*International Journal of Pharmaceutics*, 72 (1991) 149–155

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*ADONIS* 037851739100217Z

IJP 02417

# Transdermal permeability and skin accumulation of amino acids

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(Received 5 December 1990)
(Accepted 30 January 1991)

*Key words*: Amino acid; Hairless mouse skin; Permeability; pH; Skin reservoir

## Summary

The permeation coefficients of protein amino acids through full-thickness hairless mouse skin were determined by means of an infinite dose technique. The permeability coefficients were found to be very low and ranged between 1 and $50 \times 10^{-5}$ (cm/h). No significant difference between ionized and unionized transdermal transport was observed. Higher permeation coefficients were clearly attributed to membrane damage caused by alkaline solutions. A significant difference between the permeation of positively and negatively charged molecules was not obvious. The permeability also did not depend on molecular weight or on the hydrophobicity of the amino acids. Hairless mouse skin did not form a reservoir for amino acids.

## Introduction

Increasing interest exists in the administration of proteins and peptides via the transdermal route. However, the hydrophilicity and the large molecular size of such substances as well as the lipophilic nature of the stratum corneum still seem to be the main problems in the use of this route. Previous research was carried out to determine the skin permeation of elastine peptides (Menasche et al., 1981), insulin (Chien, 1982; Siddiqui et al., 1987), vasopressin (Banerjee and Ritschel, 1989), thyrotropin releasing hormone (Burnette and Marrero, 1986), and gonadotropin releasing hormone (Miller et al., 1990). However, so far it appears to be impossible to administer these substances in sufficent amounts without using penetration enhancers or iontophoretic treatment. Even the cosmetic industry is trying to use proteins and peptides to add moisturizing substances to the skin. Skin preparations containing elastin and collagen as potent moisturizing agents have been prepared (Alexander, 1986). The most recent development is a skin preparation containing several amino acids to improve the moisturizing content in the skin. Cosmetic companies such as Biotherm and Phas are marketing these amino acid-containing products.

Nevertheless, the skin permeation of smaller peptides or amino acids has not been studied systematically. The purpose of this investigation is to analyse the transdermal behaviour of amino acids.

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## Materials and Methods

*Amino acids*

Glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, aspartic acid, glutamine, glutamic acid, lysine, arginine, histidine, tryptophane, phenylalanine, tyrosine, proline, methionine and cysteine were purchased from Serva (Heidelberg, Germany). All were reagent grade and were used without further purification. $^{14}C$-labelled amino acids were obtained from Amersham Buchler (Braunschweig, Germany) and were used as received. [$^{14}C$]Cysteine was purchased from Dupont (Dreieich, Germany).

*Buffers*

The isotonic buffers shown in Table 1 were used. All buffers were prepared to be isoosmotic using analytical grade chemicals obtained from Merck (Darmstadt, Germany) and distilled water.

*Hairless mouse skin*

17–22-week-old female hairless mice, strain hr/hr -$c_3H$/Tif Bom (Bommice, Bomholtgard Breeding and Research Center Ltd, Ry, Denmark) were used for the permeation studies. They were killed with $CO_2$. Both abdominal and dorsal skin was used after careful excision and cleaning from the subcutaneous tissue.

*In vitro skin permeation*

Full-thickness freshly excised hairless mouse skin was mounted in a two-chamber, side-by-side diffusion cell with the stratum corneum facing the donor half cell. The cell was made of glass. Both donor and receiver compartment had a volume of 1.5 ml. The surface area of the membrane of the diffusion cell was 0.8 $cm^2$. The cell was then immersed in a constant temperature waterbath of $37 \pm 1°C$. The donor chamber was charged with 1.5 ml of an amino acid solution in the actual buffer. These solutions contained 10 mg/ml of the respective amino acid with the exception of aspartic acid and tyrosine. The latter amino acids were used as saturated solutions. The solution was spiked with radiolabelled amino acids (3.3 $\mu$Ci/ml). The receiver compartment was filled with 1.5 ml of the respective buffer. The contents of the diffusion cell were allowed to equilibrate and were stirred at 100 rpm. The moment of charging the donor half cell marked the beginning of the diffusion run. Before taking a sample of the donor compartment, 4 min were allowed to elapse to ensure achievement of a uniform concentration. This first sample was used as an estimation of the initial donor cell concentration. At specified time intervals, samples of 200 $\mu$l were withdrawn from the receiver compartment. An equivalent amount of the respective buffer solution (200 $\mu$l) was added to maintain a constant volume. The resulting dilution of the receiver compartment was taken into account when evaluating the permeation data. The samples were transferred immediately into vials containing 7 ml scintillation cocktail (ready protein + , Beckman Instruments, München, Germany) and the amount of the radioactivity (disintegrations per min, dpm) was determined using a liquid scintillation counter (Model LS 1501, Beckman Instruments, München, Germany).

