

The digestion of the Enks by a dipeptidyl carboxypeptidase (called an enkephalinase) was first reported by Malfroy *et al.* (1978), and Sullivan *et al.* (1978). It was immediately recognized that ACE had the specificity to carry out the hydrolysis of a dipeptide from the carboxy terminal of Enk (Scheme 1) (Erdos *et al.*, 1978), and so the question of the co-identity of the enkephalinase and ACE became a focal point of intense research. While the two enzymes have properties in common, several distinguishing features have been reproducibly observed by a number of investigators. In particular, differential inhibition of these enzymes can be obtained by using thiorphan and captopril. Thiophan is a potent inhibitor of enkephalinase (K_i=4.2 nM) while it is less potent on ACE (K_i=150 nM), and captopril is a more potent on ACE (K_i=7 nM) while being a weak inhibitor of enkephalinase (K_i=10 μM) (Roques *et al.*, 1980). Another property in which these two enzymes vary is their sensitivity to chlorine ion. Enkephalinase is inhibited or unaffected by chlorine ion while ACE is activated in the presence of chlorine ion (Swerts *et al.*, 1979). The affinity of ACE for the Enks is less than the affinity of enkephalinase for these peptides, and the distribution of ACE in brain does not parallel that of Enks or opioid receptors (Swerts *et al.*, 1979).

A number of investigators have pursued the characterization of enkephalinase from different soures (Table III) (Frederickson and Geary, 1982). Gorenstein and Snyder (1979) have described two enkephalinases from solubilized membrane fractions of rat brain. An enzyme designated enkephalinase A is dipeptidyl carboxypeptidase discussed above which cleaves the Gly(3)-Phe(4) bond, and the other (enkephalinase B) is a dipeptidyl aminopeptidase which cleaves the Gly(2)-Gly(3) bond. They have obtained completely resolved enkephalinase A activity by DEAE column chromatography from ACE activity, but it was reported that it has not been possible to obtain selective antisera for immunohistochemical studies (Gorenstein *et al.*, 1981). Studies of subcellular distribution (De La Baume *et al.*, 1981) and regional distribution (Malfroy *et al.*, 1979), however, have already demostrated a close association between enkephalinase A and nerve endings, and enkephalinase A and opiate receptors.

Almenoff *et al.*, (1981) reported that a membrane bound zinc-metalloenkephalinase from bovine pituitaries with a specificity towards bonds on the amino side of hydrophobic amino acids, cleaved Met- and Leu-Enks at the Gly-Phe bond, releasing Phe-Met and Phe-Leu. respectively. The enzyme also hydrolyzes bonds on the amino side of hydrophobic amino acids in oxytocin, bradykinin, neurotensin and several synthetic substrates. The regional distribution of this enzyme in brain, its specificity towards natural and synthetic substrates, and its sensitivity to inhibitors (phenobarbital, dithiotreitol, glutathione) suggest that the enzyme is identical to an activity referred to as enkephalinase, which has been described as dipeptidyl carboxypeptidase. The data showed that the enzyme is an endopeptidase with a specificity similar to that of a group of microbial proteases (e.g. thermolysin).

2-3. Dipeptidyl Aminopeptidase and Other Enzymes

Besides aminopeptidase and enkephalinase, some other enzymes have been reported to degrade Enks: carboxypeptidase A, a lysosomal enzyme which cleaved the C-terminal amino acid

Table III. Enkephalinases

Source and Reference	Rabbit Brain Benuck and Marks. 1980	Human Brain Arrequi *et al.*, 1979	Rat Brain Schwartz *et al.*, 1979, 1980a and b	Rat Brain Benuck *et al.*, 1981 Gorenstein *et al.*, 1979	Rabbit Kidney Benuck *et al.*, 1981
K_m	0.14 mM (leu-enkephalin)		22 μM (leu-enkephalin)	–	80 μM (leu-enkephalin)
Isolations	Triton extract of crude mitochondrial fraction	Brij 35 extract of diencephalon	Triton extract of whole brain	Triton extract of microsomal fraction	Triton extract of whole brain
Properties	Active on bradykinin Not active on A_1 or hippuryl his-leu Not effected by Cl^- Not inhibited by captopril	Captopril less potent inhibitor than on angiotensin converting enzyme	Thiorphan is a specific inhibitor ($K_i = 4$ nM) while captopril is less potent ($K_i = 10$ μM) inhibited by Cl^- Regional distribution parallels brain opiate receptor metalloenzyme (Zn^{2+})	Captopril is a weak inhibitor inhibited by Cl^- inhibited by bacitracin *o*-phenanthroline metalloenzyme (Zn^{2+})	Active on bradykinin Not active on angiotensin I or hippuryl his-leu Not inhibited by captopril Neutral pH optimum inhibited by bacitracin and phe-met

Source: Frederickson and Geary (1982)

Table IV. The Depressant Effects of the Enkephalins and Degradation Products on the Electrically Induced Contractions of the Mouse Vas Deferens

Structure	Relative activity mouse vas deferens	$T_{1/2}$ (rat plasma) min
H-Tyr-Gly-Gly-Phe-Met-OH	100	2.0
H-Tyr-Gly-Gly-Phe-Leu-OH	170	2.5
H-Gly-Gly-Phe-Met-OH	0.1	–
H-Gly-Gly-Phe-Leu-OH	0.1	–

Source: Hambrook *et al.* (1976)

from Enks (Della Bella *et al.*, 1979) and another enzyme designated enkephalinase B which hydrolyzes the Gly(2)-Gly(3) bond in Enk (Gorenstein and Snyder, 1979). The extent to which carboxypeptidase A participates in Enk degradation is not clear. Some evidence obtained in studies using inhibitors suggests a role in regulating Enk concentrations. It is unlikely, however, because of its lysosomal origin that it plays a significant role in the disposition of Enk. Enkephalinase B has not been well characterized , and more information on the specificity and properties of these enzymes is needed before its importance can be evaluated.

On the other hand, it was shown that purified acetylcholinesterase from bovine serum, bovine brain and bovine adrenal medulla has the ability to hydrolyze a number of peptides including the physiologically occurring Enks (Chubb *et al.*, 1983). The Enks lost both the amino- and carboxy-terminal amino acids, but several other peptides were not degraded. The peptidase activity was not affected by the aminopeptidase inhibitor puromycin, but it was inhibited by acetylcholine. Therefore it was concluded that acetylcholinesterase also has the capacity for a novel type of hydrolysis of peptide bonds.

2-4. Enzymatic Degradation of Enkephalins in CNS and Plasma

Early work (Hughes, 1975a) demonstrated that the Enks were subject to rapid deactivation when exposed to tissue homogenates and purified enzyme preparations such as carboxypeptidase A and leucine aminopeptidase. Using the depressant effects of the Enks on the electrically-evoked contractions of the mouse vas deferens as a bioassay, the half-life of both peptides in rat plasma was shown to be very short (Table IV) (Hambrook *et al.*, 1976). The deactivation of the Enks in rat and human plasma and in rat whole brain homogenates has been shown to take place through cleavage of the Tyr-Gly amide bond.

Enks were degraded rapidly by homogenates of rat brain and by ultrafiltrate of mouse brain supernatant with release of N-terminal tyr followed by Met (or Leu), Phe and Gly as measured by amino acid analysis and by microdansylation techniques. The slow appearance of Gly and the accumulation of Gly-Gly in digests of Enk points to the presence in brain extracts of a Gly-Gly dipeptidase which is rate limiting. Incubation of Met- or Leu-Enk with rat brain homogenate led to a 28-38% release of Tyr as the only detectable product after one min. The appearance of other cleavage products with increasing time of incubation provided additional information on the nature of other enzymes involved in the total degradation of the pentapeptide. Thus liberation of Met proceeding Phe indicates action by a carboxypeptidase. The presence of Gly-Gly indicates splitting of the Tyr(1)-Gly(2) and Gly(3)-Phe(4) bonds (Table V).

Meek *et al.* (1977) reported that the release of Tyr from Enk was at least 20 times faster than Gly or Leu, using membrane preparation obtained by homgenizing rat striata, and the enzyme acivity could be inhibited by *o*-phenanthroline. With an Enk concentration of 40 μM, Tyr release was linear with time for 40 min, at rates of 450 nmol /mg·prot./hr (Leu-Enk) and 300 (Met-Enk). With both substrates the V_{max} for Tyr release was 2.6 mmol/mg·prot./hr, and the K_m 8×10^{-5} M.

Met-Enk is the fragment of 61-65 of beta-lipot-

Table V. Cleavage of Enkephalins by Rat or Mouse Brain Extracts

Substrate	Time (min)	Percent breakdown					
		Tyr(1)	Gly(2)	Gly(2)-Gly(3)	Phe(4)	Met(5)	Leu(5)
Leu-enkephalin	1	38	0	0	0	–	0
	10	95	18	73	79	–	81
	180	100	43	48	100	–	100
Met-enkephalin	1	28	0	0	0	0	–
	10	84	21	60	66	75	–
	30	91	28	46	70	85	–

Source: Marks *et al.* (1977)

ropin; a larger fragment, beta-endorphin, has been suggested as a possible precursor of Enk. The amino terminal Tyr group in endorphin appears to be protected: when beta-endorphin (100 μM) was incubated with the opiate receptor preparation, the release of Tyr was less than 4% of that with a similar concentration of Met-Enk

Analogs of Enk containing D-amino acids in position 2 were exposed to extracts prepared from mouse brain and found to be highly resistant to breakdown (Glynbaum *et al.*, 1977). D-Ala(2)-Met-Enk alone did not prevent aminopeptidase action but retarded the action as compared to the unsubstituted pentapeptide. The presence of D-amino acids in positions 2-5 led to the production of a pentapeptide intermediate that was resistant to the action of carboxypeptidases.

On the other hand, it was suggested that Enk binding to opiate receptors is coupled to subsequent aminopeptidase degradation (Knight and Klee, 1978). Aminopeptidase action to degrade Enk was thought of as being linked to receptor occupancy as an efficient mechanism for turning off the Enk signal. The initial rate of Tyr liberation from Enk was directly proportional to protein concentration over the range of 0.5 and 5 mg of protein/ml, and the pH optimum for Enk degradation by brain membrane was near 7. They found that a great variety of peptides are inhibitors of the enzyme, perhaps because they act as competitive substrates, and that bacitracin which has been used for this purpose was only one of many such inhibitory peptides and was not the best of these. Interestingly, puromycin which can be considered to be a tyrosyl peptide analog is the most potent aminopeptidase inhibitor which they have found. Hersh (1982) has reviewed that the opioid peptides Met- and Leu-Enks appear to exert their biological effects through a receptor mediated mechanism. Three mechanisms for Enk degradation are involved to control Enk levels in the vicinity of Enk receptors. They are, 1) cleavage of the Tyr-Gly bond by aminopeptidases, 2) cleavage of the Gly-Gly bond by a dipeptidyl aminopeptidase, and 3) cleavage of the Gly-Phe bond by a dipeptidyl carboxypeptidase. When the guinea-pig ileum or myenteric plexus longitudinal muscle was incubated with [^{3}H] Leu-Enk, the major degradative product was [^{3}H]Tyr. This fact coupled with the ineffectiveness of enkephalinase and ACE inhibitors such as thiorphan and captopril to potentiate the inhibitory action of the Enks provides strong evidence that aminopeptidase is the primary degradative enzyme for inactivation of exogenous Enk in the guinea pig ileum (Geary *et al.*, 1982).

