***In Vitro* Skin Penetration and Degradation of Peptides and Their Analysis Using a Kinetic Model**

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The main purpose of this study was to estimate the net percutaneous absorption of physiologically active peptides *in vitro*. The degradation of two peptides, Leu-enkephalin (Enk) and Tyr-Pro-Leu-Gly amide (TPLG), during skin penetration and on the dermal side following penetration, and the prevention of degradation by some protease inhibitors, were investigated using rat skin *in vitro*. In addition, these permeation and degradation data were analyzed using a kinetic model. These peptides were rapidly degraded in the receptor fluid of a Franz diffusion cell (rate constant: 0.977 h^{-1} for Enk and 0.250 h^{-1} for TPLG). The addition of phenylmethylsulfonyl fluoride (PMSF) and phenanthroline and the pretreatment of skin with these inhibitors prevented almost completely any degradation in the receptor fluid and skin, respectively. The pretreatment of skin with PMSF and phenanthroline had no effect on the penetration of dextran (1000 Da). The degradation rate constant during skin penetration, calculated from the difference in the penetration rate constants *via* pretreated and untreated skins, was also high (0.037 h^{-1} for Enk and 0.050 h^{-1} for TPLG). A kinetic model including an input rate (zero-order), the permeation rate across the viable skin (first-order) and the degradation rate in skin (first-order) was sufficient to describe the apparent steady-state flux of the peptides through skin. We have, thus, established a method for measuring the true flux of peptides across skin *in vitro* and a kinetic model which simply describes the skin penetration of peptides.

Key words peptide; skin penetration; degradation; inhibition; kinetic model analysis

Recent advances in biotechnology have given rise to large numbers of bioactive peptides, which may have therapeutic potential. However, peptide-based drugs cannot be effectively delivered by oral administration because of their enzymatic degradation in the gastrointestinal tract and the intrinsically low permeability of the gastrointestinal mucosal membrane to peptides. Various parenteral options, such as rectal, pulmonary, nasal and transdermal routes, have therefore been studied as alternative methods for the administration of peptides and proteins.

The transdermal delivery of peptides offers an attractive possibility for several reasons. The main advantages of the transdermal route are the low proteolytic activity of the skin,¹⁾ and the possibility of continuously delivering drugs with short half-lives and prolonged effects by continuous absorption. Recent studies have demonstrated the ability of some peptides to cross the skin.²⁻⁸⁾ However, the amount which can be administered transdermally is quite low and, to facilitate the transdermal transport of peptides, penetration enhancers have been used in many studies. We have recently found that peptides were quickly degraded on the dermal side of the skin in an *in vitro* study.⁹⁾ Thus, it is predicted that the amount actually penetrating is much more than that reported by many workers. A permeant diffuses through the stratum corneum, viable epidermis, and dermis and may be metabolized in several skin layers. Viable epidermis has been thought to be a major site of metabolism,^{10,11)} and it is very important to exactly measure the degradation and penetration of peptides through the skin.

The present study was designed to evaluate the degradation of two peptides, Leu-enkephalin (Enk) and Tyr-Pro-Leu-Gly amide (TPLG), during skin penetration and on the dermal side following penetration separately, using specific protease inhibitors, and to determine the net flux of peptides in an *in vitro* study. In addition, this study focused on the ki-

netics of degradation of the peptides in the skin and on the dermal side, and the permeation data were analyzed using a simple kinetic model.

MATERIALS AND METHODS

Materials Enk and TPLG ([Tyr⁶]-oxytocin fragment) were obtained from Peptide Institute Inc. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively. DL-5-(4-Hydroxyphenyl)-5-phenylhydantoin (OH-DPH) and caffeine, internal standards for high performance liquid chromatography (HPLC), were purchased from Nacalai Tesque Co. (Kyoto, Japan). *n*-Octyl- β -D-thioglucoside (OTG, Wako Pure Chemical Industries, Osaka, Japan), and sodium deoxycholate (Tokyo Kasei Organic Industries, Tokyo, Japan) were used in this study. Gabexate mesylate (gabexate), phenylmethylsulfonyl fluoride (PMSF) and 1,10-phenanthroline were purchased from Ono Pharmaceutical Industries (Osaka, Japan), Sigma Chemical Co. and Kanto Chemical Co. (Tokyo, Japan), respectively. Dextran (mean molecular weight, 1000 Da) was a generous gift from Meito Sangyo Company (Nagoya, Japan). All other chemicals and solvents were of reagent grade or HPLC quality.

