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Effect of linolenic acid/ethanol or limonene/ethanol and iontophoresis on the in vitro percutaneous absorption of LHRH and ultrastructure of human epidermis

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Abstract

The effect of enhancer(s) (e.g. ethanol (EtOH), 5% linolenic acid/EtOH, and 5% limonene/EtOH) and iontophoresis was investigated on the in vitro percutaneous absorption of luteinizing hormone releasing hormone (LHRH) and ultrastructure of human epidermis by transmission electron microscopy (TEM). 5% linolenic acid/EtOH or 5% limonene/EtOH significantly enhanced ($P < 0.05$) the passive flux of LHRH through human epidermis in comparison to the control (no enhancer treated epidermis). Iontophoresis further increased the flux of LHRH through enhancer(s) treated epidermis. Iontophoretic flux of LHRH through 5% linolenic acid/EtOH and 5% limonene/EtOH treated epidermis was significantly ($P < 0.05$) enhanced in comparison to iontophoretic flux through the control epidermis. TEM is the most efficient way to visualize the ultrastructure of the stratum corneum (SC). TEM results reveal that iontophoresis in combination with enhancers (e.g. linolenic acid/EtOH or and limonene/EtOH) transformed the highly compact cells of the SC into a looser network of filaments, disrupted the keratin pattern, and resulted in swelling of SC cell layers of human epidermis. Thus, linolenic acid/EtOH or limonene/EtOH in combination with iontophoresis increased the flux of LHRH through human epidermis by disrupting keratin pattern as well as loosening and swelling of SC cell layers.

Keywords: Penetration enhancer; Iontophoresis; Linolenic acid; Limonene; Ethanol; Ultrastructure; Transmission electron microscopy; Human epidermis

1. Introduction

The potential advantages associated with transdermal drug delivery are well documented and

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include avoidance of first-pass gut and hepatic metabolism, decreased side effects, and relative advantage of drug input termination whenever required (Hadgraft, 1984). As a result of recent development in genetic engineering, large number of peptide and protein drugs are available in the market for medical use. Peptides and proteins, because of their hydrophilic nature and large molecular size, have limited permeability in the skin. Iontophoresis increases penetration of charged substances through skin under a potential gradient and has shown to enhance delivery of several drugs (Burnette and Marrero, 1986; Singh and Maibach, 1993).

Another approach is the use of chemical penetration enhancers that partition into, and interact with, skin constituents to induce a temporary, reversible increase in skin permeability (Williams and Barry, 1992). Enhancer in combination with iontophoresis increased the permeability coefficient of methotrexate and metoprolol through human epidermis (Singh and Singh, 1995; Ganga et al., 1996). A synergism of iontophoresis and ethanol as enhancer was reported on the transport of peptides through human epidermis (Srinivasan et al., 1990). However, little is known about the mechanism of action of iontophoresis in combination with enhancer as a potential means to enhance and control the transdermal delivery of peptides. The combining effect of iontophoresis and enhancer may permit the use of lower quantities of enhancer and current within the delivery system.

The specific mechanisms and paths by which molecules traverse skin during iontophoresis remain a subject of considerable debate. It is generally recognized that the stratum corneum (SC) is the principal penetration barrier. Physiological structures (appendages) in the stratum corneum associated with regions of high iontophoretic flux have been identified (Abramson and Engel, 1941; Burnette and Marrero, 1986). In contrast, direct evidence supporting paracellular transport, in which molecules are transported through the lipid bilayer region surrounding corneocytes, has been obtained by high-resolution transmission microscopy of skin tissues following iontophoretic transport of heavy metal cations (Monteiro-Riviere et al., 1994). Recently, Pechtold Louk et al. (1996) found that during iontophoresis, ion and water transport through the skin is associated, at least in part, with the stratum corneum lipid lamellae. Sharata and Burnette (1988) demonstrated that the applications of dipolar aprotic enhancers accentuated the intercellular pathway of heavy metal cations along with additional diffusion into the SC cells. This implies that changes in the SC by iontophoresis/enhancer should affect the ionic permeability of the skin. Enhancer (Oleic acid/ethanol) in combination with iontophoresis synergistically enhanced the permeability coefficient of luteinizing hormone releasing hormone (LHRH) by increasing the lipid fluidity and loosening and swelling of cell layers of the porcine SC (Bhatia et al., 1997a,b). Thus, it is increasingly important to investigate the combining effect of iontophoresis and penetration enhancers on the ultrastructural changes in the SC in order to understand the mechanism of penetration enhancement of peptides. Several studies where different techniques have been used to study the skin structure and route of skin penetration at ultrastructural level have been reported (Sharata and Burnette, 1988; Bodde et al., 1991). Transmission electron microscopy (TEM) is the most efficient way to visualize the ultrastructure of the SC.

LHRH is used clinically for inducing ovulation in women with hypothalamic amenorrhea, inducing puberty and spermatogenesis in men with hypogonadotrophic hypogonadism, and the treatment of prostatic carcinoma. It has also been used in the endometriosis and polycystic ovary syndrome (Santen, 1985). In this study, we have investigated the effect of chemical enhancer(s) (i.e. ethanol (EtOH), 5% linolenic acid/EtOH, and 5% limonene/EtOH) and iontophoresis on the in vitro permeability of LHRH and ultrastructural changes in the human epidermis.

2. Materials and methods

2.1. Materials

[^{3}H] LHRH (specific activity 51 Ci/mmol) was obtained from NEN Research Products, Wilming-