In vitro studies in the guinea pig ileum, Enk hydrolysis in this tissue is attributed to the combined action of aminopeptidases, enkephalinase and ACE (Aoki *et al.*, 1984). In the isolated perfused rat lung, aminopeptidases and ACE, but not enkephalinase, are reported to be responsible for Enk metabolism (Gillespie *et al.*, 1985), although Llorens and Schwartz (1981) found high enkephalinase activity in particular fraction from rat lung. In a study of plasma samples taken from several species, it was concluded that aminopeptidases

and dipeptidyl aminopeptidases are responsible for Enk metabolism and that dipeptidyl carboxypeptidases (e.g., enkephalinase or ACE) play only a minor role in most species investigated (Venturelli *et al.*, 1985).

The enzymes responsible for the hydrolysis of Leu-Enk in rat plasma were characterized in more detail (Weinberger and Martinez, 1988). The effects of the inhibitors bestatin (selective for aminopeptidase M), puromycin (selective for aminopeptidase MII), thiorphan (selective for enkephalinase) and captopril (selective for ACE), alone and in combination, on the rate of metabolism of Leu-Enk were assessed. In addition, the effects of these inhibitors on the rate of metabolism of [D-Ala(2)]-Leu-Enk, a Leu-Enk analog susceptible to hydrolysis by enkephalinase but not by aminopeptidase in brain tissue were investigated (Llorens *et al.*, 1982). Rat plasma was found to have its own unique pattern of Enk hydrolysis. Approximately 85-90% of the hydrolysis of Leu-Enk is attributed to the combined action of aminopeptidase M and ACE, whereas enkephalinase and aminopeptidase MII activity against Leu-Enk are not detectable. Similarly, 80-90% of the hydrolysis [D-Ala(2)]-Leu-Enk is due to the combined action of aminopeptidase M and ACE, whereas aminopeptidase MII and enkephalinase activity against this substrate also could not be detected. This is in contrast to the high susceptibility to hydrolysis by enkephalinase, and the low susceptibility to aminopeptidase activity, for [D-Ala(2)]-Leu-Enk in brain tissue. The half-life of Leu-Enk in rat plasma was determined to be 3.5 min. This is similar to the finding of a 2.4 min half-life in plasma collected from somewhat older rats (Martinez *et al.*, 1988) and to 2.5 min Leu-Enk half-life determined by Hambrook *et al.*, 1976). On the other hand, Venturelli *et al.* (1985) reported a 46-sec half-life for Leu-Enk in fractioned, as opposed to whole, rat plasma. The plasma half-life of [D-Ala(2)]-Leu-Enk was approximately 3-4 hr.

2-5. Enzymatic Degradation of Enkephalins in Mucosae

The nasal administration of peptides for systemic medication has been used for desmopressin acetate and oxytocin. However, this route of administration has recently been considered as an alternative to the parenteral for luteinizing hormone-releasing hormones, insulin, interferon and growth hormone-releasing factor.

Although many drugs are absorbed rapidly and quantitatively following nasal administration (Hussain *et al.*, 1984a; Hussain *et al.*, 1984b), the peptides have generally shown low nasal bioavailabilities. For examples, studies with insulin and nafarelin acetate have shown that considerable greater nasal than parenteral doses are required to produce similar effects (Hirai *et al.*, 1981; Anik *et al.*, 1984). It is well established that proteins and peptides are subject to degradation by proteases and peptidases during passage through the mucosal membrane (Klostermyer and Humbel, 1966) and/or while at the surface of the mucosa. For this reason, only modest improvements over the oral delivery have been observed in many reports, even in the presence of absorption enhancers. It appears that this enzymatic barrier to peptide absorption has been ignored until recent years (Stratford and Lee, 1985; Hirai *et al.*, 1981). In general, there are three types of aminopeptidase present in the nasal mucosa. These are plasma membrane-bound peptidases; aminopeptidase N and aminopeptidase A; and a cytosolic enzyme, aminopeptidase B.

Recently a study claimed that the Enk analogs, [^{3}H]Tyr-D-Ala-Gly-L-Phe-D-Leu-OH and metkephamid (Tyr-D-Ala-Gly-Phe-N-Me-Met-$NH_2 \cdot CH_3$ COOH) were efficiently and completely absorbed from the nasal cavity (Su *et al.*, 1985; Su, 1986). It should be noted, however, that the Enk was administered in unrealistically large doses (2-50 mg/kg).

The nasal absorption of Leu-Enk in rats was examined using an *in-situ* perfusion technique (Hussain *et al.*, 1985). A 60 μg/ml solution of the Enk in Ringer's buffer was circulated through the nasal cavity of anesthesized rats. The overall disappearance of Leu-Enk from nasal perfusate was found to be due to hydrolysis and that the extent of absorption, if any, was less than about 10%.

Table VI. First Order Rate Constants (10^2 k, min^{-1}) and Half-lives (min) for the Hydrolysis of Methionine Enkephalin (TGGPM), Leucine Enkephalin (TGGPL), and [D-Ala]Met-enkephalinamide (TAGPM) in Homogenates of Various Absorptive Mucosae of the Albino Rabbit

Mucosa	TGGPM		TGGPL		TAGPM	
	k (10^2)*	$t_{0.5}$	k (10^2)	$t_{0.5}$	k (10^2)	$t_{0.5}$
Nasal	4.25± 0.37 (4.06)	16.3± 1.4	3.45± 0.36 (3.40)	20.1± 2.1	0.43± 0.07 (0.41)	162.0± 26.9
Buccal	5.76± 0.51 (5.15)	12.0± 1.1	5.40± 0.64 (5.02)	12.8± 1.5	0.45± 0.05 (5.45)	153.2± 15.9
Rectal	6.14± 0.86 (7.42)	11.3± 1.6	3.97± 0.36 (4.23)	17.5± 1.6	0.61± 0.03 (0.56)	114.3± 6.0
Vaginal	3.12± 0.29 (2.34)	22.2± 2.1	2.49± 0.28 (0.23)	27.8± 3.1	0.38± 0.07 (0.36)	183.7± 36.3
Ileal	4.58± 0.60 (4.59)	15.1± 2.0	3.33± 0.23 (3.53)	20.8± 1.5	0.31± 0.02 (0.59)	226.5± 1.5

*Mean± SD for triplicate determinations. Figures within parentheses represent calculated rate constants.
Source: Dodda Kashi and Lee (1986)

However, in the presence of a 20 fold molar excess of other peptides such as L-Tyr-L-Tyr, the extent of Enk hydrolysis was considerably reduced. Thus, it was concluded that a) polar compounds such as peptides can penetrate the nasal mucosa; undergo extensive hydrolysis in the nasal mucosa; and c) the hydrolysis of Leu-Enk can be inhibited by the addition of peptidase labile peptides to the mucosal perfusate. It was proposed that the coadministration of a competing, pharmacologically inactive peptide, may be a useful approach to improving the bioavailability of nasally administered peptides.

The systemic delivery of peptides and proteins from the nasal, rectal, vaginal, and buccal mucosae has been the subject of active investigation. The pathway and rate of hydrolysis of Met-Enk, Leu-Enk and [D-Ala(2)] met-enkephalinamide (TAGPM) in homogenates of these non-oral mucosae were determined comparing to the ileal mucosa (Dodda-Kashi and Lee, 1986). Aminopeptidases appeared to contribute over 85% to the hydrolysis of Met-Enk and Leu-Enk, although dipeptidyl peptidase and dipeptidyl carboxypeptidase also played a role. This finding indicates that aminopeptidases are the principal enzymes to be inhibited in order to minimize Enk degradation while permeating various absorptive mucosae. In general, Met-Enk was somewhat more susceptible to hydrolysis than Leu-Enk, but was 10 times more so than TAGPM. These Enks were more rapidly hydrolyzed in the rectal and buccal homogenates, followed by the nasal and then the vaginal homogenats, but the differences in hydrolytic rates were small. The half-lives ranged from 11-22 min for Met-Enk, 13-28 min for Leu-Enk, and 114-227 min for TAGPM (Table VI). These rates were not substantially different from the ileal mucosa, indicating that the same enzymatic barrier to Enk absorption possibly exists in both the oral and the non-oral mucosae. Quantitatively, both Met- and Leu-Enks were hydrolyzed at three sites within their backbone resulting in the formation of Tyr, Tyr-Gly, and Tyr-Gly-Gly, presumably due to the action of aminopeptidases, dipeptidyl peptidase, and dipeptidyl carboxypeptidase, respectively.

In order to define the aminopeptidase barrier to peptide absorption from non-oral routes, the type and activity of aminopeptidase in the homogenates of conjunctival, nasal, buccal, rectal and vaginal mucosae, relative to duodenal and ileal homogenates, were characterized using 4-methyl-2-naphthylamides of leucine, alanine, arginine and glutamic acid as substrates (Stratford and Lee, 1986). Based on the pattern of substrate hydrolysis and the effect of activators and inhibitors on the rate of substrate hydrolysis, four or five aminopeptidases were estimated to be present in these mucosal homogenates. Aminopeptidase N,

a plasma membrane-bound peptidase (Vannier *et al.*, 1976) which is concentrated in the apical regions of the intestinal brush border (Maze and Gray, 1980), was present in all these mucosae to the extent of 50-100% of ileal activity, whereas aminopeptidase A, which is another plasma membrane-bound peptidase and appears to be most abundant in the ileum, was present to the extent of 4-20% of ileal activity. 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. Overall, the differences in aminopeptidase activity among the various non-oral routes were not large.

Indeed, the delivery of leuprolide 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. However, the fraction of peptide absorbed systemically from the respective routes was only 0.1%, 1.2% and 3.8% of the applied dose. 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.

The corneal epithelium of the albino rabbit, which is anticipated to be defficient in peptidase activity, was found to have 15% of the aminopeptidase activity in the ileum when determined using L-leucine, L-alanine, and L-arginine-4-methoxy-naphthylamide as substrates. The conjunctiva was 5% as active as the ileum (Stratford and Lee, 1985). This appreciable aminopeptidase acvitity is principally responsible for the more than 75% degradation, as early as 5 min post-dosing, of Leu- and Met-Enks following topical ocular administration to albino rabbit (Lee *et al.*, 1985). Unexpectedly, this *in vivo* hydrolytic rate was 50-100 times higher than the *in vitro* rate. Although the hydrolysis of Enks within the eye was unaffected by predosing the eye with bestatin, this rate of hydrolysis could be reduced by treatement with di-, tri-, tetra-, and pentapeptides or by varying the primary structure of an Enk.