Animals Male Wistar rats, weighing 220–250 g, were used throughout this experiment. A group of 4 to 9 rats was randomly selected from the population and these rats had free access to MF diet (Oriental Yeast, Tokyo, Japan) for 3–4 d prior to the experiment.

Preparation of Transdermal Formulation A liquid formulation was prepared by dissolving a peptide in a mixture of water and propylene glycol, followed by adding the enhancer (deoxycholate and OTG) solution. Details of the formulations are listed in Table 1. These enhancers were used for insuring the rapid and complete penetration of peptides. TPLG was selected as a comparatively stable peptide, from

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Table 1. Liquid Formulation of Peptides

Enk or TPLG	100 (mg)
Propylene glycol	20.0 (g)
Sod. deoxycholate	1.0 (g)
<i>n</i> -Octyl- β -thioglucoiside	1.5 (g)
Gentamicin sol.	1.0 (ml)
Dist. water ad.	100.0 (g)

an enzymatic point of view.

In Vitro Percutaneous Penetration 1) Inhibition of Degradation in Receptor Fluid: On the day before the experiment, the abdominal hair of the rats was removed with electric clippers and an electric razor. On the next day, the rats were sacrificed by decapitation and pieces (3×3 cm area) of full-thickness abdominal skin were excised. The adherent fat and other visceral debris were removed from the undersurface. The dermal side of the skin was soaked in buffer solution (0.85% NaCl–10 mM phosphate buffer, pH 7.4) for 12 h at 5 °C to equilibrate the skin.¹²⁾ Then, the skin was mounted in a Franz diffusion cell (reservoir volume 10.5 ml, 1.6 cm i.d. O-ring flange). In a degradation prevention experiment, gabexate (10 mM) and phenanthroline (1 mM in ethanol) or PMSF (5 mM) and phenanthroline (1 mM in ethanol), the concentrations being the final ones, were mixed with receptor fluid (0.85% NaCl 10 mM phosphate buffer–15% ethanol) containing the peptide (20 μ g/ml). Ethanol (15%) was used to dissolve phenanthroline. The ethanol concentration had no effect on the penetration of peptides, as reported by Ghanem *et al.*, who showed that ethanol at low levels (<25%) had little or no effect on the pore pathway.¹³⁾ The concentrations of inhibitors and the treatment time were determined based on our previous data.⁹⁾ Gentamicin sulfate solution (10 mg/ml) was added to the receptor fluid at a ratio of 1 : 100. The diffusion cell was thermoregulated by a water jacket at 37 °C. Aliquots (100 μ l) of the receptor fluid were withdrawn at intervals for 26 h and the phenanthroline in the sample was extracted with chloroform (1.0 ml) to avoid any interference with the determination, followed by centrifugation. The supernatant was stored frozen until the time of assay.

2) Inhibition of Degradation in Skin: The dermal side of the skin, prepared by the same method as described above, was soaked in buffer or 15% ethanol solution containing PMSF (5 mM) and phenanthroline (1 mM) for 12 h at 10 °C to inhibit the enzymes. Then, 0.5 ml liquid formulation was applied to the stratum corneum surface of the skin. PMSF (5 mM) and phenanthroline (1 mM) was mixed with receptor fluid containing 15% ethanol and gentamicin solution. The diffusion cell was incubated at 37 °C, and aliquots of receptor fluid were withdrawn at intervals for 26 h. In the experiment on dextran penetration, 0.5% dextran and 1% sodium deoxycholate dissolved in water (1.0 ml) (OTG was not used because it interfered with the assay) were used as the donor solution, and the dermal side of the skin was pretreated with gabexate mesylate (10 mM) and phenanthroline (1 mM) or PMSF (5 mM) and phenanthroline (1 mM) dissolved in 15% ethanol-buffer solution for 12 h at 10 °C.

