Transmucosal Delivery of Methionine Enkephalin. I: Solution Stability and Kinetics of Degradation in Various Rabbit Mucosa Extracts

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Abstract □ To evaluate the feasibility of transmucosal delivery of methionine enkephalin (Tyr-Gly-Gly-Phe-Met; Met-Enk), it is important to first investigate its physicochemical and enzymatic stability. The kinetics of degradation of Met-Enk in aqueous solution was determined at pH 2.01–9.84 and 37–45 °C by high-performance liquid chromatography. The first-order rate constant (k) was calculated, and the log k–pH profile showed that Met-Enk is most stable at pH ~5.0. Various mucosae excised from rabbit were mounted on Valia-Chien permeation cells and exposed to isotonic phosphate buffer at physiologic pHs. Mucosal and serosal extracts were collected from the donor and receptor solutions, respectively. The degradation of Met-Enk in the extracts followed first-order kinetics, but no significant difference in the degradation rates was observed between mucosal and serosal extracts, regardless of the type of mucosa used. Degradation was most rapid in the extracts of rectal mucosa, followed by vaginal and nasal mucosae. The major metabolites were Des-Tyr-Met-Enk and Tyrosine (Tyr), indicating the enzymatic hydrolysis by aminopeptidases. However, the data also suggested that dipeptidyl peptidase and dipeptidyl carboxypeptidase could play some roles in the degradation of Met-Enk. The degradation pathways of Met-Enk were further explored by concomitantly determining the formation of smaller metabolites of primary hydrolytic fragments of Met-Enk in the mucosal extracts.

A growing number of bioactive peptides have recently become available in clinically useful quantity, and increasing research activities have been initiated to investigate their potential therapeutic applications. Successful application of peptides in therapy is, however, determined by the ability of the peptides to reach the site of action and maintain their pharmacological activities for a sufficient length of time. Because of their extremely low oral bioavailability, mostly due to their rapid and extensive breakdown in the gastrointestinal tract, these peptides have been delivered primarily by parenteral administration.[1] To overcome the limitations of oral delivery and the inconvenience of parenteral administration, much attention is now being directed to search for easily accessible absorptive mucosae, such as nasal,[2–5] rectal,[6,7] buccal,[8] and vaginal[9,10] mucosae, as alternative routes for the systemic delivery of peptides and proteins. Delivery through these absorptive mucosae has several advantages over the parenteral route, especially the advantage that no invasive means for drug administration is required with mucosae.

Methionine enkephalin (Met-Enk) and leucine enkephalin (Leu-Enk), the naturally occurring analgesic pentapeptides, are known to act as neurotransmitters or neuromodulators in pain transmission.[11,12] Their analgesic activity is, however, rather short in duration and remains purely transient even when administered by intracerebroventricular injection. This is because of their rapid inactivation by the enzymes present in the plasma[13,14] and brain membrane and homogenate.[15,16] Results obtained to date have suggested that several specific enzymes are responsible for the metabolism of enkephalin. There are some differences in the relative contribution of these enzymes to the degradation of enkephalins,[17–22] which vary from one animal species to another and even among different tissues or preparations within a given species.

Similarity has been noted in the enzyme activities among the mucosae in terms of the hydrolysis of enkephalins in the various mucosal tissue homogenates.[23–25] It has been reported that aminopeptidases play a very important role in the degradation of natural enkephalins, but dipeptidyl carboxypeptidase is the primary enzyme for the hydrolysis of [D-ala^2]met-enkephalinamide (YAGFM), which, by design, is more resistant to aminopeptidase activity in the homogenates of these non-oral mucosae.[23,24] In the rat, endopeptidase activity toward YAGFM was significantly higher in the intestinal than in the buccal homogenates, whereas in the hamster there is no significant difference in activities between buccal and intestinal homogenates.[26]

With respect to the systemic delivery of enkephalins, a recent study conducted in rats showed that mekephamid, an enkephalin analogue, is rapidly and efficiently absorbed from the nasal cavity.[27] However, it should be pointed out that extremely large doses of the peptide were administered. In the albino rabbit, the conjunctival mucosa was noted to play a role as important as the nasal mucosa in the systemic absorption of YAGFM. It was also found that in addition to the permeation barrier of conjunctival and nasal mucosae, metabolism of YAGFM during its permeation through the conjunctival and the nasal mucosae is another limitation in achieving its systemic delivery.[28] On the other hand, the nasal absorption of leucine enkephalin was examined in rats, by an in situ perfusion technique, and the results suggested that the overall disappearance of leucine enkephalin is due to its extensive hydrolysis in the nasal cavity.[29,30] However, there is no literature report on the permeation or absorption enhancement of natural enkephalins through the mucosal membrane. The stability of Met-Enk in aqueous solution has not been investigated, even though leucine enkephalin was found unstable in dilute solution upon prolonged storage.[14]