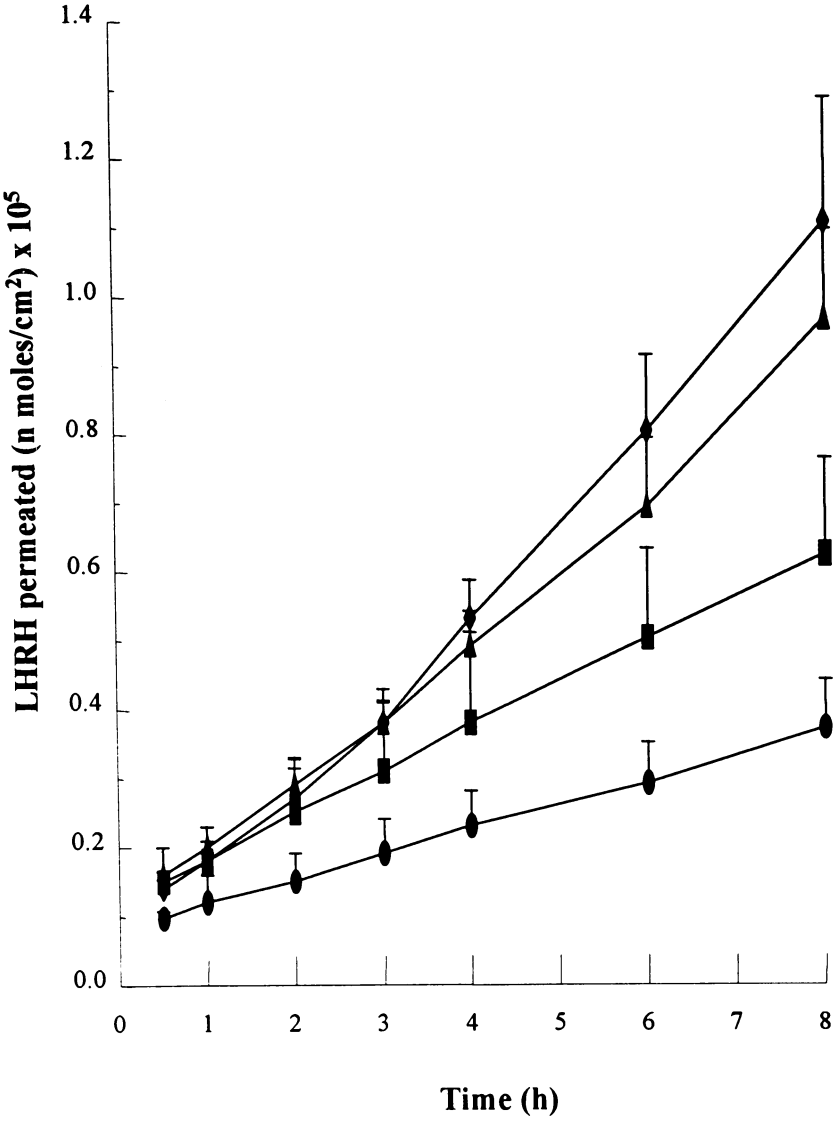

Fig. 1. The effect of 5% linolenic acid/EtOH and 5% limonene/EtOH on the in vitro passive transport of LHRH through human epidermis. The donor concentration of LHRH used was 3.92×10^{-3} nmoles/ml. Each data point is the mean $\pm$S.D. of four determinations. Key: (●) Control (no enhancer);(■) EtOH; (◆) 5% linolenic acid/EtOH; (▲) 5% limonene/EtOH.

ton, DE. Ethanol was obtained from Curtison Matison Scientific, Houston, TX. Linolenic acid, limonene, and NaCl were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals used were of analytical grade. De-ionized water (resistivity ≥ 18 MΩ) was used to prepare all solutions. The purity of radiolabelled LHRH was evaluated, prior to use, by thin-layer chromatography (TLC). The radiochemical purity of the peptide was 98%. Furthermore, electrochemical degradation of the peptide was examined by TLC during the course of the iontophoretic experiments. Cellulose plates (Alltech Associates, Deerfield, IL) were used as the stationary phase, and a mixture of *n*-butanol/acetic acid/water (4:1:1) was the mobile phase. Spots were detected

by a ninhydrin spray followed by heating for 10 min. The electrochemical degradation of LHRH following 8 h iontophoresis was found to be minimal (described elsewhere in detail, Bhatia et al., 1997a).

2.2. Preparation of epidermis

The human skin was obtained from National Disease Research Interchange (Philadelphia, PA) from autopsy of cadavers. The method of Kligman and Christophers (1963), with slight modification was adopted to remove the epidermis. The epidermis was prepared by soaking the whole skin in water at 60°C for 45 s. The skin was removed from the water, blotted dry and pinned with the dorsal side down. The intact epidermis was teased off from the dermis with forceps, washed with water and used in the in vitro transport/TEM studies.

2.3. In vitro studies

The epidermis was pretreated by immersing in the enhancer solution for 2 h and then was washed with de-ionized water. In the work of Srinivasan et al. (1990), the epidermis was pretreated with enhancers by applying both sides of the skin. We pretreated the epidermis by immersing in the enhancers. Epidermis without pretreatment was used as a control. Franz diffusion cells modified for iontophoresis were used in all transport studies. The control/pretreated epidermis was sandwiched between the cells with SC facing the donor compartment. The capacities of the donor and receiver compartments were 2 and 5 ml, respectively. The surface area of epidermis exposed to the solution was 0.785 cm^2. The donor compartment contained 1 ml of LHRH solution (0.2 μCi/ml of LHRH in 0.9% w/v sodium chloride (normal saline)), and the receiver compartment contained 5 ml of normal saline. The donor concentration of LHRH used was 3.92×10^{-3} nmoles/ml. The cells were maintained at 37 ± 0.5°C by PMC Dataplate® stirring digital dry block heater (Crown Bioscientific Inc., NJ). The content of the receiver compartment was stirred with a magnetic bar at 100 rpm. At specified intervals, 0.5-ml samples were withdrawn from the receiver compartment and an equivalent amount of normal saline (0.5 ml) was added to maintain the constant volume. Ag/AgCl electrodes ($>99.99\%$ Ag wire plated with AgCl) of 0.5 mm diameter and 4 cm length obtained from Keltronics Corporation, Oklahoma, were used. These electrodes are non-polarizable, reversible and therefore do not decompose water. The anode was placed in the donor and the cathode in the receiver for anodal iontophoresis. LHRH in normal saline is positively charged (Heit et al., 1993). A constant current of 0.2 mA/cm^2 was applied (Scepter®, Keltronics Corporation, OK, USA), with a flexible programmable voltage or current source that permits facile measurement of the voltage or current throughout the experiment. We chose 0.2 mA/cm^2 constant current in this study, since this current density has shown to be clinically safe (Burnette and Ongpipattanakul, 1988). The results were expressed as the mean $\pm$ S.D. of four experiments.

The samples were assayed by liquid scintillation counting. Each sample was mixed with 10 ml of scintillation cocktail (Econosafe®, biodegradable counting cocktail, Research Products International Corp., IL), and counted in a liquid scintillation counter (Packard, Tri Carb® 2100 TR, CT). The instrument was programmed to give counts for 10 min.

2.4. Transmission electron microscopy (TEM)

TEM was performed on the same piece of human epidermis through which in vitro transport was conducted in order to study the ultrastructural changes. Samples were immediately fixed in 2.5% gluteraldehyde in Millonig's phosphate buffer (pH 7.4). Post fixation was performed in 2% osmium tetraoxide in Millonig's phosphate buffer (pH 7.4). The samples were dehydrated in graded acetone, stored in saturated uranyl acetate in 70% acetone and embedded in Epon-araldite resin. Ultrathin transverse sections were cut on a ultramicrotome, stained with lead citrate, examined and photographed using a JEOL JEM 100 CX transmission electron microscope.