Inhibition of Degradation in Skin Homogenate Full-thickness abdominal skin was prepared as described above. The skin (3×3 cm area) was homogenized in 9.0 ml ice-cold 0.85% NaCl–10 mM phosphate buffer, pH 7.4, for 2 min

using a microhomogenizer (Physcotron NS-10, Nichion, Tokyo, Japan). After 2.5-fold dilution of the homogenate, Enk solution (40 μ g/ml, 1.0 ml) containing gentamicin solution and 30% ethanol solution (2.0 ml) containing 10 mM PMSF and 2 mM phenanthroline were added to the diluted homogenate (1.0 ml). The mixture was incubated at 37 °C for 12 h. Aliquots (150 μ l) were withdrawn periodically, and the phenanthroline in sample was extracted with chloroform. The supernatant was stored frozen until the time of the assay.

Determination of Peptide and Dextran Enk and TPLG in the sample solution were determined by HPLC. Phenanthroline in the sample was removed by extraction with chloroform (1.0 ml) and an aliquot of sample (100 μ l) was added to 50 μ l internal standard solution (OH-DPH, 10 μ g/ml for Enk; caffeine, 10 μ g/ml for TPLG). Following centrifugation, the supernatant was passed through a membrane filter (0.45 μ m, Chromatodisc 4A, Kurabo, Osaka). The filtrate (10–50 μ l) was injected onto a reversed-phase Inertsil ODS column (4.6×150 mm, Nacalai Tesque, Inc.) in a Shimadzu liquid chromatograph (model LC-10A, Kyoto, Japan) equipped with an ultraviolet (UV) detector (model SPD-10A). The mobile phases for determination of Enk and TPLG were acetonitrile: 0.05 M KH₂PO₄ (pH 3.0) (25 : 75, v/v) and acetonitrile: 0.05 M KH₂PO₄ (pH 3.0) (10 : 90, v/v), respectively, pumped at a flow rate of 1.2 ml/min. Detection was 214 nm. The detection limit for both peptides was 200 ng/ml. The concentrations of dextran were determined by the anthrone-sulfuric acid method.¹⁴⁾

Pharmacokinetic and Statistical Analyses The degradation rate constant (*k*) was calculated from the degradation data using the following equation:

$$k = \frac{2.303}{t} \log \frac{a}{a-x}$$

where *a* is the initial amount of peptide and *x* is the amount degraded in time, *t*.

The penetration rate constant was calculated from the penetration data using the following equation:

$$\text{penetration rate constant} = \frac{2.303}{t} \log \frac{A_2}{A_1}$$

where *A*₁ and *A*₂ are the amount of peptide penetrated at time *t*₁ and *t*₂ (*t* < 10 h), respectively.

The means of all data are presented with their SDs. Statistical analysis was performed using an unpaired Student's *t*-test, and a *p* value of 0.05 or less was considered to be significant.

Theoretical Treatment and Kinetic Analysis To describe the pharmacokinetics for the steady-state flux of peptides and its metabolism in skin, a pharmacokinetic model is proposed as shown in Fig. 1, which is based on the model of Shah¹⁵⁾ but includes the degradation process. The kinetic analysis is also intended to see if the kinetic parameters obtained agree well with those calculated by steady-state diffusion kinetics. In the kinetic model, *k*₀ (μ g/cm²/h) is the input rate of peptide through the stratum corneum, and this is zero-order. Therefore, *k*₀ is equal to the steady-state flux, *J*_s, and *k*₂ (h⁻¹) is the first-order rate constant for permeation of peptide across the viable epidermis while *k*₄ and *k*₅ are the degradative rate constants in viable skin and receptor fluid, respectively. Since the degradation in receptor fluid was com-

pletely inhibited and back-diffusion (k_3) was assumed to be negligible, k_5 and k_3 were eliminated from the model and equations.

