CHAPTER II

LITERATURE REVIEW

Tea tree oil

Antimicrobial activity

Antimicrobial activity of tea tree oil has been determined by many scientists. In 1995, Carson et al. used a broth micro-dilution method to examine the susceptibility of *Escherichia coli* and *Staphylococcus aureus* to essential oil of *Melaleuca alternifolia.* (Carson, Hammer and Riley, 1995:181-185) The detergent Tween 80 was used successfully to enhance the solubility of tea tree oil in the test medium. The test broth did not result in the formation of a completely homogeneous solution, but Tween 80 did enhance the solubility of tea tree oil in broth to the point where consistent results could be obtained thereby allowing the antimicrobial activity to be evaluated. Overall, *E. coli* was more susceptible to tea tree oil than *S. aureus* with all isolates having minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of $\leq$ 0.25%. In contrast, 77% of the *S. aureus* tested had an MIC of $\leq$ 0.25% while the MBCs spanned the range 0.25-2.0%. Concentration of tea tree oil, which inhibited growth of *E. coli* also inhibited glucose-dependent respiration of *E. coli* and stimulated the leakage of intracellular Potassium ion. Moreover, stationary phase cells had greater tolerance to tea tree oil when compared to exponentially grown cells. (Cox, S.D. et al., 1998:355-358; Gustafson, J.E. et al., 1998:194-198)

In addition, susceptibility of methicillin-resistant *staphylococcus aureus* to tea tree oil was determined. Using disc diffusion method, 64 isolates were methicillin-resistant *S. aureus* (MRSA) and 33 were mupirocin-resistant *S. aureus*. The MIC and MBC were 0.25% and 0.50%, respectively. These results suggest tea tree oil may be useful in the treatment of MRSA carriage. (Carson, Cookson, Farrelly, and Riley, 1995:421-424) From another experiment in 2000, the combination of a 4% tea tree oil nasal ointment and 5% tea tree oil body wash was compared with a standard 2% mupirocin nasal ointment and triclosan body wash for the eradication of methicillin-resistant *Staphylococcus aureus* carriage. The tea tree oil combination appeared to perform better than the standard combination. (Caelli et al. 2000:236-237)

Tea tree oil has been suggested for the treatment of many common ailments, including acne, thus anti-acne activity of tea tree oil was also determined. 124 patients were randomized in clinical trial to evaluate the efficacy and skin tolerance of 5% tea tree oil gel in the treatment of mild to moderate acne when compared with 5% benzoyl peroxide lotion. The results of this study showed that both 5% tea tree oil and 5% benzoyl peroxide had a significant effect in ameliorating the acne by reducing the number of inflamed and non-inflamed lesions (open and close comedones) and fewer side effects were experienced by patients treated with tea tree oil. (Bassett, Pannowitz, and Barnetson, 1990:455-458) Furthermore, in 1994, Carson and Riley determined the susceptibility of 32 strains of *Propionibacterium acnes* to the essential oil of *Melaleuca alternifolia* by using broth micro-

dilution method. As a result, the minimum bactericidal concentration of tea tree oil for five strains was 0.25%, while, for the remainder, it was 0.50%. (Carson and Riley, 1994:24-25)

Moreover, the in vitro activity of tea tree oil against a range of wild strains of microorganisms isolated from clinical specimens of leg ulcers and pressure sores. The antimicrobial effectiveness of tea tree oil is determined in terms of MIC, MBC and minimum fungicidal concentration (MFC). The isolates include methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus*, *faecal streptococci*, beta-haemolytic streptococci, coagulase-negative staphylococci, *Pseudomanas spp.* and coliform bacilli. Eleven *Candida spp.* isolates from skin and vaginal swabs also are tested. Using an agar dilution assay, the MICs of tea tree oil was 0.5-1.0% (v/v), whilst with *P. aeruginosa* it was >2% (v/v). A broth microdilution method was used to determine MIC and minimum cidal concentration (MCC) of 80 isolates. In 64 isolates, tea tree oil produced an inhibitory and cidal effect at 3% and 4% (v/v), respectively. *S. aureus* and *Candida spp.* were the most susceptible to tea tree oil, with MICs and MBCs of 0.5% and 1%, respectively. *P. aeruginosa* and the *faecal streptococci* isolates, with MICs and MBCs of >8%, were resistant to tea tree oil. (Banes-Marshall, Cawley, and Phillips, 2001:139-145)

