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Research Paper

Effect of Charge and Molecular Weight on Transdermal Peptide Delivery by Iontophoresis

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Purpose. The study was conducted to investigate the impact of charge and molecular weight (MW) on the iontophoretic delivery of a series of dipeptides.
Methods. Constant current iontophoresis of lysine and 10 variously charged lysine- and tyrosine-containing dipeptides was performed *in vitro*.
Results. Increasing MW was compensated by additional charge; for example, Lys (MW = 147 Da, +1) and H-Lys-Lys-OH (MW = 275 Da, +2) had equivalent steady-state fluxes of 225 ± 48 and 218 ± 40 nmol cm^{-2} h^{-1}, respectively. For peptides with similar MW, e.g., H-Tyr-D-Arg-OH (MW = 337 Da, +1) and H-Tyr-D-Arg-NH_2 (MW = 336 Da, +2), the higher valence ion displayed greater flux (150 ± 26 *vs.* 237 ± 35 nmol cm^{-2} h^{-1}). Hydrolysis of dipeptides with unblocked N-terminal residues, after passage through the stratum corneum, suggested the involvement of aminopeptidases. The iontophoretic flux of zwitterionic dipeptides was less than that of acetaminophen and dependent on pH.
Conclusions. For the series of dipeptides studied, flux is linearly correlated to the charge/MW ratio. Data for zwitterionic peptides indicate that they do not behave as neutral ("charge-less") molecules, but that their iontophoretic transport is dependent on the relative extents of ionization of the constituent ionizable groups, which may also be affected by neighboring amino acids.

KEY WORDS: charge/molecular weight ratio; iontophoresis; peptide; skin metabolism; zwitterion.

INTRODUCTION

The poor oral bioavailability of peptides and proteins, and the problems associated with their parenteral delivery (e.g., patient compliance and risk of infection) have led to continued interest in noninvasive delivery techniques. Transdermal iontophoresis is a century-old technique that uses a mild electric current to drive charged and uncharged molecules across the skin. Peptides, which are usually charged at physiological pH, are poor candidates for passive transdermal delivery, but are very well suited for iontophoresis. As iontophoresis also enables tight control over the drug administration kinetics, it is especially useful for the delivery of peptides that exhibit input rate-dependent pharmacological activity (1,2).

Despite the growing interest in the iontophoretic delivery of therapeutic peptides, and significant developments in the "applied" aspects of this mode of delivery, mechanistic data—especially those pertaining to peptide structure–permeation relationships—have not been forthcoming. Fundamental issues concerning the relative importance of physicochemical characteristics, which may ultimately allow the delivery efficiency of a given peptide to be estimated (if not predicted), remain elusive. Clearly, formulation optimization in the absence of such data can only proceed in an empirical and time-consuming fashion.

The work presented in this paper represents a systematic study of the factors governing transdermal iontophoretic peptide delivery, in particular, the dependence of iontophoretic transport on peptide charge and molecular weight (MW). The main objectives were: (1) to determine whether increasing peptide charge could compensate increasing MW, (2) to evaluate the impact of increasing charge in a peptide of given MW, and (3) to investigate zwitterion iontophoresis and the effect of partial charge on transport. The effect of metabolism on peptide transport, particularly with respect to the role of peptide structure in determining hydrolytic susceptibility, and the site of enzymatic degradation in the skin, was also investigated. Two series of dipeptides were selected for this study: the first was a group of unblocked lysine-containing dipeptides, the second a series of (unblocked and partially blocked) tyrosine-containing dipeptides. These peptides have low MW (~300 Da) and differ from each other with respect to their ionization state at the pH of the experiment. Coiontophoresis of acetaminophen was used to determine the respective contributions of electromigration (EM) and electroosmosis (EO) to peptide transport.

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MATERIALS AND METHODS

Materials

Lysine, tyrosine, citric acid, and acetonitrile (Acetonitrile Chromasolv® for HPLC, gradient grade) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The dipeptides H-Lys-Lys-OH, H-His-Lys-OH, H-Glu-ε-Lys-OH, H-Tyr-D-Arg-OH, H-Tyr-Lys-NH_2, H-Tyr-His-OH, H-Tyr-Gln-OH, H-Tyr-Phe-NH_2, and Ac-Tyr-Phe-OH were obtained from Bachem (Voisins-le-Bretonneux, France). The dipeptide H-Tyr-D-Arg-NH_2 was custom-synthesized by NeoMPS (Strasbourg, France). Acetaminophen, sodium hydrogen carbonate, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, dabsyl chloride, and trifluoroacetic acid (TFA) were purchased from Fluka (Saint Quentin Fallavier, France). All solutions were prepared using deionized water (resistivity > 18 MΩ cm).

Porcine ears were obtained fresh from a local abattoir (Société d'Exploitation d'Abbatage, Annecy, France). The excised skin was then dermatomed (~750 μm) on the same day and stored at −20°C for a maximum period of 2 months.

Iontophoresis Setup

The experiments were performed using vertical iontophoretic cells as described by Glikfeld *et al.* (3). These cells, in contrast to side-by-side cells, permit both electrodes to be applied to the external side of the skin [stratum corneum (SC)], thus simulating *in vivo* conditions. A flow-through system was used to sample the receiver compartment (flow rate: 3 mL h^{-1}) hourly in the studies with lysine, H-Lys-Lys-OH, H-His-Lys-OH, H-Glu-ε-Lys-OH, H-Tyr-D-Arg-OH, H-Tyr-D-Arg-NH_2, and Ac-Tyr-Phe-OH. In the case of H-Tyr-Lys-NH_2, H-Tyr-His-OH, H-Tyr-Gln-OH, and H-Tyr-Phe-NH_2, the receiver compartment was replaced manually every hour. A current of 0.34 mA (corresponding to a current density of 0.5 mA cm^{-2}) was applied for 5 h in the case of lysine and lysine-containing dipeptides, and 7 h for tyrosine dipeptides. This current was generated by a power supply (Kepco, Flushing, NY, USA, or Iomed, Salt Lake City, UT, USA). Silver and silver chloride electrodes were used as the anode and cathode, respectively. The receiver volume was ~4.7 mL. After equilibration of each compartment for 1 h with the buffer (see below), 1 mL of a buffered solution of each peptide was introduced into the appropriate donor compartment.