The mass balance equations for the model were defined as:

$$\frac{dX_2}{dt} = k_0 - k_2 X_2 \quad (1)$$

$$\frac{dX_3}{dt} = k_2 X_2 - k_4 X_3 \quad (2)$$

where X_2 and X_3 are the amount ($\mu\text{g}/\text{cm}^2$) of peptide in the stratum corneum and in the viable epidermis/receptor/sink, respectively. Using the initial conditions, $X_2 = X_3 = 0$, we can obtain

$$X_3 = \frac{k_0}{k_4} + \frac{k_0}{k_2 - k_4} e^{-k_2 t} - \frac{k_0 k_2}{k_4 (k_2 - k_4)} e^{-k_4 t} \quad (3)$$

when inhibitors are present ($k_4 = 0$), X_3 is given as:

$$X_3 = k_0 t + \frac{k_0}{k_2} (e^{-k_2 t} - 1) \quad (4)$$

In Eqs. (3) and (4), t is limited as:

$$0 < t < \frac{X_0}{k_0} \quad (5)$$

where X_0 is the applied amounts per unit area ($\mu\text{g}/\text{cm}^2$). For

Fig. 1. Schematic Figure Depicting the Kinetic Model for *in Vitro* Percutaneous Permeation

k_0 , absorption rate across the stratum corneum at zero-order; k_2 , the rate constant for permeation across the viable epidermis; k_3 , the rate constant for degradation (metabolism) in viable skin; k_4 , the rate constant for degradation in receptor fluid. In skin pretreated with inhibitors and in the receptor fluid with inhibitors, the degradation (k_4 and k_5) was assumed to be zero.

the simultaneous multiline fitting of two time-courses after application of peptides to pretreated and untreated skins, we used WinNonlin software (Pharsight Corp., Mountain View, CA, U.S.A.).

RESULTS AND DISCUSSION

Degradation and Inhibition of Peptides in Receptor Fluid The viable epidermis possesses enzyme activities,^{10,11} and cutaneous metabolism may influence the percutaneous fate of topically applied peptides. Enzyme inhibition is important for determining the net permeability of peptides. To evaluate the degradation of peptides on the dermal side after penetration through skin, the degradation of peptides in the receptor fluid of the Franz diffusion cell was estimated with or without inhibitors. Both peptides in the absence of inhibitors were rapidly degraded (Fig. 2), and 90% degradation was observed within 10 h, although Enk decreased more rapidly than TPLG. The degradation rates followed first-order kinetics and the rate constants (k) are given in Table 2. The presence of PMSF and phenanthroline almost completely prevented the degradation. Since PMSF is an inhibitor of serine proteases, including trypsin and chymotrypsin, and phenanthroline is an inhibitor of metalloenzymes, which include aminopeptidases and endopeptidases,¹⁶ both peptides would be hydrolyzed by aminopeptidases, endopeptidases and serine proteases which dissolved out of viable skin.⁹ The combination of gabexate plus PMSF had a weaker inhibitory effect than PMSF plus phenanthroline. The k values in the presence of PMSF and phenanthroline were about one-two hundredth of the values without inhibitors. Consequently, PMSF and phenanthroline almost completely inhibited these enzymes.

Table 2. First-Order Rate Constants for Degradation of Peptides in Receptor Fluid

	Rate constant (h^{-1})	
	Enk	TPLG
Without inhibitor (control)	0.977 ± 0.102	0.250 ± 0.034
PMSF + phenanthroline ^{a)}	0.004 ± 0.002^c	0.002 ± 0.000^c
Gabexate + phenanthroline ^{b)}	0.009 ± 0.005^c	0.064 ± 0.010^c

a) PMSF (5 mM) + phenanthroline (1 mM). b) Gabexate (10 mM) + phenanthroline (1 mM). c) $p < 0.01$ compared with the control. Values are expressed the mean $\pm$ S.D. ($n = 3-9$).