Each major components of tea tree oil were also determined. In 1995, the disc diffusion method was used to determine the susceptibility of a range of microorganisms to 1,8-cineole, 1-terpinen-4-ol, *p*-cymene, linalool, α-terpinene, γ-terpinene, α-terpineol and terpinolene. Using this method, each of the individual components

demonstrated some antimicrobial activity. The growth of *Pseudomonas aeruginosa* was inhibited by terpinen-4-ol only. *Bacteroides fragilis, Candida albicans* and *Clostidium perfringens* were inhibited by all of the components tested. Terpinen-4-ol was active against all the test organisms, while linalool and α-terpineol failed to inhibit *Pseudomonas aeruginosa* only. The least inhibitory component was *p*-cymene, which inhibited only three of the test organisms (*Bacteroides fragilis, Candida albicans* and *Clostidium perfringens*).(Carson and Riley, 1995:264-269) Moreover, each component of tea tree oil was also determined by using thin layer and gas-liquid chromatography (TLC and GLC). (Raman, Weir, and Bloomfield, 1995:242-245) Terpinen-4-ol, α-terpineol and α-pinene were found to be active against *Staphylococcus aureus, Staph. epidermidis* and *Propionibacterium acnes* whereas cineole was inactive against these organisms. The MIC values of the three active compounds increased in the order α-terpineol < terpinen-4-ol < α-pinene for all three organisms. MIC values of the tea tree oil components were lower for *P. acnes* than for the two *staphylococci.* This study supports the use of tea tree oil in the treatment of acne.

Antifungal activity

In 1992, one hundred and four patients was used completed a randomized, double-blind trial to evaluate the efficacy of 10% w/w tea tree oil cream compared with 1% tolnaftate and placebo creams in the treatment of tinea pedis. The tea tree oil group and the tolnaftate group showed significant improvement in clinical condition when compared

to the placebo group. Tea tree oil cream (10% w/w) appeared to reduce the symptomatology of tinea pedis as effectively as tolnaftate 1% but is no more effective than placebo in achieving a mycological cure. (Tong, Altman, and Barnetson, 1992:145-149)

Another study assessed the efficacy and tolerability of topical application of 1% clotrimazole solution compared with that of 100% *Melaleuca alternifolia* oil or tea tree oil for the treatment of toenail onychomycosis. 117 patients with distal subungual onychomycosis received twice daily application of either 1% clotrimazole solution or 100% tea tree oil for 6 months. Debridement and clinical assessment were performed at 0, 1, 3, and 6 months. After 6 months of therapy, the two treatment groups were comparable based on culture cure. As a result, clotrimazole had 61% cure rate, whereas tea tree oil had 60% cure rate. In conclusion, these two preparations provided improvement in nail appearance and symptomatology. The use of a topical preparation in conjunction with debridement is an appropriate initial treatment strategy. (Buck, Nidorf, and Addino, 1994:601-605)

In 1996, the in vitro antifungal activity of tea tree oil has been evaluated against 26 strains of various dermatophyte species, 54 yeasts, among them 32 strains of *Candida albicans* and other *Candida spp.* as well as 22 different *Malassezia furfur* strains. Tea tree oil was found to be able to inhibit growth of all clinical fungal isolates. For the investigated dermatophytes MIC values from 1,112.5 to 4,450.0 μg/ml were demonstrated. Both *C. albicans* strains and the other strains belonging to the genus Candida and Trichosporon appeared to be slightly less susceptible to tea tree oil in vitro. However, their MIC

values, which varied varied from 2,225.0 to 4,450.0 μg/ml (mean 4,080 μg/ml), indicated moderate susceptibility to the essential oil of *M. alternifolia*. The lipophilic yeast *M. furfur* seemed to be most susceptible to tea tree oil, with MIC values between 556.2 and 4,450.0 μg/ml (mean 1,261.5 μg/ml). In comparison with tea tree oil, in vitro susceptibility against miconazole, an established topical antifungal, was tested. As expected, very low MIC values for miconazole were found for dermatophytes, yeasts and *M. furfur* (mean 0.2, 1.0 and 2.34 μg/ml, respectively). It is suggested that the in vivo effect of tea tree oil ointment in the therapy of fungal infections of the skin and mucous membranes as well as in the treatment of dandruff, a mild form of seborrheic dermatitis, may be at least partly due to an antifungal activity of tea tree oil. (Nenoff, Haustein, and Brandt, 1996:388-394)

In 1998, tea tree oil was investigated for activity against 81 *C. albicans* isolates and 33 non-albicans Candida isolates. By the broth microdilution method, the minimum concentration of oil inhibiting 90% of isolates for both *C. albicans* and non-albicans Candida species was 0.25% (v/v). The minimum concentration of oil killing 90% of isolates was 0.25% for *C. albicans* and 0.5% for non-albicans Candida species. Fifty-seven Candida isolates were tested for sensitivity to tea tree oil by the agar dilution method; the minimum concentration of oil inhibiting 90% of isolates was 0.5%. Tests on three intra-vaginal tea tree oil products showed these products to have MICs and minimum fungicidal concentrations comparable to those of non-formulated tea tree oil, indicating that the tea tree oil contained in these products has retained its anticandidal activity. These data indicate that this essential

oil is active against *Candida spp.*, suggesting that it may be useful in the topical treatment of superficial candida infections. (Hammer, Carson, and Riley, 1998:591-595)