In a study (Dodda Kashi and Lee, 1985b) on the kinetic and pathway of hydrolysis of Met-Enk, Leu-Enk and [D-Ala(2)] met-enkephalinamide (TAGPM) in homogenates of anterior segment tissues of the albino rabbit eye using reversed phased HPLC, both Met- and Leu-Enks were equally susceptible to hydrolysis with a half-life ranging from 11-50 min and were 11-23 times more susceptible to hydrolysis than TAGPM. All three peptides were hydrolyzed most rapidly in the corneal epithelium, followed by the iris-ciliary body, conjunctiva, corneal stroma, lens, and tears. Aminopeptidases were responsible for over 90% of the hydrolysis of Met- and Leu-Enks in tissue homogenates, while dipeptidyl peptidase and dipeptidyl carboxypeptidase were responsible for the remainder. In contrast, dipeptidyl carboxypeptidase was principally responsible for the hydrolysis of TAGPM, which is resistant to aminopeptidase action.

2-6. Enzymatic Degradation of Enkephalins in Skin

Recently, the permeation through hairless mouse skin of Leu-Enk was investigated using a gradient HPLC method, and it was found that the major metabolite is the Tyr fragment resulting from aminopeptidase activity, and that endopeptidase cleaving the Gly-Phe and a carboxypeptidase also contributed to the metabolism. On the other hand, metabolism of the enzymatically stable Leu-Enk analogs, [D-Ala(2)] Leu-Enk and its amide, in the skin was less than for Leu-Enk, but still significant. [D-Ala(2)] leu-enkephalinamide was observed to be more stable against hydrolysis than [D-Ala(2)] Leu-Enk (Choi *et al.*, 1989a).

In the homogenates of hairless mouse skin, the rate of degradation was found to be in the order of Phe-Leu>Leu-Enk>Gly-Phe-Leu>Gly-Gly-Phe-Leu>Tyr--Gly, and Tyr-Gly-Gly> [D-Ala(2)] leu-enkephalinamide. At least two types of aminopeptidase activity appeared to be responsible for metabolizing the N-terminal amino acid successively to complete degradation of Leu-Enk. Endopeptidase activity in the homogenates, if any, was negligible (Choi *et al.*, 1989b).

Aminopeptidase activity and Enk metabolism in the homogenates of cultured human keratinocytes and human skins were compared using leucine,

tyrosine, lysine and aspartic acid derivatives of β-naphthylamine (Shah and Borchardt, 1989). The homogenates of fore skin, breast skin and Clonetics cells yielded comparable enzymatic activities. Hydrolysis of Leu-Enk gave similar K_m values (50 μM), apparent first order rate constants (0.301 min^{-1}) and a similar metabolite profile in the homogenates of cultured human keratinocytes and human epidermis.

3. Inhibitors of Enkephalin Degradation

The Enks are rapidly hydrolyzed by enzymatic activities present in brain, plasma, GI tract (Okada *et al.*, 1982; Kerchner *et al.*, 1983), mucosae (Dodda Kashi and Lee, 1986a) such as nasal (Su *et al.*, 1985), buccal, rectal, vaginal and ocular (Stratford and Lee, 1985; Dodda Kashi and Lee, 1986b) tissues. The rapid dagradation probably accounts for the low and transient analgesia (Belluzzi *et al.*, 1976) and bioavailability (Lee *et al.*, 1985; Su *et al.*, 1985) of the Enks. The prevention of this hydrolysis is therefore required in order to enhance the membrane permeability, bioavailability and the interaction of the Enks with opiate receptors.

An useful approach to evaluating the significance of various metabolic pathway of Enk disposition is to investigate the pharmacological effect and/or degradation of Enks alone or conjunction with enzymatic inhibitors. Such studies have been performed both in whole animal and in tissue preparations.

3-1. Aminopeptidase Inhibitors

The antibiotic puromycin (0.1-1.0 mM) is an effective inhibitor of aminopeptidase in brain and ileum homogenates (Barclay and Phillips., 1978; Vogel and Altstein, 1979; Sullivan *et al.*, 1980). Vogel and Altstein (1979) have shown that puromycin effectively inhibits the degradation of Leu-Enk by enzymatic activity present in rat brain homogenates and guinea pig ileum, and potentiates the inhibition of guinea pig ileum contractions produced by Leu-Enk. Puromycin does not inhibit the hydrolysis of Enk by either leucine aminopeptidase or rat serum. Bacitracin (IC_{50}: 20 μM), a less potent aminopeptidase inhibitor, can produce a dose dependent, naloxone-reversible analgesia in mice tested in the tail-flick assay with the drug administration by the i.c.v. route (Simmons and Ritzman, 1980). It was found that the K_m is 80 μM and IC_{50} is 0.8 μM (Barclay and Phillips, 1978).

The degradation of Leu-Enk by an aminopeptidase in rat whole brain supernatant was inhibited by two brain peptides and two bacterial peptides, amastatin and bestatin, were slightly more potent than somatostatin and substance P. Amastatin and bestatin exhibited non-competitive kinetics; somatostatin and substance P were competitive inhibitors. Therefore it was suggested that the known analgesic properties of somatostatin and substance P when given i.c.v. may be due to inhibition of degradation of endogenous opioid peptides. Amastatin, a tetrapeptide, is 100-fold more potent than puromycin (Barclay and Phillips, 1980).

D-leucine (at 80 μg/m*l*), another aminopeptidase inhibitor, can potentiate Met-Enk effects on the longitudinal muscle of the guinea pig ileum and can potentiate stress-induced anagesia in rodents (Carenzi *et al.*, 1980).

Puromycin is a general inhibitor of aminopeptidases which hydrolyze amino acid 2-naphthylamides or amino acid 4-nitroanilides, and bestatin is a more selective inhibitor of leucine aminopeptidases and aminopeptidase B with substrate specificity for N-teriminal Leu containing peptides and peptides containing N terminal hydrophobic residues (Umezawa and Aoyagi, 1979). Both agents are capable of inhibiting Enk degradation in broken cell preparations from brain. However, only bestatin enhanced the pharmacological response to Enk in the guinea pig ileum and ileal longitudinal muscle. Concentration-response curves to bestatin indicated that the enhanced physiological effect of the Enks was coupled to inhibition of [^{3}H] Tyr formation from [^{3}H] Leu- or [^{3}H] Met-Enk (Cohen *et al.*, 1983).

In the presence of thiorphan an enkephalinase inhibitor, bestatin aminopeptidase inhibitor potently inhibited the hydrolysis of [^{3}H] Leu-Enk by slices from rat striatum with an IC_{50} value of about 0.2 μM, whereas puromycin was approxima-

tely 1000 times less potent on this preparation. *In vivo* bestatin or thiorphan (but not puromycin) significantly protected [^{3}H] Met-Enk administered i.c.v. to mice from hydrolysis and coadministration of these two peptides inhibitors resulted in a strong reduction in the appearance of hydrolytic products in brain. In a parallel fashion the antinociceptive activity of Met-Enk in the mouse hot plate test was additively potentiated by bestatin and thiorphan but not puromycin. Finally both bestatin and thiorphan themselves displayed antinociceptive properties on either the hot-plate jump test or the phenylbenzoquinone writhing test. It was concluded that a bestatin-sensitive aminopeptidase activity together with the enkephalinase activity plays a critical role in the inactivation of both endogenous and exogenous Enks (Chaillet *et al.*, 1983).

The effect of the inhibition of aminopeptidase and enkephalinase A on the pain threshold of mice and rats was studied using bestatin and thiorphan, and it was shown that both enzymes are relevant to the catabolism of Enks in vivo; however, their simultaneous activation requires particular conditions. Only concomitant intracerebral treatment with both inhibitors led to an increase in the threshold of animal pain, whereas, in the presence of exogenous peptides, the concomitant injection of both inhibitors in mice ilicited an analgesic response greater than the sum of the effects of each single inhibitor (Carenzi *et al.*, 1983b).

Even though amastatin, bestatin and puromycin are potent inhibitors of aminopeptidases of rat brain, inhibiting the hydrolysis of Leu-Enk with inhibitor concentrations required for 50% inhibition (IC_{50}) of 5, 20 and 200 nM, respectively, these materials are much less effective inhibitors of the enkephalinase (IC_{50} values of 300, 70 and 100 μM, respectively). The enkephalinase activity was inhibited by various barbiturates, e. g., secobarbital or pentobarbital (IC_{50} of 8 and 20 μM, respectively). The barbiturates did not significantly inhibit the hydrolysis of Leu-Enk by aminopeptidase or enkephalinase B activities. Both aminopeptidase and enkephalinase seem to be metalloproteases, and thier activity is inhibited by metal chelating agents. The combination of a hydrophobic dipeptidyl moiety and a transition metal ion-chelating moiety in the same molecule resulted in a very efficient inhibitors of enkephalinase. Thus the N-phosphorylated dipeptides (K_2PO_3-Leu-Phe and phosphoramidon) and the mercaptoacetyl dipeptides (SH-CH_2CO-Leu-Phe and SH-CH_2CO-Phe-Leu) inhibited enkephalinase activity with IC_{50} values of 0.3, 1.0, 1.5 and 70 nM, respectively. Much higher concentrations were needed to inhibit the aminopeptidase (IC_{50} values 100, >1000, 80, and 150 μM, respectively), indicating the high selectivity of these inhibitors toward enkephalinase activity (Vogel and Altstein, 1982).

Effect of the proteolytic enzyme inhibitors, bestatin (30 μM) or D-phenylalanine (20 mM) on the degradation and transport of [D-Ala(2)]-D-Leu (5)] Enk was investigated using everted rat jejunum sections. The presence of these inhibitors significantly reduced degradation of the peptide; however no accumulation of the peptide occurred in the mucosal tissue. Thus it was suggested that accumulation of this peptide from the GI tract probably occurs either by passive or facilitated diffusion (Kerchner and Geary, 1983).

The available evidence indicates that aminopeptidase (aminoenkephalinase) is the major enzyme that inactivates Enk released from presynaptic sites (Horsthemke *et al.*, 1983). Hui *et al.*, (1982, 1983) have reported that several neuropeptides are potent inhibitors of membrane aminoenkephalinase. Peptides with arginine at the amino end is especially effective. Proctolin (Arg-Tyr-Leu-Pro-Thr-OH), which acts as a neurotransmitter or modulator in insects and causes contraction of the rodent ileum at 10^{-8} M, also has arginine as its N-terminal amino acid.

Proctolin was 10-fold stronger than bestatin and 2-fold stronger than amastatin (IC_{50}=0.17 μM) as a most potent inhibitor of aminoenkephalinase. Like puromycin, proctolin did not inhibit leucine aminopeptidase or aminopeptidase M; thus it is more selective than bestatin (Table VII) (Hui *et al.*, 1985).