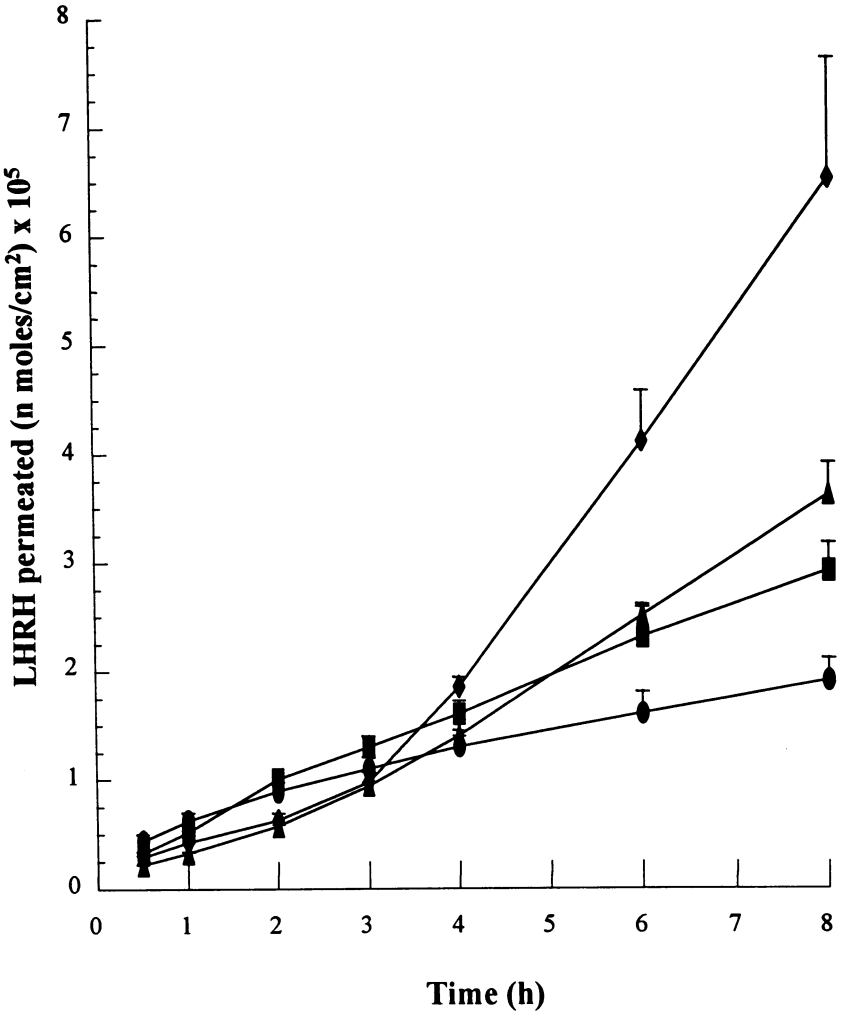

Fig. 2. The effect of 5% linolenic acid/EtOH and 5% limonene/EtOH on the in vitro iontophoretic transport of LHRH through human epidermis. The donor concentration of LHRH used was 3.92×10^{-3} nmoles/ml. Each data point is the mean $\pm$ S.D. of four determinations. Key: (●) Control (no enhancer); (■) EtOH; (◆) 5% linolenic acid/EtOH; (▲) 5% limonene/EtOH.

2.5. Data treatment

The receiver compartment concentration of LHRH was corrected for sample removal by using the equation given by Hayton and Chen (1982):

$$C'_n = C_n\left(\frac{V_t}{V_t - V_s}\right)\left(\frac{C'_{n-1}}{C_{n-1}}\right)$$

where, C'_n and C_n are the corrected and measured LHRH concentrations, respectively. V_t is the total volume of the receiver compartment and V_s is the volume of the sample. C'_{n-1} and C_{n-1} are the corrected and measured LHRH concentration before sampling, respectively. The cumulative amount of LHRH permeated per unit skin surface area was plotted against time and the slope of the linear portion of the plot was estimated as the steady-state flux.

Statistical comparisons were made using ANOVA and Duncan's multiple range test with a help of SAS program. The probability value of less than 0.05 was considered to be significant.

Table 1
Flux and enhancement factor of LHRH due to 5% linolenic acid/EtOH, 5% limonene/EtOH and iontophoresis through human epidermis[a]

Enhancer	Flux (n moles/cm^2 per h) $\times 10^6$ (mean $\pm$ S.D., $n=4$)		Enhancement factor		
	Passive	Iontophoresis	E_1	E_2	E_3
Control (no enhancer)	0.43 ± 0.09	1.55 ± 0.14	—	—	—
EtOH (ethanol)	0.57 ± 0.08	3.37 ± 0.27[b,c]	1.33	2.17	7.84
5% C18:3/EtOH (5% linolenic acid in ethanol)	1.33 ± 0.16[d,e]	11.72 ± 2.86[b,c,f]	3.10	7.56	27.25
5% limonene/EtOH	1.21 ± 0.21[d]	5.72 ± 0.99[b,c,f]	2.81	3.70	13.30

[a] E_1 = [Flux with enhancer treated epidermis (passive)]/[Flux with no enhancer treated epidermis (passive)]; E_2 = [Iontophoretic flux with enhancer treated epidermis]/[Iontophoretic flux with no enhancer treated epidermis]; E_3 = [Iontophoretic flux with enhancer treated epidermis]/[Flux with no enhancer treated epidermis (passive)].
[b] Significantly ($P<0.05$) different from iontophoresis alone (no enhancer).
[c] Significantly ($P<0.05$) different from the corresponding passive permeability.
[d] Significantly ($P<0.05$) different from control (passive no enhancer).
[e] Significantly ($P<0.05$) different from ethanol (passive).
[f] Significantly ($P<0.05$) different from ethanol (iontophoresis).

3. Results and discussion

Figs. 1 and 2 demonstrate in vitro passive and iontophoretic transport profiles, respectively, of LHRH through 5% linolenic acid/EtOH and 5% limonene/EtOH pretreated human epidermis. Pretreatment of epidermis either with 5% linolenic acid/EtOH or 5% limonene/EtOH increased the passive transport of LHRH. The flux and enhancement factors of LHRH are given in Table 1. 5% linolenic acid/EtOH and 5% limonene/EtOH significantly ($P < 0.05$) increased the flux of LHRH in comparison with control (no enhancer treated epidermis).

Iontophoresis further increased the transport of LHRH through the above enhancer pretreated epidermis. The iontophoretic flux of LHRH through 5% linolenic acid/EtOH and 5% limonene/EtOH pretreated epidermis was significantly enhanced ($P < 0.05$) in comparison to the control epidermis (Table 1). Also, a significant enhancement ($P < 0.05$) in the iontophoretic flux of LHRH was found through either 5% linolenic acid/EtOH or 5% limonene/EtOH pretreated epidermis in comparison with their passive flux. Furthermore, iontophoretic flux of LHRH through 5% linolenic acid/EtOH and 5% limonene/EtOH pretreated epidermis was significantly greater ($P < 0.05$) in comparison with iontophoretic flux of LHRH through EtOH pretreated epidermis signifying the enhancement effect of linolenic acid and limonene beyond EtOH alone.