Furthermore, another study was conducted to determine the activity of tea tree oil against 58 clinical isolates *Candida albicans, Trichophyton rubrum, Trichophyton mentagrophytes, Trichophyton tonsurans, Aspergillus niger, Penicillium species, Epidermophyton floccosum,* and *Microsporum gypsum.* Tea tree oil showed inhibitory activity against all isolates tested except one strain of *E. floccosum.* These in vitro results suggest that tea tree oil may be useful in the treatment of yeast and fungal mucosal and skin infections. (Concha, Moore, and Holloway 1998:489-492)

In 2000, the activities of ketoconazole, econazole, miconazole and tea tree oil against 54 Malassezia isolates were determined by agar and broth dilution methods. Ketoconazole was more active than both econazole and miconazole, which showed very similar activities. *M. furfur* was the least susceptible species. *M. sympodialis, M. slooffiae, M. globosa,* and *M. obtusa* showed similar susceptibilities to the four agents. (Hammer, Carson, and Riley, 2000:467-469)

Antiviral activity

Antiviral activity of tea tree oil and eucalyptus oil against herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) was tested in vitro on RC-37 cells using a plaque reduction assay. (Schnitzler, Schon, and Reichling, 2001:343-347) The 50% inhibitory concentration (IC50) of tea tree oil for herpes simplex virus

plaque formation was 0.0009% and 0.0008% and the IC50 of eucalyptus oil was determined at 0.009% and 0.008% for HSV-1 and HSV-2, respectively. Australian tea tree oil exhibited high levels of virucidal activity against HSV-1 and HSV-2 in viral suspension tests. At noncytotoxic concentrations of tea tree oil plaque formation was reduced by 98.2% and 93.0% for HSV-1 and HSV-2, respectively. Noncytotoxic concentrations of eucalyptus oil reduced virus titers by 57.9% for HSV-1 and 75.4% for HSV-2. Virus titers were reduced significantly with tea tree oil, whereas eucalyptus oil exhibited distinct but less antiviral activity. In order to determine the mode of antiviral action of both essential oils, cells were pretreated before viral infection or viruses were incubated with tea tree oil or eucalyptus oil before infection, during adsorption or after penetration into the host cells. Plaque formation was clearly reduced, when herpes simplex virus was pretreated with the essential oils prior to adsorption. These results indicated that tea tree oil and eucalyptus oil affect the virus before or during adsorption, but not after penetration into the host cell. Thus tea tree oil and eucalyptus oil are capable to exert a direct antiviral effect on HSV. Although the active antiherpes components of Australian tea tree and eucalyptus oil are not yet known, their possible application as antiviral agents in recurrent herpes infection is promising.

Anti-inflammatory activity

Anti-inflammatory properties of tea tree oil were evaluated. The ability of tea tree oil to reduce the production in vitro of tumor

necrosis factor-alpha (TNFalpha), interleukin (IL)-1beta, IL-8 IL-10 and prostaglandin E2 (PGE2) by lipopolysaccharide (LPS)-activated human peripheral blood monocytes was examined. As a result, the water soluble components of tea tree oil at concentrations equivalent to 0.125% significantly suppressed LPS-induced production of TNFalpha, IL-1beta and IL-10 (by approximately 50%) and PGE2 (by approximately 30%) after 40 h. Gas chromatography/mass spectrometry identified terpinen-4-ol (42%), α-terpineol (3%) and 1,8-cineole (2%, respectively, of tea tree oil) as the water soluble components of tea tree oil. When these components were examined individually, only terpinen-4-ol suppressed the production after 40 h of TNFalpha, IL-1beta, IL-8, IL-10 and PGE2 by LPS-activated monocytes. In summary, the water-soluble components of tea tree oil can suppress pro-inflammatory mediator production by activated human monocytes. (Hart et al., 2000:619-626)

Human skin

The skin, the heaviest single organ of the body, combines with the mucosal linings of the respiratory, digestive, and urogenital tracts to form a capsule which separates the internal body structures from the external environment. This flexible, self-repairing shell defends the stable internal milieu of living tissues, bathed in their body fluids, from a hostile external world of varying temperature, humidity, radiation, and pollution. The integument not only physically protects the internal organs and limits the passage of substances into and out of the body but also stabilizes temperature and blood pressure with its

circulation and evaporation systems. The skin mediates the sensations of touch, pain, heat, and cold; it expresses the redness of anger and embarrassment, the sweating of anxiety, and the pallor of fear; and the integument identifies individuals through the characteristics of the hair, odor, texture, and color shades particular to man.

In the light of the many requirements, which the skin must fulfill, it is not surprising that anatomists find that the integument is a very unhomogeneous organ. For an average 70 kg human with a skin surface area of 1.8 m^2, a typical square centimeter covers 10 hair follicles, 12 nerves, 15 sebaceous glands, 100 sweat glands, 3 blood vessels with 92 cm total length, 360 cm of nerves and $3x10^6$ cells.

