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Cutaneous metabolism of a dipeptide influences the iontophoretic flux of a concomitant uncharged permeant

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Abstract

Passive and iontophoretic transport of the model dipeptide tyrosine-phenylalanine (TyrPhe) that is subject to cutaneous metabolism and the uncharged glucose derivative benzyl-2-acetamido-2-deoxy-α-D-glucopyranoside (BAd-α-Glc) used as electroosmosis marker through heat-separated human epidermis was investigated in vitro. TyrPhe and BAd-α-Glc were used separately and in combination in order to determine their interaction in terms of permeability and the influence of skin metabolism of TyrPhe on permeation rate and tissue retention of itself and of BAd-α-Glc. TyrPhe was chemically and electrochemically stable but underwent considerable degradation in the epidermis under reflection boundary conditions with generation of degradation products tyrosine (Tyr) and phenylalanine (Phe) confirming cutaneous metabolism of TyrPhe in heat-separated human epidermis, which was more pronounced at pH 4.5 than at pH 3.0. As a result, no reproducible epidermis permeation of TyrPhe at pH 3 and no permeation at all at pH 4.5 was measured regardless of the presence of BAd-α-Glc, accompanied by increased levels of Tyr and Phe compared to blank runs. Low temperature (4 °C) at both pH values and addition of *o*-phenanthroline at pH 3 but not at pH 4.5 yielded reproducible TyrPhe permeation and blank, i.e., endogenous levels of Tyr and Phe evidencing inhibition of degradation. Constant voltage anodal iontophoresis marginally reduced BAd-α-Glc flux at pH 3 and 4.5 compared to the passive flux. In combination with TyrPhe, iontophoretic flux of BAd-α-Glc was increased markedly compared to the passive one when TyrPhe was metabolized in the tissue, while no such increase was observed when TyrPhe metabolism was inhibited. The increase of BAd-α-Glc iontophoretic flux was accompanied by a considerable decrease of the BAd-α-Glc amount retained in the epidermis. The presence of the generated Tyr and Phe, therefore, appears to be related to a decrease of the BAd-α-Glc amount retained in the epidermis upon application of an electrical voltage and an enhancement of its iontophoretic flux. Thus, an interaction between the concurrent permeants at the level of tissue retention induced by metabolism can influence the apparent iontophoretic permeation.

Keywords: Iontophoresis; Cutaneous; Electroosmosis; Peptides; Amino acid; Skin; Metabolism; Transdermal drug delivery; Permeability; Transport; Tissue retention

1. Introduction

Iontophoresis is a non-invasive physical method to increase and modulate the permeation rate of ionized but also unionized compounds through skin by the application of an external electric field to the skin. Peptide drugs are potential candidates for systemic iontophoretic delivery because of the charges they usually carry and because they exhibit a rather small passive permeability for the highly structured, lipophilic stratum corneum barrier of the skin due to their charge related hydrophilicity and their frequently large molecular size. The epidermis is a charged permselective membrane with an isoelectric point (p*I*) of well above 4 (Marro et al., 2001; Luzardo-Alverez et al., 1998; Hoogstraate et al., 1994) and a negative fixed charge of the tissue at physiological pH (Brunette and Ongpipattanakul, 1987). This characteristic has as a result that the epidermis favors at physiological pH the permeation of positively charged compounds compared to negatively charged ones of comparable size. Furthermore, electroosmotic flow through the epidermis upon application of an electric field takes place from the anodal to the cathodal electrode at physiological pH which results in convective movement of the dissolved permeants, ionized and unionized alike. Convective solvent flow was shown to be predominant for the iontophoretic transport of neutral, polar compounds or compounds with high molecular weight (Kirjavainen et al., 2000; Pikal and Shah, 1990; Hirvonen and Guy, 1998). Lowering the pH of solutions bathing the epidermis below its p*I* reverses the permselectivity and the direction of the electroos-

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motic flow in accord with the tissue assuming positive fixed charges (Marro et al., 2001; Delgado-Charro and Guy, 1994; Lopez et al., 2001). A pH in the acidic range may be necessary for the iontophoresis of drugs that are weak organic bases or have two ionizable groups exhibiting zwitterionic behavior, such as peptides, in order to render them positively charged. The net ionic valence of such compounds will depend on their pK_a and the pH of the solution. For pH values approaching the isoelectric point of the epidermis, the pH must be optimized taking into account the ionic valence of the compound and the electroosmotic flow with respect to their impact on iontophoretic flux.

Apart from its function as physical barrier, the skin also plays an important role as a metabolic barrier capable of degrading a wide range of substances upon permeation. Although structure and function of the skin have been well characterized, the extent and the exact localization of enzyme activity remains still uncertain. The epidermis contains many enzymes, which are involved in the differentiation process of its viable layers. A large variety of low molecular weight compounds such as amino acids and sugars found in the stratum corneum may be derived from endogenous metabolism (Schaefer and Redelmeier, 1996). Enzymatic activity in the dermis is primarily associated with the sebaceous glands and the hair follicles. Cutaneous metabolism of drugs was found to take place in epidermal as well as in dermal tissue and peptide drugs were reported to be susceptible to degradation by the enzymes of the epidermis (Bucks, 1984; Steinsträsser and Merkle, 1995; Boderke et al., 1998). It has been suggested, finally, that heat-separated epidermis exhibits a lower metabolic activity than intact epidermis (Täuber and Rost, 1987).

In the present study the iontophoresis of a model dipeptide, tyrosine-phenylalanine (TyrPhe), which is subject to metabolic degradation in cutaneous tissue is investigated under constant voltage conditions. The unionized glucose derivative benzyl-2-acetamido-2-deoxy-α-D-glucopyranoside (BAd-α-Glc) was used as electroosmosis marker in order to quantitatively delineate the contributions of the direct effect of the electric field on ions (termed electromigration) and that of convective solvent flow to the iontophoresis of the peptide. The two compounds were used separately and in combination in transport experiments in order to discover potential interactions between them in terms of permeability. Such interactions while not expected for passive permeability on theoretical grounds, may well occur in case of facilitated or active transport processes such as iontophoresis. Determination of the extent of metabolism of TyrPhe during permeation and assessment of the impact of this metabolism on the passive and iontophoretic permeation of the dipeptide itself and of BAd-α-Glc conclude the objectives of the study.