The effect of bestatin and puromycin on substrate hydrolysis in both commercially purified ami-

Table VII. Effect of Peptidase Inhibitors on the Metabolism of Met-Enkephalin

	IC_{50}(50% Inhibition Concentration, μM)		
	Puromycin	Bestatin	Proctolin
Cytosol Aminoenkephalinase	0.85	0.9	0.44
Membrane Aminoenkephalinase	1.6	1.0	0.1
Leucine Aminoenkephalinase	>50.0	0.1	>50.0
Aminoenkephalinase M	>50.0	4.6	42.0
Synaptosomal Plasma Membranes	0.72	0.18	0.65

To obtain 50% breakdown of the Met-Enkephalin, 300 ng of cytosol aminoenkephalinase, 20 ng of membrane aminoenkephalinase, 5 μg of leucine aminopeptidase, 380 ng of aminopeptidase M, or 8.6 μg of synaptosomal plasma membrane was used in each incubation. The IC_{50} values were determined by measuring the disappearance of the Met-Enkephalin with HPLC. Source: Hui *et al.*, (1985)

nopeptidase preparations and various mucosal homogenates is shown in Table VIII (Stratford and Lee, 1986). Bestatin and puromycin were considered to inhibit aminopeptidase N and B in the mucosal tissues. And it was suggested that leucine aminopeptidase may not be a dominant aminopeptidase in any of the mucosal tissues studied.

Hussain *et al.* (1989) reported that α-aminoboronic acid derivatives, potent and reversible inhibitors of aminopeptidases, at nanomolar concentrations, inhibited greatly the degradation of Leu-Enk in the nasal perfusate. Enzyme inhibition was greater with boroleucine and borovaline than that observed with boroalanine. Boroleucine was 100 times more effective in enzyme inhibition than bestatin and 1000 times more effective than puromycin.

Choi *et al.* (1989b) reported the inhibitory effects of various compounds including metabolites of Leu-Enk on its metabolism in the hairless mouse skin homogenates. Puromycin and amastatin showed the highest inhibitory effect. However, when their inhibitory effects were tested in skin diffusion experiments in the presence of 10 mM *n*-decylmethyl sulfoxide, Phe-Leu showed the highest inhibitory effect in terms of the amount of parent compound permeated. From the results, it was suggested that the complex proteolytic enzyme activities in skin diffusion are different from those in skin homogenates.

3-2. Carboxypeptidase A Inhibitors

The amino acid, D-Phe, is a weak inhibitor of

Table VIII. Percent Inhibition of Mucosal Aminopeptidase Activities by 0.01 mM Bestatin and 0.1 mM Puromycin

Tissue	Bestatin				Puromycin			
	Leu	Ala	Glu	Arg	Leu	Ala	Glu	Arg
Conjunctival	28.1	73.2	25.5	46.8	1.8	28.4	5.9	2.3[c]
Nasal	38.1	71.5	1.4	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.

Source: Stratford and Lee, (1986)

carboxypeptidase A. Carenzi *et al.*, (1980) have reported that this compound potentiated the inhibition by Met-Enk of guinea pig longitudinal muscle contractions, and potentiated the analgesic response to Met-Enk in the rat hot plate jump assay and stress-induced analgesia in rats. Ehrenpreis *et al.* (1978) reported a pain reduction in chronic pain patients.

3-3. Dipeptidyl Carboxypeptidase Inhibitors

The potent and selective inhibitors of enkephalinase A is thiorphan (3-mercapto-2-benzylpropienyl glycine, K_i=4.2 nM) (Roques *et al.*,1980). Thiorphan administered i.c.v. or systemically significantly potentiated the analgesic effect of [D-Ala(2)] Met-Enk in the analgesic test using mouse tail withdrawal. Blocking enkephalinase activity by i.c.v. thiorphan or i.p. acetorphan results in an increased analgesic effect of electrostimulation as measured by the 50℃ wet tail flick test (Malin *et al.*, 1989).

Captopril, an ACE inhibitor which is a less potent inhibitor of enkephalinase (K_i=10 μM) (Roques *et al.*, 1980), has been evaluated in a number of systems. The results of these experiments seem initially contradictory, but may reflect the variety of protocols employed to evaluate the activity of this compound.

A variety of peptides chemically unrelated to enkephalins are relatively good inhibitors (IC_{50} in the micromolar range) of enkephalinase. However, it appears that enkephalinase does not exhibit an extremely stringent specificity towards various peptides (Fournie-Zaluski *et al.*, 1979a).

Carboxyalkyl compounds (Fournie-Zaluski *et al.*, 1982) derived from the Phe-Leu and corresponding to the general formula C_6H_5-CH_2-CH(R)-CO-Leu have been found to inhibit the breakdown of the Gly(3)-Phe(4) bond of [^{3}H] Leu-Enk or [^{3}H] [D-Ala(2)] Leu-Enk resulting from the action of the mouse striatal metallopeptidase (enkephalinase or ACE). One of them N-[(R, S)-2-carboxy, 3-benzylpropanoyl]-L-Leu exhibits an inhibitory potency (K_i=0.34 μM) and a very high specificity for enkephalinase since it is at least 10,000 fold more potent on this enzyme than on ACE. This compound is able to prevent *in vitro* degradation of endogenous Enks and exhibits analgesic properties in mice. Therefore this compound can be considered as one of a new series of enkephalinase inhibitors, to add to thiorphan, N-carboxymethyl-dipeptides (Patchett *et al.*, 1980), barbiturates (IC_{50}; 4×10^{-4} M-8×10^{-6} M) (Altstein *et al.*, 1981), phosphoryl-Leu-Phe (IC_{50}: 0.3 nM) (Altstein *et al.*, 1982), and hydroxamic acids (IC_{50}; 3.1-8.4 nM) (Hudgin *et al.*, 1982) which also inhibit Enk degradation.

Bestatin and high concentration of puromycin increase the depressing effect of Met-Enk on the twitch response of the electrically stimulated guinea pig ileum. Thiorphan (enkephalinase A inhibitor) is hardly effective, but phelorphan (mercaptoacetyl-Phe-Phe) (enkephalinase A, enkephalinase B and soluble aminopeptidase activity inhibitor) potentiates the effect of Enk dose-dependently and in low concentrations (0.01-1 μM). Thus it was demonstrated that enkephalinase B and the membrane-bound aminopeptidase, but not the soluble aminopeptidase or enkephalinase A hydrolyze Enks in the isolated guinea-pig ileum (Van Amsterdam *et al.*, 1988).

4. Degradation and Stabilization of Met-Enk

4-1. Stability of Met-Enk in Buffered Solution

The physicochemical stability of Met-Enk was evaluated by studying its degradation profiles in various buffered solutions as a function of solution pH, environmental temperature and drug concentration (Chun and Chien, 1991a). In the early stage of incubation at 37℃, none of the solutions showed any precipitation, aggregation or surface adsorption .

The degradation profile of Met-Enk was observed to follow the apparent first order kinetics. One typical plot is shown in Figure 1, which indicates that the first-order degradation kinetics of Met-Enk is temperature-dependent and the rate of degradation increases with the increase in environmental temperature. The results summarized in Table IX indicate that the rate constant of degradation varies not only with temperature but also

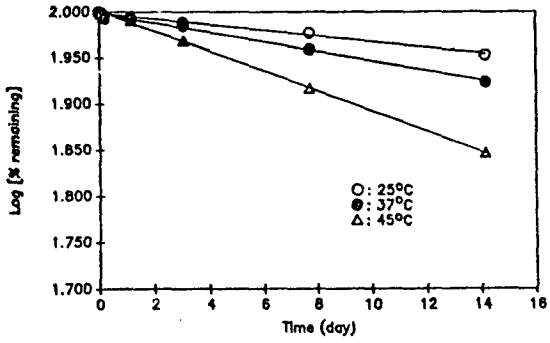


Figure 1—Apparent first-order degradation of Met-Enk (50 μg/ml) in borate buffer (pH 9.84, μ=0.12) at various temperatures.

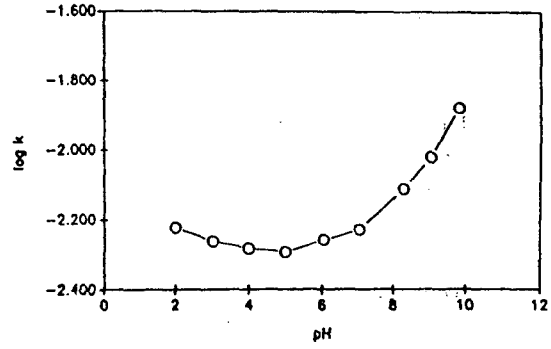


Figure 2—Log k-pH profile for the stability of Met-Enk at 37℃.

Table IX. Apparent First-Order Rate Constants (k) and Half-lives ($t_{1/2}$) for the Degradation of Met-Enk in Aqueous Solution as a Function of pH and Temperature

pH	k ($day^{-1} \times 10^3$)			$t_{1/2}$(days)		
	25℃	37℃	45℃	25℃	37℃	45℃
2.01	ND	5.99	7.11	ND	115.7	97.5
4.99	ND	5.10	5.57	ND	135.7	124.4
7.07	ND	5.92	13.48	ND	117.1	51.4
9.84	8.51	13.19	22.60	81.43	52.5	30.7

ND: Not determined

with solution pH.

The log k-pH profile is shown in Figure 2. The degadation of Met-Enk indicates that Met-Enk is at maximum stability in the vicinity of pH 5. The effect of solution pH on the degradation of Met-Enk is much greater in the pH range higher than 5 than that in the range lower than 5. However, the log k-pH profile shown in Figure 2 did not yield a unity slope value in both the acidic and alkaline regions studied (pH 2.01-9.84), indicating the complexity of the mechanism involved in the degradation of Met-Enk molecule.

Met-Enk molecule has three ionizable groups: one aromatic hydroxyl group (pKa=3.68), one amino group (pKa=7.77) of tyrosine residue at the N-terminal, and a carboxylic acid group (pKa=3.68) at the C-terminal of the peptide (Ishimitsu and Sakurai, 1982). Therefore, Met-Enk could exist in various ionic forms, depending upon solution pH, and all of them may have different propensities to degradation. Experimentally, it was observed that after incubation at 37℃ for one month, several additional peaks, which could be the degradation products of Met-Enk, were observed in the chromatograms obtained.

Similarly, it was reported that upon prolonged storage at 4℃, the dilute solution of leucine enkephalin has been observed to increase in Rf value. The phenomenon was explained as caused by the conversion of N-terminal tyrosine into an indole derivative, a mechanism which is apparently similar to the chemical and enzymatic oxidation of tyrosine derivatives. Since tyrosine is positioned at the N-terminal of Met-Enk molecule, the cyclization seems to be implicated as one of the degradation mechanisms (Dukler *et al.*, 1971). Consequently, the appearance of other peaks is considered to be due to the cleavage, cyclization (Vogel *et al.*, 1978) and oxidation (Wilchek *et al.*, 1968) of Met-Enk, and any further change of its degradation products. The appearance of different peaks at different pH's and temperatures indicates that the mechanism of degradation is very complex. Therefore, the elucidation of pathways and mechanisms for the degradation of Met-Enk requires further extensive studies.