The objective of this series of investigations is to evaluate the feasibility of the transmucosal delivery of Met-Enk via various mucosal membranes. To achieve this objective, the physicochemical stability of Met-Enk was first evaluated in various buffered solutions. Then, the enzymatic degradation of Met-Enk was studied in the mucosal and serosal extracts of various mucosae of rabbit as an animal model.[31]

Experimental Section

Materials—Met-Enk (Tyr-Gly-Gly-Phe-Met, as acetate salt), des-tyr(1)-methionine enkephalin [as acetate salt, Des-(Tyr)-Met-Enk], Phe-Met, Tyr-Gly, Tyr-Gly-Gly, Gly-Phe-Met, Tyr, and Phe were obtained from Sigma Chemical Company (St. Louis, MO) and were used as received. Except purified water (18 MΩ), which was prepared from Nanopure System (Barnstead, Boston, MA) and used for the preparation of all the test solutions and acetonitrile (HPLC grade), all other reagents (Fisher Scientific, Springfield, NJ) were of analytical grade and used as received. Stock solutions of Met-Enk and its hydrolytic fragments were prepared fresh daily.

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High-Performance Liquid Chromatography (HPLC) Assay of Met-Enk and Its Metabolites—The HPLC system used consisted of a Waters model 590 pump, a WISP 710B autoinjector, and a Kratos Spectroflow 783 UV detector set at a wavelength of 214 nm. A stainless steel column (4.6 × 150 mm) packed with an octylsilane reversed-phase support (Ultrasphere C8, 5 μm, Beckman Instruments, Irvine, CA) in line with a guard column (Ultrasphere C8, 4.6 × 45 mm) was used for separation. A mixture (25:75, v/v) of acetonitrile and phosphate buffer (0.05 M, adjusted to pH 3.0 with phosphoric acid) containing 0.1% sodium heptanesulfonate was used as the mobile phase. The flow rate was set at 1.0 mL/min. Under these conditions, Met-Enk and its metabolites were well resolved (the chromatographic peaks detected will be reported later in the pertinent sections). Met-Enk and its metabolites in the samples were quantitated with the calibration curve established from a series of seven standard solutions. Peak area was computed with a Waters Data Module. The coefficient of variance for the HPLC assay was <0.5% for Met-Enk and its metabolites.

Solution Stability Studies—Met-Enk solutions (100 μg/mL) were prepared in various buffer solutions: KCl · HCl buffer (pH 2.01), McIlvaine buffer (pH 3.03 and 3.99), Sørensen phosphate buffer (pH 4.99, 6.06, 7.07, and 8.31), and Sørensen sodium borate buffer (pH 9.06 and 9.84). The resultant solutions were filtered through a membrane filter (Gelman Acrodisc CR PTFE, 0.45 μM). Each Met-Enk solution was stored in stability cabinets (set at 25, 37, and 45 °C) for 2–4 weeks. Samples (100 μL each) were periodically taken from each solution and then mixed well with 900 μL of 0.05 M phosphate buffer (pH 4.5). Met-Enk concentration in the sample was determined by assaying 20 μL of the resultant mixture by the HPLC procedure outlined above.

Dissection of Mucosa Specimens—Six female New Zealand White rabbits (Davidson Mill Breeding Labs, Jamesburg, NJ), weighing 2.5–3.5 kg each, were used. They were sacrificed by an intravenous injection of 1 mL of Beauthanasia-D Special solution (which contains pentobarbital sodium at 390 mg/mL and phenytoin sodium at 50 mg/mL, Schering Corp., Kenilworth, NJ) into a marginal ear vein. Mucosae were excised immediately within 30 min by scraping (with a no. 10 Surgical blade) in the following order: vaginal, rectal, and nasal mucosa. Vaginal and rectal mucosae were obtained by first cutting along the center cartilage, followed by excising the mucosal tissues. Rectum was cut off at 5 cm from the anus. Nasal mucosa was collected by making an incision from the tip of the nose all the way up with a surgical scissor and cutting the cartilage along the length of the lateral wall of the nose on each side of the nasal septum from the bottom with a heavy-duty scissor. This was followed by cutting the nasal septum across the top and the bottom with the surgical scissor and pulling off the nasal bone anteriorly to fully expose the nasal cavity. The nasal cartilage plate, which is covered with nasal membrane, was carefully removed by making three cuts from the top, bottom, and along side (while it was held at the top by small pointed forceps to avoid damage of nasal membrane). Nasal membranes were separated from the underlying cartilage by carefully pushing off cartilage, with the gloved thumb fingertip, from the lower end.