Acetaminophen (15 mM) was added to the donor solution as a marker for EO flow: as a polar but uncharged molecule, it is transported by EO during transdermal iontophoresis, its passive delivery being negligible. The latter was confirmed in a control experiment in which the measured passive flux was ~3 nmol cm^{-2} h^{-1} when delivered from a 15 mM solution in 30 mM $NaHCO_3$/133 mM NaCl (pH 7.4) into a receiver containing 150 mM $NaHCO_3$/133 mM NaCl (pH 7.4) for 5 h. All experiments were replicated with $n = 5$ at least, using skin sections from as many different animals as possible.

Iontophoresis Buffers

The buffers used were either (1) 30 mM $NaHCO_3$/133 mM NaCl, pH 7.4 (donor), 150 mM $NaHCO_3$/133 mM NaCl, pH 7.4 (receiver) for lysine, H-Lys-Lys-OH, H-His-Lys-OH, and H-Glu-ε-Lys-OH; or (2) phosphate-buffered saline (PBS), pH 7.4 (16.8 mM Na_2HPO_4/1.4 mM KH_2PO_4/136.9 mM NaCl) in all compartments, used with H-His-Lys-OH, H-Glu-ε-Lys-OH and tyrosine-containing dipeptides; or (3) citrate buffer (10 mM citric acid/133 mM NaCl, pH 4.5) for certain experiments with H-Tyr-His-OH and H-Tyr-Gln-OH. Buffers were selected to minimize interference in the peptide assay.

Dipeptides

Lysine- and tyrosine-containing dipeptides were studied, as well as the cationic amino acid lysine (Table I). Ionization states of the molecules are reported as discrete values of charge (−1, 0, +1, +2) at pH 7.4 (for lysine and all the dipeptides), and at pH 4.5 for H-Tyr-His-OH (i.e., a pH at which histidine is almost completely positively charged (pK_a of His ~6–6.5), rendering it more suitable for iontophoretic delivery). Note that the ionization state of the dipeptides has been estimated from the pK_a values of the constituent amino acids. However, the pK_a values may differ slightly when these amino acids are incorporated in dipeptides, because of a change in their microenvironment (4,5). Charge/MW ratio was calculated by dividing the peptide valence by the molecular weight (expressed in Da).

Theory

According to the Nernst–Planck theory, the total flux (J_{TOT}) of a molecule during iontophoresis is given by (6):

$$J_{TOT} = J_P + J_{EM} + J_{EO} \quad (1)$$

where J_P is the passive flux, and J_{EM} and J_{EO} are the contributions of EM and EO, respectively. In this study, J_{TOT} and J_{EO} were deduced from the receiver concentrations of the amino acid/peptide and acetaminophen, respectively. Because J_P is negligible for the molecules delivered during this study, J_{EM} can be directly estimated from J_{TOT} and J_{EO}, and the contribution of each transport mechanism can be separated.

Table I. Dipeptides Physicochemical Properties

Amino acid/Dipeptide	MW	Charge (at pH 7.4)	Charge/MW ($\times 10^{-3}$)
H-Lys-OH	146	+1	6.9
H-Lys-Lys-OH (KK)	274	+2	7.3
H-His-Lys-OH (HK)	283	+1	3.5
H-Glu-ε-Lys-OH (EεK)	275	Zwitterion	0
H-Tyr-D-Arg-OH (YdR)	337	+1	3
H-Tyr-D-Arg-NH_2 (YdR-NH_2)	336	+2	6
H-Tyr-Lys-NH_2 (YK-NH_2)	308	+2	6.5
H-Tyr-His-OH (YH)	318	0^a/$+1^b$	0^a/3.1^b
H-Tyr-Phe-NH_2 (YF-NH_2)	327	+1	3.1
Ac-Tyr-Phe-OH (Ac-YF)	370	−1	−2.7
H-Tyr-Gln-OH (YQ)	309	Zwitterion	0

[a] Donor pH = 7.4.
[b] Donor pH = 4.5.

The fraction of the total charge carried by an iontophoretically delivered peptide is represented by its transport number t_{PEP} (2,7,8):

$$t_{PEP} = (c_{PEP}\ z_{PEP}\ u_{PEP}) \Big/ \sum (c_i z_i u_i) \quad (2)$$

where c_{PEP}, z_{PEP}, and u_{PEP} represent the concentration, valence, and mobility, respectively, of the peptide, and c_i, z_i, and u_i represent the corresponding values for the other charge carriers in the system. The transport number is specific to each species under given conditions and is a measure of the transport efficiency of a molecule. It can be determined from the steady-state flux using Faraday's law (2,6,8–11):

$$J_{EM,\ PEP} = (I\ t_{PEP})/(AF\ z_{PEP}) \quad (3)$$

where $J_{EM,\ PEP}$ is the electromigration flux of the peptide, I is the applied current, F is Faraday's constant, and A is the contact area.

Combining Eqs. (1)–(3) and introducing $v_{A\rightarrow C}$ to represent the convective solvent flow from anode to cathode (cm s^{-1}) gives:

$$J_{TOT,\ PEP} = \left[\frac{I}{AF}\frac{u_{PEP}}{\sum c_i z_i u_i} + v_{A\rightarrow C}\right] c_{PEP} \quad (4)$$

where $J_{TOT,\ PEP}$ represent the total flux of the peptide.

Analysis

Although all samples were assayed by reverse-phase high-pressure liquid chromatography (RP-HPLC), the detection method depended on the nature of the dipeptide.

Lysine- and Lysine-Containing Dipeptides

As lysine does not possess a UV chromophore, an alternative method was required for its detection after chromatographic separation. Although the peptide bond in the lysine-containing dipeptides, H-Lys-Lys-OH and H-His-Lys-OH, has a UV absorbance at ~210 nm, cleavage of this linkage during iontophoresis (see below), meant that transport had to be estimated from the free lysine concentrations in the receiver compartment.

We used precolumn derivatization with dimethylaminoazobenzene sulfonyl chloride [dabsyl chloride (DABS Cl)] for the detection of lysine and the three lysine-containing dipeptides. Dabsylation involves a reaction between the amine groups (of amino acids and peptides) and the sulfonyl chloride group of DABS Cl to yield a sulfonamide derivative, which has an absorbance maximum at λ = 440 nm. Dabsylation conditions in the literature (12–16) were optimized with respect to the ionic strength and type of buffer, DABS Cl concentration and the solvent, heating temperature and duration, and sample dilution.

