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In vitro permeation of human skin by multipolar ions

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Summary

The percutaneous absorption at various pH values of aspartic acid, histidine, and lysine was studied as models of multipolar ions. For all three amino acids and all pH values studied, the permeabilities were in the range of $1.2–4.7 \times 10^{-8}$ cm s^{-1}. The time lags were of the order of 20–30 h. While these data for fluxes are consistent with a weakly selective porous mechanism of transport, the diffusion constants, which are typical for permeation of nonionic compounds through stratum corneum, are quite small and suggest extensive restriction, tortuosity or binding.

Introduction

Charged species are believed to be very poor penetrants across skin, other biological membranes and nonporous polymers. The permeation coefficient has been estimated to be 10^4 times smaller than for respective uncharged species (Swarbrick et al., 1984).

Percutaneous absorption of inorganic electrolytes through the skin has been extensively studied (Wahlberg, 1968; Lansdown, 1973; Boddé et al., 1991). However, passive transport of organic electrolytes across skin has been largely neglected and such investigations would be central to the transdermal or iontophoretic delivery of peptides or other multipolar ionic drugs (Wearley et al., 1990; Green et al., 1991; Ruland and Kreuter, 1991; Mazzenga et al., 1992). Amino acids are not only model organic electrolytes, but are also used in skin care products with the purpose of increasing water holding capacity of stratum corneum by analogy to natural moisturizing factor which also contains amino acids. Systematic studies on their penetration had not been performed until recently when Ruland and Kreuter (1991) measured the in vitro permeability through and accumulation in hairless mouse skin of 20 amino acids. Other investigators (Wearley et al., 1990; Green et al., 1991) examined the iontophoretic transport of amino acids and passive transport was treated as a reference state. Dramatic increases in skin transport of phenylalanine and other zwitterions from aqueous ethanolic donor solutions have also been achieved by forming salts (Mazzenga et al., 1992). A porous

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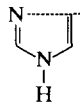
TABLE 1

Physicochemical characteristics of the amino acids studied

Amino acid	Molecular weight	Solubility [a] (g/100 ml)	Log P [c]	pI [d]
Aspartic acid $HOOC-CH_2-CH(NH_2)COOH$	133.1	0.5	−3.47	2.77
Histidine 	155.2	4.3 [b]	−2.90	7.59
Lysine $NH_2-(CH_2)_3-CH(NH_2)COOH$	146.2	very freely	−3.05	9.74

[a] Solubility in water at 25°C at pI (Green, et al., 1991).
[b] Handbook of Chemistry and Physics (1991).
[c] Octanol/pH 7.4 Hepes buffered saline distribution coefficient (Green, et al., 1991).
[d] Isoelectric point (Jakubke and Jeschkeit, 1977).

pathway of the transport has been proposed for these data.

In the present study, three amino acids, aspartic acid, histidine, and lysine, were selected as models of multipolar ions and their percutaneous absorption was investigated at various pH values. The values of pH were selected to be either close to the isoelectric points (pI) or to be sufficiently different from the pI that the dominant species were charged. Table 1 presents physicochemical characteristic of the compounds under investigation, and Table 2 shows the pH values at which permeation was studied and the degree of ionization.

TABLE 2

Percent ionized of the amino acids

Amio acid	pH	Net charge				
		+2	+1	0	−1	−2
Aspartic acid	3.4	–	2.93	62.14	34.93	~0
	7.3	–	~0	~0	99.50	~0
Histidine	5.0	~0	90.91	9.09	~0	–
	7.3	~0	4.77	93.96	1.27	–
	8.5	~0	~0	82.40	17.60	–
Lysine	7.3	~0	97.81	2.19	~0	–
	8.9	~0	52.88	46.04	1.08	–

The percents ionized were calculated using the following equation: % ionized = $100/[1 + \text{antilog}(pK_a - pH)]$ (Florence and Attwood, 1988).

Materials and Methods

Reagents

L-Aspartic acid, DL-histidine and L-lysine with declared purity at least 97% were purchased from Aldrich Chemical Co. (Milwaukee, WI). Gentamicin sulfate and sodium phosphate were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium chloride, sodium hydroxide, and the other constituents of buffers – potassium hydrogen phthalate and magnesium chloride, were obtained from Fisher Scientific (Springfield, NJ). Mallinckrodt Inc. (Paris, KY) was the source of hydrochloric acid. All chemicals were reagent grade and used as received.