*Data analysis*

In the present studies, the permeation rates were assayed for the respective amino acids at two different pH values. One pH value was adjusted at the isoelectric point of the respective amino acids and the other at the physiological pH of 7.4. The theory underlying the calculation of permeability coefficients has been previously discussed in detail (Flynn et al., 1974; Albery and Hadgraft, 1979; Flynn, 1985). Since sink conditions were maintained throughout each experiment, the equation derived from Fick's law can be used for the calculation of the permeability coefficients. The per-

TABLE 1

*Buffer compositions*

| Buffer Components | pH |
|---|---|
| $NaH_2PO_4$/ $Na_2HPO_4$ | 5.2; 5.4; 5.6; 5.7; 6.0; 6.2; 7.4; 7.6 |
| $Na_2B_4O_7$/ $H_3BO_3$ | 9.8; 10.8 |
| $KH_2PO_4$/ HCl. conc. | 2.8 |

meability data were plotted as amounts of permeant (dpm) collected in the receiver compartment as a function of time. The permeability coefficient for a given run was calculated from the pseudo-steady-state portion using:

$$J_T = PA\,\Delta C \quad (1)$$

where $J_T$ is the total flux (dpm $h^{-1}$), $P$ the permeability coefficient (cm/h), $A$ the diffusional area ($cm^2$), and $\Delta C$ the concentration differential across the membrane. Since $J_T = V(dc/dt)$, the permeability coefficient can be calculated from:

$$P = \frac{V(dc/dt)}{A\,\Delta C} \quad (2)$$

where $V$ represents the half cell volume ($cm^3$) and $dc/dt$ is the steady-state slope in dpm $h^{-1}$ $cm^{-3}$.

*Skin reservoir*

This study was designed to allow the further estimation of an eventual skin reservoir of amino acids. Therefore, the skin samples from each run were assayed. After the end of each experiment, the skin patches were removed carefully from the top of the half cells and immersed in distilled water. After several washings, the skin patches were dried with pieces of cellulose and were placed in glass vials containing 1 ml tissue solubilizer BTS-450 (Beckman Instruments, München, Germany). The samples were kept at 60°C until they were completely dissolved. The samples were decolorized with 50 μl $H_2O_2$ 30% (Merck, Darmstadt, Germany). Subsequently, 70 μl neat acetic acid (Merck, Darmstadt, Germany) were added to eliminate chemiluminiscence. After addition of 10 ml ready organic cocktail (Beckman Instruments, München, Germany) the amount of radioactivity was determined by scintillation counting.

## Results and Discussion

Our experiments were designed to distinguish between charged and uncharged amino acid transport across hairless mouse skin. For this reason, the transport of amino acids was determined at the respective isoelectric points and at physiological pH 7.4. Table 2 shows the permeation coefficients for each amino acid at the two pH values. Fig. 1 depicts the permeation coefficients of the amino acids measured at pH 7.4. Arginine shows a relatively high permeability, followed by alanine and glutamine, whereas asparatic acid, isoleucine and threonine exhibit lower (factor 2–5) permeabilities. Fig. 2 shows the permeation coefficients of the amino acids measured at their respective isoelectric points. Here, lysine, arginine and phenylalanine are the most permeable substances (factor 7–10) with respect to the other amino acids. Glycine, threonine and asparatic acid are also more permeable (factor 2–3) than the other amino acids. Most of the permeation coefficients of the various amino acids are basically of the same order of magnitude ($2–5 \times 10^{-5}$ cm/h). These orders are approximately 100–1800 fold smaller than the values observed for $n$-alkanols (methanol-octanol: $2–90 \times 10^{-3}$ cm/h) by Dürrheim et al. (1980). The amino acid permeabilities, however, are in good agreement with data obtained by Banerjee and Ritschel (1989) for vasopressin permeability through rat skin or by Ackermann and Flynn (1987) for other hydrophilic compounds like glucose, urea, glycerol and thiourea through the skin of nude mice.