The optimized procedure for lysine analysis was as follows. In an Eppendorf tube (Treff®), 600 μL of a solution of DABS Cl (7.5 mM in acetonitrile prepared just before use) was mixed with 300 μL of a solution containing lysine or the dipeptide in 150 mM $NaHCO_3$ + 133 mM NaCl solution (pH 7.4). The mixture was then heated in a water bath (15 min at 70°C) and allowed to cool. The samples were diluted using 600 μL of a 1:1 mixture of 50 mM phosphate buffer pH 7 (prepared by adding 2 N NaOH to a solution of 50 mM phosphoric acid in water) and ethanol, and then centrifuged (7000 rpm for 10 min). A portion (50 μL) of the supernatant was injected into the HPLC system. These samples were stable at room temperature for several weeks. The HPLC system consisted of a 600E Controller pump, a 717-plus Autosampler Injector, an In-Line Degasser, and a UV 2487 dual λ Detector (Waters, Saint-Quentin Yvelines, France). A C18 Nucleosil 120-3 column (L: 125 mm; ID: 4 mm; Macherey-Nagel, France) was used at ambient temperature. The mobile phase, comprising 50 mM phosphate buffer (pH 7)/acetonitrile (45:55), was delivered at a flow rate of 1 mL min^{-1}. As lysine has two amine groups, two peaks were attributed to the amino acid, due to the mono- and bisdabsylated lysine (as previously reported 14,16–18). Only the second peak (which eluted at ~7.6 min) was considered because of its greater area and better separation. DABS Cl also reacts with phenol moieties (19–22), and dabsylation of the phenol group of acetaminophen under the same conditions (peak at ~9.2 min) enabled the simultaneous assay of lysine and the EO marker. The limits of detection (LOD) and quantification (LOQ) for lysine were 0.2 and 0.5 μM, respectively; the relative standard deviation (RSD) of the repeatability was <0.5% and the accuracy was within 2%.

During assay development, the choice of buffer in the receiver phase (type and ionic strength) was found to affect the quality of derivatization. For example, use of PBS gave rise to parasite peaks that could not be separated from that of lysine. For this reason, an extemporaneously prepared bicarbonate buffer was employed [150 mM $NaHCO_3$ + 133 mM NaCl solution (pH 7.4) in the receiver, and 30 mM $NaHCO_3$ + 133 mM NaCl solution (pH 7.4) in the electrode compartments].

Tyrosine and Tyrosine-Containing Dipeptides

As tyrosine is a fluorescent amino acid (λ_{ex} = 275 nm, λ_{em} = 305 nm), its detection, as well as that of the tyrosine-containing dipeptides, did not require derivatization. These compounds were therefore quantified using the HPLC system described above but with fluorescence detection (Waters 474 Scanning Fluorescence Detector). The separation was performed on a C18 Nucleosil 100-5 Nautilus column (L: 125 mm; ID: 4.6 mm; Macherey-Nagel) with a 98:2 mixture of 0.1% trifluoroacetic acid (TFA) in H_2O (pH 2.5)/acetonitrile. The flow rate was 1 mL min^{-1}, the temperature was adjusted to 30°C, and 50 μL of sample were injected. Tyrosine and acetaminophen eluted at ~5 and 13 min, respectively, although the latter was detected (in the same run) by UV at 243 nm, again enabling a simultaneous analysis of the peptides and acetaminophen. The limits of detection and quantification for tyrosine were 0.07 and 0.2 μM, respectively, with a coefficient of variation (CV) for the precision of <1.7%; the CV for the accuracy was also <1.7%. In the case of acetaminophen, the LOD and LOQ were 0.2 and 0.8 μM, respectively, with a CV for the precision of <2.9%; the corresponding CV for the accuracy was <0.9%.

Tyrosine-containing dipeptides that also comprised the unnatural amino acid, D-Arg, were resistant to metabolism and could be assayed directly. For example, in the case of H-Tyr-D-Arg-OH, the LOD and LOQ were 0.3 and 0.9 μM, respectively; the CV for the precision and the accuracy were <2.4% and <1.9%, respectively.

Data Treatment

All experiments were conducted with a minimum of five replicates using skin from different pig ears. Statistical analysis of the data was conducted either using Student's *t* test or analysis of variance. The level of statistical significance was fixed at $p < 0.05$.

RESULTS AND DISCUSSION

Hydrolysis

Peptide hydrolysis was observed during iontophoresis of H-Lys-Lys-OH, H-His-Lys-OH, H-Tyr-Lys-NH_2, H-Tyr-His-OH, H-Tyr-Phe-NH_2, and H-Tyr-Gln-OH. During iontophoretic transport across the skin, these dipeptides were completely degraded into their constituent amino acids, which were detected in the receiver phase and quantified. To identify the site of this enzymatic activity, three experiments were performed.

In the first, dermatomed skin was mounted in a vertical diffusion cell and a solution of H-Lys-Lys-OH (200 μM in 150 mM $NaHCO_3$/133 mM NaCl, pH 7.4) was placed in the receiver compartment, as well as in the two electrode compartments (20 mM in 30 mM $NaHCO_3$/133 mM NaCl, pH 7.4). After 4 h in the absence of current, analysis revealed that although the peptide in the two electrode compartments (in contact with the SC) had remained intact, only free lysine was detected in the receiver compartment. This implied that the peptide was stable in solution at room temperature, and also when in contact with the outer layer of the skin, but was completely degraded when in contact with the dermis, perhaps as a result of the release of hydrolytic enzymes into the receiver solution.

In the second experiment, H-Lys-Lys-OH (20 mM in 30 mM $NaHCO_3$/133 mM NaCl) was iontophoresed across porcine skin while samples were taken every 20 min to monitor degradation kinetics. Only free lysine could be detected in the receiver compartment, even after only 20 min of current application. The dipeptide present in the donor compartment remained intact, demonstrating that neither current application nor electrode chemistry influenced peptide stability.