Tritium labelled amino acids were purchased from NEN Research Products (DuPont Co., Boston, MA): L-[2,3-^{3}H]aspartic acid and L-[*ring*-2,5-^{3}H]histidine with declared radiochemical purity 99%, L-[4,5-^{3}H]lysine with purity 97.8%. Since tritiated water was formed during storage, the radiochemicals were evaporated to dryness in a stream of nitrogen, directly prior to preparation of the experimental solutions. Tritiated water with specific activity 1 mCi/g was obtained from NEN

Research Products. Small quantities of [^{14}C]lysine and [^{14}C]histidine were obtained from NEN as a check on the measurements with ^{3}H-labelled amino acids.

Amino acids and buffer solutions

The following aqueous solutions of amino acids were prepared: 0.3% aspartic acid solutions of pH 3.4, 0.5% solution of aspartic acid of pH 7.3, 1.0% solutions of histidine of pH 5.0, 7.3 and 8.5 and 1.0% solutions of lysine of pH 7.3 and 8.9. The amino acids were weighed and dissolved in sterile water, 0.5 mg/ml of gentamicin sulfate was added, and the pH was adjusted with either 1 or 0.1 M of either NaOH or HCl. The resulting solution was filtered through a 0.45 μm pore size sterile syringe filter (Corning Glass Works, Corning, NY) and stored at +4°C for no longer than 1 month. Radiolabelled amino acids were dissolved in these solutions to obtain approx. 10 μCi/ml.

The receiver solution was 0.9% NaCl solution to which 0.05% gentamicin was added in order to prevent bacterial growth. The pH was adjusted to 7.3 with 0.1 M NaOH and the saline was filtered using a 0.2 μm sterilization filter unit (type S, Nalge Co., Rochester, NY). Steady-state fluxes have been demonstrated to be stable over 4 days under these conditions.

For analytical procedure, pretreatment and water permeation studies, the following buffers were prepared: pH 3.4 and 5.0, 0.05 M phthalate buffers; pH 7.3, 0.05 M phosphate buffer; and pH 8.9, 0.01 M borate buffer. Sodium chloride was used to make them isotonic, and 0.1% gentamicin was added. For pretreatment experiments and for water permeation studies, the buffers were diluted four times with sterile water to obtain hypotonic solutions of tonicity similar to that of the aqueous solutions of amino acids. As an eluant for separation of amino acids from tritiated water on an ion exchange column 0.01 M phthalate buffer pH 5.0 was employed.

Permeation studies

Dermatomed human cadaver skin obtained from the thigh (500 μm thickness) was stored at −25°C. Just prior to the experiment, the skin was thawed at room temperature. Skin from four individuals (age 18–55 years) was used.

The skins were mounted in two-chamber glass diffusion cells (type LG-1084-SPC, Lab. Glass Apparatus Inc., Berkeley, CA). The penetration area was 1.0 cm^2, the volume of the receiver chamber was 3 ml, and the receiver solution was isotonic saline. The temperature of the receiver chamber was maintained at 37°C, and the fluid was stirred continuously with a magnetic stirrer (approx. 300 rpm). A period of equilibration of 5 h was allowed and then the receiver solution was replaced with fresh solution and 300 μl of the

TABLE 3

Transport parameters for amino acids [a]

	Aspartic acid		1% Lysine		1% Histidine		
	0.5%	0.3%					
pH:	7.3	3.4	7.3	8.9	5.0	7.3	8.5
Charge:	(−)	(+ −)	(+)	(+ −)	(+)	(+ −)	(+ −)
C_{ss} (mg cm^{-3})	4.93	2.93	9.60	9.06	9.90	9.80	9.90
J_{ss} (mg cm^{-2} h^{-1})	0.47 ± 0.31	0.40 ± 0.12	1.62 ± 1.0	4.80 ± 1.0	0.43 ± 0.12	0.56 ± 0.19	0.44 ± 0.29
K_p (cm s^{-1})(×10^{-8})	2.60 ± 1.8	3.70 ± 1.1	4.70 ± 2.8	13.80 ± 3.2	1.24 ± 0.32	1.54 ± 0.54	1.25 ± 0.82
t_L (h)	36.7 ± 5.1	30.6 ± 6.7	26.0 ± 8.1	23.0 ± 4.8	21.0 ± 2.5	21.5 ± 4.0	19.5 ± 1.2
D (cm^2 s^{-1})(×10^{-11})	3.15 ± 0.44	3.78 ± 0.83	4.40 ± 1.40	5.0 ± 1.0	5.51 ± 0.66	5.40 ± 1.0	5.93 ± 0.36

[a] All parameters are presented as mean values ± S.D.