Fig. 2. Degradation Profiles of Enk (A) and TPLG (B) in Receptor Fluid

Points and vertical bars represent the mean $\pm$ S.D. ($n = 3-9$). $\circ$, without inhibitor; $\blacktriangle$, PMSF (5 mM) and phenanthroline (1 mM); $\blacksquare$, gabexate (10 mM) and phenanthroline (1 mM). The concentration of peptide added to the receptor fluid was $20 \mu\text{g}/\text{ml}$.

Fig. 3. Inhibition of Skin Metabolism by PMSF and Phenanthroline

Enk, 10 µg/ml; PMSF 5 mM and phenanthroline 1 mM. Points and vertical bars represent the mean $\pm$ S.D. ($n=4$).

These results demonstrate that the peptides in the receptor fluid which are in contact with the dermis are rapidly degraded and, since peptides penetrating *via* viable skin are quickly degraded, apparently negligible penetration of peptides will occur without inhibitors. Consequently, abolishing degradation in the receptor fluid is essential for determining the true permeation of peptides and the *in vitro* penetration data reported previously may underestimate the permeation of peptides.

Inhibition of Skin Metabolism by PMSF and Phenanthroline To estimate the inhibitory effect of PMSF plus phenanthroline on skin enzymes, the degradation of Enk in skin homogenates was measured in the presence of 5 mM PMSF and 1 mM phenanthroline. The result is shown in Fig. 3. There was no degradation of Enk for 12 h. Thus, it was concluded that both inhibitors could completely inhibit the proteolytic enzymes in skin and viable tissues where the peptides were metabolized, thus effectively abolishing degradation in the skin. Imanidis *et al.* have reported that the combinations of PMSF plus *p*-hydroxymercuribenzoate plus phenanthroline completely inhibited leuprolide hydrolysis in rat intestinal homogenates.¹⁶⁾

Effect of Pretreatment with Inhibitors on Penetration To estimate the effect of pretreatment of the skin with inhibitors on the penetration of peptides, the penetration of dextran (1000 Da) through rat skin pretreated with inhibitors was measured using the same method as for the peptide penetration. The results are shown in Fig. 4. The penetration of dextran through normal skin (control) was approximately the same as that *via* pretreated skin. Consequently, the penetration of peptides is largely unaffected by pretreatment with inhibitors.

In Vitro Penetration of Peptides Two enhancers, deoxycholate and OTG, were used in this study to enhance the penetration,¹⁷⁾ since Enk only slightly penetrated the skin without enhancers.⁹⁾ When the *in vitro* skin penetration of these peptides was measured without protease inhibitors in the receptor fluid, no peptide was detected in the receptor fluid by this analysis method, indicating massive degradation of peptides in skin and receptor fluid. Therefore, two potent inhibitors, PMSF and phenanthroline, were added to the receptor fluid to prevent degradation. The penetration profiles of Enk and TPLG are shown in Fig. 5. Appreciable amounts of

Fig. 4. Penetration Profiles of Dextran through Skin Pretreated with Inhibitors

Points and vertical bars represent the mean $\pm$ S.D. ($n=4-5$). ○, normal skin; ●, skin pretreated with gabexate (10 mM) and phenanthroline (1 mM); △, skin pretreated with PMSF (5 mM) and phenanthroline (1 mM).

these penetrated through the skin from the liquid formulation, with a comparatively short lag-time (2–3 h), although TPLG penetrated through the skin at a much slower rate than Enk. The apparent flux (J_s) of Enk was approximately 3 times greater than that of TPLG. The penetration kinetics reached a plateau in about 12–15 h. This indicated that the penetration of both peptides through the skin was very rapid and complete in about 15–20 h. The differences in penetration parameters between the two peptides is probably due to differences in their intrinsic permeability and polarity; TPLG has an amide group at its terminal and is basic. Counter ions such as fixed, negatively-charged end groups (*e.g.*, carboxylic and phosphate groups) contained in intercellular lipids^{18,19)} would potentiate cationic binding by electrostatic interaction and prevent diffusion into other regions.²⁰⁾ Thus, TPLG being cationic would partly bind to the negative-charged groups and penetration would be retarded to some extent. The detailed mechanism for the low penetration of TPLG must, however, await further study.