In a third iontophoretic study, free lysine and H-His-Lys-OH (10 mM in 30 mM $NaHCO_3$/133 mM NaCl, pH 7.4) were separately iontophoresed across porcine skin for 4 h. In both cases, only free lysine was detected in the receiver compartment at the end of the experiment; however, the receiver levels of lysine subsequent to dipeptide iontophoresis were threefold lower than those after lysine iontophoresis. The disparity between these levels of lysine in the two experiments suggested that the permeating species in each case was not the same. That is, in the case of H-His-Lys-OH, the dipeptide had presumably been cleaved *after* passage (in the intact form) across the SC, the rate-limiting barrier for iontophoretic delivery. As H-His-Lys-OH has twice the molecular weight but the same charge as lysine, it was reasonable to assume that its transport would be less than that of the free amino acid. Because hydrolysis occurred after the rate-limiting step, it was possible to estimate dipeptide transport from the free amino acid concentrations quantified in the receiver compartment.

Every peptide that was subsequently used was first tested for its susceptibility to enzymatic hydrolysis by placing a 100 μM peptide solution in contact with the skin for 4 h (in the absence of current) using the same buffer as that for iontophoresis. The following dipeptides were completely degraded when in contact with the dermis: H-Lys-Lys-OH, H-His-Lys-OH, H-Tyr-Lys-NH_2, H-Tyr-His-OH, H-Tyr-Phe-NH_2, H-Tyr-Gln-OH. Note that during the iontophoresis of H-Tyr-Gln-OH in citrate buffer at pH 4.5, intact peptide could be detected in the receiver, in addition to free tyrosine, suggesting a pH-dependent activity of the enzyme(s). It is possible that the lower, nonphysiological pH reduced enzyme catalytic efficiency. In those cases where partial hydrolysis was observed, the total flux was calculated from the sum of free amino acid and dipeptide present in the receiver phase.

When benzamidine, a trypsin inhibitor, was added (in excess) to the receiver phase, no reduction in catabolism was observed, suggesting that trypsin-like skin enzymes were not responsible for peptide hydrolysis. Other specific enzyme inhibitors were not investigated, because it was thought that multiple enzymes were most probably involved in peptide metabolism. It was hypothesized that the addition of a mixture of different inhibitors would increase the number of competing ions, thus reducing iontophoretic delivery efficiency, and making definitive conclusions about the metabolic mechanisms impossible.

Peptides resistant to skin catabolism were also identified. The H-Glu-ε-Lys-OH dipeptide, in which the peptide bond involves the ε-NH_2 from the lysine side chain rather than the α-NH_2, was not cleaved by skin enzymes. Two dipeptides that contained a D-amino acid, H-Tyr-D-Arg-OH and H-Tyr-D-Arg-NH_2, were also resistant to enzymatic attack. Blocked peptides were stable only when the blocking group was at the N-terminal end of the peptide. For example, Ac-Tyr-Phe-OH was stable, whereas H-Tyr-Phe-NH_2 was degraded. These results suggested that the enzymes in question were aminopeptidases, which have been previously reported to be present in human and animal viable epidermis (23–27). This conclusion was further supported by experiments investigating the stability of three other dipeptides: Ac-Phe-Tyr-NH_2, H-Tyr-Phe-OH, and Ac-Tyr-Phe-OMe. Although Ac-Phe-Tyr-NH_2 was stable, H-Tyr-Phe-OH was metabolized. Ac-Tyr-Phe-OMe was peptidase-resistant (i.e., the peptide bond remained intact), but the methyl ester was hydrolyzed, confirming significant esterase activity in the skin. Furthermore, the tyrosine released after hydrolysis of the tyrosine-containing dipeptides was subject to further degradation, perhaps catalyzed by enzymes released from the skin and present in the receiver phase. Hence for these experiments, the receiver compartment was sampled manually and the solutions withdrawn were analyzed immediately thereafter.

Endogenous Amino Acid Release

The skin is rich in several amino acids including lysine and tyrosine. These endogenous amino acids were released into the receiver both passively and during iontophoresis; release was most pronounced during the first and second hours of current application. As described above, many of the lysine- and tyrosine-containing dipeptides were subject to considerable enzymatic hydrolysis during their transit across the skin. Thus, control experiments, featuring the same conditions as for dipeptide iontophoresis, but in the absence of any peptide, were conducted to quantify the release of endogenous amino acids (Fig. 1). This quantity of lysine or tyrosine was then subtracted from the total amount of amino acid found in the receiver after each dipeptide iontophoresis experiment. Although endogenous lysine release was still apparent after 5 h of current application (~4 nmol cm^{-2} h^{-1}), this was not the case for tyrosine, which could not be detected after 2 h.

Cationic Dipeptides

The iontophoretic fluxes of the cationic substrates investigated in this study are reported in Fig. 2a, and represent the total fluxes (J_{TOT}) observed during iontophoresis at steady-state (SS), i.e., after 5 h of current application in the case of lysine and lysine-containing dipeptides, and 7 h for tyrosine-containing dipeptides. The fluxes reported for H-His-Lys-OH and H-Tyr-His-OH were those obtained with respective donor buffers of PBS (pH 7.4) and citrate/133 mM NaCl (pH 4.5). Calculation of the acetaminophen flux enabled separation of the EO and EM contributions to peptide transport according to Eq. (1). The acetaminophen flux was shown to be constant for all experiments with the cationic dipeptides (57.4 ± 12.8 nmol cm^{-2} h^{-1} at the end of current application, n = 37, donor concentration = 15 mM); that is, no inhibition of EO was induced by the dipeptides studied; therefore, the differences in J_{TOT} could be attributed solely to variations in EM.

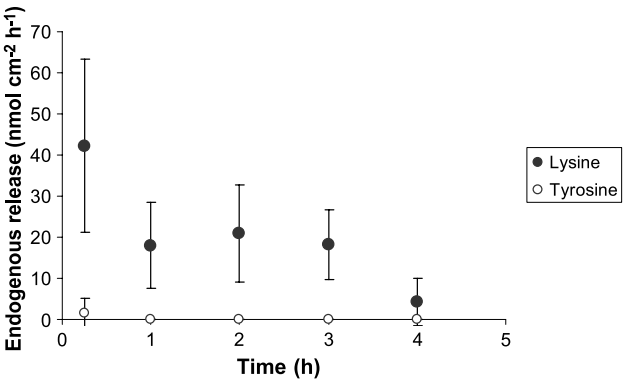

Fig. 1. Release of endogenous lysine and tyrosine from the skin as a function of time upon iontophoresis of buffer solution. The release of lysine was determined using a 150 mM $NaHCO_3$/133 mM NaCl (pH 7.4) receiver solution (30 mM $NaHCO_3$/133 mM NaCl, pH 7.4, in the upper compartments); tyrosine was measured with PBS (pH 7.4) in all the compartments ($n \geq 5$; mean ± SD).