Experiments were carried out in vitro using heat-separated human epidermis at pH 3, 4.5 and 7.2 chosen to provoke a change of direction of the electroosmotic flow and unveil the interplay of it with the varying ionic valence of the peptide in terms of iontophoretic permeation. The dipeptide was used at pH 3 and 4.5 in order to guarantee its having a net positive ionic valence and allow the use of anodal iontophoresis. The choice of pH is primarily dictated by the pK_a of the given drug. TyrPhe was deliberately chosen as a model of an unmodified, unprotected peptide. Cathodal iontophoresis with a net negative ionic valence of the dipeptide was not studied in this work because at no pH it was possible under these conditions to attain cathode-to-anode electroosmotic flow. Constant voltage iontophoresis was employed because it allows treatment of the iontophoretic transport of concomitant permeants independently of each other. Thus, physiologic tonicity of the solutions may be used and iontophoretic enhancement of the studied compounds will not depend on the presence of other ions that may also be extracted from the tissue or be produced by chemical and enzymatic processes such as permeant degradation. Furthermore, constant voltage conditions are appropriate for model assisted theoretical analysis of the enhancement factor derived from the Nernst–Planck equation and leading to strategies for predicting iontophoretic behavior (Kochhar and Imanidis, 2003; Imanidis and Luetolf, in press; Altenbach et al., 2005). Clearly, for highly efficient drug delivery, constant current iontophoresis with a minimum amount of concurrent electrolytes is the method of choice. The constant voltage approach was employed here, however, because it served better the purpose of the present study.

2. Materials and methods

2.1. Chemicals

Tyrosine-phenylalanine (TyrPhe), a model dipeptide with a molecular weight of 328.4 was purchased from Bachem AG (Bubendorf, Switzerland). Its pK_a was determined by potentiometric titration. Tyrosine (Tyr) with a molecular weight of 181.2 and pK_a 2.2, 9.1 and 10.1 and phenylalanine (Phe) with a molecular weight of 165.2 and pK_a 2.2 and 9.2 were purchased from Fluka BioChemika (Buchs, Switzerland) and Sigma Chemical Co (St. Louis, MO, USA), respectively. Benzyl-2-acetamido-2-deoxy-α-D-glucopyranoside (BAd-α-Glc) (Toronto Research Chemicals, North York, Canada) a glucose derivative with a molecular weight of 311.3 was used as electroosmosis marker. The metallopeptidase inhibitor *o*-phenanthroline HCl monohydrate was purchased from Fluka BioChemika (Buchs, Switzerland).

2.2. Buffer

A Universal buffer was used at pH 3, 4.5 and 7.2. It was composed of citric and phosphoric acid, each at a concentration of 6.67 mM and boric acid at a concentration of 11.5 mM. The acids were all dissolved in double distilled water and the mixture was titrated to the desired pH using sodium hydroxide. For a final osmolarity of 300 mOsmol, the appropriate amount of sodium chloride was added to the buffer. All chemicals were of analytical reagent grade.

2.3. Skin

Human cadaver skin of postmortem biopsies excised within 24 h of death from the abdominal region of female donors was supplied by the Department of Pathology, University Hospital,

Basel and kept at −70 °C until use. Epidermal membrane including the stratum corneum and the viable epidermis was isolated from the underlying dermis by heat treatment and mechanical separation (Kochhar and Imanidis, 2003; Sims et al., 1991). Preparation of the membrane, its mounting to the diffusion cell, a gravitational leaking test and an electrical test of integrity were carried out using the procedures reported previously (Kochhar and Imanidis, 2003). Skin from 16 different donors was used. The average thickness of the isolated epidermis was 43 μm with a standard deviation of 9.7 μm. The specimens were not randomized but their electrical resistance was used to normalize the measured fluxes.

2.4. Diffusion cells and iontophoretic equipment

Custom made two-chamber symmetrical "side-by-side" glass diffusion cells outfitted with a pair of working Ag/AgCl electrodes and a pair of reference Ag/AgCl electrodes combined with membrane reaching capillaries described in detail previously (Kochhar and Imanidis, 2003) were used for permeation experiments. The anode was placed in the donor and the cathode in the receiver compartment of the cells. The current source built in the Department of Physics, University of Basel, was connected to the four electrodes and supplied direct current. Iontophoresis was carried out under constant voltage conditions. The electric current flowing through the epidermal membrane was continuously measured and recorded electronically using a Digital Chart Recorder (DCR 520, W + W Instruments AG, Basel, Switzerland).

2.5. Protocol of permeation experiments

A typical experiment involved three stages. The "passive I" stage lasted for 44 h during which the baseline passive permeability was measured at steady state. Samples of 0.2 ml were collected from the receiver chamber every 90 min. In the subsequent iontophoretic stage, which lasted for 3 h, a fixed electrical potential difference of 250 mV was applied across the membrane and samples of 0.2 ml were taken every 20 min from the receiver chamber. Finally, in the "passive II" stage, post-iontophoretic passive permeability was measured over 22 h during which samples of 0.2 ml were drawn every 90 min from the receiver chamber. All these samples were replenished with fresh buffer and analyzed with no further treatment by HPLC-MS. At the beginning of each of the above stages, 0.05 ml samples were drawn from the donor chamber and not replaced. These samples were diluted 400-fold before analysis by HPLC-MS. At the end of passive I and II stages, a potential difference of 250 mV was applied for 5 min to record the electrical resistance and check the integrity of the membrane. At the end of each experiment, the absence of significant pH and osmolarity shifts in both chambers was verified.

The same buffer was used in both chambers of the diffusion cell. The TyrPhe concentration in the donor solution was approximately 3 and 2 mM at pH 3 and 4.5, respectively, and the BAd-α-Glc concentration was 13 mM at all pH values. From the concentrations determined in the receiver solution, the cumulative amount of permeation through the epidermal membrane as a function of time was computed. Fluxes of TyrPhe and BAd-α-Glc were determined for each experimental stage whereas for the degradation products Tyr and Phe which were produced in the membrane, time-averaged rates of appearance in the receiver solution over the entire duration of the experiment were computed and expressed as flux. Blank experimental runs involved the use of human epidermis and the same protocol as above but both compartments of the diffusion cells contained solely buffer solution.