Choi *et al.* studied the penetration of Enk through hairless rat skin, and reported that the peptide penetrated the skin along with its metabolites such as Phe-Leu and Gly-Gly-Phe-Leu in the presence of *n*-decylmethyl sulfoxide. However, they did not calculate the flux.²¹⁾

The penetration data obtained by their method, however, did not consider the degradation in skin. To estimate the degradation of peptides in that tissue, rat skin was pretreated with PMSF and phenanthroline and the penetration of peptides was measured (Fig. 5). The cumulative amounts of peptides penetrating through the pretreated skin were greater than those through the untreated skin. This indicates that these peptides were partly degraded by the peptidases during the passage through the skin. The penetration parameters are listed in Table 3. The J_s (27.87 and 7.12 µg/cm²/h) of Enk and TPLG were significantly greater than those (19.26 and 5.98 µg/cm²/h) through the untreated skin. This data demonstrates that the degradation of peptides in crossing the viable epidermis and dermis is significant, and degradation constitutes a clear barrier to the percutaneous permeation of peptides. The degradation rate constants, obtained from the difference in the penetration rate constants with or without in-

Fig. 5. Penetration Profiles of Enk (A) and TPLG (B) through Normal Skin and Skin Pretreated with PMSF and Phenanthroline

Points and vertical bars represent the mean $\pm$ S.D. ($n=4-5$). $\circ$, normal skin; $\bullet$, skin pretreated with PMSF (5 mM) and phenanthroline (1 mM). Applied amount of peptides was $260.5 \mu\text{g}/\text{cm}^2$.

Table 3. *In Vitro* Skin Penetration Parameters for Enk and TPLG

Parameter	Enk		TPLG	
	Normal skin	Pretreated skin ^{a)}	Normal skin	Pretreated skin ^{a)}
Lag-time (h)	2.26 ± 0.34	1.85 ± 0.17	2.32 ± 0.19	1.94 ± 0.27
Penetration rate (J_s) ($\mu\text{g}/\text{cm}^2/\text{h}$)	19.26 ± 2.68	27.87 ± 1.74^b	5.98 ± 0.27	7.12 ± 0.13^b

^{a)} The skin was pretreated with PMSF (5 mM) and phenanthroline (1 mM). ^{b)} $p < 0.05$ compared with the normal skin. Values are expressed as the mean $\pm$ S.D. ($n=4-5$).

hibitors, in skin were 0.037 h^{-1} for Enk and 0.050 h^{-1} for TPLG. Consequently, the extent of the first-pass skin metabolism during steady-state flux was 31% for Enk and 16% for TPLG, calculated from the difference in the fluxes.

It has been reported that there are no proteolytic enzymes in the skin, and presystemic metabolism should be minimal.²²⁾ However, in our *in vitro* experiment, extensive skin metabolism was observed as mentioned above. In the *in vivo* situation, the blood sink is effectively located at the interface of the viable epidermis and dermis. Therefore, the residence time for metabolism to occur in the *in vivo* skin tissue may be shorter than that for metabolism *in vitro*. As a result, the extent of first-pass skin metabolism *in vivo* tends to be much less than that *in vitro*.

Analysis of Permeation Data Using the Model The diffusion model has widely been used to describe the *in vitro* percutaneous permeation of drugs.^{23,24)} However, it is mathematically very complex, and its biophysical significance has not been thoroughly established. An alternative approach is the analysis of drug permeation across skin as a kinetic process. Guy *et al.*²⁵⁾ and Guy and Hadgraft²⁶⁾ developed a linear pharmacokinetic model to describe the absorption of various drugs across the skin. These models described the plasma concentration–time profiles following transdermal application of many drugs fairly well. Shah applied the kinetic model to *in vitro* percutaneous permeation data and compared it with the diffusion model used earlier.¹⁵⁾ This kinetic model described these data very well with a smaller sum of squared deviations than that for the diffusion model. Therefore, we used the kinetic model to describe the *in vitro* percutaneous permeation of peptides and to simplify the analysis of the permeation data.