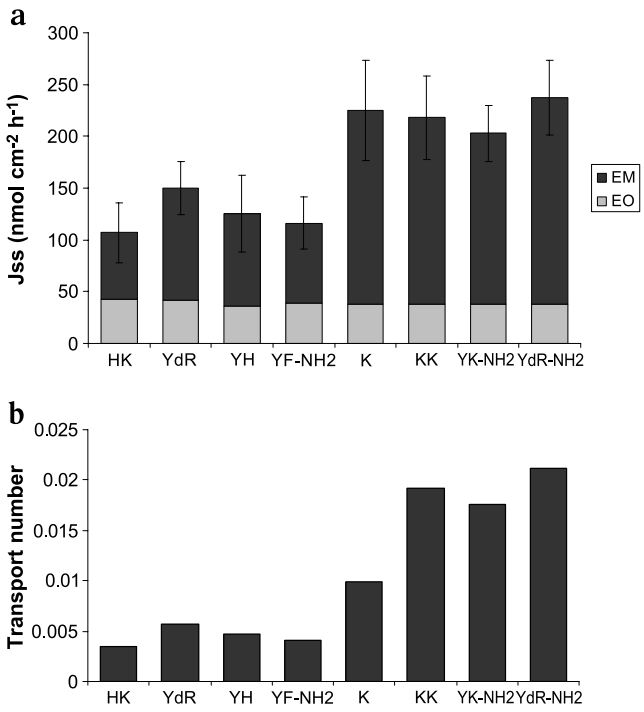

Fig. 2. (a) Steady-state iontophoretic fluxes (J_{SS}) of lysine and cationic dipeptides, where single-letter amino acid codes have been used for clarity: F = Phe, H = His, K = Lys, R = Arg, Y = Tyr. The respective contributions of electromigration (EM) and electroosmosis (EO) are shown ($n \geq 5$; mean ± SD). (b) Transport numbers of lysine and cationic dipeptides. Divalent molecules have approximately double the transport number of lysine, although their iontophoretic fluxes are the same.

The molecules represented in Fig. 2a can be separated into two groups: the first, with steady-state fluxes of ~125 nmol cm^{-2} h^{-1}, comprising H-His-Lys-OH, H-Tyr-D-Arg-OH, H-Tyr-His-OH, and H-Tyr-Phe-NH_2; the second with steady-state fluxes of ~220 nmol cm^{-2} h^{-1} and comprising lysine, H-Lys-Lys-OH, H-Tyr-Lys-NH_2, and H-Tyr-D-Arg-NH_2. In addition to their similar delivery rates, molecules within each group were also characterized by similar charge/MW ratios (Table I). These results demonstrate that an increase in charge can compensate for an increase in MW. For example, a comparison of lysine and H-Lys-Lys-OH transport reveals that the approximately twofold increase in molecular weight (149 to 274 Da) was compensated by a doubling of charge (J_{SS} = 225 ± 48 and 218 ± 40 nmol cm^{-2} h^{-1}, respectively); in contrast, H-His-Lys-OH, with approximately the same molecular weight as H-Lys-Lys-OH but half the charge, had a significantly lower flux (107 ± 29 nmol cm^{-2} h^{-1}). For dipeptides of a given molecular weight, e.g., H-Tyr-D-Arg-OH (MW = 337 Da, +1) and H-Tyr-D-Arg-NH_2 (MW = 336 Da, +2), the increment in charge led to a significant difference in total flux. Moreover, none of the dipeptides (or lysine) inhibited EO; the average EO contribution to peptide transport, $J_{EO,\ PEP}$, was 38.3 ± 8.6 nmol cm^{-2} h^{-1}. Hence, the difference in total flux was attributable entirely to an increased EM contribution. In the case of lysine, H-Lys-Lys-OH, and H-His-Lys-OH, $J_{EM,\ PEP}$ was estimated to be 187, 180, and 64 nmol cm^{-2} h^{-1}, respectively. For H-Tyr-D-Arg-OH and H-Tyr-D-Arg-NH_2, $J_{EM,\ PEP}$ was estimated to be 108

Table II. Iontophoretic Delivery of the Zwitterion H-Tyr-Gln-OH Under Different Experimental Conditions

Donor compartment	pH[a]			Steady-state flux (nmol cm^{-2} h^{-1})	
	Anode	Cathode	Receiver	Peptide	Acetaminophen[b]
Anode	7.4	7.4	7.4	5.4 ± 4.3	66.1 ± 3.7
Anode	4.5	7.4	7.4	7.3 ± 3.8	55.2 ± 12.7
Cathode	7.4	7.4	7.4	4.9 ± 0.6	–
Cathode	4.5	4.5	4.5	21.9 ± 10.1	−76.9 ± 28.0
Control (passive)	7.4	–	7.4	4.9 ± 0.6	

[a] Solutions at pH 7.4 contained PBS, while solutions at pH 4.5 were buffered with citrate +133 mM NaCl.
[b] Negative value indicates solvent flow in cathode-to-anode direction.

and 199 nmol cm^{-2} h^{-1}, respectively. As the EM contribution is dependent on electrical mobility, this suggests that an increase in peptide charge resulted in a corresponding increase in mobility.