Permeation experiments were carried out at 37 °C except when suppression of enzymatic activity in the tissue was intended in which case a temperature of 4 °C was employed (Higo et al., 1992). The enzyme inhibitor *o*-phenanthroline was added at pH 3.0 and 4.5 in a concentration of 0.5 mM to the donor and the receiver solution of the diffusion cells.

Statistical significance of the difference between iontophoretic and passive fluxes was examined by double-sided *t*-test.

2.6. Stability of TyrPhe and BAd-α-Glc

Chemical stability of TyrPhe was determined at pH 3.0 and 4.5 in the same buffer and concentration used in permeation experiments under continuous stirring by magnetic stirrer at 37 °C, room temperature and 4 °C over a period of 95 h. Samples were withdrawn every 10–12 h.

Electrochemical stability of TyrPhe and BAd-α-Glc was determined in the diffusion cells employed for iontophoresis experiments at pH 4.5 and 37 °C using a stack of 75 membrane filters (Isopore™ Membrane Filter, Filter Type 0.1 μm VCTP, Millipore AG) instead of human epidermis. Buffer and TyrPhe and BAd-α-Glc concentration were the same as in the donor solution of the permeation studies but both chambers of the diffusion cell contained the same solution of the compounds under investigation. Constant voltage of 250 mV was applied for 8 h and samples were taken every hour.

Cutaneous metabolism of TyrPhe and BAd-α-Glc was studied with the reflection boundary experimental set-up at 37 °C (Boderke et al., 1998). Heat-separated epidermis with the viable epidermis side facing the donor compartment and the stratum corneum side facing an impermeable barrier consisting of a plastic foil was mounted in the diffusion cells used for the permeation studies without including the electrodes as shown schematically in Fig. 1. The same buffer as in the other stability experiments and a concentration of 1.22 μM for TyrPhe and 4.7 μM for BAd-

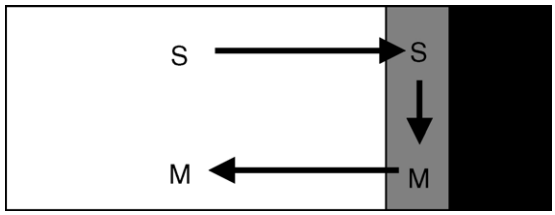

Fig. 1. Schematic representation of reflection boundary experimental set-up consisting of donor compartment (left), heat-separated epidermis (middle) and impermeable barrier of plastic foil (right). S, substrate; M, metabolite.

α-Glc were used, these being of the same order of magnitude as the concentrations detected in the receiver solution. Samples were withdrawn roughly every 10 h. In separate experiments, 1 ml of BAd-α-Glc solution was incubated in a vial under shaking at 37 °C with a piece of epidermis of similar size as that used in the permeation experiments and 100 μl samples were withdrawn every 8–17 h for a period of 70 h. In these experiments, the generation of benzyl alcohol, the potential degradation product of BAd-α-Gl, was monitored.

Samples of the stability experiments were diluted to various degrees chosen such as to allow detection of the starting compounds and the potential degradation products by HPLC-MS.

2.7. Skin extraction

After completion of the permeation experiments, the epidermal membrane that had a total surface area of approximately 4.5 cm^2 was dismounted from the diffusion cell and pulverized at liquid nitrogen temperature. A Freezer mill, type 6750 (SPEX CertiPrep, Inc., New Jersey, USA) was used with the following settings: P Cool T3 (freezing time 10 min), Run T1 (milling time 2–5 min), Cycles (one cycle). After milling, the pulverized tissue was taken up in HPLC mobile phase, briefly ultrasonicated and centrifuged at 10,000 rpm for 10 min. The supernatant was analyzed by HPLC-MS for the permeants and possible degradation products. The recovery of the method was tested by spiking epidermal tissue with a known amount of the compounds and subjecting it to the same extraction procedure. It was found to be 60 ± 9%. The results are reported "per specimen" rather than being referred to the volume (or weight) of the tissue because of the rather small variation of the thickness of the isolated epidermis. Statistical significance of the difference of the extracted amount of BAd-α-Glc between different experimental conditions and of the extracted amount of Tyr and Phe compared to the blank was examined by double-sided *t*-test.

2.8. Assay

Assay of all compounds was performed by HPLC-MS (Hewlett Packard and Agilent 1100 system). A Spherisorb ODS2 chromatography column (CC 125 mm × 2 mm, 5 μm, Macherey and Nagel, Switzerland) was used with mobile phase consisting of 95% aqueous ammonium acetate 10 mM solution and 5% acetonitrile set to pH 3.2 with acetic acid und run in isocratic modus. All compounds were eluted within 16 min. The settings of the mass spectrometer were as follows: API-ES ionization source, SIM mode, positive polarity, drying gas flow 10 l/min, nebulizer pressure 30 psig, drying gas temperature 350 °C, fragmentor variable 40–80 V, capillary voltage 4000 V, peak width 0.2 min, cycle time 1.2 s/cycle. TyrPhe was detected at *m*/*z* 329, Tyr at *m*/*z* 182, 204 and 226, Phe at *m*/*z* 166, BAd-α-Glc at *m*/*z* 312. Benzyl alcohol was detected in UV at 258 nm using a CC 125 mm × 2 mm Lichrospher 100–5 RP-18 ec column and a acetonitrile/water 15/85 mobile phase. Quantification was always performed against a set of external standard solutions.

3. Results and discussion

3.1. Integrity of epidermal membrane

The integrity of the epidermis during permeation experiments was monitored by continually measuring the electrical resistance of the membrane. This was possible with the employed four-electrode diffusion cells and power unit (Kochhar and Imanidis, 2003). Monitoring membrane integrity was essential because of the long duration of the experiments. Electrical resistance is a sensible measure of integrity because it is immediately related to the permeation barrier function of the epidermis and is quite sensitive because it responds to changes of permeation of small electrolyte ions through the membrane.