4-2. Enzymatic Degradation of Met-Enk in Various Mucosa Extracts

In view of its susceptibility to enzymatic degra-

dation, it is important to understand the ability of Met-Enk to survive the enzymatic barrier on and in the various biological mucosae when the mucosal delivery is aimed. Met-Enk was chosen for this study primarily because of the fact that while the pathway of its metabolism in the brain (Hambrook *et al.*, 1976, Hersh *et al.*, 1981) and elsewhere in the body have been well characterized, there are very few reports on the metabolism of Met-Enk at the non-oral mucosal sites (Dodda-Kashi and Lee, 1986a). In this investigation (Chun and Chien, 1991a), the kinetics and pathway of Met-Enk degradation in the extracts of nasal, rectal and vaginal mucosae were thus investigated over a duration of 6 hr by simultaneously monitoring the disappearance of Met-Enk and the appearance of its metabolites as a function of incubation period.

The results of control experiments confirmed that Met-Enk is stable, physicochemically, in the isotonic phosphate buffer used to extract the enzymes from each mucosal membrane. The disappearance profiles of Met-Enk in the extracts of nasal, rectal and vaginal mucosae of New Zealand White rabbits are shown in Figure 3. The results indicate that Met-Enk degrades most rapidly in the rectal extracts (with mean half-life of 10.31-14.10 min), followed by vaginal (38.27-45.98 min) and nasal (63.84-74.09 min) extracts, indicating a considerable variation in peptidase activities among the mucosal extracts, but degradation rates were similar between the extracts from the mucosal and serosal surfaces, regardless of the mucosa extracted.

Figure 4 compares the concentration profiles of Met-Enk as well as its hydrolytic fragments upon incubation with various mucosa extracts (Chun and Chien, 1991a). The results appear to suggest that Met-Enk is cleaved primarily at the Tyr-Gly bond to yield Tyr as its main hydrolytic fragment in all the mucosal extracts, but the amount of Tyr-Gly fragment yielded by cleavage at the Gly-Gly bond is relatively small. It should be noted, however, that both in the nasal mucosal and serosal extracts, relatively larger amount of Tyr-Gly fragment was formed compared with those in rectal and vaginal extracts. Especially, considerable amount of Tyr-Gly-Gly was detected only in the nasal extracts, as resulted from the cleaveage at the Gly-Phe bond.

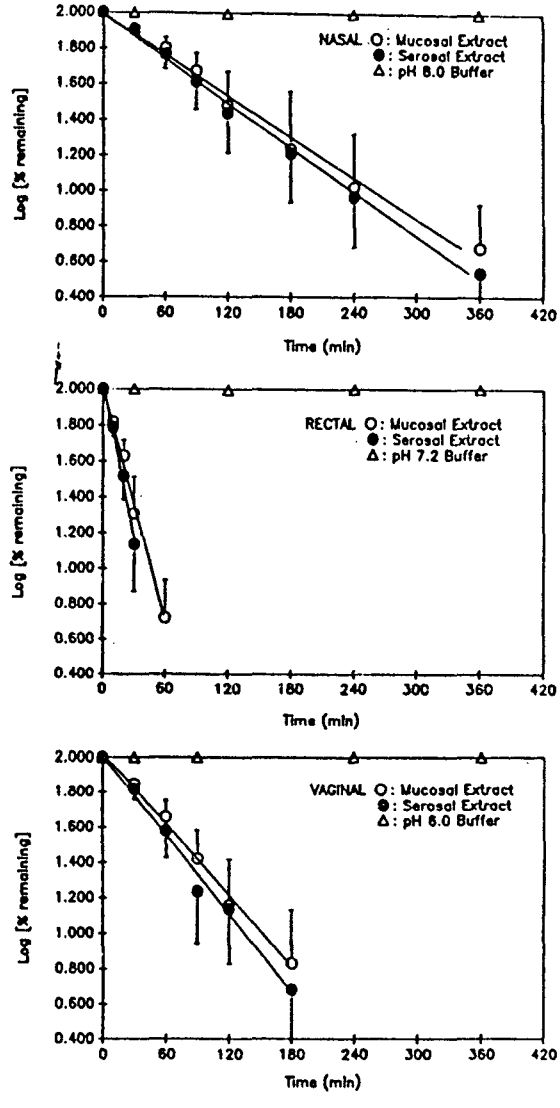


Figure 3—Disappearance of Met-Enk in the mucosal and serosal extracts of various mucosae of NZW rabbits at 37℃. Each point indicates mean (± SE) of six rabbits. The disappearance profile of Met-Enk in the buffer at the specific mucosal pH is also shown for comparison.

The important difference among various mucosal extracts is that the formation of Tyr-Gly in

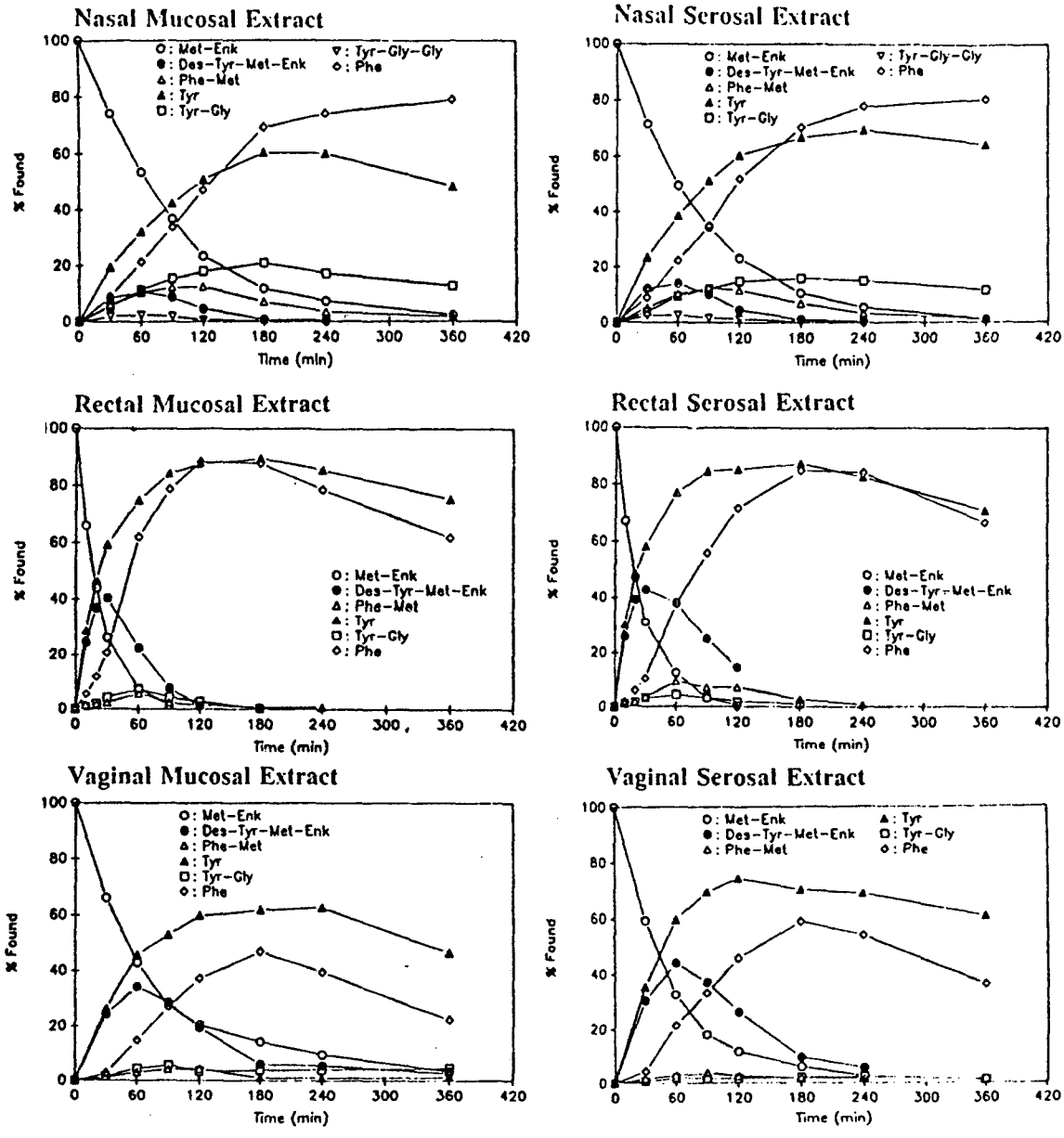


Figure 4—Time course for the degradation of Met-Enk and appearance of its metabolites in the extracts of various mucosae of NZW rabbits at 37℃. Each point is the data obtained from three rabbits.

the nasal, rectal and vaginal extracts at 90 min after incubation was in the range of 15.4(± 2.2)% (mean± SE), 4.3(± 1.8)% and 3.4(± 1.5)%, respectively, and the formation of Tyr was 42.4(± 11.5)%, 84.4(± 7.9)% and 52.8(± 12.5)%, respectively. Similar results were obtained in the serosal extracts of the mucosae. These results indicate some differences in the relative contribution of aminopeptidases and enkephalinase B to the overall Met-Enk degradation, depending on the type of mucosa. Consequently, nasal extracts showed the highest enkephalinase B activity, followed by vaginal and then rectal mucosae. In addition, nasal extracts showed enkephalinase A activity even though the

Table X. First-Order Rate Constants ($hr^{-1} \times 10^2$) for the Degradation of Met-Enk in Various Rabbit Mucosa Extracts at 37℃ in the Absence and Presence of Various Enzyme Inhibitors

Inhibitor	Nasal extract		Rectal extract		Vaginal extract	
	Mucosal	Serosal	Mucosal	Serosal	Mucosal	Serosal
None[a]	85.80(± 18.22)[b]	100.74(± 33.53)	311.16(± 51.37)	327.72(± 54.73)	124.00(± 37.62)	146.46(± 38.76)
0.01% TM (A)[c]	13.87(± 4.09)	10.40(± 2.94)	46.30(± 3.41)	26.92(± 6.70)	12.21(± 4.24)	10.92(± 3.35)
A+50 μM AM (B)	10.11(± 3.78)	7.83(± 1.94)	9.77(± 0.93)	1.84(± 0.36)	1.27(± 0.33)	0.99(± 0.11)
A+50 μM TP (C)	7.47(± 2.71)	6.85(± 2.23)	17.87(± 3.98)	6.57(± 2.03)	4.80(± 1.93)	5.03(± 1.26)
A+5 mM EDTA (D)	0.88(± 0.06)	1.02(± 0.07)	0.66(± 0.02)	0.45(± 0.11)	0.66(± 0.13)	0.43(± 0.02)
A+B+C	0.66(± 0.03)	0.85(± 0.02)	2.67(± 1.44)	0.46(± 0.04)	0.53(± 0.02)	0.52(± 0.01)
A+B+D	0.26(± 0.05)	0.28(± 0.03)	0.38(± 0.09)	0.37(± 0.03)	0.27(± 0.10)	0.36(± 0.03)
K_o/k_{min}[d]	332.7	359.8	816.7	893.0	452.2	402.4

[a]Each extract was prepared by extracting each mucosal membrane of rabbits for 24 hrs with isotonic phosphate buffer at physiological pH.
[b]Expressed as means± S.E. of the data obtained from three rabbits.
[c]Extraction was carried out using the isotonic phosphate buffer containing 0.01% thimerosal from the beginning.
[d]Ratios of rate constants for None to those for A+B+D.
Source: Chun and Chien (1991b)

activity was lower (2.4± 0.3% Tyr-Gly-Gly at 60 min after incubation). Although it was reported (Dadda Kashi and Lee, 1986a) that Tyr-Gly-Gly has been formed at the same rates in the homogenates of nasal, rectal and vaginal mucosae, the formation of Tyr-Gly-Gly is not obsereved in rectal and vaginal extracts of New Zealand White rabbits, as may be attributed to the weak enkephalinase A activity, and/or further rapid disappea rance of Tyr-Gly-Gly fragment formed.