Because the skin is the most accessible tissue of the body, it is easily damaged mechanically, chemically, biologically and by radiation. The tissue was often exposed to organic solvents, detergents, chemical residues, and pollutants and to contact allergens produced by bacteria, yeasts, molds, fungi, and plants. Insects and animals sting and bite it. Toiletries, cosmetics, topical and systemic drugs, together with a myriad of skin diseases, may all harm the skin.

Anatomy and Physiology

The human skin comprises two distinct but mutually dependent tissues, the stratified, avascular, cellular epidermis and an underlying dermis of connective tissue. At the bottom of the dermis lies the fatty, subcutaneous layer. In transverse section, the dermoepidermal junction undulates because a series of thickened epidermal ridges (the rete ridges) project downwards into the dermis. The ridges inscribe

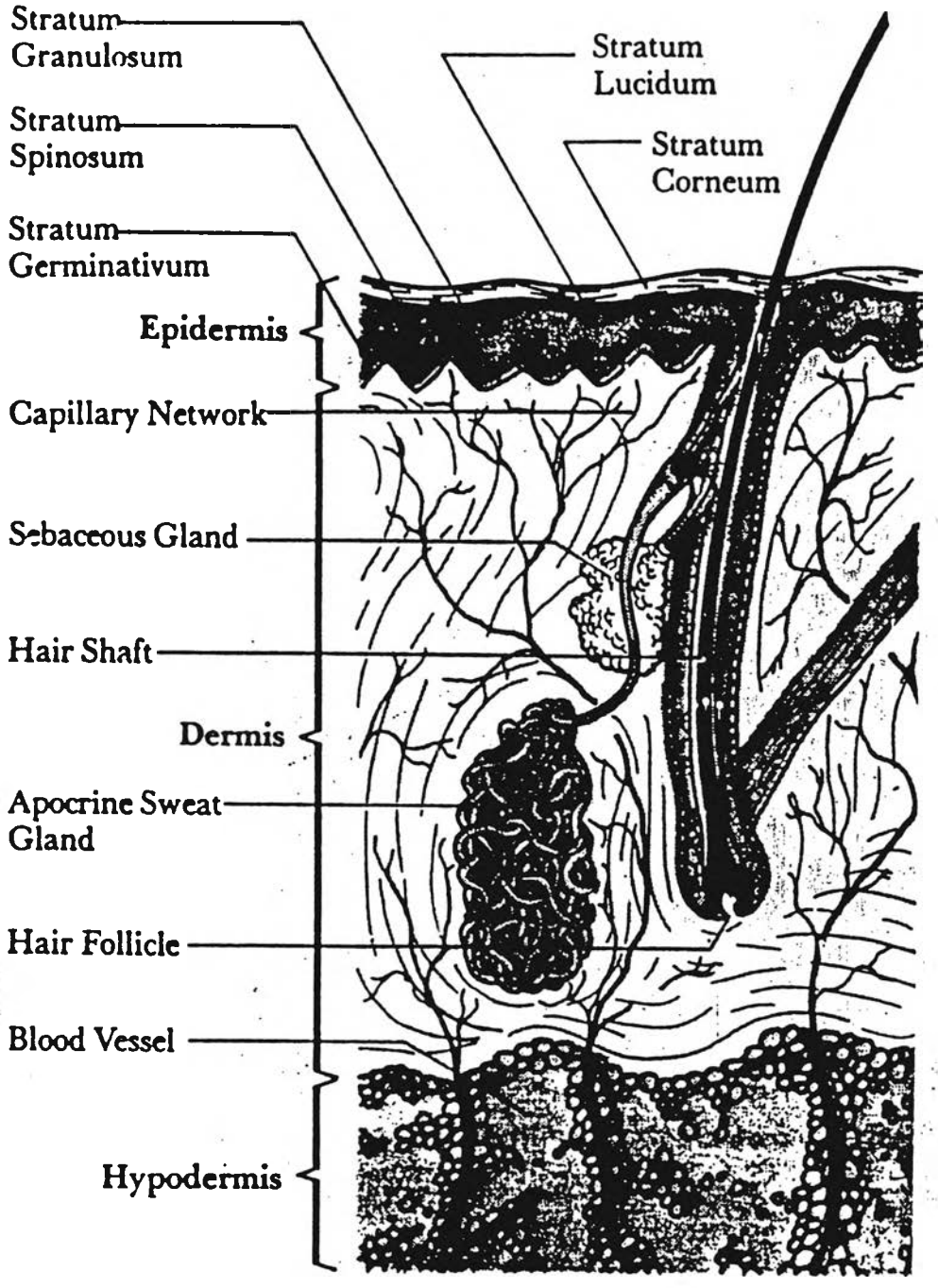

Figure 1 A cross-sectional view of human skin, showing various skin tissue layers and appendages.(Chien, Y.W. 1987:3)

characteristic patterns in different regions of the body, which can be seen best in split skin preparation.