In Fig. 2, membrane resistance during the 3 h of iontophoresis is shown for several epidermis specimens encompassing different experimental conditions. Typically, the resistance remained constant or showed a small decline. In absolute terms, membrane resistance values varied between 20 and 150 kΩ cm^2, the majority being around 40 kΩ cm^2. These values are in agreement with established electrical resistance values of human epidermis in isotonic electrolyte solution. Experiments that did not conform with this resistance pattern were discarded. These results demonstrate that it was possible to guarantee the integrity of the epidermis under the present experimental conditions. The post-iontophoretic passive stage was used previously (Kochhar and Imanidis, 2003; Imanidis and Luetolf, in press) to verify that no irreversible alteration of the epidermis due to the electric field typically took place under the present experimental conditions.

3.2. Permeation and metabolism of TyrPhe

TyrPhe was found to have a pK_a of 3.5, corresponding to the carboxylic group. The pK_a of the amino group is >7. Accordingly, the average net ionic valence of this dipeptide at pH 3 and 4.5 was calculated to be +0.76 and +0.091, respectively, for practically fully protonated amino group. Therefore, permeation experiments of TyrPhe at these two pH values were carried out using anodal iontophoresis. Since experiments were carried out in the acidic pH range and cathodal iontophoresis was not included in the study, accurate knowledge of the lower pK_a value was of essence. The experimental protocol involved a rather long passive permeation stage in order to safely establish the baseline passive permeability coefficient. This passive stage was followed by a 3-h iontophoretic stage that was demonstrated previously to be sufficiently long to determine the steady state iontophoretic flux (Kochhar and Imanidis, 2003). Permeation profiles of the passive and the iontophoretic stages were invariably linear.

In Table 1, the passive and iontophoretic fluxes of the dipeptide TyrPhe and the time averaged fluxes of the amino acids Tyr and Phe through epidermal membrane are reported. The amount of the amino acids in the receiver compartment of the diffusion cells increased steadily during the permeation experiment, but no uniform distinction in the pattern between passive and iontophoretic stages could be made. Therefore, the amino acid results are reported as average fluxes over the entire experimental duration.

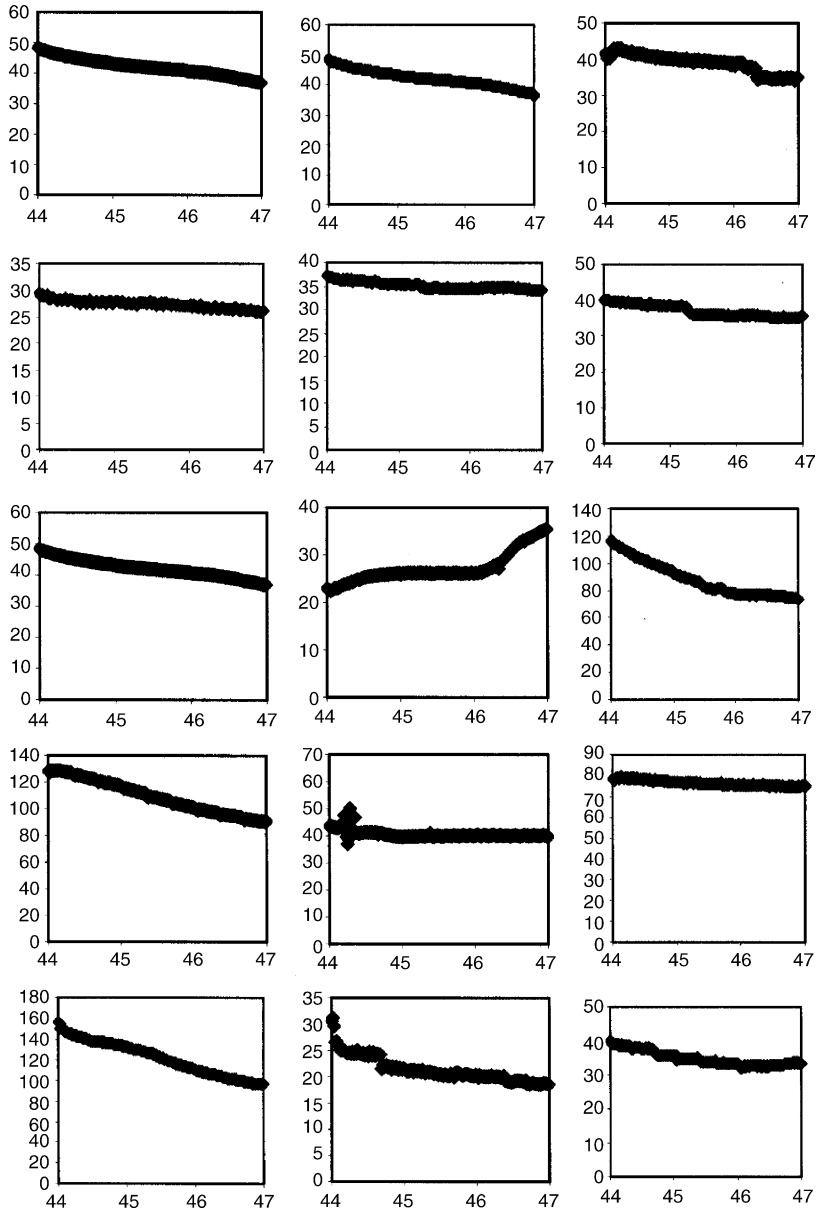

Fig. 2. Course of electrical epidermis resistance during iontophoresis stage (250 mV, 3 h) of different permeation experiments; the first three rows denote experiments at pH 3, the last two rows at pH 4.5; *x*-axes show time in hours and *y*-axes membrane resistance in kΩ cm^2.

No reproducible permeation of TyrPhe was measured at pH 3 when the dipeptide was used in the presence of BAd-α-Glc or alone (latter data not shown). TyrPhe was either not detectable at all or it first appeared in the receiver solution at varying time points later in the course of the experiment. Under the same conditions at pH 4.5, TyrPhe permeation was never detectable. In the same experiments, a consistent trend towards elevated amino acid amounts measured in the receiver solution compared to the blank runs was noted. It is worth pointing out that in the blank runs a notable amount of endogenous Tyr and Phe was released from the epidermis. This trend for elevated amino acid amounts suggest that the failure to detect dipeptide fluxes may have been because of hydrolytic cleavage of its peptide bond, whereby the constituent amino acids are formed. No amino acids were detected in the donor solution either because of the strong dilution of this solution prior to the assay or because of limited diffusion of the amino acids through the stratum corneum.