From above results, it can be inferred that iontophoresis is synergistic with enhancers such as 5% linolenic acid and 5% limonene in combination with EtOH to provide an additional driving force to maintain and control the target flux of LHRH. The enhancer would moderate the iontophoretic regimen required to achieve the target flux, thus improving the tolerability of the skin to the iontophoretic regimen. Electrical current could also be used to modulate the transport over and above the effect of an enhancer. For the transdermal delivery of high molecular weight peptides, such as LHRH, iontophoresis can be coupled with enhancer such as 5% linolenic acid and 5% limonene in combination with EtOH, which would increase the permeability of the peptide through the skin.

TEM micrographs of human epidermis are shown in Figs. 3–10. Fig. 3A and B shows the ultrastructure of control human epidermis. Closely packed SC cells layers and a tightly packed keratin pattern are observed in the control epidermis (Fig. 3A). Also, Fig. 3a shows the cytoplasmic details of epidermal cells, which in-

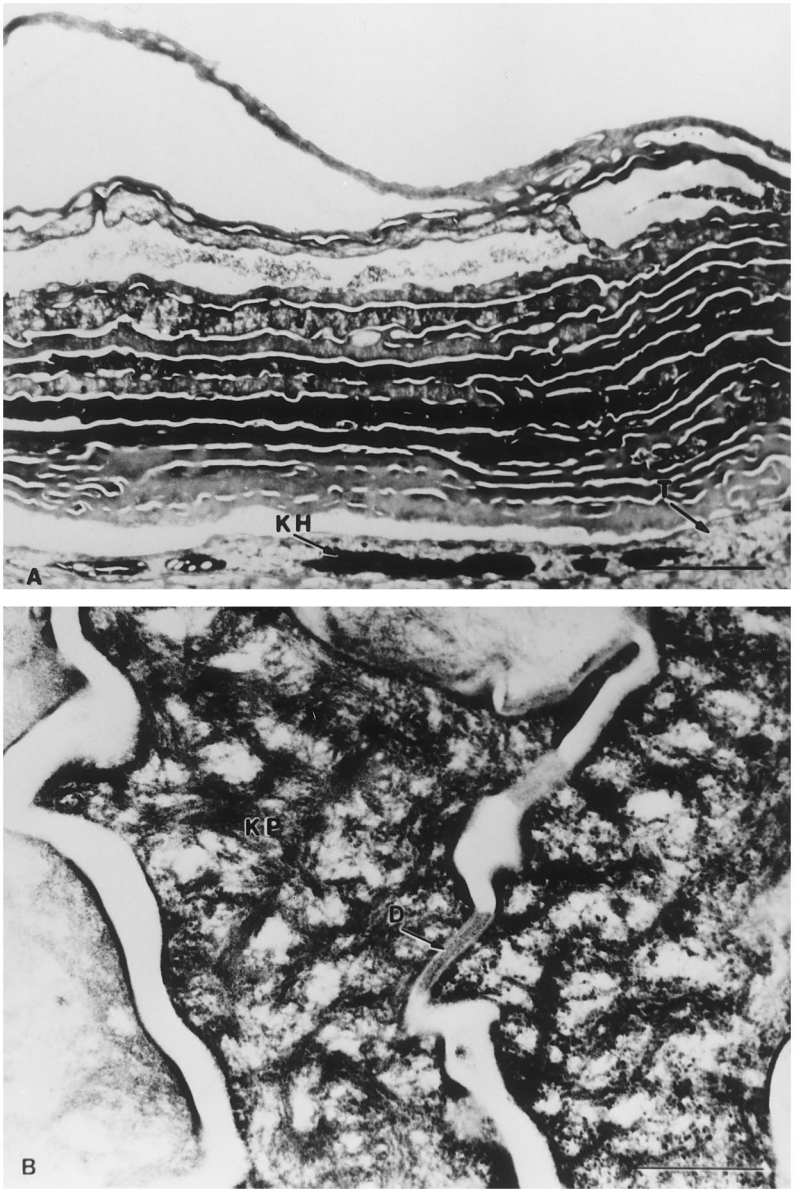

Fig. 3. Transmission electron micrograph of control human epidermis (passive). (A) calibration bar, 3 μm. At the bottom of the illustration appears an epidermal cell containing keratohylin (KH) and tonofilaments (T). Tonofilaments are characteristic feature of epidermal cell and are present throughout the cytoplasm of a fully developed epidermal cell. (B) Calibration bar, 0.5 μm. 'KP' represents keratin pattern that results from a dense filament-matrix protein complex that is normally seen in stratum corneum cells. Note the desmosome (D) joining the two stratum corneum cells.

clude a granular cell containing keratohyalin granules. Only upper one to two flattening cells of living epidermis are shown and the boundaries between them are defined by a somewhat linear array of desmosomes. Note the tonofibrils appearing in the epidermal cells. Fig. 4A and B shows an increase in the intercellular space in the epidermis exposed to 8 h iontophoresis as compared with