Human skin displays two main types. Hairy skin encloses hair follicles and sebaceous glands, but there are no encapsulated sense organs. Glabrous skin of the palms and the soles constructs a thick epidermis with a compact stratum corneum, but the integument lacks hair follicles and sebaceous glands and the dermis supports encapsulated sense organs. Ridges groove hairless skin into individually unique configurations termed dermatoglyphics. Besides providing identification, for example, fingerprints, dermatoglyphics may aid diagnosis or they may indicate that a patient has an increased tendency to develop certain diseases, for example, alopecia areata or psoriasis.

A. The Epidermis

The multilayered envelope of the epidermis varies in thickness, depending on cell size and the number of cell layers, ranging from about 0.8 mm on the palms and the soles down to 0.06 mm on the eyelids. Cells which provide epithelial tissue differ from those of all other organs in that as they ascend from the proliferative layer of basal cells they change in an ordered fashion from metabolically active and dividing cells to dense, dead, keratinized protein.

1. The basal layer (stratum germinativum) and the dermoepidermal junction

The basal sells are nucleated, columnar, and about 6 μm wide, with their long axis at right angles to the dermoepidermal junction; they connect by cytoplasmic intercellular bridges.

Mitosis of the basal cells constantly renews the epidermis and this proliferation in healthy skin balances the loss of dead horny cells from the skin surface. The epidermis thus remains constant in thickness. Although there are difficulties in calculation epidermal turnover times, workers use tritiated thymadine to selectively label nuclear DNA and thereby they estimate that a cell from the basal layer takes at least 14 days to reach the stratum corneum. In the rapidly proliferating epidermis of psoriasis, the transit time is only 20 days. Radioactive glycine studies indicate that the normal turnover time in the stratum corneum is some 13 or 14 days, with the residence time in psoriaticstratum corneum shortening to 2 days. Therefore, the total turnover times, from the basal layer to shedding, average 28 days in healthy skin and only 4 days in psoriatic skin. The mitotic rate also increases within 24 to 36 hr of injuries such as radiation damage or removal of the stratum corneum by adhesive tape stripping, scrapings, and incisions.

The basal cell layer also includes melanocytes, which produce and distribute melanin granules to the keratinocytes in a complex interaction. The skin requires melanin for pigmentation, a protective measure against radiation. Melanocytes lose their activity in vitiligo and hyperactive melanocytes produce tanning, cholasma, freckles, moles and malignant melanomas.

Below the basal cell layer lies the complex dermoepidermal junction, which constitutes an anatomic functional unit. In electron micrographs, the junction spans four components: (1) the basal cell plasma membrane with its specialized attachment devices, the

hemidesmosomes; (2) the lamina lucida; (3) the basal lamina; and (4) the fibrous components below the basal lamina, which include anchoring fibrils, dermal microfibril bundles, and collagen fibrils. The "basement membrane" revealed by light microscopy corresponds to the fibrous zone below the basal lamina.

The junction serves the three functions of dermal-epidermal adherence, mechanical support for the epidermis, and control of the passage of cells and some large molecules across the junction. Thus, diseases, which operate at this level, can markedly reduce the adhesion of the epidermis to the dermis. Investigators who use suction to produce blisters in the lamina lucida conclude that the major stabilizing force at the dermo-epidermal junction is a highly viscous bond.

2. The prickle cell layer: the keratinocytes (stratum germinativum)

As the cells produced by the basal layer move outward, they alter morphologically and histochemically. The cells flatten and their nuclei shrink, called polygonal cells prickle cells because they interconnect by fine prickles. Each prickle encloses an extension of the cytoplasm, and the opposing tips of the prickles of adjacent cells adhere to form intercellular bridges, the desmosomes. These links maintain the integrity of the epidemis. Between the desmosomes, a capillary space full of tissue fluid separates neighboring cells and the void permit nutrients and oxygen to pass outward. The desmosomes can break and reform to allow migrating melanocytes and leukocytes to pass.

3. Thc granular layer (stratum granulosum)

As the keratinocytes approach the surface, they manufacture basic staining particles, the keratohyalin granules. It was suggested that these granules represent an early form of keratin, but they may be cell organelles partially destroyed by hydrolytic enzymes. This keratogenous of transitional zone is a region of intense biochemical activity and morphological change. The dynamic operation manufactures the keratin to form the horny layer by an active rather than by a degenerative process. The term "transitional zone" is convenient in that we can define a region between living cells and dead keratin, even when no granules form. This is the condition in psoriasis when the epidermis produces a parakeratotic tissue. Polypeptide building blocks of keratin, formed in the prickle cell layer, aggregate in the transitional zone to form insoluble, fibrous keratin molecules.

4. The stratum lucidum

In the palm of the hand and the sole of the foot, an anatomically distinct poorly staining hyaline zone forms a thin, translucent layer immediately above the granular layer. This region is the stratum lucidum.