Analysis of the epidermis specimens at the end of the experiment confirms the above view (Table 2). Intact TyrPhe was recovered from the epidermis when it was used alone or in the presence of BAd-α-Glc at both pH values demonstrating that the dipeptide penetrated into the tissue. The levels of Tyr and Phe, however, were higher compared to those of the blank runs, the difference being statistically significant at pH 4.5 ($p < 0.05$ for Tyr (4.04 μg/specimen versus 1.45 μg/specimen) and $p < 0.1$ for Phe (3.27 μg/specimen versus 1.06 μg/specimen) by *t*-test) and statistically marginal at pH 3 ($p < 0.2$ for Tyr (2.39 μg/specimen versus 1.02 μg/specimen) and $p < 0.2$ for Phe (2.54 μg/specimen versus 0.9 μg/specimen) by *t*-test). From the over all consistently

Table 1
Flux, *J*, of TyrPhe, Tyr and Phe at pH 3.0 and 4.5 for different donor compositions[a]

pH	Donor composition	J ($\times 10^6$ μg/s/cm^2)				
		TyrPhe			Tyr[e]	Phe[e]
		Passive I	Iontophoresis	Passive I[f]		
3.0	TyrPhe + BAd-α-Glc	ND[b]	ND[b]	ND[b]	1.87 (0.40)	1.67 (0.11)
3.0	TyrPhe + BAd-α-Glc + phenanthroline	1.11 (0.48)	3.04 (2.49)	0.95 (0.44)	NM[c]	NM[c]
3.0	TyrPhe + BAd-α-Glc at 4 °C	1.33 (0.32)	2.31 (0.94)	1.08 (0.36)	1.21 (0.21)	0.92 (0.13)
3.0	Blank	NA[d]	NA[d]	NA[d]	1.02 (0.23)	0.74 (0.012)
4.5	TyrPhe + BAd-α-Glc	ND[b]	ND[b]	ND[b]	3.17 (1.06)	1.27 (0.43)
4.5	TyrPhe + BAd-α-Glc + phenanthroline	ND[b]	ND[b]	ND[b]	NM[c]	0.93 (0.20)
4.5	TyrPhe + BAd-α-Glc at 4 °C	2.69 (1.69)	1.08 (0.77)	1.01 (0.37)	1.94 (0.53)	0.72 (0.11)
4.5	Blank	NA[d]	NA[d]	NA[d]	1.91 (0.21)	0.84 (0.14)

[a] Mean values and standard error of the mean in parenthesis ($n = 3$–8).
[b] ND, not detectable.
[c] NM, not measured.
[d] NA, not applicable.
[e] Average flux over 69 h.
[f] Flux of passive I stage interpolated to a membrane electrical resistance of 60 kΩ cm^2 using a log–log linear correlation of passive permeability vs. resistance data with slope −1.04 and $r = 0.67$ for pH 3.0 and 4.5.

higher levels of Tyr and Phe compared to blank, hydrolysis of TyrPhe in the epidermis may be inferred.

The degradation of TyrPhe was quantitatively assessed by incubations with epidermal membrane using the reflection boundary experimental arrangement (Fig. 1). A decrease of TyrPhe concentration was evident, which was stronger at pH 4.5 than at pH 3 (Fig. 3). BAd-α-Glc included as a control showed a marginal concentration change. In separate incubation experiments of BAd-α-Glc with epidermal membrane, generation of <5% benzyl alcohol, the product of glycosidase action on this glucose derivative, in 70 h was found for both pH values, confirming that under these conditions this compound was practically stable. The mass balance of the degradation reaction of TyrPhe can be deduced from the data given in Table 3. In this Table, the absolute decrease of the TyrPhe concentration at the end of the incubation time is compared with the concentration

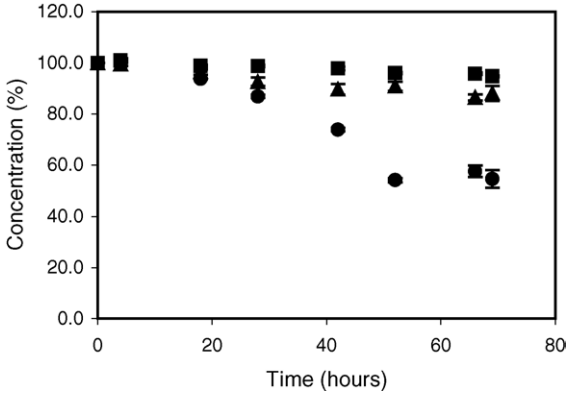

Fig. 3. Concentration of TyrPhe and BAd-α-Glc of enzymatic stability study in % of zero time value under reflection boundary conditions. Points and bars show mean and standard error of the mean, respectively ($n = 4$). Key: (●) TyrPhe pH 4.5, (▲) TyrPhe pH 3, (■) BAd-α-Glc pH 3.

Table 2
Amount of TyrPhe, Tyr, Phe and BAd-α-Glc extracted from the epidermis at the end of permeation experiments for different donor compositions[a]

pH	Donor composition	Amount (μg/skin sample)			
		TyrPhe	Tyr	Phe	BAd-α-Glc
3.0	TyrPhe + BAd-α-Glc	4.96 (0.60)	2.39 (0.71)	2.54 (0.81)	4.97 (1.67)
3.0	TyrPhe + BAd-α-Glc + phenanthroline	10.20 (0.61)	1.20 (0.49)	1.23 (0.63)	21.48 (3.82)
3.0	TyrPhe + BAd-α-Glc at 4 °C	9.40 (1.08)	1.10 (0.32)	0.94 (0.61)	17.90 (3.98)
3.0	TyrPhe	4.23 (1.40)	NM[b]	NM[b]	NA[c]
3.0	BAd-α-Glc	NA[c]	NM[b]	NM[b]	22.43 (4.18)
3.0	Blank (4 and 37 °C)[d]	NA[c]	1.02 (0.50)	0.90 (0.40)	NA[c]
4.5	TyrPhe + BAd-α-Glc	1.66 (0.93)	4.04 (0.87)	3.27 (1.10)	7.48 (1.21)
4.5	TyrPhe + BAd-α-Glc + phenanthroline	0.94 (0.40)	3.90 (1.20)	4.10 (1.60)	12.50 (4.70)
4.5	TyrPhe + BAd-α-Glc at 4 °C	2.80 (0.18)	1.93 (0.65)	1.53 (0.38)	22.63 (5.20)
4.5	BAd-α-Glc	NA[c]	NM[b]	NM[b]	24.10 (5.18)
4.5	Blank (4 and 37 °C)[d]	NA[c]	1.45 (0.50)	1.06 (0.43)	NA[c]
7.2	BAd-α-Glc	NA[c]	NM[b]	NM[b]	22.53 (5.84)

[a] Mean values and standard error of the mean in parenthesis ($n = 3$–11). Statistical analysis is reported in the respective text passages.
[b] NM, not measured.
[c] NA, not applicable.
[d] Average values of both temperatures.