Three theories are mainly discussed to explain the mechanism of peptide transport through the skin: (1) the pH-partition hypothesis favouring the absorption of the unionized species (Swarbrick et al., 1984); (2) the hypothesis of Siddiqui et al. (1985), who suggest that both unionized as well as ionized species can penetrate the skin; and (3) the possibility of skin permeation by ion pair formation (Barker and Hadgraft, 1981; Siddiqui et al., 1985).

According to the pH-partition hypothesis, drug permeation through predominantly lipophilic membranes by passive diffusion is greater for the unionized species compared to the ionized species (Swarbrick et al., 1984). Our experiments show that this hypothesis holds true for most amino acids with the exception of alanine, leucine, glutamine, methionine and histidine. In the previously mentioned study with vasopressin, Banerjee

TABLE 2

*Permeability coefficients of amino acids determined at physiological pH value of 7.4 and at the pH value of the respective isoelectric points*

| Amino acid | pH value | Permeability coefficient ($\times 10^{-5}$) (cm/h) $x$ | Mean of experiments $n$ | SD (%) ±SD | |
|---|---|---|---|---|---|
| Glycine | pH 6.0 | 11.9 | 3 | 33.1 | ** |
| | pH 7.4 | 3.8 | 5 | 14.0 | |
| Alanine | pH 6.0 | 3.7 | 4 | 36.8 | ns |
| | pH 7.4 | 5.5 | 3 | 16.2 | |
| Serine | pH 5.6 | 3.6 | 5 | 23.2 | ns |
| | pH 7.4 | 3.0 | 4 | 28.7 | |
| Proline | pH 6.3 | 3.3 | 3 | 33.6 | ns |
| | pH 7.4 | 2.7 | 3 | 37.9 | |
| Valine | pH 6.0 | 4.5 | 3 | 20.8 | ns |
| | pH 7.4 | 1.3 | 3 | 27.8 | |
| Threonine | pH 6.2 | 11.8 | 3 | 12.8 | ** |
| | pH 7.4 | 1.3 | 3 | 31.2 | |
| Isoleucine | pH 6.0 | 5.2 | 4 | 18.3 | ** |
| | pH 7.4 | 1.3 | 4 | 29.7 | |
| Leucine | pH 6.0 | 1.6 | 3 | 33.2 | ns |
| | pH 7.4 | 2.9 | 3 | 44.3 | |
| Asparagine | pH 5.4 | 4.1 | 3 | 24.5 | ns |
| | pH 7.4 | 3.5 | 4 | 33.5 | |
| Asparatic acid | pH 2.8 | 8.6 | 4 | 25.4 | ** |
| | pH 7.4 | 0.8 | 3 | 11.9 | |
| Glutamine | pH 5.6 | 3.2 | 3 | 31.0 | ns |
| | pH 7.4 | 5.0 | 3 | 22.4 | |
| Lysine | pH 9.8 | 38.9 | 3 | 23.3 | ** |
| | pH 7.4 | 2.1 | 3 | 9.3 | |
| Glutamic acid | pH 2.8 | 4.9 | 3 | 27.1 | ** |
| | pH 7.4 | 1.0 | 3 | 21.5 | |
| Methionine | pH 5.6 | 1.7 | 3 | 42.9 | * |
| | pH 7.4 | 3.1 | 4 | 16.9 | |
| Histidine | pH 7.6 | 1.6 | 3 | 37.7 | ns |
| | pH 7.4 | 2.0 | 3 | 33.2 | |
| Phenylalanine | pH 5.4 | 24.4 | 4 | 13.8 | ** |
| | pH 7.4 | 3.0 | 4 | 3.0 | |
| Arginine | pH 10.8 | 32.6 | 3 | 25.7 | ns |
| | pH 7.4 | 10.0 | 4 | 26.7 | |
| Tyrosine | pH 5.6 | 2.6 | 3 | 44.8 | ns |
| | pH 7.4 | 1.6 | 3 | 6.1 | |
| Tryptophan | pH 5.7 | 1.9 | 4 | 27.2 | ns |
| | pH 7.4 | 1.5 | 4 | 31.8 | |
| Cysteine | pH 5.2 | 3.4 | 3 | 16.0 | * |
| | pH 7.4 | 1.9 | 3 | 14.6 | |