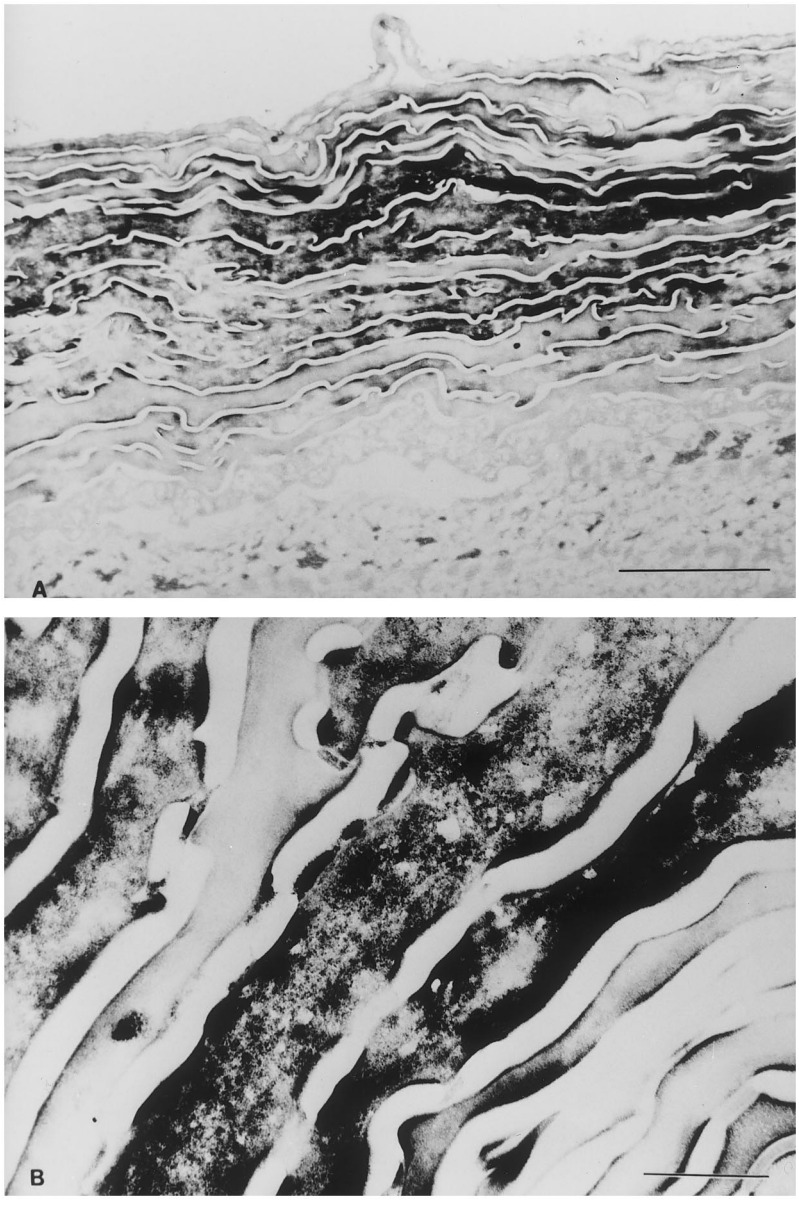

Fig. 4. Transmission electron micrograph of control human epidermis (iontophoresis). (A) calibration bar, 3μm. (B) Keratin pattern in stratum corneum cell layers (calibration bar, 0.5 μm).

control epidermis (no enhancer or iontophoresis treatment). The ultrastructure of EtOH-treated epidermis is shown in Fig. 5A and B. The SC layers are closely packed with total decrease in the thickness of epidermis. Fig. 6A and B shows the ultrastructure of epidermis treated with EtOH followed by 8 h iontophoresis. We did not observe swelling/loosening of SC cell layers and the keratin pattern was preserved. The ultrastructure of epidermis treated with 5% linolenic acid/EtOH is shown in Fig. 7A and B. Loosening and swelling of SC cell layers and disruption of keratin pattern

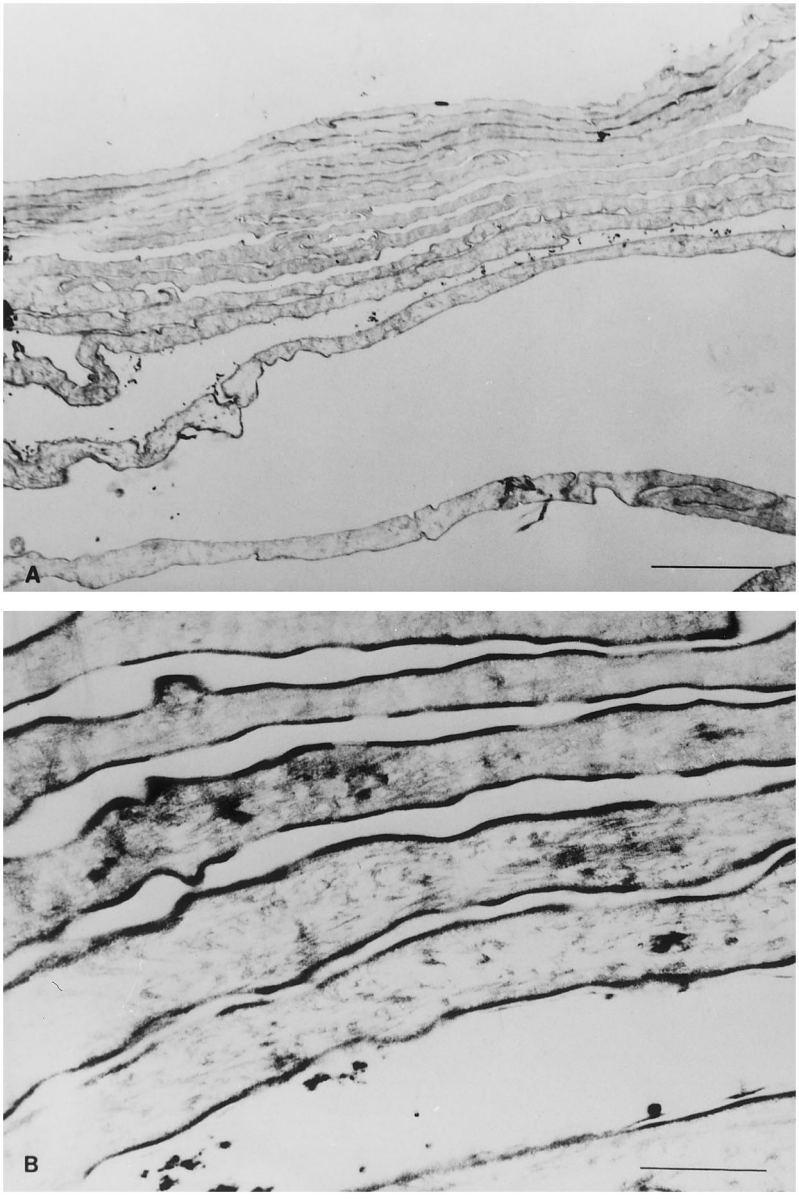

Fig. 5. Transmission electron micrograph of ethanol treated human epidermis (passive). (A) calibration bar, 3 μm. Stratum corneum cell layers have shrunk. (B) Keratin pattern in stratum corneum cell layers (calibration bar, 0.5 μm).

are observed. Treatment of epidermis with 5% linolenic acid /EtOH followed by iontophoresis (Fig. 8A and B) resulted in the loss of intercellular material, a disruption of keratin pattern, and further swelling of SC cell layers. Also, we noted a large increase of intercellular space between the SC cells indicating increased free volume. Fig. 9A and B shows the ultrastructure of SC treated with 5% limonene/EtOH. Again, we observe loosening and swelling of SC cell layers. Fig. 10A and B shows the ultrastructure of human epidermis treated with 5% limonene/EtOH followed by 8 h iontophoresis. We observed a disruption of keratin pattern and further swelling/loosening of SC