Table 3
Concentration of TyrPhe, Tyr and Phe of the enzymatic degradation study with reflection boundary experimental set-up at 70 h[a]

pH		Concentration (μM)			
		Decrease	Generated with TyrPhe	Blank	Generated blank corrected
3.0	TyrPhe	0.15 (0.034)			
3.0	Tyr		0.99 (0.023)	0.74 (0.035)	0.13 (0.023)
3.0	Phe		0.85 (0.012)	0.69 (0.020)	0.16 (0.012)
4.5	TyrPhe	0.55 (0.044)			
4.5	Tyr		1.53 (0.032)	0.86 (0.043)	0.67 (0.032)
4.5	Phe		1.26 (0.024)	0.66 (0.023)	0.60 (0.024)

[a] Mean values and standard error of the mean in parenthesis ($n = 3$–4).

of Tyr and Phe generated in the solution during the incubation. The concentration of Tyr and Phe generated in the presence of the dipeptide, when corrected for the concentration of the amino acids produced endogenously (blank values), accounted exactly for the decrease of the TyrPhe concentration. TyrPhe was found to be chemically stable under the employed experimental conditions. Also, electrochemical stability was verified after application of a voltage of 250 mV for 8 h to the solution (data not shown). These results provide evidence that degradation of TyrPhe in the epidermis took place by enzymatic hydrolysis. The presence of peptidases in the epidermis has already been reported in the literature (Steinsträsser and Merkle, 1995). Degradation was roughly three-fold greater at pH 4.5 compared to pH 3.

From these results, substantial metabolism of TyrPhe in human cadaver epidermis taking place during permeation can be concluded. The lack of measurable transdermal permeation of this dipeptide is therefore the result of its metabolism in the epidermis. Metabolism is more pronounced at pH 4.5 than at pH 3 probably reflecting a difference of enzymatic activity between these two pH values. This is directly evidenced by the results of the reflection boundary experiment and is consistent with the larger amount of Tyr and Phe recovered from the tissue at pH 4.5 compared to pH 3 (Table 2), even more so when this is related to the recovered amount of the dipeptide. The consistently not detectable permeation at pH 4.5 and the in this respect more variable result at pH 3 can also be ascribed to this difference of enzymatic activity between the pH values, considered on average over all skin specimens.

The metabolism of TyrPhe is potentially catalyzed by enzymes such as dipeptidase, carboxypeptidase and aminopeptidase that have been found in the epidermis (Higo et al., 1992). These are typically metalloenzymes. Therefore, the addition of *o*-phenanthroline, a chelating agent, was investigated as a means to inhibit degradation. At pH 3, it was possible to inhibit enzymatic cleavage of TyrPhe by 0.5 mM *o*-phenanthroline as indicated by the reproducible permeation across epidermis (Table 1), the larger amount of intact dipeptide extracted from the tissue compared to the composition without *o*-phenanthroline and the nearly blank levels of Tyr and Phe extracted from the tissue (Table 2). At pH 4.5, however, the lack of epidermal permeation and elevated tissue levels of Tyr and Phe demonstrate that, at least at this concentration, addition of *o*-phenanthroline could not inhibit hydrolysis of TyrPhe. This appears to be in line with the stronger metabolism at pH 4.5 compared to pH 3 discussed above.

Metabolic degradation of TyrPhe at both pH values was completely suppressed at 4 °C. This is evidenced by the amounts of Tyr and Phe found in the receiver solution and recovered from the epidermis, that were all at the level of the blank runs (Table 2). Furthermore, TyrPhe permeation was measurable in a reproducible fashion (Table 1) and the amount of intact TyrPhe extracted from the tissue was larger compared to the experiments at 37 °C at the same pH. Therefore, iontophoresis of TyrPhe under the present experimental conditions could be studied without interference from cutaneous metabolism only at this low temperature.

The exact values of flux (where available) and epidermal accumulation of TyrPhe as well as the difference of iontophoretic permeation enhancement between the used pH values are discussed elsewhere using appropriate normalization of the data and taking into account the net ionic valence of the dipeptide and the electroosmotic flow (Altenbach et al., 2005). It is shown there that iontophoretic enhancement under constant voltage in an isotonic solution may be too small for practical purposes but provides quantitative information about the factors contributing to iontophoresis. The present results reassert that the epidermis exhibits considerable metabolic activity even under in vitro conditions and can elicit considerable first pass drug metabolism in dermal and transdermal delivery. The relative impact of this metabolism on delivery rate will conceivably diminish as the flux of the permeant increases due to the saturable nature of the enzymatic reaction.

3.3. Iontophoresis of BAd-α-Glc

BAd-α-Glc, a glucose derivative, is used as a reporter of electroosmotic flow in the iontophoresis experiments. This compound is practically unionized at all employed pH values because the pK_a of the acetyl-amino group is typically around unity. Frequently, poly-alcohols such as mannitol being highly hydrophilic and electrically neutral molecules, have been employed for this purpose. In Bad-α-Glc, glucose is substituted with a benzyl group making the compound detectable by UV spectroscopy. The *n*-octanol/aqueous buffer partition coefficient of BAd-α-Glc was found to be 0.110 and 0.133 at pH 4.5 and 3, respectively (Altenbach et al., 2005), confirming that the compound was quite hydrophilic thus warranting its use as electroosmosis marker. Also, BAd-α-Glc is not subject to glucolysis because of its 2-position substitution and is stable towards epidermal glycosidases as shown above. Finally, the acetyl-amino