At first glance, these results may seem paradoxical. That is, under constant current conditions, increased drug charge should reduce iontophoretic delivery efficiency because fewer molecules need to be transported to carry the same amount of charge. Fundamentally, this assumes that the transport number [Eq. (3)] remains constant and is not influenced by the charge. However, this does not seem to be the case. For example, a comparison of the transport data for lysine and H-Lys-Lys-OH reveals that a twofold increase in charge is paralleled by an equivalent increase in transport number (Fig. 2), resulting in a similar flux (i.e., mass transport) when iontophoresed under the same conditions. Figure 2b shows two distinct groups of dipeptides; molecules within each group are characterized by similar values of transport numbers, ionization state, and charge/MW ratio: (1) H-His-Lys-OH, H-Tyr-D-Arg-OH, H-Tyr-His-OH, and H-Tyr-Phe-NH_2; and (2) H-Lys-Lys-OH, H-Tyr-Lys-NH_2 and H-Tyr-D-Arg-NH_2. Lysine has a lower transport number than the divalent dipeptides because it only carries one positive charge.

Zwitterionic Dipeptides

The transport of two zwitterionic dipeptides (H-Tyr-Gln-OH and H-Glu-ε-Lys-OH), which contain both positive and negative charge centers at physiological pH, was also investigated. Zwitterions are often considered as neutral, "charge-less" molecules; if this was indeed the case, the dipeptides would presumably be transported by EO alone, and their permeation rate would be equivalent to that of acetaminophen when delivered from the anode (using identical donor concentrations). However, experiments revealed that their iontophoretic delivery, under various experimental conditions, was far less than that expected. Indeed, no transport of H-Glu-ε-Lys-OH was observed after either anodal (13 h) or cathodal (6 h) iontophoresis, when the receptor was sampled uniquely at the end of the experiment. For H-Tyr-Gln-OH, anodal delivery was no different from passive transport (Table II). Although cathodal delivery of H-Tyr-Gln-OH at pH 4.5 was higher than the passive control, transport was still significantly less than that predicted from the cathode-to-anode acetaminophen flux.

The results, particularly the negligible anodal delivery of these dipeptides, suggest that the zwitterions are behaving as (partially) negatively charged molecules at pH 7.4. To understand their transport behavior, the *relative extents* of dissociation of each ionizable group as a function of pH and the resulting net charge on the molecule must be taken into consideration (Fig. 3). At pH 7.4, the carboxylate and amine groups of H-Tyr-Gln-OH [estimated isoelectric point (p*I*) = 5.64] are ionized at >99.99% and 98.09%, respectively (assuming pK_a values are equal to those of the free amino acids), and the molecule is partially negatively charged. Despite this apparently small difference (1.9%), capillary electrophoresis measurements confirmed its anionic character (Abla *et al.*, personal communication). Moreover, although these calculations can be made, to a first approximation, using the dissociation constants of the constituent (free) amino acids, it is quite plausible that the latter do not reflect the true pK_a values of the dipeptides. Although it may be argued that the hypothesized anionic nature of these zwitterions is inconsistent with their insignificant cathodal transport, it is possible that the EM flux was opposed, in this case, by an EO flow of the same magnitude.

When H-Tyr-Gln-OH was delivered by cathodal iontophoresis at pH 4.5 (in all compartments), transport was higher relative to the passive control (Table II). Although porcine skin is expected to be essentially uncharged and nonpermselective at pH 4.5 on the basis of its reported p*I* of 4.4 (28), the cathodal delivery of acetaminophen in this study, where the receptor was also buffered at pH 4.5, suggested the

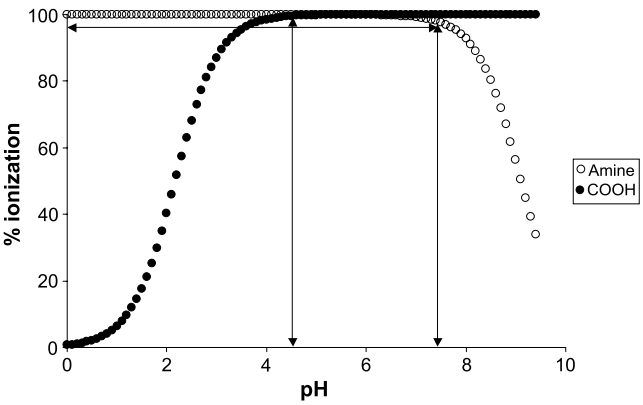

Fig. 3. Ionization profile of H-Tyr-Gln-OH calculated using the pK_a values of the amine and carboxylic acid groups in free tyrosine and glutamine, respectively. At pH 4.5, the carboxylic acid group is 99% ionized.

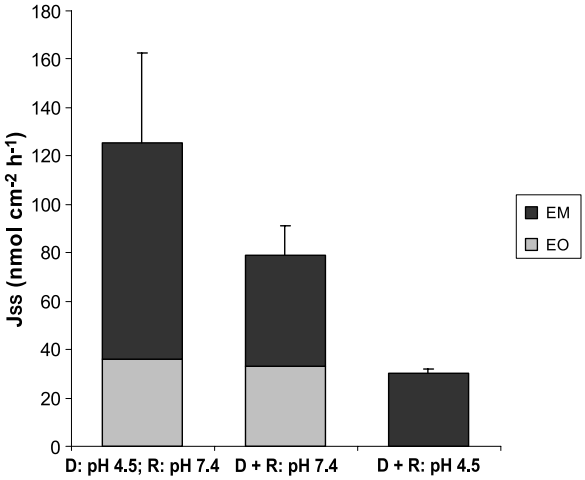

Fig. 4. Contributions of electromigration (EM) and electroosmosis (EO) to the steady-state iontophoretic flux (J_{SS}) of H-Tyr-His-OH delivered from the anodal compartment as a function of pH in the donor (D) and receiver (R) compartments; the fluxes were significantly different (ANOVA, $p < 0.05$) ($n \geq 5$; mean ± SD).

presence of considerable EO in the cathode-to-anode direction. Nevertheless, the total flux of H-Tyr-Gln-OH remained lower than that of acetaminophen because the dipeptide was now partially positively charged (formulation pH < p*I* of H-Tyr-Gln-OH) and not conducive to cathodal iontophoresis.