Levels of significance between permeability determination at the respective isoelectric point and at pH 7.4 according to Student's $t$-test: ns, not significant; * significant ($p \ll 0.05$), ** highly significant ($p \ll 0.01$).

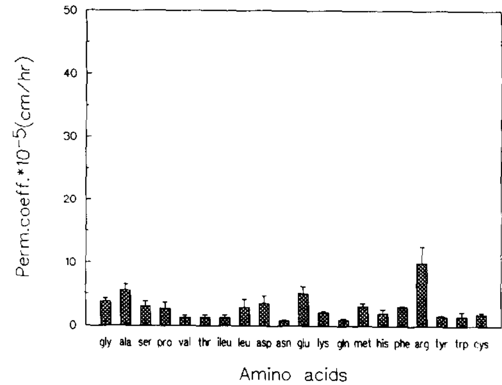


Fig. 1. Permeation coefficients of the various amino acids measured at pH 7.4 (mean of 3–5 experiments ± SD).

and Ritschel (1989) explained the diminished permeation of vasopressin at pH values close to its isoelectric point by an increasing aggregation tendency of this peptide. Ritschel and Ritschel (1984) and Toniolo et al. (1985) also found increasing self-association of peptides corresponding to the decreasing solubility at the isoelectric point. These findings together with our observations in the present study show that, due to self-association or other interactions in the case of amino acids or peptides, the unionized species does not always yield the highest permeability through membranes such as skin.

So far, it is very difficult to specify the actual penetration mechanism. Arginine and lysine show a significant increase in permeability by a factor

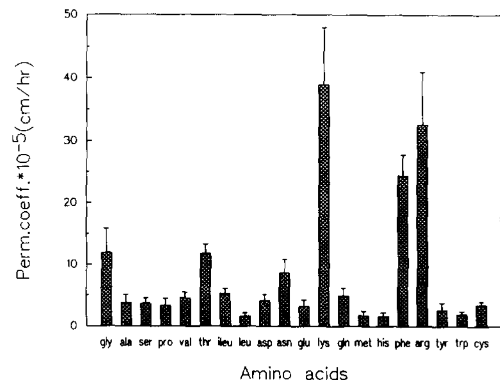


Fig. 2. Permeation coefficients of the various amino acids measured at their respective isoelectric points (mean of 3–5 experiments ± SD).

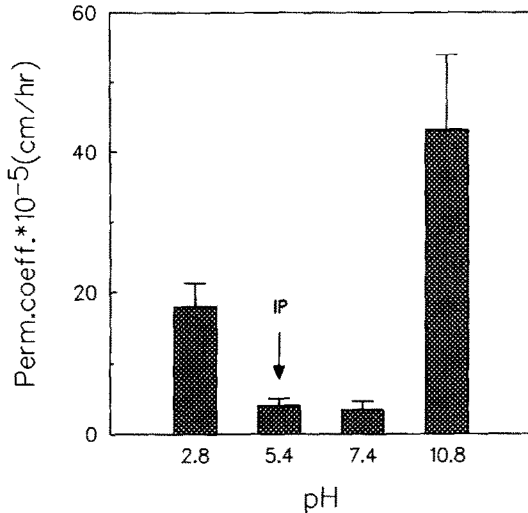


Fig. 3. Effect of different pH values on the permeation of asparagine through hairless mouse skin (mean of 3–5 experiments ± SD).