5. The horny layer (stratum corneum)

As the final stage of differentiation, epidermal cells construct the most superficial layer of the epidermis, the stratum corneum. Man owes his ability to survive in a non-aqueous environment to the almost impermeable nature of this refractory horny layer. On general body areas the membrane provides 10-15 layers of much flattened,

keratinized dead cells, stacking them in highly organized units of vertical columns. The horny layer may be only 10 μm thick when dry (less than half the thickness of an average piece of paper), but it swells in water to several times this thickness. When dry, it is a very dense tissue around 1.5 g cm^{-3}. Each thin, polygonal cell measures approximately 0.5 to 1.5 μm thick, with a diameter ranging from 34 μm on the forehead to 46 μm on the thigh axilla. The cells lie tangential to the skin surface and interdigitate their lateral edges with adjacent cells so as to form cohesive laminae. Besides containing keratin, the cells are the final repositories of the end products of epidermal metabolism. They enclose sebaceous and sweat gland secretions, all accommodated in a highly organized structure.

Horny layer can be distinguished in two types by anatomical site, function, and structure. The horny pads of the palms and soles adapt for weight bearing and friction, and the membranous stratum corneum over the remainder of the body is flexible but impermeable. The horny pads are at least 40 times thicker than the membranous horny layer, and the cells stack vertically in a much less regular fashion. The cells of the pads contain less water-soluble substances, they are very brittle when dry, and they are more permeable to water and to chemicals.

Horny layer has an average daily loss from the whole body surface of 0.5 to 1 g. Thus, the skin functions economically, demanding insignificant amounts of body nitrogen.

The stratum corneum plays a crucial role in controlling the percutaneous absorption of drug molecules. The selective permeability

of its elegant structure provides a central theme in many aspects of the study of the biopharmaceutics of topical products.

6. Other cells of the epidermis

Langerhans' cells are dendritic cells with a lobular nucleus, a clear cytoplasm containing characteristic Langerhans' cell granules, and well-developed endoplasmic reticulum, Golgi complex, and lysosomes. Langerhans' cells may be concerned with the organization and function of the squamous epidermis. In recent years, much evidence has been presented that these cells are also involved in the immune response in the skin; thus they bind antigens, probably modify them, and transport them to the lymph nodes for lymphocyte activation.

B. The Dermis

The dermis (or corium), at 3 to 5 mm thick, is much wider than the overlying epidermis, which it supports, and the corium thus makes up the bulk of the skin. The dermis consists essentially of a matrix of connective tissue woven from fibrous proteins (approximate composition: collagen, 75%; elastin 4%; and reticulin 0.4%), which embed in an amorphous ground substance of mucopolysaccharide providing about 20% of the mass. Blood vessels, nerves, and lymphatics cross this matrix and skin appendages (eccrine sweat glands, apocrine glands, and pilosebaceous units) penetrate it. In man, the dermis divides into a superficial, thin, papillary layer (composed of narrow fibers), which forms a negative image of the ridged lower surface of the epidermis, and a thick underlying reticular layer made of wide collagen fibers. In regions of the body such as the penis,

scrotum, nipple and perineum, the reticular layer contains smooth muscle fibers which, when contracted, produce wrinkling.

1. Dermal components

a. Collagen Fibroblasts secrete collagen into the surrounding tissue in the form of a precursor, soluble tropocollagen. This is a rod-shaped molecule in the form of a triple polypeptide helix, with a molecular weight of 300,000 to 360,000, a length of about 280 nm, and a width of only 1.5 nm. In living skin, tropocollagen molecules aggregate to form, in sequence, filaments (composed of three to five molecules), microfibrils, and fibrils. Bundles of these fibrils assemble into the collagen fibers, which can be seen in light microscopy as colorless, branching, wavy bands with faint longitudinal striations.

Collagen is the generic name for a family of proteins, which accounts for a third of total human protein. It is the main fibrous constituent of skin, bone, cartilage, tendon, and ligament; collagens from different connective tissues differ only slightly in their amino acid composition. Collagen lacks cystine, but it is rich in the amino acids glycine, proline, and hydroxyproline.

b. Elastin In humans, the dermis abounds with elastic fibers which stretch relatively easily and which revert to their original shape when the stress subsides, the elastic fibers from a framework in the dermis, so that the mechanical properties of connective tissues depend on the presence of both the collagen and the elastic fibers.

Elastic fibers incorporate two components, an inner amorphous medulla composed of the protein elastin (derived from a precursor, tropoelastin) and an outer cortex consisting of non-elastin protein

microfibrils. These two portions differ markedly in amino acid composition, and neither resembles collagen. Elastin possesses the unique amino acids desmosine and isodesmosine, which probably form a special kind of cross-link within the molecule; the resulting product is insoluble and very stable. Adult animals retain their elastin for life.

c. Ground substance The amorphous ground substance, in which the cells and fibers lie, contains a variety of lipid, protein, and carbohydrate materials. The most important are the mucopolysaccharides hyaluronic acid and dermatan sulfate (chondroitin sulfate B), together with a small amount of chondroitin-6-sulfate. The molecular weight of the hyaluronic acid depends on the tissue source and the method of preparation; it may be several millions. Dermatan sulfate has a molecular weight of not more than 40,000. Both compounds are single-chain, linear polymers.

d. Cells Fibroblasts are the most numerous cells inhabiting loose connective tissue. Mast cells and histiocytes also occur.