Preparation of Mucosal and Serosal Extracts—Mucosal and serosal extraction was carried out in Valia-Chien permeation cells.[32] Freshly excised specimens of various mucosae were mounted over the cell opening (0.64 cm^2) between the two half-cells, with the mucosal epithelium facing the donor half-cell. Both the donor and receptor half-cells were filled with 3.5 mL of isotonic phosphate buffer (0.07 M) at physiologic pH (pH 6.0 for vaginal mucosa, pH 7.2 for rectal mucosa, and pH 8.0 for nasal mucosa), and mucosal and serosal extracts were collected separately by exposing the mucosal and serosal surfaces of each mucosa, respectively, to the isotonic phosphate buffer at 37 °C for 8 h. The extraction procedure for every mucosa was repeated three times. The mucosal and serosal extracts, three each, from each mucosa were combined individually and stored in the refrigerator. All the extracts were used for enzymatic degradation studies of Met-Enk within 1 h of the final extraction.

Kinetic Studies of Met-Enk Degradation in Mucosal and Serosal Extracts—The kinetics of degradation of Met-Enk in the mucosal extracts were investigated by adding 50 μL of Met-Enk solution (147.3 μM) into 950 μL of each extract and incubating in a shaker bath at 37 °C for up to 360 min with constant shaking (60 rpm). At each of the predetermined time intervals, 100 μL were withdrawn from the incubation solution and added to 400 μL of pH 2.2 phosphate buffer to terminate the enzymatic reaction. Twenty microliters of the resultant solution were sampled and assayed for Met-Enk and its metabolites by the HPLC conditions outlined above. The assay of Met-Enk and its metabolites, Des(Tyr)-Met-Enk and Phe-Met, was conducted with the same mobile phase outlined earlier, and their chromatographic peaks were identified separately at retention times of 7.62, 5.30, and 4.85 min, respectively. On the other hand, chromatographic peaks of other metabolites, Tyr-Gly-Gly, Tyr-Gly, Tyr, and Phe, were separated with a 12:88 (v/v) mixture of acetonitrile and 0.05 M phosphate buffer (pH 3.0), which also contained 0.1% sodium heptanesulfonate; the peaks of the metabolites were detected separately at retention times of 4.25, 4.68, 2.99, and 5.46 min, respectively. The concentrations of Met-Enk and its metabolites were quantitated with the calibration curves established from the reference standards for each compound.

Kinetic Studies of Degradation of Major Hydrolytic Fragments of Met-Enk in Mucosal Extracts—To investigate the mechanisms and pathways involved in the enzymatic degradation of Met-Enk, the degradation kinetics of primary hydrolytic fragments of Met-Enk was also studied in the same mucosal extracts. First, 50 μL each of Des(Tyr)-Met-Enk, Phe-Met, Tyr-Gly, and Tyr-Gly-Gly solutions (147.3 μM each) was added to 950 μL of each mucosal extract and incubated in the water bath at 37 °C with constant shaking (60 rpm). Samples were taken at predetermined time intervals and assayed by HPLC as outlined above.

Results and Discussion

Solution Stability of Met-Enk—The physicochemical stability of Met-Enk was evaluated by studying the kinetics of its degradation in various buffered solutions as a function of solution pH, environmental temperature, and drug concentration. In the early stage of incubation at 37 °C, none of the solutions showed any precipitation, aggregation, or surface adsorption. The degradation profile of Met-Enk followed first-order kinetics. The first-order degradation rate was temperature dependent and increased with increasing environmental temperature. The temperature-dependent degradation occurred in all the buffer solutions studied. Results (Table I) indicate that the degradation of Met-Enk varies not only with temperature but also with solution pH. A linear Arrhenius plot was obtained for the degradation of Met-Enk, indicating that an activation energy of 8.97 kcal/mol is needed for the degradation at pH 9.84. The variation in Met-Enk concentration had only a slight effect on degradation rate (Figure 1); the higher the initial concentration, the lower the degradation rate.

Studies of the effect of pH on the solution stability of Met-Enk (Figure 2) indicate that Met-Enk has maximum solution stability at pH ~5. The effect of pH on the solution stability of Met-Enk in the pH range above 5 is much greater than that in

Table I—Effect of pH and Temperature on the Apparent First-Order Rate Constants and Half-lives for the Degradation of Met-Enk in Aqueous Solution

pH	Rate Constant × 10^3 (day^{-1}) at:			Half-life (days) at:		
	25 °C	37 °C	45 °C	25 °C	37 °C	45 °C
2.01	ND[a]	5.99	7.11	ND	115.7	97.5
4.99	ND	5.10	5.57	ND	135.9	124.4
7.07	ND	5.92	13.48	ND	117.1	51.4
9.84	8.51	13.19	22.60	81.4	52.5	30.7

[a] ND, Not determined.