Table 4
Flux, *J*, of BAd-α-Glc at pH 3.0 and 4.5 for different donor compositions[a]

pH	Donor composition	J ($\times 10^5$ μg/s/cm^2)		
		Passive I	Iontophoresis	Passive I[b]
3.0	BAd-α-Glc + TyrPhe	0.57 (0.35)	2.52 (2.18)	0.39 (0.27)
3.0	BAd-α-Glc + TyrPhe + phenanthroline	0.25 (0.15)	−0.057 (0.055)[c]	0.065 (0.019)
3.0	BAd-α-Glc + phenanthroline	3.67 (2.60)	1.15 (0.76)	0.23 (0.084)
3.0	BAd-α-Glc + TyrPhe at 4 °C	0.064 (0.018)	0.039 (0.047)	0.035 (0.0065)
3.0	BAd-α-Glc	0.40 (0.11)	0.20 (0.32)	0.57 (0.28)
4.5	BAd-α-Glc + TyrPhe	0.15 (0.030)	0.69 (0.25)[c]	0.30 (0.026)
4.5	BAd-α-Glc + TyrPhe + phenanthroline	0.063 (0.012)	0.22 (0.074)[c]	0.043 (0.012)
4.5	BAd-α-Glc + TyrPhe at 4 °C	2.61 (1.48)	2.26 (1.97)	0.36 (0.017)
4.5	BAd-α-Glc	0.75 (0.35)	0.41 (0.16)	0.40 (0.12)
7.2	BAd-α-Glc	4.13 (1.90)	24.9 (5.45)[d]	0.37 (0.10)

[a] Mean values and standard error of the mean in parenthesis ($n = 3$–9).
[b] Flux of passive I stage interpolated to a membrane electrical resistance of 60 kΩ cm^2 using the regression line of Fig. 4.
[c] Iontophoretic flux significantly different than passive flux, $p \leq 0.1$.
[d] Iontophoretic flux significantly different than passive flux, $p < 0.02$ (*t*-test).

group enables the detection of the compound by positive mode electrospray mass spectroscopy.

Steady state passive and iontophoretic fluxes were obtained with the employed experimental protocol (see above) and are reported in Table 4. Passive epidermal permeability of BAd-α-Glc was inversely proportional to the electrical resistance of the membrane regardless of pH as demonstrated in Fig. 4. This behavior has also been observed for other hydrophilic molecules (Kochhar and Imanidis, 2003; Peck et al., 1995). This relationship was used to correct for skin-to-skin variability by referring all passive flux values to a membrane resistance of 60 kΩ cm^2 (Table 4). No difference in the adjusted passive permeability between pH 3, 4.5 and 7.2 was observed within experimental error as would be expected for an unionized compound. The presence of *o*-phenanthroline or TyrPhe individually did not appear to affect passive BAd-α-Glc fluxes while their combination might have caused a reduction of passive flux; the present data basis, however, is not broad enough to allow a definite conclusion in this respect. Also, no systematic effect of temperature could be observed after correction based on the 4 °C electrical membrane resistance.

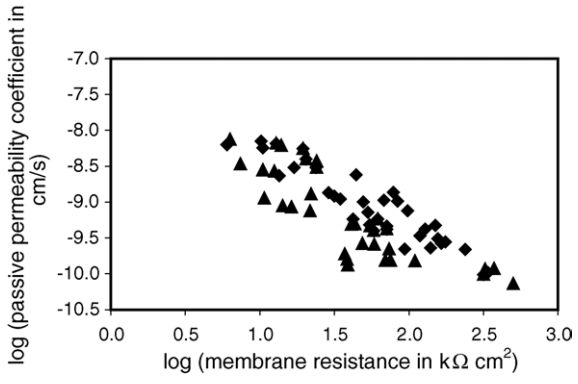

Fig. 4. Correlation between passive permeability of BAd-α-Glc and electrical epidermis resistance. Each point represents data of one diffusion cell. (♦) pH 3.0, (▲) pH 4.5. Linear regression gives a slope of −1.08 and $r = 0.70$ for pH 3.0, and a slope of −1.09 and $r = 0.86$ for pH 4.5.

The effect of iontophoresis on BAd-α-Glc permeation is evaluated by comparing iontophoretic fluxes with the corresponding raw, i.e., unadjusted passive fluxes (Table 4). For BAd-α-Glc used alone, iontophoretic flux at pH 7.2 was significantly greater than the passive one, this being in line with the established notion that at this pH electroosmotic flow and therefore convective transport of solutes takes place in the anode-to-cathode direction due to the negative fixed charges of the epidermal membrane. At pH 4.5 and 3, iontophoretic fluxes seemed to be diminished compared to the passive ones, this trend, however, was not statistically significant. This suggests no electroosmotic flow or, if anything, a slight one taking place in the cathode-to-anode direction at this pH, this being consistent with the proposed isoelectric point of human epidermis of 4.5–5 (Marro et al., 2001; Luzardo-Alverez et al., 1998).

The presence of TyrPhe in the donor solution had a striking effect on the iontophoresis of BAd-α-Glc depending on donor composition. For the compositions BAd-α-Glc + TyrPhe pH 3, BAd-α-Glc + TyrPhe pH 4.5, and BAd-α-Glc + TyrPhe + *o*-phenanthroline pH 4.5, iontophoretic fluxes were several fold greater than passive fluxes (statistical significance shown for the last two compositions) contrary to the result obtained when BAd-α-Glc was used alone at these pH values. For all other compositions at pH 3 and 4.5 of Table 4, no effect or a flux retardation of BAd-α-Glc was achieved by iontophoresis which was statistically significant in one instance. This result is similar to the one obtained when BAd-α-Glc was used alone. It is noteworthy, that especially at pH 3, iontophoretic fluxes of individual experiments varied between positive, negative and zero values reflecting a rise, a reduction and no change, respectively, of the permeant concentration in the receiver compartment. While this variation stems from experimental error, the results do suggest that on average iontophoretic flux did not differ significantly from zero. This is a remarkable result indicating that the magnitude of convective transport due to electroosmosis from cathode to anode could have been such as to abolish the passive flux due to the concentration gradient between donor and receiver compartment.