2. The vascular supply

The dermis needs a rich blood supply, which regulates temperature and pressure, delivers nutrients to the skin and removes waste products, mobilizes defense forces, and contributes to skin color. Branches from the artery network (the arterial plexus) convey blood to the hair follicles, the sweat glands, the subcutaneous fat, and the dermis itself.

The blood supply reaches to within 0.2 mm of the skin surface, so that it readily absorbs and systemically dilutes most chemicals,

which penetrate past the stratum corneum and the viable epidermis. The vascular surface available for the exchange of materials between local tissues and the blood is about 1-2 cm^2 per cm^2 of skin surface, with a blood flow rate of about 0.05 ml min^{-1} per cm^3 of skin.

Of particular relevance to biopharmaceutical studies is the fact that this generous blood volume usually functions as a "sink" with respect to the diffusing molecules, which reach it during the process of percutaneous absorption. This sink condition ensures that the penetrant concentration in the dermis remains near zero and therefore the concentration gradient across the epidermis is maximal. As the concentration gradient provides the driving force for diffusion, an abundant blood supply assists percutaneous absorption. However, vasoconstrictors such as topical steroids may restrict the local circulation and vasodilators such as the nicotinic acid esters may widen the capillaries to increase further the blood flow.

The lymphatic system forms a vascular network with the primary function of removing plasma proteins from the extravascular spaces, together with particulate and other matter.

3. The neural supply

A liberal nerve supply serves the skin, with great variations from region to region. The face and the extremities are richly innervated, whereas the bank of the trunk carries a sparse network. Cutaneous nerves, nerve endings, and capillaries modulate the sensations of pain, pruritus (itching), touch, and temperature. A point made by Winkelman (1961) is that the skin is a physiological paradox, as it serves two mutually exclusive functions; protection from the

environment (which requires minimal sensory perception), and recording of the surroundings (needing sensitive nervous responses).

C. The Subcutaneous Tissue

The subcutaneous fat (hypoderm, subcutis) spreads all over the body as a fibrofatty layer, with the exception of the eyelids and the male genital region. The sheet of fat lies between the relatively flexible skin and the unyielding, deep fascia, and its thickness varies with the age, sex, endocrine, and nutritional status of the individual. The cells manufacture and store lipids in large quantities and bundles of collagen fibers weave between aggregates of fat cells to provide flexible linkages between the underlying structures and the superficial skin layers. The subcutis provides a thermal barrier and a mechanical cushion; it is a site of synthesis and a depot of readily available high-energy chemicals.

The skin performs many varied functions. These are brief main functions of skin (Barry, 1983:1-40)

1. To contain body fluids and tissues – the mechanical function.
2. To protect from potentially harmful external stimuli – the protective or barrier function: (a) microorganisms; (b) chemicals; (c) rediation; (d) heat; (e) electrical barrier; or (f) mechanical shock.
3. To receive external stimuli, i.e., to mediate sensation; (a) tactile (pressure); (b) pain; or (c) heat.
4. To regulate body temperature.
5. To synthesize and to metabolize compounds

6. To dispose of chemical wastes (glandular secretions).
7. To provide identification by skin variations.
8. To attract the opposite sex (apocrine secretions are evolutionarily defunct in this role)
9. To regulate blood pressure.

Stratum Corneum

In order to fully under stand the form and function of the stratum corneum it is helpful to know the processes involved in its formation from the living cells of the epidermis. Traditionally, several layers of living cells, constituting the Malpighian region, have been named either for their location or their appearance: the stratum corneum forms the straum lucidum (or compactum) and the stratum disjunctum. Although these terms or their English equivalents are still used, it is important to realize that the entire architecture of the epidermis constitutes a dynamic system in which each cell changes continuously during its passage from the basal layer where it is formed to the surface of the horny layer where it is discarded.

Study of the epidermis includes the morphology of the changing cell types, the dynamics of the differentiation process, the arrangement and chemical composition of the subcellular structures, the metabolic processes occurring in epidermal cells, and the location, composition, and physical properties of the epidermal barrier. All of these are discussed as a basis for evaluating the physical and pharmacological aspects of transdermal drug delivery.