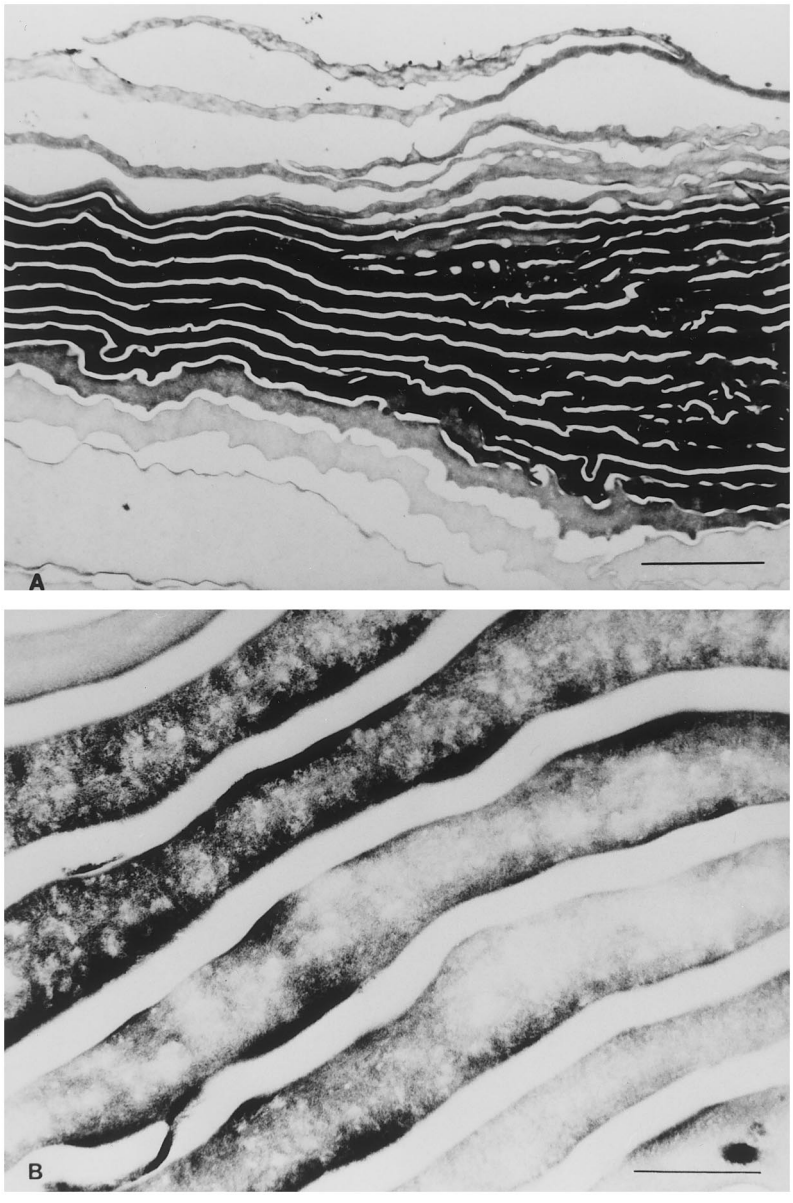

Fig. 6. Transmission electron micrograph of ethanol treated human epidermis (iontophoresis). (A) Calibration bar, 3 μm. Stratum corneum cell layers have shrunk. (B) Keratin pattern in stratum corneum cell layers (calibration bar, 0.5 μm).

cell layers. Also, note that the morphological changes (measured in terms of loosening/swelling of SC cells, disruption of keratin pattern, and increase of intercellular space between SC cells) seem to be more prominent in 5% linolenic acid /EtOH in combination with iontophoresis in comparison to 5% limonene/EtOH in combination with iontophoresis.

TEM has been successfully utilized to support findings and mechanistic deductions proposed following in vitro/in vivo transport experiments (Turner and Nonato, 1997). The present TEM

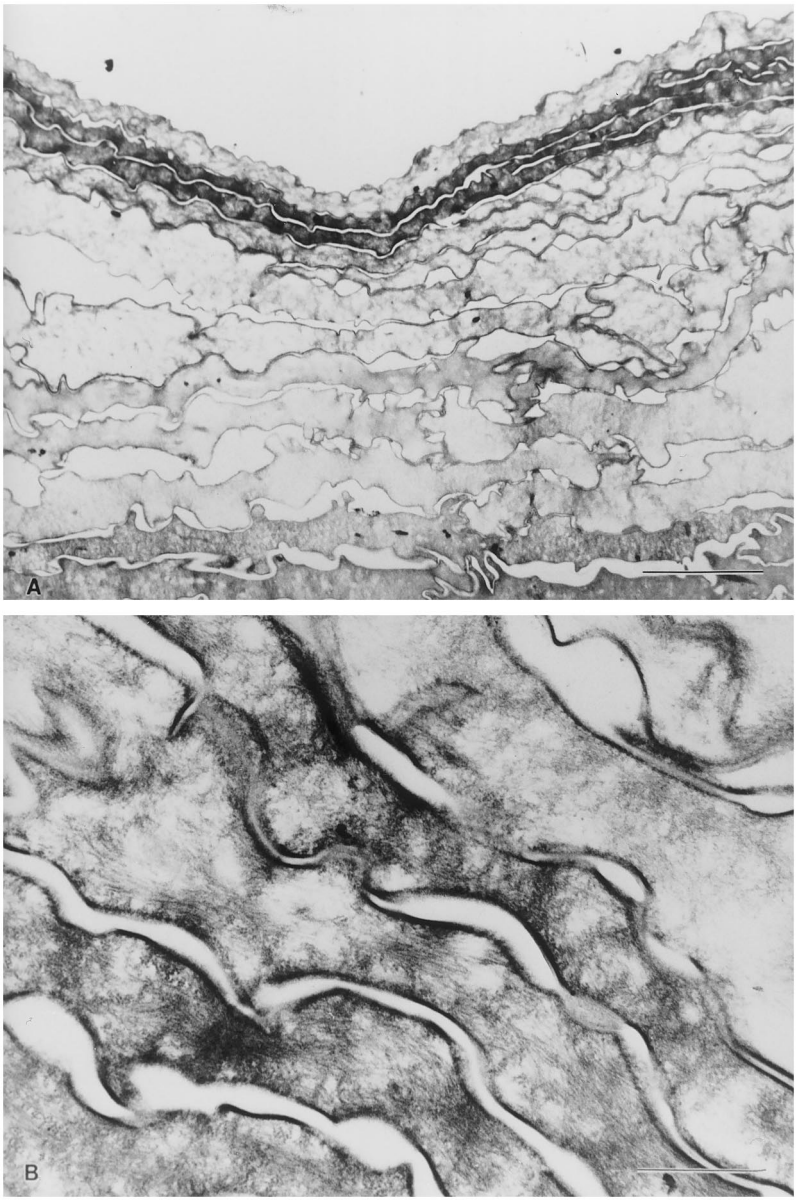

Fig. 7. Transmission electron micrograph of linolenic acid/ethanol treated human epidermis (passive). (A) Calibration bar, 3μm. (B) Keratin pattern in stratum corneum cell layers (calibration bar, 0.5 μm).

results depict that the epidermis treated with EtOH alone and EtOH/iontophoresis resulted in shrinking of SC cells (Figs. 5 and 6). In vitro transport studies showed that EtOH/iontophoresis significantly ($P < 0.05$) increased LHRH flux in comparison to control (Table 1). Recent FT–IR spectroscopic studies in our laboratory on the stratum corneum treated with EtOH for 2 h showed a decrease in C–H stretching absorbances (Bhatia et al., 1997a). These findings are in agreement with the findings of Bommannan et al. (1991) who reported a gradient of lipid concentration and conformational order, with a predominance of the less structured lipids near the surface