The difference between the employed donor compositions with respect to the effect of iontophoresis on the flux of BAd-α-Glc was evaluated in light of the fact that TyrPhe was metabolized in the epidermis. For the compositions BAd-α-Glc + TyrPhe pH 3, BAd-α-Glc + TyrPhe pH 4.5, and BAd-α-Glc + TyrPhe + *o*-phenanthroline pH 4.5, which produced an apparent iontophoretic flux enhancement, a hydrolysis of TyrPhe to Tyr plus Phe was found (see previous section) whereas for all other compositions this hydrolysis was inhibited by the low temperature or (at pH 3) by the addition of *o*-phenanthroline. *o*-Phenanthroline itself did not affect the iontophoretic behavior of BAd-α-Glc (Table 4). These results clearly demonstrate that the generation of the metabolic products of TyrPhe, i.e., the amino acids Tyr and Phe, but not the presence of the dipeptide itself must have been responsible for the effect of iontophoresis on the flux of BAd-α-Glc.

A first attempt to interpret the iontophoretic enhancement of BAd-α-Glc observed at pH 3 and 4.5 when TyrPhe was hydrolyzed to Tyr and Phe might involve the convective transport due to electroosmosis which would then have to take place in the anode-to-cathode direction. This would further entail that the amino acids reversed the direction of the electroosmotic flow in this pH range by shifting the isoelectric point of the epidermis towards lower values. This has been reportedly accomplished by negatively charged compounds which bind to the tissue (Hirvonen and Guy, 1998; Kochhar and Imanidis, 2003). Tyr and Phe, however, could not be exerting this effect because they are partly positively charged at the pH range studied here. Further, no possible perturbation of the stratum corneum structure by Tyr or Phe has been reported nor is considered likely for these physiological compounds at levels in the epidermis found to exceed the endogenous ones by a mere factor of 2–3 (Table 2).

A mechanistic understanding of the observed phenomenon may be gained by considering the accumulation of BAd-α-Glc in the epidermis (Table 2). When BAd-α-Glc was used alone, the amount of this compound recovered from the tissue was >20 μg per specimen. This was true at pH 7.2 at which anode-to-cathode electroosmotic flow took place as well as at pH 3 and 4.5 at which no statistically significant effect of electroosmotic flow was noted. For all donor compositions at pH 3 and 4.5 exhibiting no hydrolysis of TyrPhe and showing no effect of iontophoresis on the flux of BAd-α-Glc (including the significant flux retardation observed in one instance), the amount of BAd-α-Glc recovered from the tissue was comparable to that of the experiments involving BAd-α-Glc alone. For the compositions which showed hydrolysis of TyrPhe and iontophoretic enhancement of BAd-α-Glc, the recovered amount of BAd-α-Glc was significantly reduced, i.e., for BAd-α-Glc + TyrPhe pH 3 to 4.97 μg/specimen versus 22.43 μg/specimen, $p < 0.01$, for BAd-α-Glc + TyrPhe pH 4.5 to 7.48 μg/specimen versus 24.1 μg/specimen, $p < 0.02$, and for BAd-α-Glc + TyrPhe + *o*-phenanthroline pH 4.5 to 12.5 μg/specimen versus 24.1 μg/specimen, $p < 0.2$ (*t*-test). It appears, therefore, that in the presence of Tyr and/or Phe generated by the hydrolysis of TyrPhe, a reduction of the amount of BAd-α-Glc retained in the epidermis took place upon iontophoresis. Since the amount of this permeant accumulating in the epidermis is considerable compared to the amount permeating into the receiver solution, a variation of the former can influence the determined flux values. It is shown in a subsequent manuscript that Tyr and Phe added a priori to the donor solution exerted exactly the same effect on tissue retention of BAd-α-Glc as the amino acids generated by hydrolysis of TyrPhe (Altenbach et al., 2005).

These results demonstrate a clear correlation between the apparent iontophoretic enhancement of BAd-α-Glc and the amount of this compound that is recovered from the tissue. It may therefore be suggested that the permeation enhancement of BAd-α-Glc upon iontophoresis for the above three compositions is due to a promoted release of this compound from the epidermis into the receiver compartment and not an anode-to-cathode electroosmotic flow. The results also indicate that the decrease of tissue retention and the associated iontophoretic enhancement are related to the presence of the metabolically generated amino acids Tyr and Phe. Notably, the iontophoretic flux relative to the passive flux, i.e., the apparent iontophoretic enhancement rather than the passive flux itself was found to systematically correlate with the reduced accumulation of BAd-α-Glc in the tissue. Thus, the reduction of the retained BAd-α-Glc amount in the presence of Tyr and/or Phe appears to be induced by the application of the electrical voltage. This reduction of tissue accumulation is obviously not annihilated by a build up in the passive stage succeeding iontophoresis.

The mechanism by which, firstly, BAd-α-Glc is retained in the epidermis and, secondly, this retention is decreased and the apparent permeation enhanced in the presence of Tyr and/or Phe upon epidermal iontophoresis is scantly understood. Nevertheless, some interesting characteristics of the process may be pointed out: (i) the endogenously present Tyr and Phe, representing one-third to one-half of the total amino acid amount recovered from the tissue, do not seem to produce the same effect; (ii) the dipeptide TyrPhe does not produce the effect of Tyr and Phe; (iii) importantly, the decrease of the amount of BAd-α-Glc retained in the epidermis in the presence of Tyr and/or Phe is triggered by iontophoresis; (iv) the release of BAd-α-Glc into the receiver solution relevantly affects the measured permeability because the amount retained in the tissue is considerable compared to the permeating amount of this rather poorly permeable compound; (v) this release outweighs a possible cathode-to-anode convective transport due to electroosmosis taking place in this pH range when it comes to determining the apparent iontophoretic permeation of the compound. The prospective examination of this property of the amino acids for the purpose of confirming it using different permeants is the subject of subsequent investigations (Altenbach et al., 2005).

4. Conclusions

The dipeptide TyrPhe is extensively metabolized in the epidermis. The generated metabolic products Tyr and Phe promote iontophoretic enhancement at pH 3 and 4.5 of the glucose derivative BAd-α-Glc used as an unionized electroosmosis marker. This may be taking place by an interference of the amino acids with the retention of BAd-α-Glc in the epidermal tissue during iontophoresis. The results underline the possibility of perme-

ability interactions in iontophoretic delivery between permeants or the products of their cutaneous metabolism.

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