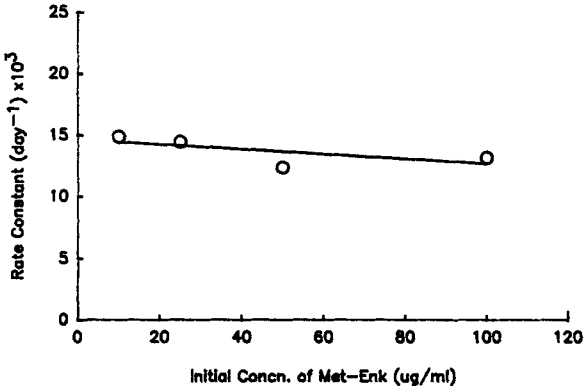

Figure 1—Effect of the initial concentration of Met-Enk on the rate constant for Met-Enk degradation at pH 9.84. Key: (○) 25 °C; (●) 37 °C; (△) 45 °C.

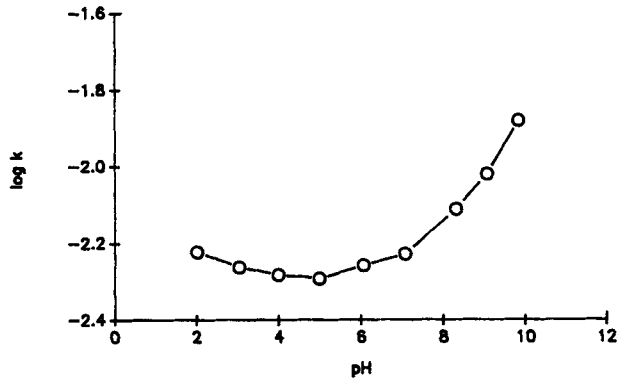

Figure 2—Log k–pH profile for the stability of Met-Enk at 37 °C.

the range below pH 5. However, the log k–pH profile (k is rate constant, Figure 2) did not yield a slope value of unity in either the acidic or alkaline regions studied, suggesting the complexity of the mechanism involved in the degradation of Met-Enk, an ionizable molecule.

In the neutral pH region, an unusual phenomenon was noted in the preliminary studies using a nonsterile buffered solution; that is, ~35% of Met-Enk was degraded within 3 days at pH 7.0 and 37 °C, with the formation of some turbidity. This observation suggests the growth of some microorganisms in the nonsterile solution at neutral pHs and their possible involvement in the degradation of Met-Enk. These findings suggest that the aqueous solution of Met-Enk prepared for stability and/or permeation studies must be prevented from any microbial contamination.

The Met-Enk molecule has three ionizable groups: one aromatic hydroxyl group [pK_a (K_a is the dissociation constant) = 9.89], the amino group (pK_a = 7.77) of the tyrosine residue at the N-terminal, and the carboxylic acid group (pK_a = 3.68) at the C-terminal of the peptide.[33] Therefore, Met-Enk could exist in various ionic forms, depending on solution pH, and all of them may have different propensities to degradation. Following incubation at 37 °C for 1 month, several additional peaks, which could be the degradation products of Met-Enk, were detected in the HPLC chromatograms. For example, chromatographic peaks were detected with retention times of 8.70 min for Met-Enk solution at pH 3.03, of 3.95 and 4.76 min for pH 4.99 and 6.06 solutions, respectively, of 4.77 min for pH 7.07 solutions, and of 8.35 min for pH 9.06 and 9.84 solutions, whereas the Met-Enk peak appeared at 6.67 min. On the other hand, Met-Enk solution stored at 45 °C for 1 month showed more degradation peaks with retention times at 4.78, 5.19, 8.00, and 8.35 min for pH 7.07. Furthermore, at pH 11.0, four other peaks were observed at retention times of 3.86, 4.59, 5.23, and 8.09 min within 3 days. The detection of peaks with retention times longer than that of Met-Enk (6.67 min) suggests the formation of degradation products that are more hydrophobic than Met-Enk, possibly due to a reduction of the charges on the molecule.

Similarly, it was reported that upon prolonged storage at 4 °C, Leu-Enk in diluted solution increased in its R_f value. The phenomenon was explained as caused by the conversion of tyrosine at the N-terminal into an indole derivative, a mechanism that is apparently similar to the chemical and enzymatic oxidation of tyrosine derivatives.[14] Because tyrosine is positioned at the N-terminal of Met-Enk molecule, cyclization is implicated as one of the degradation mechanisms.[34] Consequently, the appearance of other peaks is considered to result from the cleavage, cyclization,[14] and oxidation,[35,36] of Met-Enk, and any further change of its degradation products. The observation of different peaks at different pHs and temperatures suggests that the mechanism for the degradation of Met-Enk is a very complex one. The total elucidation of the complex degradation mechanisms is expected to be a rather difficult task.