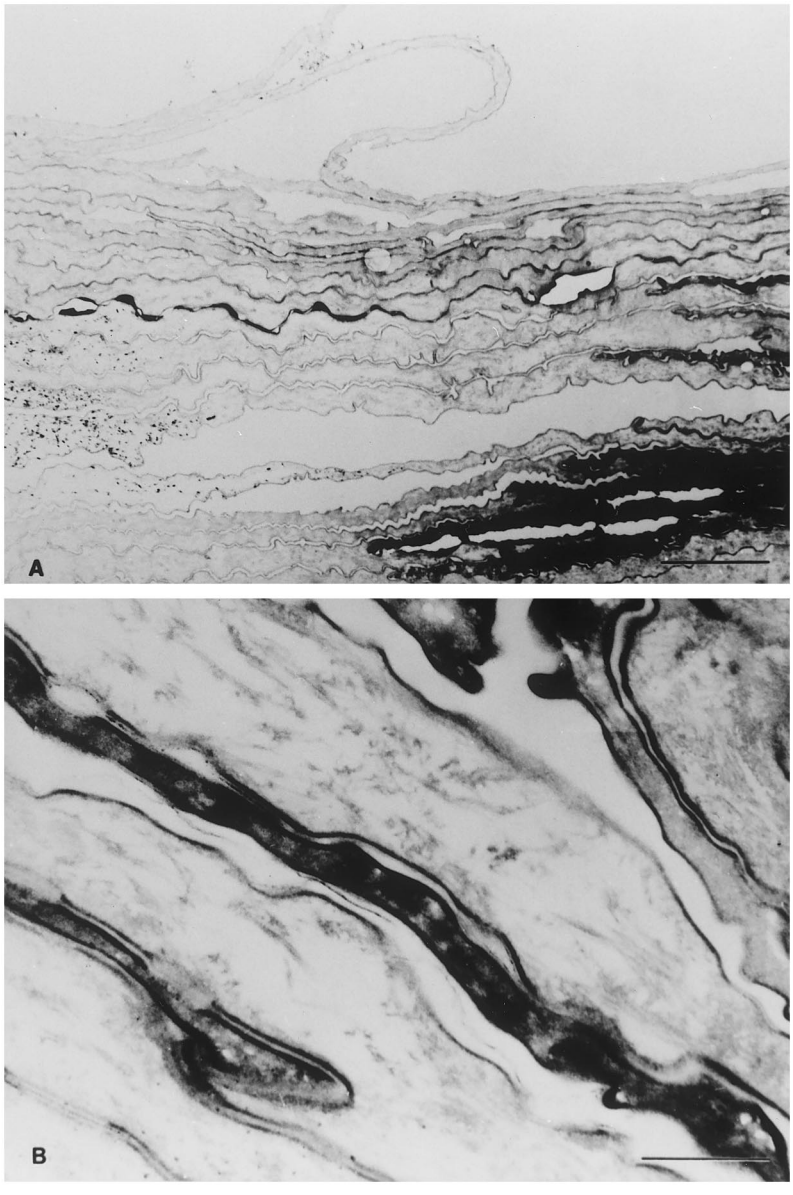

Fig. 8. Transmission electron micrograph of linolenic acid/ethanol treated human epidermis (iontophoresis). (A) Calibration bar, 3 μm. Stratum corneum cells has swollen and loosened. There is an increase in the intercellular space. (B) Keratin pattern in stratum corneum cell layers (calibration bar, 0.5 μm). Keratin pattern has been disrupted.

and extraction of lipids following in vivo dermal pretreatment with EtOH. Also, Goates and Knutson (1994) showed that at high concentration (75% v/v) EtOH leads to lipid and protein extraction. Hence, an increase in the flux of LHRH due to EtOH/iontophoresis can be correlated with lipid extraction. However, absolute EtOH also causes dehydration of the SC cell layers. Megrab et al. (1995) reported that higher concentration of EtOH causes dehydration of the SC cells. This explains shrinking of SC cell layers following treatment with EtOH/iontophoresis. Therefore,

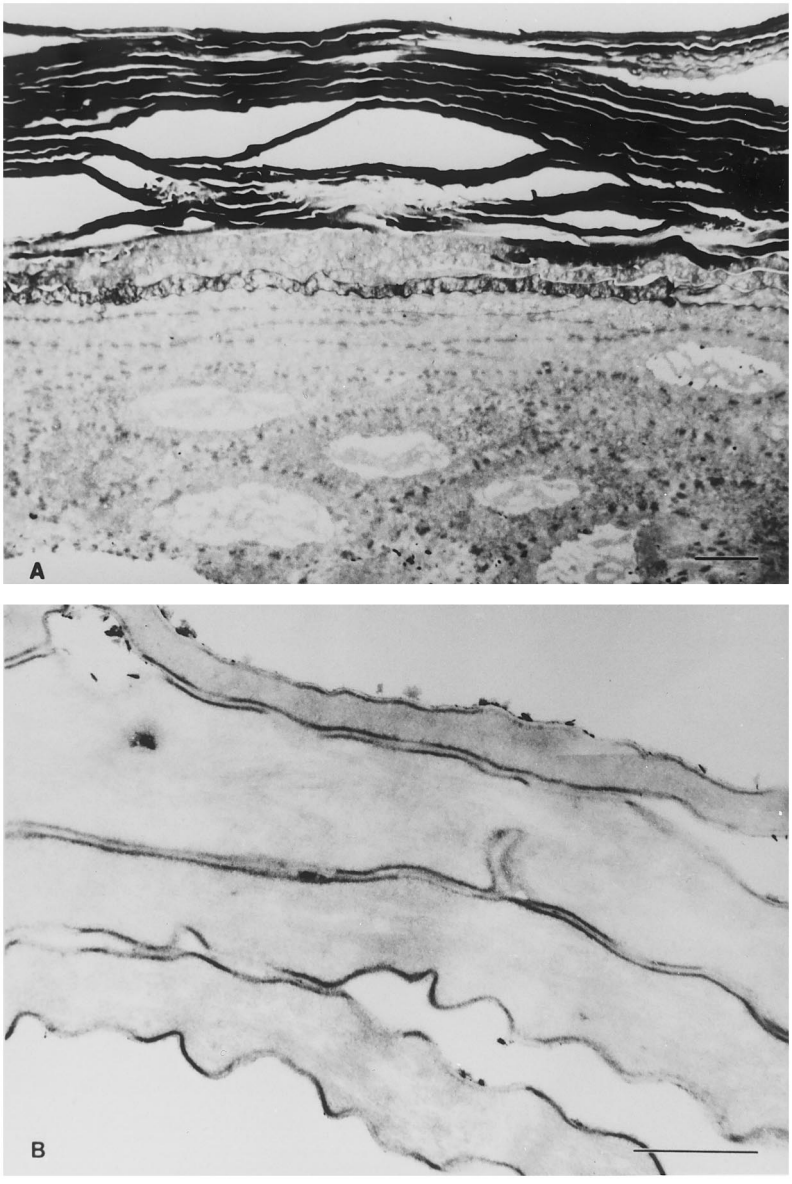

Fig. 9. Transmission electron micrograph of limonene/ethanol treated human epidermis (passive). (A) Calibration bar, 3 μm. (B) Keratin pattern in stratum corneum cell layers (calibration bar, 0.5 μm).

the enhancement in flux of LHRH due to EtOH/iontophoresis was due to lipid extraction and not due to disorder of SC cell layers.