Therefore, it is apparent that zwitterionic peptides are complex candidates for iontophoresis and that their successful delivery requires judicious formulation. Studies should include accurate measurement of the dipeptide dissociation constants, which, as discussed above, may differ from those of the constituent amino acids. Formulation at a pH equal to the p*I* of the zwitterions, such that its net charge is zero (i.e., it effectively behaves as a neutral "charge-less" molecule), may facilitate electroosmotic transport from the anodal compartment. However, as the pH across the skin progressively increases (from ~5 at the surface to 7.4 in the viable layers), the zwitterion's ionization state may change as a function of its local microenvironment. Hence, a molecule with zero net charge in the anodal (donor) compartment may attain a net partial negative charge in the skin, at which point the EO driving force would be opposed by EM back toward the anode. Although blocking the N and C termini of zwitterionic peptides to "neutralize" these molecules for more efficient EO transport is also feasible, Ac-Phe-Tyr-NH_2 could not be delivered because of the very poor solubility of this blocked peptide in PBS (pH 7.4).

Effect of Donor pH: H-Tyr-His-OH

Iontophoretic delivery of H-Tyr-His-OH was studied from formulations at pH 4.5 (citrate buffer/133 mM NaCl) and pH 7.4 (PBS) (Fig. 4); in both cases, the receiver compartment was maintained at pH 7.4. The acetaminophen flux, and hence the contribution of EO to dipeptide transport, was the same irrespective of donor pH—suggesting that the pH of the solution bathing the dermal layer controlled *skin* pH. Given that the pK_a of free histidine is ~6–6.5, it is only weakly cationic at pH 7.4, but fully protonated at pH 4.5. The increased cationic character of H-Tyr-His-OH at pH 4.5, and consequent increase in electrical mobility, was manifest by a greater EM flux: $J_{EM,\ PEP}$ (89.2 nmol cm^{-2} h^{-1} at pH 4.5 *vs.* 46.2 nmol cm^{-2} h^{-1} at pH 7.4). However, when the dermal compartment was buffered at pH 4.5, a value close to the skin p*I*, the concomitant change in skin permselectivity was translated into a reversal of EO flow in the cathode-to-anode direction. Under these conditions, the measured dipeptide flux ($J_{TOT,\ PEP}$) results from two opposing forces (EM from anode-to-cathode and EO from cathode-to-anode), and Eq. (4) becomes:

$$J_{\mathrm{TOT,\ PEP}} = \left[\frac{I}{AF}\frac{u_{\mathrm{PEP}}}{\sum c_i z_i u_i} - v_{C\rightarrow A}\right] c_{PEP} = J_{\mathrm{EM,\ PEP}} - J_{\mathrm{EO,\ PEP}} \quad (5)$$

For comparison, note that the cathodal delivery of this dipeptide using PBS pH 7.4 in all compartments was negligible (4.8 ± 1.7 nmol cm^{-2} h^{-1}). In summary, the skin pH was determined by the milieu in contact with the dermal phase; thus, although formulation pH can be modified to optimize drug ionization and solubility, it might not be able to change skin permselectivity.

Effect of Donor Buffer Composition: H-His-Lys-OH

As shown earlier (Fig. 2a), the iontophoretic flux of H-His-Lys-OH from a donor comprising PBS (pH 7.4) into a receiver compartment with 150 mM $NaHCO_3$/133 mM NaCl (pH 7.4) was 106.9 ± 29.3 nmol cm^{-2} h^{-1} after 5 h. This equates to approximately 50% of the lysine and H-Lys-Lys-OH transport rates, and is reasonably consistent with the value predicted from its charge/MW ratio. However, when the donor formulation was changed to 30 mM $NaHCO_3$/133 mM NaCl (pH 7.4), the steady-state flux was approximately halved (59.1 ± 9.7 nmol cm^{-2} h^{-1}). Therefore, the bicarbonate-containing formulation seemed to decrease the peptide delivery efficiency, despite having an equivalent concentration of competing cations (~170 mM Na^+) and lower overall ionic strength (0.163 cf. 0.246 mol l^{-1}). It is also worth noting that, whereas histidine seemed to be in part positively charged in H-Tyr-His-OH at pH 7.4 ($J_{TOT,\ PEP}$ at pH 7.4 > $J_{EO,\ PEP}$), it seemed to be uncharged in H-His-Lys-OH at the same pH. The pK_a of histidine is reported to

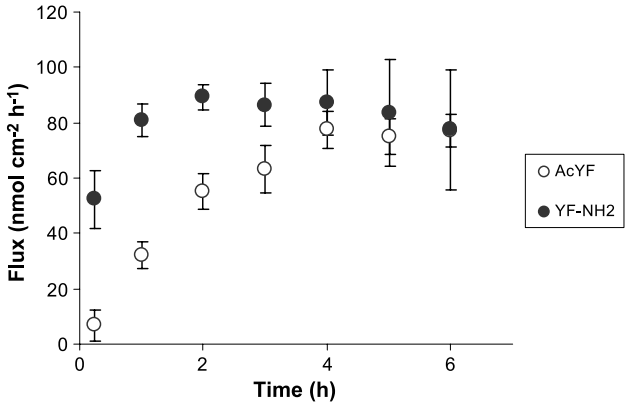

Fig. 5. EM flux of anionic Ac-Tyr-Phe-OH and cationic H-Tyr-Phe-NH_2 ($n \geq 5$; mean ± SD).

vary as a function of the environment, and is influenced by the nature of the neighboring amino acids and the ionic strength of the medium (29,30). Therefore, it is possible that the substitution of lysine by tyrosine and, more importantly perhaps, the location of the imidazole group in H-Tyr-His-OH at the C-terminal, and close to the electron-rich carboxylate group, modifies the acidity of the proton and, hence, the degree of ionization.

Anionic Dipeptide: Ac-Tyr-Phe-OH

Ac-Tyr-Phe-OH has the same amino acid sequence as H-Tyr-Phe-NH_2; but whereas the former is acetylated at the N-terminal, the latter is blocked at the C-terminal by amidation; thus H-Tyr-Phe-NH_2 is positively charged whereas Ac-Tyr-Phe-OH carries a negative charge at physiological pH. These peptides were used, therefore, to investigate whether the EM contribution to their electrotransport was the same. Electromigration of the anion was determined using Eq. (5), assuming that cathodal delivery was opposed by EO.