The data in Figure 2 and Table I suggest that Met-Enk has sufficient physicochemical stability in the buffered solutions with physiologic pHs of 6–8 prepared for enzymatic degradation studies of Met-Enk in the following sections.

Degradation of Met-Enk in Various Mucosa Extracts—Whereas the metabolism of Met-Enk in the brain[15,16,18–20] and several other tissues has been well characterized, there are very few reports on the metabolism of Met-Enk at nonoral mucosal sites.[23] To better simulate the actual mucosal permeation in live animal and to determine the amount of Met-Enk degraded enzymatically before and after permeation, we chose to use the mucosal and serosal extracts, not the homogenate, of the mucosae. The extent and pathway of the degradation of Met-Enk in the extracts of various mucosae were investigated by monitoring the disappearance of Met-Enk and the appearance of its metabolites over an incubation period of 6 h.

The appearance profiles of Met-Enk in the mucosal and serosal extracts of nasal, rectal, and vaginal mucosae of New Zealand White rabbits apparently followed a first-order kinetics. For comparison, control experiments were also conducted in the isotonic phosphate buffer used in the extraction of enzymes from each of the mucosae, and the results confirmed that Met-Enk is physicochemically stable. The rate constants for the enzymatic degradation of Met-Enk (Table II) 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. These results suggest a considerable variation in peptidase activities among the extracts from different mucosae. On the other hand, there is no significant difference in degradation rates between the extracts from the mucosal and the serosal surfaces, regardless of the mucosa extracted. Whereas the hydrolysis of Met-Enk in the homogenates of nasal, rectal, and vaginal mucosae was reported to have small difference in their rates, the hydrolysis in the extracts of mucosae in the present investigation show more distinct differences than that in the homogenates in the hydrolytic rates among the nasal, rectal, and vaginal mucosae (Table II).

The concentration profiles of Met-Enk and its hydrolytic fragments upon incubation with various extracts (Figure 3) indicate that Met-Enk is degraded extensively in all the extracts, with the formation of several hydrolytic fragments. The data suggest that the Tyr-Gly bond is cleaved rapidly and extensively to yield Tyr, the main hydrolytic fragment, with high concentrations found in all the mucosal extracts. A much lower concentration of Des(Tyr)-Met-Enk, which reached the peak level within 30–60 min and then declined due to its further degradation to smaller fragments, was formed. The amount of Tyr-Gly fragment yielded by cleavage at the Gly-Gly bond is

Table II—Apparent First-Order Rate Constants (k) and Half-lives ($t_{1/2}$) for the Degradation of Met-Enk in Extracts of Various Absorptive Mucosae

Mucosa[a]	Extract[b]	$k \times 10^3$, (± SE) min^{-1}	$t_{1/2}$ (± SE), min
Nasal	Mucosal	9.35 (± 2.57)	74.09 (± 20.36)
	Serosal	10.86 (± 3.71)	63.84 (± 21.83)
Rectal	Mucosal	49.15 (± 9.25)	14.10 (± 2.65)
	Serosal	67.19 (± 14.7)	10.31 (± 2.26)
Vaginal	Mucosal	15.07 (± 4.18)	45.98 (± 12.75)
	Serosal	18.11 (± 5.43)	38.27 (± 11.48)

[a] Excised freshly from six New Zealand White rabbits just prior to extraction. [b] Exposed mucosal and serosal surfaces separately to the isotonic phosphate buffer solution adjusted to the physiological pHs for each mucosa (nasal, 8.0; rectal, 7.2; vaginal, 6.0).

relatively small. It should be noted, however, that in both the mucosal and serosal extracts of nasal mucosa, a relatively large amount of the Tyr-Gly fragment was formed as compared with that in the rectal and vaginal extracts. In particular, a considerable amount of Tyr-Gly-Gly was detected only in the nasal extracts, resulting apparently from the cleavage of Met-Enk at the Gly-Phe bond.

The formation of Tyr, Tyr-Gly, and Tyr-Gly-Gly fragments in the extracts of absorptive mucosae studied suggests the presence of, respectively, aminopeptidases, dipeptidyl peptidase (enkephalinase B), and dipeptidyl carboxypeptidase (enkephalinase A).[19,23,37] The observed extensive formation of Tyr indicates that aminopeptidases play a principal role in the hydrolytic degradation of Met-Enk. Similar observations were also