J_{ss}, steady state-flux; K_p, apparent permeability coefficient; D, apparent diffusion constant calculated from $D = l_s^2/6t_L$, where l_s is the thickness of the barrier and 50 μm was estimated for the thickness of hydrated stratum corneum.

radioactive amino acid solution was applied onto the skin. The donor side of the cell was covered by Parafilm (American National Can., Greenwich, CT). At 14, 24, 38, 48, 62, 72, and 86 h, the receiver fluid was entirely removed and replaced with fresh saline that had been preequilibrated to 37°C. The sampled receiver solutions were subjected to the appropriate analytical procedure to measure the concentration of the penetrant. The values of pH of the donor solutions were recorded (Micro pH Electrode PHM-146, Lazar Research, Los Angeles, CA) during the experiment in reference diffusion cells in which unlabelled solution was placed on the skin surface. The values of pH of these solutions remained stable within ± 0.2 units throughout the experiments.

The thickness of hydrated stratum corneum of 50 μm, the value used in Table 3, was based on a measured weight of 5 mg/cm^2 for hydrated stratum corneum, and the assumption of unit density. This uptake of water is consistent with the literature (Anderson et al., 1973).

Penetration of histidine at pH 5.0 and 7.3 was studied after skin pretreatment with a buffer of the same pH and tonicity as the amino acid solution. The skin area was exposed for 24 h to 500 μl of the buffer under occlusion (Parafilm), and after carefully removing all of this solution, the permeation experiment was initiated as previously described.

Percutaneous absorption of tritiated water

Into 300 μl of the respective buffer (pH 3.4, 5.0, 7.3, and 8.9), 3 μl tritiated water was added, and the solution was applied to the donor side of the skin. The experiments ($n = 6$) were conducted utilizing flow-through cells with 0.9% NaCl receiver solution that was pumped continuously at a flow rate of 1.5 ml/h. During the 86 h experiment fractions were collected every 3 h by a fraction collector (Retriever III, ISCO Inc., Lincoln, NE) and the amounts of radioactivity in the fractions were measured. Evaporation was negligible during this period. The donor compartment was completely replaced with fresh solution every 10 or 14 h.

Permeation of tritiated water remained at the same level for the duration of the 86 h experiment. pH did not influence the absorption rate ($p < 0.05$, ANOVA test). The barrier was intact since the measured K_p for water was $1.5–3.0 \times 10^{-3}$ cm h^{-1}, which is within the limits suggested by Bronaugh and Stewart (1986).

Analytical

The contributions of the radioactive amino acids were measured after separation of in situ formed tritiated water by the following procedure:

An ion exchange column (1×7 cm) was prepared using mixed bed resin AGR 501-X8 (D) (Bio-Rad Lab, Richmond, CA). The collected receptor fluid fractions were adjusted to pH 5 with 1 M HCl. The volume of 0.5 ml was placed on the column, and the elution of water from the column performed with 0.025 M phthalate buffer pH 5.0 until no radioactivity was detected in the eluent. Approx. 9 ml of the buffer was used. The total radioactivity of the 0.5 ml sample and the radioactivity eluted from the column were measured; the amount of the radiolabelled amino acids in the sample was calculated as the difference between these two values. Tritiated water (300–30 000 dpm) and standard solutions of amino acids (300–30 000 dpm) were placed on the column in order to estimate accuracy and reproducibility of this separation technique. The assay was repeated 10 times. In 10 experiments when tritiated water was eluted from an ion exchange column, $96.4 \pm 3.2\%$ of the initial radioactivity was recovered in the eluent. When ^{14}C-amino acids were dissolved in buffer solution at pH 5.0 and placed on the column, no significant radioactivity was found in the eluent. Typical contents of tritiated water ranged from 0.5–80% of the radioactivity in the receiver and donor samples. To substantiate these measurements, samples of selected receiver and donor solutions were also evaporated to dryness, and the radioactivity in the residue was measured. The mean ratio ($n = 43$) of the radioactivity measured in the residue to that by the ion exchange column method was 0.99 ± 0.23, but there was a trend for this ratio to increase with the content of tritiated water in the sample.