Comparison of $J_{EM(YF-NH_2)}$ and $J_{EM(Ac\text{-}YF)}$ (Fig. 5) shows that the time taken to reach steady state was greater for the negatively charged Ac-Tyr-Phe-OH, with the result that $J_{EM(YF-NH_2)}$ was more than twofold greater than $J_{EM(Ac\text{-}YF)}$ after 1 h of iontophoresis. However, the EM fluxes at steady-state were similar for the two dipeptides. Further analysis suggested that the discrepancy between H-Tyr-Phe-NH_2 and Ac-Tyr-Phe-OH was attributable to the former attaining steady state more quickly (~2 h) than the other cationic dipeptides studied which, in agreement with Ac-Tyr-Phe-OH, also took ~4 h.

Relationship Between Charge/MW Ratio and Flux

The EM fluxes of the molecules studied were linearly correlated to their charge/MW ratio (Fig. 6). To include the zwitterions, H-Tyr-Gln-OH and H-Glu-ε-Lys-OH, in this analysis, they were attributed an arbitrary charge of −0.5 based on their transport data and ionic mobilities (Abla *et al.*, personal communication). Moreover, as their total cathodal iontophoretic flux, J_{TOT}, at pH 7.4, was negligible, it can be argued that "cathode-to-anode" EM must be approximately equal to the anode-to-cathode solvent flow. Therefore, the mean value of J_{EO} observed with the cationic dipeptides (approximately −38 nmol cm^{-2} h^{-1}) was used to estimate J_{EM} for the zwitterions; this J_{EM} was considered to be negative because it describes transport from cathode to anode. As none of the molecules studied here interacted with the membrane and the electroosmotic contribution (J_{EO}) was effectively constant, the observed differences in transport were due exclusively to variations in the electromigratory component (J_{EM}). In addition to the dipeptides studied here, transport data for lidocaine, propranolol, and quinine are included in Fig. 6 (31). These molecules have similar molecular weights to the amino acids/peptides studied here and, as Fig. 6 shows, their iontophoretic fluxes are in good agreement with the current data despite differences in the composition of the donor electrode formulation.

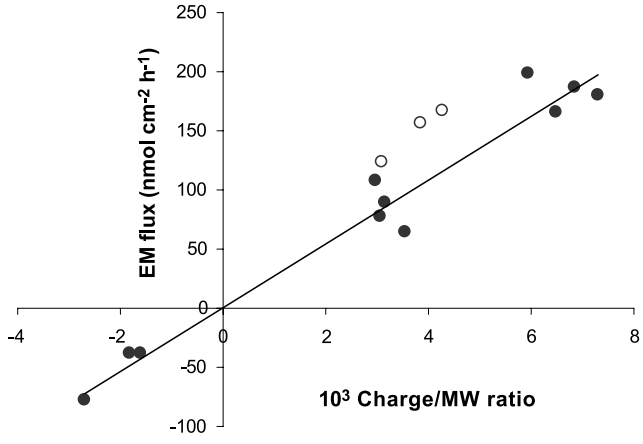

Fig. 6. Correlation between the EM flux of lysine and all dipeptides studied in this work (full circles), and quinine, propranolol, and lidocaine from [31] (open circles), and their corresponding charge/MW ratio. For dipeptides: EM flux = 27.02{charge/MW 10^{-3}}, r^2 = 0.96. When adding the nonpeptidic drugs: EM flux = 28.99{charge/MW}, r^2 = 0.91.

CONCLUSIONS

The results presented here demonstrate that for the amino acids and dipeptides studied, iontophoretic flux was strongly governed by the charge/MW ratio and not, exclusively, by either parameter in isolation. That is, with respect to EM, an increase in molecular weight was compensated by a corresponding increase in charge. Moreover, at a given molecular weight, increasing peptide charge enhanced EM flux. These findings have implications for the selection of drug candidates for iontophoresis and for the evaluation of the relative merits of structurally related compounds. For example, monovalent cations should not be taken as *de facto* superior candidates to di- and trivalent molecules, because the higher charge/MW ratios of the latter may render them more mobile (assuming that they do not increase propensity to interact with the membrane). The presence of multiple charge centers in higher valence ions could considerably modify their transport number compared to the univalent species.

What do these results teach us about the molecular weight limit for drugs that can be delivered by iontophoresis? We have shown that compensation of molecular weight by charge certainly holds for relatively low molecular compounds (MW ~ 300 Da); ionic mobility decreases with increasing molecular weight and although increasing charge can offset this to some extent, this may be insufficient above a given molecular weight. Furthermore, the probability of interaction with the transport pathway will also increase with increasing molecular weight (and hence molecular volume) and this would obviously hinder delivery. The literature shows that calcitonin, human parathyroid hormone, and growth hormone releasing factor analogs—monovalent peptides with molecular weights in the 3.5- to 5-kDa range—can be delivered without additional barrier impairment (32–39). Published investigations on the transport of higher molecular weight, multiply charged species are rare, although the delivery of cytochrome *c* (MW = 12.4 kDa, multiple charges) across excised human skin has been reported (40,41). Further studies are obviously required to address this issue more completely. However, it must be remembered that, as

peptides and proteins increase in size, they are more likely to have tertiary and quaternary structure that is essential for pharmacological effect; the delivery conditions must therefore ensure that biological activity is maintained.

The transport data for the histidine-containing dipeptides indicate the influence of neighboring amino acids and the local microenvironment on the acidity of the imidazole group; this may have implications for the delivery of peptides containing charged residues for which selection of the formulation pH might be critical to achieve the desired ionization state of the molecule. On a related note, the different transport rates of H-His-Lys-OH observed from formulations at the same pH but using different buffering systems also serve to highlight that interactions with the buffer may further complicate formulation design. Similarly, the work on zwitterion transport has shown that these molecules should not be regarded as neutral species *per se* and that the individual pK_a values of the ionizable groups of the component amino acids, as well as the effect of the local environment, should be considered in the determination of the net charge.

Finally, although catabolic enzymatic activity in the skin is considerably less than that in the gastrointestinal tract and liver, an enzymatic barrier to transdermal delivery is evident. Enzymatic hydrolysis seemed to occur after passage through the stratum corneum, the rate-limiting barrier to transport. It was also observed that amide bonds containing ε-NH_2 groups from the lysine side chain were more resistant to hydrolysis than peptide linkages with the α-NH_2 group. Furthermore, dipeptides containing D-amino acids, and those with blocked N-termini, were also less susceptible to enzymatic attack. The results using selectively blocked dipeptides point to the involvement of aminopeptidases during passage across the skin.

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