of 7–10 at their isoelectric points. This is attributed to the relatively high pH values at these isoelectric points. Scheuplein (1965) found that alkaline solutions destroy the skin membrane. Our pH profile of asparagine further strengthens this possibility. Fig. 3 clearly demonstrates the significant increase in permeability at very high pH values. Phenylalanine, an aromatic, relatively hydrophobic amino acid, is the only other amino acid which permeates quite well at its isoelectric point even without any enhancement by alkaline solutions. An explanation for this greater permeability was not found.

Closer examination of the permselective properties of the skin (Burnette and Ongipipattanakul, 1987) shows that positively charged molecules permeate more favorably as long as the skin has a net negative charge at physiological pH values. This would explain the relative high permeation coefficient in comparison to the other amino acids of the positively charged arginine at pH 7.4. However, it does not explain the considerably lower permeation coefficient of lysine, the only other amino acid that is positively charged at pH 7.4. According to the assumption that positively charged species penetrate the skin better than neutral or negatively charged species, the permeation coefficient of lysine should be of the same order of that of arginine.

In a further attempt to explain the mechanisms of amino acids permeating through the skin, the amino acids were arranged in different groups according to their chemical properties. Arrangement according to molecular weights exhibited only a singular relationship, namely an inverse relationship between permeation coefficients and

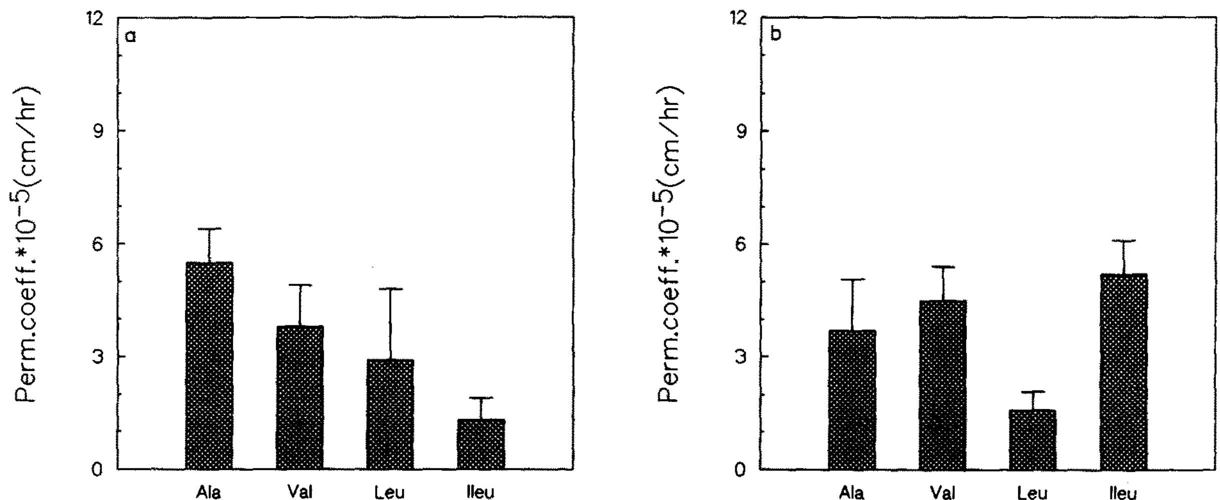


Fig. 4. Permeation of aliphatic amino acids through hairless mouse skin measured (a) at pH 7.4, (b) at the pH values of the respective isoelectric points and arranged in increasing molecular weight (mean of 3–5 experiments ± SD).

molecular weight within the aliphatic group (Fig. 4). Since a similar relationship is observable neither at the respective isoelectric points (Fig. 4) nor with any other amino acid group, we conclude that the relationship between molecular weight and permeability at pH 7.4 seems to be incidental. Scheuplein et al. (1969) confirmed that within a narrow range there is slight correlation between the size of a molecule and the penetration rate because the diffusion constants through hydrated stratum corneum are similar with many low molecular mass compounds. Siddiqui (1989) also suggested that molecular masses in the range of 90–250 Da do not have any significant impact on skin permeation coefficients. A direct correlation between increasing lipophilicity and increasing permeation rates as in the case of the aliphatic alcohols (Scheuplein, 1965) is also not observable for amino acids. The amino acid data agree with those obtained by Ackermann and Flynn (1987) for urea, thiourea, glycerol and glucose. Ackermann and Flynn also could not find a correlation between permeability and ether-water partition coefficients.