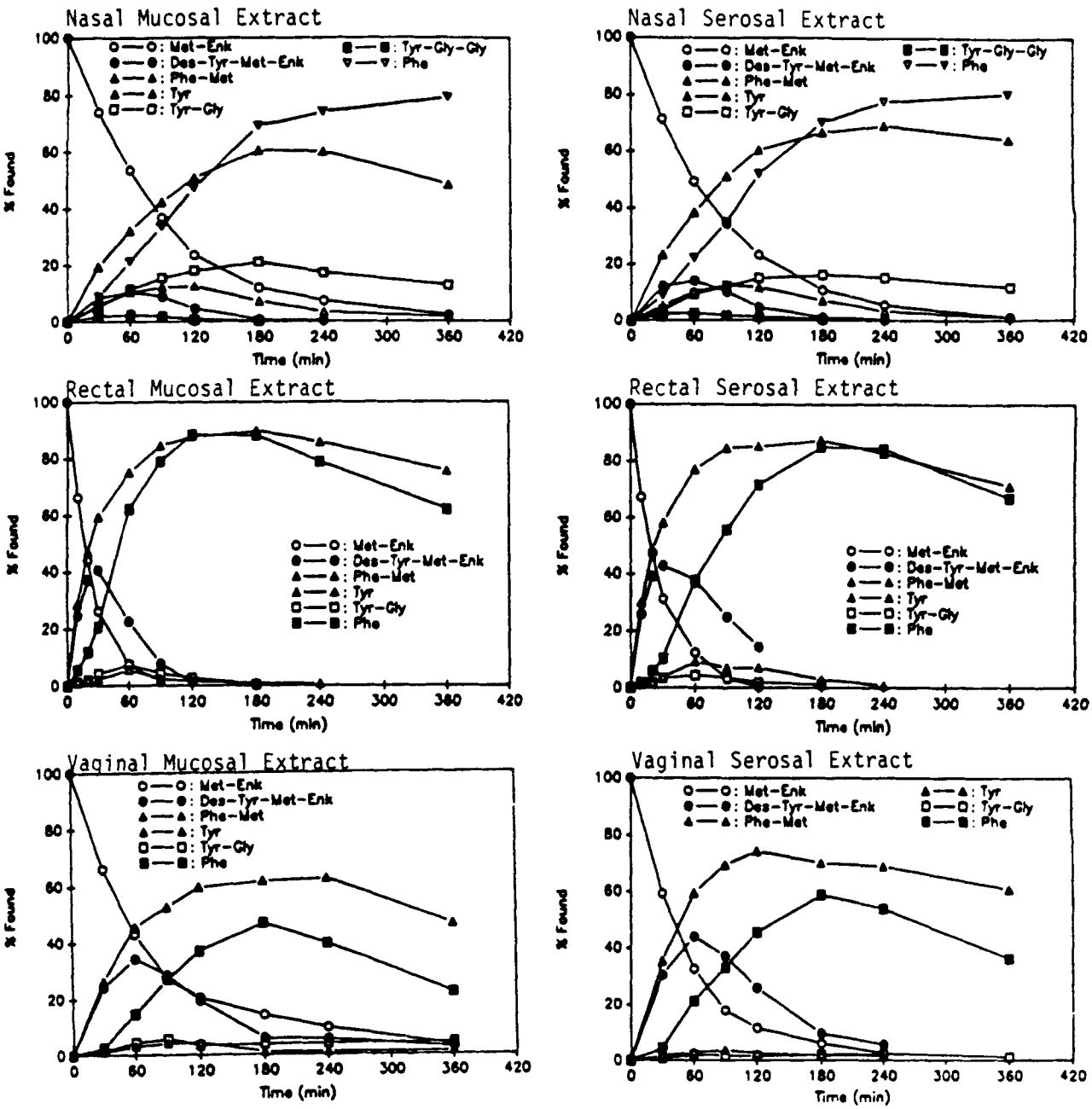

Figure 3—Time course for the disappearance of Met-Enk and the appearance of its metabolites in the extracts of various mucosae of New Zealand White rabbits at 37 °C. Each data point indicates the mean of the data obtained from three rabbits.