We found that the treatment of epidermis either with linolenic acid/EtOH in combination with iontophoresis or limonene/EtOH in combination with iontophoresis resulted in swelling/loosening of SC cell layers and disruption of keratin pattern (Figs. 7–10). Pretreatment of human epidermis either with linolenic acid/EtOH or limonene/EtOH resulted in the expansion of normal SC cytoplasm and creation of more porous intracellular structure. Pretreatment of SC with linolenic acid/EtOH or limonene/EtOH in combination

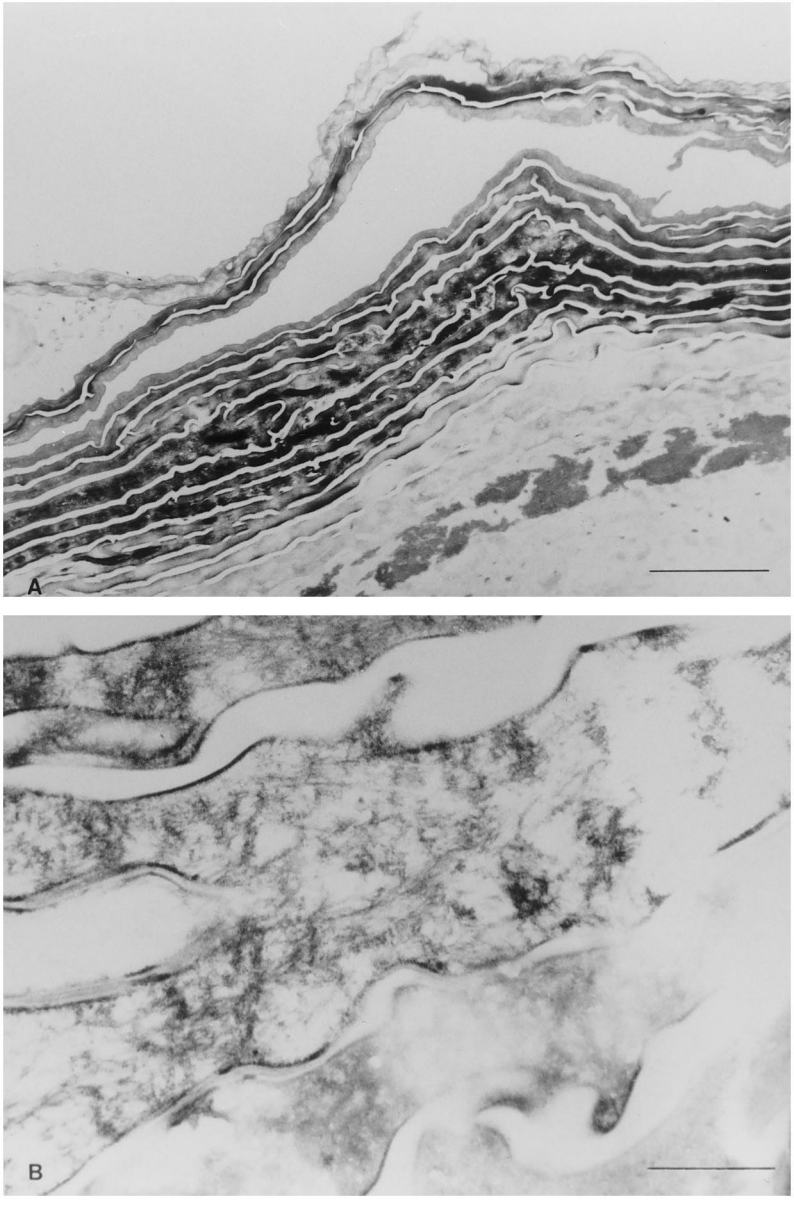

Fig. 10. Transmission electron micrograph of limonene/ethanol treated human epidermis (iontophoresis). (A) Calibration bar, 3 μm. Stratum corneum cells has swollen and loosened. There is an increase in intercellular space. (B) Keratin pattern in stratum corneum cell layers (calibration bar, 0.5 μm). Keratin pattern has been disrupted.

with iontophoresis resulted in further swelling of SC cell layers and creation of a looser network of filaments. One explanation for the observed swelling is the enhancer-induced breakdown and solubilization of macromolecular components (such as the filaggrin matrix protein of the SC) into smaller particles, resulting in a higher osmotic activity within the cells (Sharata and Burnette, 1988). Thus, the combined chemical and osmotic effects on the cytoplasmic compartments could result in an increased free volume for distribution and decreased diffusional resistance within the SC.

There has been much conjecture on the route of drug permeation through the SC. In the 'brick and mortar' view of the SC, with protein cells surrounded by intercellular lipid bilayers, both intercellular and transcellular diffusion are possible (the transcellular route must still cross intervening lipid regions) (Barry, 1987). Bodde et al. (1991) used in situ precipitation technique to investigate the transport pathway of mercury in the SC as a function of time. The authors concluded that mercury predominantly followed intercellular route but was also taken up intracellularly in the SC cells. Neelissen et al. (1993) found, using electron microscopy and immunohistochemistry methods, that transport of topically applied estradiol and norethindrone across human SC followed intercellular route but were also found within apical corneocytes (SC cells). The above reports indicate that the transcellular route plays a role, at least in part, in the transport of hydrophilic and lipophilic solutes.

Scanning electron microscopy (SEM) of hairless rat skin pretreated with dodecyl azone in combination with 55% EtOH revealed an increase in the distance between the SC cells (Osigo et al., 1992). It was postulated that *n*-octyl-β-D-thioglucoside increased the permeation of fluorescein isothiocyanate-glucoside by forming new 'pores' produced by exfoliation of cell membranes. However, it was unclear whether this implied that the disruption of the SC cell membrane provided the pathway through the SC cell or whether it resulted in disorganization of the intercellular lipid lamellae (Osigo et al., 1994). Hofland et al. (1995), using freeze-fracture electron microscopy, investigated the effect of three commercially available liposomal formulations (NAT 50, NAT 89, and NAT 106) on the ultrastructure of human SC in vitro. The authors found that apart from acting on SC lipids, the NAT 106 also resulted in the swelling of SC cells.

We believe that swelling and alterations within the keratinized protein fibrils by linolenic acid/EtOH or limonene/EtOH in combination with iontophoresis will increase diffusional volume within the SC protein domain (this is evident from our TEM results). Lipid extraction by EtOH would occur, which might be in conjunction and/or independent of alterations within protein domains. Such alterations would lead to a compromise of SC barrier function due to increase in free volume for distribution and creation of permeability defects in the SC. These pathways created would be utilized by iontophoresis, especially for enhanced transport of ionic and charged solutes such as LHRH. In conclusion, linolenic acid/EtOH or limonene/EtOH in combination with iontophoresis increased the LHRH permeability through human epidermis by loosening and swelling of SC cell layers.

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