*Epidermal reservoir*

The epidermis is a continuous, stratified keratinizing epithelium. Up to two thirds of the total mass consists of keratin filaments which differ widely in molecular weight. However, their chemical properties are very similar. In most species the amino acid compositions are similar in that the filaments contain low amounts of tryptophan and cysteine but relative high contents of serine, glutamic acid, and glycine (Table 3).

As mentioned in the Introduction, there is increasing interest in enhancing the amount of these substances as a part of the NMF (natural moisturizing factor) within the skin. Consequently, a reservoir formation for amino acids would be very helpful for the development of moisturizing creams. Table 3 clearly shows that in our study a considerable accumulation of amino acids in the skin is not observable. The investigations of Malkinson and Ferguson (1955) and Vickers (1963) were the first to suggest the possibility of reservoir formation for certain drugs within the skin. Substances with high diffusivities and high solubilities in the stratum corneum are good canditates for the build-up of a depot. In general, those physico-chemical factors that promote percutaneous absorption also potentiate reservoir formation. The hydrophilic amino acids do not possess a high solubility in the predominantly hydrophobic stratum corneum. As our results show, they also do not associate to a major degree with the keratinous skin elements.

TABLE 3

*Mean ( ± SD) amounts in % of the initial total radioactivity of the amino acids determined in the respective skin patches after each run (9 h)*

| Amino acid | Amount in skin (%) | Amino acid composition of skin keratin (%) |
|---|---|---|
| Glycine | 0.04 ± 0.01 | 26.9 |
| Glutamic acid | 0.13 ± 0.08 | 14.3 |
| Serine | 0.05 ± 0.02 | 12.4 |
| Aspartic acid | 0.03 ± 0.02 | 7.7 |
| Leucine | 0.05 ± 0.04 | 7.0 |
| Alanine | 0.03 ± 0.02 | 4.2 |
| Arginine | 0.09 ± 0.05 | 3.9 |
| Lysine | 0.07 ± 0.05 | 3.6 |
| Threonine | 0.05 ± 0.02 | 3.1 |
| Tyrosine | 0.11 ± 0.07 | 2.7 |
| Phenylalanine | 0.13 ± 0.03 | 2.7 |
| Valine | 0.05 ± 0.03 | 2.5 |
| Isoleucine | 0.04 ± 0.02 | 2.1 |
| Methionine | 0.04 ± 0.02 | 1.8 |
| Histidine | 0.07 ± 0.06 | 1.2 |
| Proline | 0.11 ± 0.07 | 1.1 |
| Half-cysteine | 0.09 ± 0.04 | 0.8 |
| Tryptophan | 0.06 ± 0.02 | 0.6 |

The initial activity for all amino acids was normalized to $10^{-7}$ dpm. In the second column, the amino acid composition of the skin keratin in % is also given source: Goldsmith (1983).

## Conclusions

The permeability coefficients of amino acids were found to be essentially in the same order ($2–5 \times 10^{-5}$ cm/h). Ionization of amino acids did not significantly influence the permeability. A difference between the permeability of positively and negatively charged amino acids also was not obvious. Because of the relatively low range of molecular weights of the amino acids, a relationship

between permeability and molecular weight of these amino acids was not observable. All these results and the fact that the hydrophobicity did not correlate with permeability lead to the assumption that the skin does not act as a simple lipoidal barrier. It seems likely that amino acids mainly permeate through hydrophilic and water-filled regions. Due to their low permeability coefficients, amino acids cannot be administered transdermally in significant amounts without penetration enhancers or iontophoretic treatment.

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