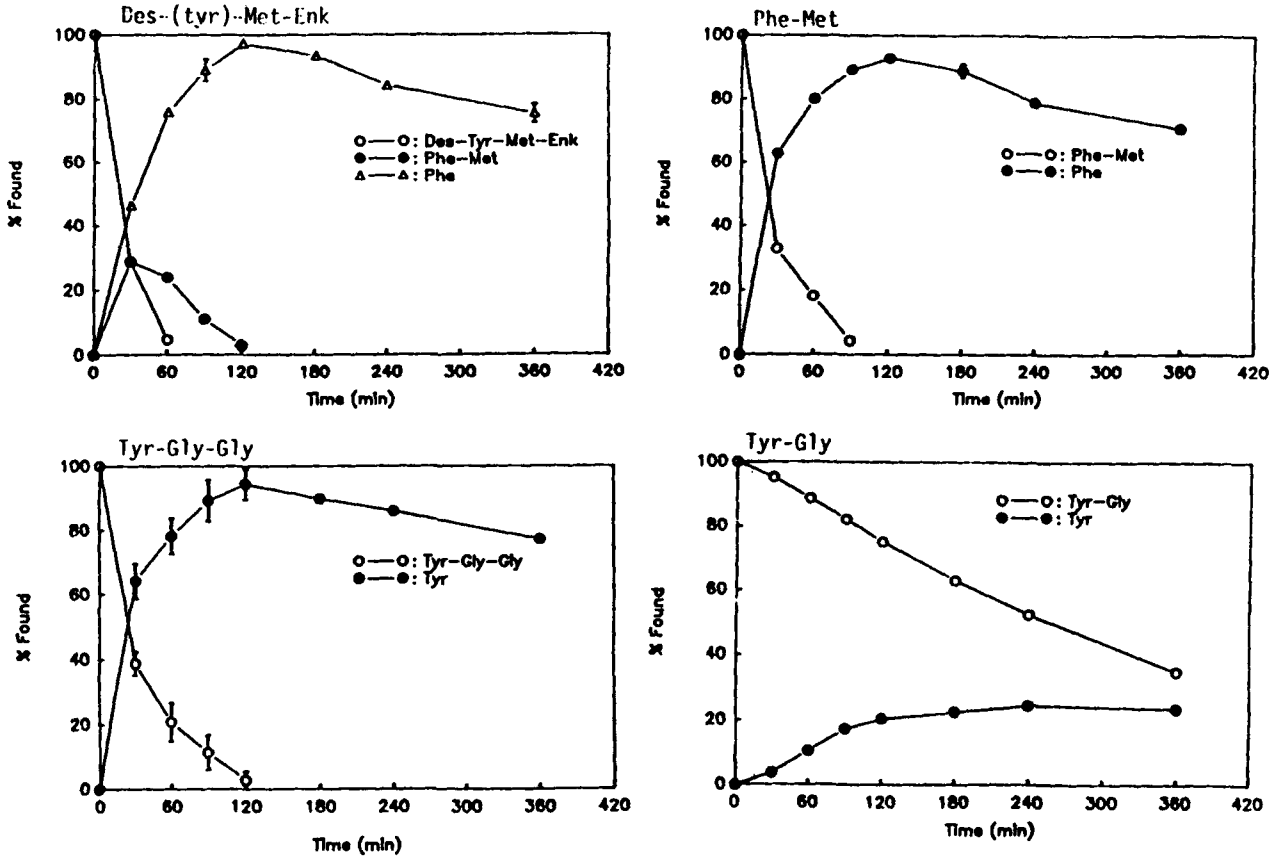

Figure 4—Time course for the disappearance of the various primary metabolic fragments of Met-Enk and the appearance of smaller metabolites in the rabbit nasal mucosa extracts at 37 °C.

reported in brain membrane,[16] ileal homogenates,[21] eye anterior segment tissue homogenates,[24] and nonoral mucosal homogenates.[23] This observation suggests that this family of enzymes have to be inhibited to stabilize Met-Enk molecules for mucosal delivery. This does not imply that the inhibition of aminopeptidases alone would totally protect Met-Enk molecule, because the other enzymes, enkephalinase A and B, could also degrade Met-Enk at the Gly-Phe and Gly-Gly bond, respectively, beyond the Tyr-Gly bond that is cleaved by aminopeptidases.

One important difference among various mucosal extracts is the formation of Tyr-Gly in the nasal, rectal, and vaginal extracts, after incubation for 90 min, which falls in the range [mean ± standard error (SE)] of 15.4 (± 2.2), 4.3 (± 1.8), and 3.4 (± 1.5)%, respectively, whereas the formation of Tyr is in the range of 42.4 (± 11.5), 84.4 (± 7.9), and 52.8 (± 12.5)%, respectively. Similar results were also obtained in the serosal extracts of these mucosae. These results imply differences in the relative contribution of aminopeptidases and enkephalinase B to the overall degradation of Met-Enk depending on the type of mucosa. The data suggest that nasal mucosa could have the highest enkephalinase B activity, followed by vaginal and then rectal mucosae. In addition, nasal extracts show enkephalinase A activity (with the formation of Tyr-Gly-Gly), even though the activity is lower [2.4 (± 0.3)% at 60 min]. Although Tyr-Gly-Gly has been reportedly formed at the same rate in the homogenates of rabbit nasal, rectal, and vaginal mucosae,[23] the formation of Tyr-Gly-Gly has not been observed in rectal and vaginal extracts. This difference may be attributed to the existence of weak enkephalinase A activity and/or further rapid disappearance of Tyr-Gly-Gly fragment soon after its formation.