The amount of radioactivity (disintegrations

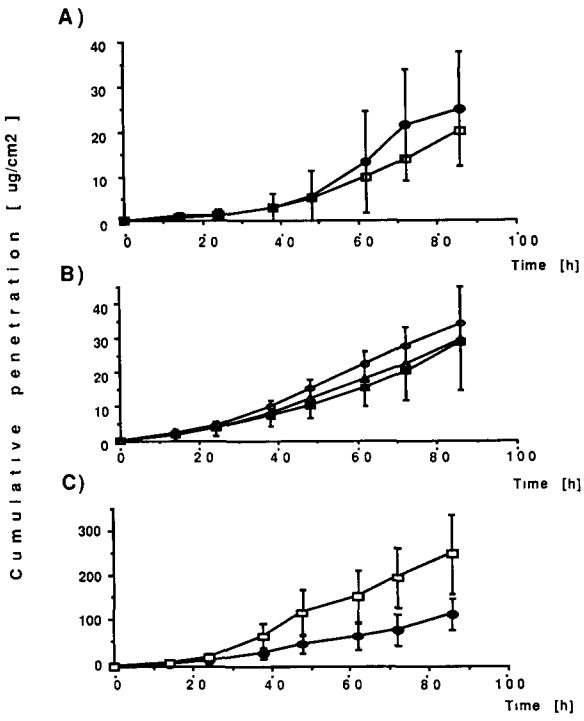


Fig. 1. (A) Aspartic acid penetration ($n = 7$) from 0.3% aqueous solution at pH 3.4 (□) and from 0.5% solution at pH 7.3 (●). (B) Histidine penetration ($n = 6$) from 1.0% aqueous solutions at pH 5.0 (○), pH 7.3 (■), and at pH 8.5 (△). (C) Lysine penetration from 1.0% aqueous solutions at pH 7.3 (●, $n = 5$) and pH 8.9 (□ $n = 5$); means ± SD.

per minute, dpm) was determined using a liquid scintillation counter (Tri-Carb 4640, Packard, Downers Grove, IL). Universol TM ES (ICN Biomedicals, Irvine, IL) was employed as scintillation liquid.

ANOVA and Student's t-test were used for statistical analyses of the data.

Results

Skin penetration profiles of the three amino acids from aqueous solutions at various pH values are presented in Fig. 1. No statistical ($p < 0.05$, ANOVA) differences in the rates of penetration (Table 3) were observed for aspartic acid or histidine throughout the pH range of 3.4–8.5. However, at pH 8.9, the steady-state flux of lysine was greater than at pH 7.3 ($p < 0.05$). The transport parameters are presented in Table 3. The lag times observed were 19.5–21.5 h for histidine, 23–26 h for lysine, 31–37 h for aspartic acid, and were independent of pH ($p < 0.05$, t-test).

Equilibration of the skin for 24 h with an appropriate buffer did not change the penetration profile of histidine. At pH 5.0 the flux was found to be 0.36 ± 0.21 $\mu g\ cm^{-2}\ h^{-1}$ and t_L 20.8 ± 1.0 h ($n = 5$), while at pH 7.3 flux was 0.41 ± 0.12 $\mu g\ cm^{-2}\ h^{-1}$ and t_L 18.5 ± 2.4 h ($n = 6$). These values did not differ significantly ($p < 0.05$, t-test) from those obtained in the experiments when pretreatment was not performed.

Discussion

Under the conditions of this study, the passive transport of ionized species across skin was observed. The calculated apparent permeability coefficients for all three amino acids were in the same range: $1.2–4.7 \times 10^{-8}$ cm s^{-1}. The values given by Wearley et al. (1990) for glycine, alanine, leucine and valine were $1.1–5.2 \times 10^{-8}$ cm s^{-1}. Kreuter and Ruland (1991) studied 20 amino acids and the resulting permeability coefficients for all of them were within the range $0.2–10 \times 10^{-8}$ cm s^{-1}. Although these values are in agreement with the current results, the duration of the prior experiments was quite short, e.g., the experiment described by Wearley et al. was conducted for 12 h. The time lags in the present study were at least 19 h. In contrast to this study, in the two other works hairless mouse skin had been used. This may be the reason for the shorter lag time (Wearley et al., 1990).