The rank order for the rate of formation of hydrolytic fragments varies from one mucosa to another, which is Tyr≫Phe>Des-Tyr-Met-Enk>Tyr-Gly≥Phe-Met>Tyr-Gly-Gly for nasal mucosal extracts, Tyr>Des-Tyr-Met-Enk≫Phe≫Tyr-Gly> Phe-Met for rectal mucosal extracts, and Tyr> Des-Tyr-Met-Enk≫Phe≫Tyr-Gly>Phe-Met for vaginal mucosal extracts.

4-3. Protection of Met-Enk from Enzymatic Degradation in Various Mucosa Extracts

The inhibition of enzymatic degradation of Met-Enk was kinetically investigated in the nasal, rectal and vaginal extracts of rabbits in the absence and presence of a single or combined inhibitors such as puromycin (PM), amastatin (AM), thiorphan (TP), disodium ethylenediaminetetraacetate (EDTA) and thimerosal (TM) by analyzing the parent peptide and its hydrolytic fragments by HPLC (Chun and Chien, 1991b). The degradation of Met-Enk was fastest at around pH 7, indicating that the activity of enkephalin-degrading enzymes is optimal at this pH. However, at the pH lower or higher than pH 7, the degradatiion was greatly decreased and no hydrolytic fragment was detected at pH 3.19 and 4.0 after 6 hrs. AM alone inhibited the enzymatic degradation of Met-Enk with the IC_{50} values of 3.5 and 0.22 μM for the rectal and vaginal extracts, whereas PM was approximately 14.2 and 26.8 times less potent than AM, respectively. In the nasal extracts, the effects of both aminopeptidase inhibitors were smaller. Even at 50 μM, TP alone revealed only small increase of Met-Enk stability in the various mucosal extracts, however, EDTA inhibited the enzymatic hydrolysis considerably by blocking the enkephalinase A and B and, to some extent, aminopeptidase, which are known as metallopeptidases. On the other hand, TM was found to be a new and potent enkephalinase B and aminopeptidase inhibitor, and to be more potent than AM in the inhibition of Met-Enk degradation in various mucosal extracts. Furthermore, as shown in Table X, the addition of TM to a combination of AM and EDTA protected Met-Enk from the enzymatic degradation in the nasal, rectal and vaginal extracts more than 90% after 24 hrs of incubation, by almost completely inhibiting all enkephalin-degrading enzymes

present in the incubation mixtures.

4-4. Conclusions

Met-Enk is unstable in aqueous solution upon prolonged storage, but it can be kept intact for a few days. On the other hand, however, Met-Enk is most susceptible to the attact by aminopeptidases in the extracts of nasal, rectal and vaginal mucosae, although dipeptidyl aminopeptidase and dipeptidyl carboxypeptidase also play some role in the degradation of Met-Enk. It is, therefore, suggested that, in order to deliver Met-Enk through the nasal, rectal and vaginal routes in the intact form, it is nesessary to control the activity of aminopeptidases in the mucosal cavity and tissue. To some degree, the activity of dipeptidyl peptidase and dipeptidyl carboxypeptidase has to be reduced too. Enzymatic degradation studies of peptide drugs in the extracts of various mucosae may provide some useful insights for studying the "first-pass" degradation of peptide drugs in the non-oral cavities. Overall, these findings would facilitate the design of a strategy to overcome the enzymatic barrier of the mucosal cavity and membrane to improve the efficiency of transmucosal peptide delivery.

The combination of inhibitors such as TM/AM /EDTA and TM/AM/TP which can inhibit simultaneously the enzymes responsible for enkephalin degradation in the nasal, rectal and vaginal mucosal extracts may provide useful tools for the potential delivery of Met-Enk through the mucosal membranes, and furthermore in the pharmacological and clinical studies on the mechanism of action, and behavioral effects of the enkephalinergic peptides, as combined inhibitors can fully prevent unwanted enzymatic degradation of Met-Enk over a period of 24 hrs.

Reference

1) Almenoff J., Wilk S. and Orlowski, M., Membrane bound pituitary metallo-endopeptidase: Apparent identity to enkephalinase. *Biochem. Biophys. Res. Commun.*, **102**, 206-214 (1981)
2) Altstein, M., Blumberg, S. and Vogel, Z., Phosphoryl-Leu-Phe: A potent inhibitor of the degradation of enkephalin by enkephalinase. *Eur. J. Pharmacol.*, **76**, 200-300 (1982)
3) Altstein, M., Mittman, S. and Vogel, Z., The effect of barbiturates on the degradation of enkephalin by brain enzymes. *Life Sci.*, **28**, 185-191 (1981)
4) Anik, S., McRae, G., Nerenberg, C., Woeden, A., Foreman, J., Yu, H.J., Kushinsky, S., Jones, R. and Vickery, B., Nasal absorption of nafarelin acetate, the decapeptide [D-Nal(2)(6)] LHRH, in rhesus monkeys I. *J. Pharm. Sci.*, **73**, 684-685 (1984)
5) Aoki, K., Kajiwara, M. and Oka, T., The role of bestin-sensitive antipeptidase, angiotensin converting enzyme and thiorphan-sensitive enkephalinase in the potency of enkephalins in the guinea-pig ileum. *Jpn. J. Pharmacol.*, **36**, 59-65 (1984)
6) Arima, H., Irie, T. and Uekama, K., Differences in the enhancing effects of water soluble β-cyclodextrins on the release of ethyl 4-biphenyl acetate, an anti-inflammatory agent from an oleaginous suppository base. *Int. J. Pharm.*, **57**, 107-115 (1989)
7) Arrequi, A., Lee, C.M., Emsom, P.C. and Iversen, L., Separation of human brain angiotensin-converting enzyme from enkephalin degrading activity. *Eur. J. Pharmacol.*, **59**, 141-144 (1979)
8) Barclay, R.K. and Phillips, M.A., Inhibition of the enzymatic degradation of leu-enkephalin by puromycin. *Biochem. Biophys. Res. Commun.*, **81**, 1119-1123 (1978)
9) Barclay, R.K. and Phillips, M.A., Inhibition of enkephalin-degrading aminopeptidase activity by certain peptides. *Biochem. Biophys. Res. Commun.*, **96**, 1732-1738 (1980)
10) Beaumont, A. and Hughes, J., Biology of opioid peptides. *Ann. Rev. Pharmacol. Toxicol.*, **19**, 245-267 (1979)
11) Belluzzi, J.D., Grant, N., Garsky, V., Sarantakis, D., Wise, C.V. and Stein, L., Analgesia induced in vivo by central administration of enkephalin in rat. *Nature*, **260**, 625-626 (1976)
12) Benuck, M., Berg, M.J. anbd Marks, N., A distinct peptidyl dipeptidase that degrades enkephalin: exceptionally high activity in rabbit kidney. *Life Sci.*, **28**, 2643-2650 (1981)
13) Benuck, M. and Marks, N., Characterization of a distinct membrane bound dipeptidyl carboxy-

peptidase inactivating enkephalin in brain. *Biochem. Bicphys. Res. Commun.*, **95**, 822-828 (1980)

14) Bogan, N., Brecha, N., Gall, C. and Karten, H.J., Distribution of enkephalin-like immunoreactivity in the rat main olfactory bulb. *Neurosci.*, **7**, 895-906 (1982)

15) Bower, J.B., Guest, K.P. and Morgan, B.A., Enkephalin: Synthesis of two pentapeptides isolated from porcine brain with receptor-mediated opiate agonist activiy. *J. C. S. Perkin I.* 2488-2492 (1976)

16) Carenzi, A., Biacin, F., Frigeni, V. and Della Bella, D., On the enzymatic degradation of enkephalins: Pharmacological implications. In: Advances in Biochemical Psychopharmacology, Vol 22, pp. 237-246, ed. E. Costa and M. Trabucchi, Raven Press, New York.

17) Carenzi, A., Frigeni, V., Reggiani, A. and Della Bella, D., Effect of inhibition of neuropeptidases on the pain threshold of mice and rats. *Neuropharmacol.*, **22**, 1315-1319 (1983b)

18) Chaillet, P., Marcais-Collado, M., Costentin, J., Yi, C.C., De La Baume, S. and Schwartz, J.C., Inhibition of enkephalin metabolism by, and antinociceptive activiy of, bestatin, an aminopeptidase inhibitor. *Eur. J. Pharmacol.*, **86**, 329-36 (1983)

19) Choi, H.K., Flynn, G.L. and Amidon, G.L., Transdermal delivery of bioactive peptides: The effect of n-decylmethyl sulfoxide and pH on enkephalin transport. *Pharm. Res.*, **6**(9) S-84, PD 830 (1989a)

20) Choi, H.K., Flynn, G.L. and Amidon, G.L., The transdermal delivery of bioactive peptides: The effect of inhibitors on enkephalin metabolism and transport, *Pharm. Res.*, **6**(9) S-148, PD 1086 (1989b)

21) Chun, I.K. and Chien, Y.W., Transmucosal delivery of methionine enkephalin I. Kinetics of degradation in buffered solution and metabolism in various mucosal extracts. *J. Pharm. Sci., Submitted* (1991a)

22) Chun, I.K. and Chien, Y.W., Unpublished data (1991b)

23) Cohen, M.L., Geary, L.E. and Eiley, K.S., Enkephalin degradation in the guinea-pig ileum: Effect of aminopeptidase inhibitors, puromycin and bestatin. *J. Pharmacol. Exp. Ther.*, **224**, 379-385 (1983)

24) Coletti-Previero, M.A., Mattras, H., Descamps, B. and Previero, A., Purification and substrate characterization of a human enkephalin-degrading aminopeptidase. *Biochem. Biophys. Acta.* **657**, 122-127 (1981)

25) Coletti-Previero,M.-A., Mattras, H., Descamps, B. and Previero, A., Purification and substrate characterization of a human enkephalin-degrading aminopeptidase. *Biochem. Biophys. Acta*, **657**, 122-127 (1981)