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

To explore the pathways for the enzymatic degradation of Met-Enk, the degradation of primary metabolic fragments (i.e., Des-(Tyr)-Met-Enk, Tyr-Gly-Gly, Tyr-Gly, and Phe-Met) was also studied in the extracts of these absorptive mucosae. One typical disappearance profile of primary metabolic fragments and the appearance of their smaller metabolites in the nasal extracts are shown in Figure 4. The first-order rate constants of their disappearance in the mucosal extracts are summarized in Table III. The data (Figure 4) suggest that the tetrapeptide fragment Des(Tyr)-Met-Enk undergoes further hydrolysis (it is believed to be cleaved at the Gly-Phe bond by the action of dipeptidyl peptidase with the formation of two dipeptide fragments) that is similar to the hydrolysis observed in rat brain cortical synaptosomes[38] and in albino rabbit mucosal homogenates.[23] One of these fragments was Phe-Met, which probably was further cleaved rapidly to yield Phe as one of the products because there was no evidence of Phe-Met accumulation. In fact, the Phe-Met fragment was rapidly degraded with the formation of Phe as one of the products in the extracts of all the mucosae studied in the order rectal > nasal >> vaginal extracts. On the other hand, the Tyr-Gly fragment was degraded to form Tyr, but at a very slow rate when compared with other fragments studied. These results suggest that the Tyr-Gly fragment should be detectable in all the extracts. The Tyr-Gly-Gly fragment formed in the nasal extract was rapidly hydrolyzed after 60 min and could not be detected after 120 min (Figure 3). This is further substantiated by the relatively high rate constants of degradation obtained (Table III). The Tyr-Gly-Gly fragment was cleaved to yield Tyr as the major degradation product, but not

Table III—First-Order Rate Constants for the Degradation of Primary Metabolic Fragments of Met-Enk in the Extracts of Various Rabbit Mucosae at 37 °C

Mucosal Extract	Rate Constant (± SD), min^{-1}			
	Des-(Tyr)-Met-Enk	Phe-Met	Tyr-Gly-Gly	Tyr-Gly
Nasal	51.01 (±1.62)	33.29 (±1.23)	20.11 (±3.76)	3.05 (±0.17)
Rectal	29.20 (±0.46)	36.07 (±3.71)	41.55 (±5.11)	2.45 (±0.06)
Vaginal	5.68 (±0.02)	13.14 (±0.18)	4.75 (±0.83)	2.34 (±0.09)

[a] Data were obtained by incubating individual fragment, in triplicate, in the combined solution of the mucosal and serosal extracts.

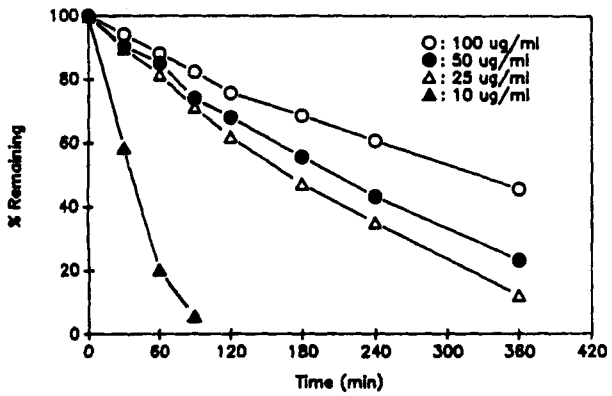

Figure 5—Effect of the initial Met-Enk concentration on its degradation in the nasal mucosa extracts at 37 °C.

to Tyr-Gly fragment. The results suggest that Tyr-Gly-Gly is cleaved at the Tyr-Gly peptide bond.

In Figure 5, the effect of initial Met-Enk concentration on the time course of its degradation in the nasal mucosa extracts is demonstrated. It is interesting to note that an increase in the concentration of Met-Enk markedly retarded its degradation, indicating that saturation of the enzymes could occur. This means that the degradation of Met-Enk can be minimized when it is administered in high concentration.

In summary, Met-Enk is unstable physicochemically in aqueous solution upon prolonged storage, but it can stay intact for a few days. On the other hand, Met-Enk is most susceptible to the hydrolytic degradation by aminopeptidases in the extracts of nasal, rectal, and vaginal mucosae, even though dipeptidyl peptidase and dipeptidyl carboxypeptidase could also play some role in its degradation. It is thus suggested that to deliver Met-Enk through the nasal, rectal, and vaginal routes in the intact form, it is critically important to control the activity of aminopeptidases in the mucosal cavity and tissue. To some degree, the activities of dipeptidyl peptidase and dipeptidyl carboxypeptidase must also be reduced. Enzymatic degradation studies of peptide drugs in the extracts of various mucosae, as conducted in this investigation, may provide some useful insights in studying the "first-pass" degradation of peptide drugs in the nonoral absorptive mucosae. Overall, these findings would facilitate the design of a viable strategy to overcome the enzymatic barrier in the mucosal cavity and membrane to improve the efficiency of transmucosal peptide delivery.

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