Ionization of the studied compounds did not influence permeability. The permeabilities of these multipolar ions did not differ significantly from those of the charged species. The same flux was obtained for histidine at pH 5.0 when the compound was positively charged, as at the same concentration at pH 8.5 when the net charge was negative. A similar conclusion had been reached by Ruland and Kreuter (1991). Permeation coefficients for the particular ions calculated on the

basis of ionization of molecules (Table 2) and apparent fluxes are as follows: aspartic acid charge (0) -4.63 and charge (-1) -2.65×10^{-8} cm s^{-1}; histidine charge $(+1)$ -1.16, charge (0) -1.61 and charge (-1) -1.16×10^{-8} cm s^{-1}; lysine charge $(+1)$ -4.17 and charge (0) -27.6×10^{-8} cm s^{-1}. Note that at p*I*, the zwitterionic form still possesses charged groups, although the net charge is 0, while at lower or higher pH, a multipolar ion is formed. Thus, skin appears weakly selective for transport based on charge (DeNuzzio et al., 1990), and the flux does not depend on pH over this range. However, the barrier properties of skin may have been altered at pH 8.9 when the higher flux of lysine was measured. Although increased permeation of water at pH 8.9 was not observed in this study, alkaline solutions of pH 9 or greater may change the epidermal barrier (Thune et al., 1988) and the impedance also reflects these changes (Allenby et al., 1969).

To optimize the transdermal delivery of such multipolar ions, the observation of a permeability that is independent of pH provides a simple approach. The maximum flux through skin should occur at that pH where the solubility in aqueous solution is optimized. That is, permeation should be minimal from saturated solutions of p*I* and would be greater in the salt forms (Mazzenga et al., 1992).

These data are consistent with little or no effect of hydrophilicity or the degree of ionization on transport, i.e., the key variable appeared to be concentration in the aqueous donor. A porous mechanism of transport is consistent with these data and with much of the data of Ruland and Kreuter (1991). In contrast, the difference in permeability of threonine and phenylalanine through hairless mouse skin in the range of pH 5.4–7.4 of almost a factor of 10 observed by these authors is not consistent with a weakly selective porous pathway.

Since the molecular weight of amino acids under investigation was almost the same (Table 1) the effect of the molecular size of the penetrant could not be studied. Ruland and Kreuter (1991) did not find within a group of 20 amino acids any relationship between the molecular weight and permeability, which can be expected for the molecular weights ranging between 90 and 250.

While the present data might also be interpreted as exchange of tritium, (1) the method of separation and its consistency with the results from evaporation, (2) the agreement with data in the literature for C^{14}-amino acids, (3) the long time lags, and (4) the agreement with C^{14}-amino acid penetration results in the current laboratory support the interpretation as actual measures of amino acid transport. In particular, the fluxes and time lags for [^{14}C]histidine at pH 5.0 and 7.3 were 0.8 ± 0.6 and 0.24 ± 0.09 $\mu g/cm^2$ per h ($n = 5$) and 20 and 20–30 h, respectively. For [^{14}C]lysine, the steady-state flux ($n = 5$) and time lag were at pH 8.9, 2.6 ± 1.7 $\mu g/cm^2$ per h and 20–30 h and at pH 7.3, 0.6 ± 0.5 $\mu g/cm^2$ per h and 20–30 h, respectively. Given the variation in skin permeation, the agreement with the results presented in Table 3 is quite favorable.

Skin appendages have been suggested as a pathway of penetration for ions (Scheuplein, 1967; Barry, 1983). The values of the time lag, t_L, for this pathway are expected to be short (Scheuplein, 1967). The much longer values of t_L for histidine, lysine and aspartic acid imply that the observed steady-state permeation occurs through either a highly restricted or tortuous pathway. It is possible that in vitro skin appendages are occluded due to swelling of stratum corneum (Barry, 1983) or to sebum. Further studies are necessary to define the actual path. Since the pretreatment of the skin for 24 h with hypotonic buffers did not change the penetration profiles for histidine, the conclusion may be reached that the observed t_L reflects the rate of diffusion rather than hydration of stratum corneum. Large values of t_L might be explained by restricted pores, tortuosity, or binding of amino acids in the epidermis or dermis. Wearley et al. (1990) suggested binding of amino acids in skin.

The calculated apparent diffusion constants (Table 3) are the same magnitude as those characteristic for penetration through stratum corneum of nonionic compounds. For example, Scheuplein (1965) reported $D = 10^{-10}$ cm^2 s^{-1} for alkanols. In contrast, the diffusion constants

of amino acids in water are in the range of $7-12 \times 10^{-6}$ cm^{-2} s^{-1} (Kuu et al., 1992).

Two types of pathways for ionic transport through skin have been proposed: (1) a tortuous route through mechanistic 'pores' (Scheuplein, 1965; Berner and Cooper, 1987; Mazzenga et al., 1992) or (2) a 'partitioning' mechanism (Guy and Hadgraft, 1988; Potts et al., 1991). Support for both positions may be gleaned from the literature (Flynn, 1989). The present results do not permit a definitive conclusion, but tend to support a porous mechanism.

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