Pharmacokinetic analysis was performed on the basis of a

compartmental description of the observed data with the assumption that the permeation and degradation processes are first-order. Although a model with a first-order transfer rate constant (k_1) was initially tried to fit the data over the observation period, this model resulted in a large Akaike's Information Criteria (AIC) and standard error of the parameters. This model did not describe the data well. When the transfer from donor solution to stratum corneum was assumed to be zero-order (k_0), a good fit was obtained between the observed and predicted curves, as indicated by the smaller CV% (Table 4). Therefore, in this study, the analysis of the steady-state flux was performed by the model depicted in Fig. 1. This kinetic model differed significantly from the model proposed by Guy *et al.*,²⁵⁾ which contained a first-order rate constant for diffusion across the stratum corneum (k_1) and for back-diffusion from the viable epidermis into the stratum corneum (k_3).

Fig. 6 shows the cumulative amount of peptide permeated and the curve predicted by the kinetic model. The kinetic model describes the permeation data reasonably well, as judged from the fit of the curves to the data, in both situations with and without degradation in viable skin. The optimized values of the rate constants so obtained are listed in Table 4. The k_2 for Enk was $0.339 \pm 0.109 \text{ h}^{-1}$ and k_4 was $0.070 \pm 0.016 \text{ h}^{-1}$. This indicates that the degradation of peptide in the viable skin is more extensive than that predicted from penetration data. The k_0 and k_4 estimated using the model approximately agreed with J_s ($27.87 \mu\text{g}/\text{cm}^2/\text{h}$) and the degradation rate (0.037 h^{-1}), respectively, calculated from the penetration data, indicating the appropriateness of this model. On the other hand, the k_0 ($22.21 \mu\text{g}/\text{cm}^2/\text{h}$, see Table 4) for TPLG was greater than the observed J_s ($7.12 \mu\text{g}/\text{cm}^2/\text{h}$). The reason for the difference could be due to the

Fig. 6. Penetration Profiles of Peptides and Curves Predicted by the Model

(A), Enk; (B), TPLG. ● (pretreated skin) and ■ (normal skin), observed values shown in Fig. 5. Solid and dotted lines are curves predicted by the kinetic model.

Table 4. Absorption Rate and Rate Constants of Peptides Obtained by the Kinetic Model

Parameter (h^{-1})	Enk	CV%	TPLG	CV%
k_0 ($\mu\text{g}/\text{cm}^2/\text{h}$) ^{a)}	28.17 ± 3.41	12.1	22.21 ± 12.80	57.6
k_2 (h^{-1})	0.339 ± 0.109	32.2	0.069 ± 0.048	70.3
k_4 (h^{-1})	0.070 ± 0.015	22.6	0.084 ± 0.018	21.2

a) Absorption rate or input rate.

retention of this peptide in the viable skin, as shown by the slow penetration data (Fig. 5), although the rate of input would be large. This is also suggested by the small value of k_2 . However, the k_4 for TPLG was similar to the obtained value (0.050 h^{-1}).

When PMSF and phenanthroline were present in the receptor fluid, both inhibitors would gradually permeate into the viable skin, and the possibility that the degradation of peptides would be inhibited to some extent with time cannot be ruled out. However, our data show that partial inhibition of peptides with both inhibitors did not have a significant effect on the kinetic analysis of peptide penetration.

Consequently, the model presented reasonably describes the steady-state flux of peptides through rat skin, and the results presented here demonstrate the usefulness of the kinetic approach.

In conclusion, the peptides were partly degraded in the skin and largely in the receptor fluid after penetration. Pretreatment of the skin with PMSF (5 mM) and phenanthroline (1 mM), and the addition of these inhibitors to the receptor fluid, almost completely prevented the degradation, resulting in the true flux of peptides *in vitro*. The kinetic model including k_0 (input), k_2 (permeation across the viable epidermis) and k_4 (degradation in skin) is adequate for describing the penetration of peptides.

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