26) Coy, D.H., Kastin, A.J., Schally, A.V., Morin, O, Caron, N.G., Labrie, F., Walker, J.M.,Fertel, R., Bertson G.G. and Sandman, C.A., Synthesis and opioid activities of stereoisomers and other D-amino acid analogs of methionine-enkephalin. *Life Sci.*, **73**, 632-638 (1976)

27) Craves, F.P., Law, P.Y., Hunt, C.A. and Loh, H. H., The metabolic disposition of radiolabeled enkephalins in vitro and in situ. *J. Pharmacol. Exp. Ther.*, **206**, 492-506 (1978)

28) Craviso, G.L. and Musacchio. J., Inhibition of enkephalin degradation in the guinea pig ileum. *Life Sci.*, **23**, 2019-2030 (1978)

29) Cupo, A., Niel, J.P., Miolan, J.P., Jule, Y. and Jarry, Th., Enkephalins in the inferior mesenteric ganglion of the cat and in the area of the lower digestive tract innervated by this ganglion : Quantification by radio-immunoassay and characterization by high pressure liquid chromatography. *Neuropeptides*, **12**, 257-263 (1988)

30) De La Baume, S., Patey, G. and Schwartz, J.C., Subcellular distribution of enkephalin-dipeptidyl carboxypeptidase (enkephalinase) in rat brain. *Neurosci.*, **5**, 315-321 (1981)

31) Dodda-Kashi, S. and Lee, V.H.L., Enkephalin hydrolysis in homogenates of various absorptive mucosae of the albino rabbit : Similarities in rats and involvement of aminopeptidases. *Life Sci.*, **38**, 2019-2028 (1986a)

32) Dodda-Kashi, S. and Lee, V.H.L., Hydrolysis of enkephalins in homogenates of anterior segment tissues of the albino rabbit eye. *Invest. Opthalmol. Vis. Sci.*, **27**, 1300-1303 (1986b)

33) Dukler, S., Wilchek, M. and Lavie, D., Oixdation of tyrosine and its peptides with potassium nitrosodisulphonate. *Tetrahedron*, **27**, 607-614 (1971)

34) Ehrenpreis, S., Comaty, J.E. and Myles, S.B., Naloxone reversible analgesia produced by d-

phenylalanine in mice. In: Characteristics and Function of Opioids, pp. 417-418, ed., J.M. Van Ree and L. Terenius, Elsevier, Amsterdam, 19 78.

35) Erdos, E.G., Johnson, A.R. and Boyden, N.T., Hydrolysis of enkephalin by cultured human endothelial cells and by purified peptidyl dipeptidase. *Biochem. Pharm.*, **27**, 843-838 (1978)

36) Fournie-Zaluski, M.C., Perdrisot, R., Gacel, G., Swerts, J.P., Roques, B.P. and Schwartz, J.C., Inhibitory potency of various peptides on enkephalinase activity from mouse striatum. *Biochem. Biophys. Res. Commum.*, **91**, 130-135(1979)

37) Fournie-Zaluski, M.C., Soroca-Lucas, E., Waksman, G., Llorens, C., Schwartz, J.C. and Roques, B.P., Differential recognition of enkephalinase and angiotensin-converting enzyme by new carboxyalkyl inhibitors. *Life Sci.*, **31**, 2947-2954 (1982)

38) Fredrickson, R.C.A. and Geary, L.E., Endogenous opioid peptides: Review of physiological, pharmacological and clinical aspects. *Prog. Neurobiol.*, **19**, 19-69 (1982)

39) Geary, L.E., Kathryn, S.W., William, L.S. and Marlene, L.C., Degradation of exogenous enkephalin in the guinea-pig ileum : Relative importance of aminopeptidase, enkephalinase and angiotensin converting enzyme activity. *J. Pharmacol. Exp. Ther.*, **221**, 104-111 (1982)

40) Gillespie, M.N., Krechniak, J.W., Crooks, P.A., Altiere R.J. and Olson, J.W., Pulmonary metabolism of exogenous enkephalins in the isolated perfused rat lungs. *J. Pharmacol. Exp. Ther.*, **232**, 675-681 (1985)

41) Giros, B., Gros, C., Solhonne, B. and Schwartz, J.C., Characterization of aminopeptidases responsible for inactivating endogenous (met 5) enkephalin in brain slices using peptidase inhibitors and anti-aminopeptidase M antibodies. *Mol. Pharmacol.*, **29**, 281-287 (1986)

42) Gorenstein, C. and Snyder, S.H., Two distinct enkephalinases: Solubilization, partial purification and separation from angiotensin converting enzyme. *Life Sci.*, **25**, 2065-2070 (1979)

43) Gorenstein, C. and Snyder, S.H., Enkephalinases. *Proc. R. Soc. Lond.*, **B210**, 123-132 (1980a)

44) Gorenstein, C. and Snyder, S.H., Characterization of enkephalinases. In : Endogenous and Expogenous Opiate Agonists and Antagonists, pp. 345-348, ed., E.L. Way, Pergamon Press, New York (1980b)

45) Gorenstein, C., Tran, V.T., and Snyder, S.H., Brain peptidase with a unique neuronal localization: The histochemical distribution of dipeptidyl-aminopeptidase II. *J. Neurosci.*, **1**, 1096-1102 (1981)

46 Grynbaum, A., Kastin, A.J., Coy, D.H. and Marks, N., Breakdown of enkephalin and endorphin analogs brain extracts. *Brain Res. Bull.*, **2**, 479-484 (1977)

47) Hambrook, J.M., Morgan, B.A., Rance, M.J. and Smith, C.F.C., Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates. *Nature*, **262**, 782-783 (1976)

48) Hayashi, M., Monkey brain arylamidase II, Further characterization and studies on mode of hydrolysis of physiologically active peptides. *J. Biochem.* (Tokyo), **84**, 1363-1372 (1978)

49) Hayashi, M. and Oshima, K., Purification and characterization of arylamidase from monkey brain. *J. Biochem.* (Tokyo), **81**, 631-639 (1975)

50) Hazato, T., Shimamura, M., Komura, T. and Katayama, T., Partial purification of two distinct enkephalin-degrading aminopeptidases from human cerebrospinal fluid. *Biochem. Int.*, **10**, 813-819 (1985)

51) Hersh, L.B., Degradation of enkephalins: The search for an enkephalinase. *Mol. Cell. Biochem.*, **47**, 35-43 (1982)

52) Hersh, L.B. and McKelvy, J.F., An aminopeptidase from bovine brain which catalyzes the hydrolysis of enkephalin. *J. Neurochem.*, **36**, 171-178 (1981)

53) Hersh, L.B., Smith, T.E. and McKelvy, J.F., Cleavage of endorphins to des-tyr endorphins by homogeneous bovine brain aminopeptidase. *Nature*, **286**, 160-162 (1980)

54) Hill, R.G. and Pepper, C.M., The depression of thalamic nociceptive neurones by D-ala (2), D-leu-enkephalin. *Eur. J. Pharmcol.*, **47**, 223-225 (1978)

55) Hirai, S., Yashiki, T. and Mima, H., Effect of surfactants on the nasal absorption of insulin in rats. *Int. J. Pharm.*, **9**, 165-172 (1981)

56) Horstemke, B., Hamprecht, B. and Bauer, K., Heterogenous distribution of enkephalin-degrading peptidases between neuronal and glial cells. *Biochem. Biophys. Res. Commun.*, **115**, 423-

429 (1983)

57) Hudgin, R.L., Charleson, S.E., Zimmerman, M., Mumford, R. and Wood, P.L., Enkephalinase: Selective peptide inhibitors, *Life Sci.*, **29**, 2593-2601 (1982)

58) Hughes, J., Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. *Brain Res.*, **88**, 295-308 (1975a)

59) Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.T., Morgan, B.A. and Morries, H.R., Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature*, **158**, 295-308 (1975b)

60) Hui, K.S., Graf, L. and Lajtha, A., β−Endorphin inhibits met−enkephalin breakdown by a brain aminopeptidase: Structure-activity relationships. *Biochem. Biophys. Res. Commun.*, **105**, 1482-1487 (1982)

61) Hui, K.S., Hui, M., Banay-Schwartz, M., DeGuzman, T., Ling. N. and Lajtha, A., Enkephalin-containing polypeptides are potent inhibitors of enkephalin degradation. *Peptides*, **4**, 639-646 (1983)

62) Hui, K.S., Hui, M.P.P., Ling, N. and Lajtha, A., Proctolin: A potent inhibitor of aminoenkephalinase. *Life Sci.*, **36**, 2309-2315 (1985)

63) Hussain, A.A., Intranasal delivery of drugs. *Proceed. Intern. Sym. Control. Rel. Bioact. Mater.*, **16**, 16-17 (1985)

64) Hussain, A., Faraj, J., Aramaki, Y. and Truelove, J.E., Hydrolysis of leucine enkephalin in the nasal cavity of the rat-A possible factor in the low bioavailability of nasally administered peptides. *Biochem. Biophys. Res. Commun.*, **133**, 923-928 (1985)

65) Hussain, A., Kimura, R., Huang, C.-H. and Kashihara, T., Nasal absorption of naloxone and buprenorphine in rats. *Int J. Pharm.*, **21**, 233-237 (1984)

66) Hussain, A., Kimura, R., Huang, C.-H. and Mustafa, R., Nasal absorption of ergotamine tartrate in rats. *Int J. Pharm.*, **21**, 289-294 (1984)

67) Hussain, M.A., Shenvi, A.B., Rowe, S.M. and Shefter, E., The use of α-aminoboronic acid derivatives to stabilize peptide drugs during their intranasal absorption. *Pharm. Res.*, **6**, 186-189 (1989)

68) Ishimitsu, T. and Sakurai, H., Structure-ionization relationships of enkephalin and related fragments in aqueous solution. *Int. J. Pharm.*, **12**, 271-274 (1982)

69) Kerchner, G.A. and Geary, L.E., Studies on the transport of enkephalin-like oligopeptides in rat intestinal mucosa. *J. Pharmacol. Exp. Ther.*, **226**, 33-38 (1983)

70) Klostermyer, H. and Humbel, R.E., The chemistry and biochemistry of insulin, *Angew, Chem. Int. Ed. Engl.*, **5**, 807-822 (1966)

71) Knight, M. and Klee, W.A., The relationship between enkephalin degradation and opiate receptor occupancy. *J. Biol. Chem.*, **253**, 3843-3847 (1978)

72) Lee, V.H.L., Stratford, R.E., Carson, L.W. and Dodda-Kashi, S., Effect of ocular aminopeptidases on ocular absorption of enkephalins. *Invest. Ophthalmol. Vis. Sci.*, Suppl., **26**(3), 106 (1985b)

73) Llorens, C., Malfroy, B., Schwartz, J.C., Gacel, G., Roques, B.P., Roy, J., Morgat, J.L., Javoy-Agid, F. and Agid, Y., Enkephalin-dipeptidyl carboxypeptidase (enkephalinase) activity : Selective radioassay, properties, and regional distribution in human brain, *J. Neurochem.*, **39**, 1081-1089 (1982)

74) Llorens, C., and Schwartz, J.C., Enkephalinase activity in the rat peripheral organs, *Eur. J. Pharmacol.*, **69**, 113-116 (1981)

75) Malfroy, B., Swerts, J.P., Guyon, A., Roques, B.P. and Schwartz, J.C., High-affinity enkephalin-degrading peptidase in brain is increased after morphine. *Nature*, **276**, 523-526 (1978)

76) Malfroy, B., Swerts, J.P., Llorens, C. and Schwartz, J.C., Regional distribution of a high-affinity enkephalin-degrading peptidase (enkephalinase) and effects of lesions suggest localization in the vicinity of opiate receptors in brain, *Neurosci. Lett.*, **11**, 329-334 (1979)

77) Malin, P.H., Lake, J.R., Hamilton, R.F. and Skolnick, M.H., Augmented analgesic effects of enkephalinase inhibitors combined with transcanial electrostimulation. *life Sci.*, **44**, 1371-1376 (1989)

78) Marks, N., Glybaum A. and Neidle, A., On the degradation of enkephalins and endorphins by rat and mouse brain extracts, *Biochem. Biophys. Res. Commun.*, **74**, 1552-1559 (1977)

79) Martinez, J.L.Jr., Weinberger, S.B. and Schulteis, G., Enkephalins and learing and memory: A review of evidence for a site of action outside

the blood-brain barrier. *Behav. Neural Biol.*, **49**, 192-221 (1988)

80) Maze, M. and Gray, G.M., Intestinal brush border aminooligopeptidases: Cytosol precursors of the membrane enzyme. *Biochem.*, **19**, 2351-2358 (1980)

81) Meek, J.L., Yang, H.-Y.T. and Costa, E., Enkephalin catabolism in vitro and in vivo. *Neuropharmacol.*, **16**, 151-154 (1977)

82) Okada, H., Yamazaki, I., Ogawa, Y., Hirai, S., Yashiki, T. and Mima, H., Vaginal absorption of a potent luteinizing hormoner-eleasing hormone analog (leuprolide) in rats I: Absorption by various routes and absorption enhancement. *J. Pharm. Sci.*, **71**, 1367-1371 (1982)

83) Pardridge, W.M. and Meitus, L.J., Enkephalin and blood-brain barrier: studies of binding and degradation in isolated brain microvessels. *Endocrinology*, **109**, 138-143 (1981)

84) Patchett, A.A., Harries, E., Tristraum, E.W., Wyvratt, M.J., Wu, M.T., Taub, D., Peterson, E.R., Ikeler, T.J., Broeke, J.T., Payne, L.G., Ondeyka, D.L., Thorsett, E.D., Grenlee, W.J., Lohr, N.S., Hoffsommer, R.D., Joshua, H., Ruyle, W.V., Rothrock. J.W., Aster, S.D., Maycock, A.L., Robinson, F.M., Hirschmann, R., Sweet, C.S., Ulm, E. H., Gross, D.M., Vassil, T.C. and Stone, C.A., A new class of angiotensin-converting enzyme inhibitors. *Nature*, **288**, 280-283 (1980)

85) Pert, C.B., Pert, A., Chang, J.K. and Fong, B.T. W., [D-Ala (2)]-met-enkephalinamide: A potent, long-lasting synthetic pentapeptide analgesic. *Science*, **194**, 330-332 (1976)

86) Roemer, D. and Pless, J., Structure activity relationship of orally active enkephalin and analogues as analgesics. *Life Sci.*, **24**, 621-624 (1979)

87) Roques, B.P., Fournie-Zaluski, M.C., Soraca, E., Lecomte, J.M., Malfroy, B., Llorens, C. and Schwartz, J.C., The enkephalinase inhibitor thiorphan shows antinoniceptive activity in mice. *Nature*, **288**, 286-288 (1980)

88) Schachter, M., Enkephalins and endorphins. *Brit. J. Hosp. Med.*, **25**, 128-136 (1981)

89) Schnebli, H.P., Phillips, M.A. and Barclay, R.K., Isolation and characterization of an enkephalin-degrading amino-peptidase from rat brain. *Biochim. Biophys. Acta*, **569**, 89-98 (1979)

90) Schulteis, G., Weinberger, S.B. and Martinez, J. L.Jr., Plasma uptake and in vivo metabolism of [leu]-enkephalin following its intraperitoneal administration of rats. *Peptides*, **10**, 913-919 (1989)

91) Schwartz, J.C., Malfroy, B. and De La Baume, S., Biological inactivation of enkephalins and the role of enkephalin-dipeptidyl-carboxypeptidase (enkephalinase) as neuropeptidase. *Life Sci.*, **29**, 1715-1740 (1981)

92) Schwyzer, R., Gremlich, H.U., Gysin, B., Sargent, O.F. and Fringeli, H.P., Specific interactions between peptide hormones and artificial lipid membranes. *Pept.: Struct. Funct., Pro. Am. Pept. Symp.*, 8th, 657-664 8th (1983)

93) Shah, P.K. and Borchardt, R.T., Cultured human kerationcytes as an in vitro model to study peptide metabolism in human skin. *Pharm. Res.*, **6**, S-148, PD 1085 (1989)

94) Simmons, W.H. and Ritzman R.L., An inhibitors of opioid peptide degradation produces analgesia in mice. *Pharmc. Biochem. Behav.*, **13**, 715-718 (1980)

95) Snyder, S.H. and Childers, S.R., Opiate receptors and opiate peptides. *Ann. Rev. Neurosci.*, **2**, 35-65 (1979)

96) Stratford, R.E. and Lee, V.H.L., Aminopeptidase activity in albino rabbit extraocular tissues relative to small intestine. *J. Pharm. Sci.*, **74**, 731-734 (1985)

97) Stratford, R.E. Jr. and Lee, V.H.L., Aminopeptidase activity in homogenates of various absorptive mucosae in the albino rabbit: Implications in peptide delivery. *Int. J. Pharm.*, **30**, 73-82 (1986)

98) Stratford, R.E. Jr., Carson, L.W., Dodda-Kashi, S. and Lee, V.H.L., Systemic absorption of ocularly administered enkephalinamide and its inulin in the albino rabbit: Extent, pathways and vehicle effects. *J. Pharm. Sci.*, 77 838-842 (1988)

99) Su, K.S.E., Intransal delivery of peptides and proteins. *Pharm. Int.*, 8-11 (1986)

100) Su, K.S.E., Campanale, K.M., Mendelsohn, L.G., Kerchner, G.A. and Gries, C.L., Nasal delivery of polypeptides I: Nasal absorption of enkephalins in rats. *J. Pharm. Sci.*, **74**, 394-398 (1985)

101) Sullivan, S., Akil, H. and Barchas, J.D., *In vivo* degradation of enkephalin: Evidence for cleavage at the gly-gly bond. *Commun. Psychopharmac.*, **53**, 209-210 (1978)

102) Sullivan, S., Akil, H. and Barchase, J.D., Enke-

phalinase: Selective inhibition and partial characterization. *Peptides*, **1**, 31-35 (1980)

103) Swerts, J.P., Perdrisot, R., Patey, G., De La Baume, S. and Schwartz, J.C., Enkephalinase is distinct from brain angiotensin convreting enzyme. *Eur. J. Pharmacol.*, **57**, 279-281 (1979)

104) Traficante, L.J., Rotrosen, J., Siekierski, J., Tracer, H. and Gershon, S., Enkephalin inactivation by N-terminal tyrosine cleavage: Purification and partial characterization of a highly specific enzyme from human brain. *Life Sci.*, **26**, 1697-1706 (1980a)

105) Traficante, L.J., Rotrosen, J., Siekierski, J., Tracer, H. and Gershon, S., Antibiotics as inhibitors of enkephalin degradation. *Pharmc. Res. Commun.*, **10**, 575-580 (1980b)

106) Uekama, K. and Otagiri, M., Cyclodextrins in drug carrier systems. CRC Crit. Rev. in Ther. Drug Carrier Systems, **3**, 1-40 (1987)

107) Umezawa, H. and Aoyagi, T., Activities of proteinase inhibitors of microbial origin, In: Proteinase in Mammalian Cells and Tissues, ed., A.T. Barret, p. 653, Elsevier/North Holland Biomedical Press, New York, 1979.

108) Van Amsterdam, J.G.C., Van Buuren, K.J.H., Krielaart, M.-J., Zuiderveld, O.P. and Tijms, R.P. J.H., Effect of inhibitors of enkephalin degradation in the isolated guinea-pig ileum. *Life Sci.*, **43**, 1529-1536 (1988)

109) Wagner, G.W. and Dixon, J.E., Inhibitors of rat brain aminopeptidase. *J. Neurochem.*, **37**, 709-713 (1981)

110) Wagner, G.W., Tavianini, M.A., Herrmann, K.M. and Dixon, J.E., Purification and characterization of an enkephalin aminopeptidase from rat brain. *Biochem.*, **20**, 3884-3890 (1981)

111) Wilchek, M., Spande, T., Milne, G. and Witkop, B., The nonenzymdtic conversion of tyrosine into mono- and dihydroxyindoles. *Biochem.*, **7**, 1777-1786 (1968)

112) Vannier, C.H., Louvard, D., Maroux, S. and Desnuelle, P., Structural and topological homology between porcine intestinal and renal brush border aminopeptidase. *Biochem. Biophys. Acta.* **455**, 185-199 (1976)

113) Venturelli, F., Roscetti, G., Possenti, R., Vita, F. and Roda, L.G., Peripheral enkephalin hydrolysis in different animal species: A comparative study. *Neurochem. Res.*, **10**, 333-342 (1985)

114) Vogel, Z. and Altstein, M., The effect of puromycin on the biological activity of leu-enkephalin. *FEBS Lett.*, **98**, 44-48 (1979)

115) Vogel, Z. and Altstein, M., Inhibitors of enkephalinase and of aminopeptidase, In: Analg. Enkephalinases: Quo Vadis ? Symp., ed., R. Biogegrain, pp. 213-221 (1982)

116) Vogel, Z., Miron, T., Altstein, M. and Wilchek, M., Spontaneous inactivation of enkephalin. *Biochem. Biophys. Res. Commun.*, **85**, 226-233 (1978)

117) Weinberger, S.B. and Martinez, J.L. Jr., Characterization of hydrolysis of [leu]enkephalin and D-ala(2)-[L-leu]enkephalin in rat plasma. *J. Pharmacol. Exp. Ther.*, **247**, 129-135 (1988)

118) Zlokovic, B.V., Begley, D.J. and Chain-Eliash, D. G. Blood-brain barrier permeability to leucine-enkephalin, D-alanine^2-D-Leucine^5-enkephalin and their N-terminal amino acid (tyrosine). *Brain Res.*, **336**, 125-132 (1985)