Production of Stratum Corneum

A. Gross Epidermal Anatomy

In addition to the structural cells that eventually produce the stratum corneum, epidermis contains a variety of other cell types that have their own specific biological functions. These include the melanocytes, Langerhans cells, and Merkle cells. However, these cells are relatively sparsely distributed and can be presumed to have little effect on the physical properties of the epidermis that govern barrier function or reservoir effects. Nevertheless, for those whose concerns include pigment formation, sensory reception, or the immunological system, special study of these cells would be essential and reviews may be found in several recent publications. Likewise, other reviews should be consulted for discussions of the epidermal appendages, including the hair follicle and its associated sebaceous glands and apocrine glands, and the eccrine sweat glands. These structures do not appear to provide a significant route of entry of substances from the skin surface. The following discussion is therefore limited to the keratinocyte and its journey from formation in the basal layer of the epidermis to its incorporation into the horny layer and its eventual loss from the skin surface.

The outermost portion of the integument, the stratum corneum, consists of layers of terminally differentiated keratinocytes embedded in a matrix of lipid bilayers. This stratum of the epidermis serves as a barrier, which both prevents desiccation of the underlying tissues and excludes the entry of noxious substances from the environment. The

production of this protective covering is the principal function of the living epidermis.

Examination of the skin surface reveals that the stratum corneum is neither continuous nor homogeneous. Some regions, most notably the fingertips, bottoms of the toes, and the palmar and plantar surfaces, display extensive systems of lines and ridges, or dermatoglyphics, whereas the remainder of the skin surface is relatively smooth. Close examination reveals sweat pores and hairs penetrating through the stratum corneum. Although it has occasionally been suggested that these various openings through the stratum corneum could be exploited to bypass the barrier, it appears that the cross sectional area of the pores is so small as to be negligible. Furthermore, the outward movement of sweat or sebum would tend to flush out anything, which did penetrate.

When examined microscopically, the individual cells of the stratum corneum can be seen to be roughly hexagonal in shape and to overlap at the edges with neighboring cells. The projected area of the corneocytes varies slightly from one anatomical region to another, being least on the hands and heels and greatest in the axillary regions. There si also a slight increase with age in the projected area of corneocytes from any given anatomical region.

B. Replication

Epidermal cell replication is confined to the basal and immediately suprabasal cells. The roughly cuboidal basal cell contains a prominent nucleus, is relatively undifferentiated, and has a much smaller projected area than the thin, flat hexagonal cell of the stratum

corneum. In fact, from 10 to 25 basal cells may lie beneath a single stack of horny cells.

Two distinct basal cell populations have been identified, which differ in appearance and function. One population, called serrated basal cells, ahs numerous well-developed projections that extend into the papillary dermis, and are thought to serve principally in anchoring the epidermis to the dermis. These serrated basal cells contain tonofilaments,a nd have low mitotic activity. A second type of basal cell is non-serrated, has a smoother border, and lacks tonofilaments, but contains abundant melanosomes that may serve to protect the prominent nucleus from ultraviolet radiation. The non-serrated basal cells are thought to represent a stem cell population. In accord with this suggestion are the findings that they are relatively undifferentiated, they give rise to highly proliferative daughter cells in the immediately suprabasal layer, and they undergo more frequent replication during wound healing. Thus, the non-serrated basal cell undergoes a round of replication to produce another stem cell, which remains on the basal lamina, and a suprabasal amplifying cell, which may undergo several rounds of replication to produce a number of daughter cells, all of which may then enter into terminal differentialtion.

Owing to the heterogeneity of the basal cell population and the replicative population, measurement and interpretation of keratinocyte cell cycle kinetics is complicated. However, transit times through the viable epidermis and the stratum corneum can be reasonable estimated. For human epidermis, it has been determined that it takes

about 2 weeks for a cell to complete differentiation and another 2 weeks are spent in transit through the stratum corneum.

C. Differentiation

The morphologically different layers of the epidermis represent different stages in the terminal differentiation process. The first noticeable step in this process is the appearance of prickles, or spines, on the cell surface. These spinous cells are also more nearly round in shape, contain more keratin filaments, and are connected by more desmosomes than the basal cells. At this stage of differentiation, the keratinocyte has a complete complement of internal organelles, including a nucleus, nucleoli, an endoplasmic reticulum, a Golgi apparatus, and mitochondria as well as a few bundles of keratin filaments.

As differentiation proceeds, the cells become noticeably flatter, there is a shift from 3.5- to 4.5- nm thick filaments to mostly 7- to 10-nm thick filaments, and keratohyalin granules appear as the most prominent feature of the cytoplasm. Accordingly, keratinocytes at this stage of differentiation are called granular cells. These keratohyalin granules are not membrane bound and appear as more or less round, electron-dense bodies at the periphery or embedded in an irregular, less electron-dense matrix.

Whereas the keratohyalin granules are easily recognized under the light microscope, electron microscopic examination reveals a second class of granules in the granular cell. These smaller round or ovoid inclusions were measuring from 0.1 to 0.5 μ in length. They contain stacks of flattened lipid vesicles, which appear as disks within

a single bounding membrane. The lamellar granules first become noticeable in the spinous cells and become more numerous in the granular layer. In the final stages of differentiation, the lamellar granules migrate to the apical side of the cell, their bounding membrane fuses with the plasma membrane, and their contents are disgorged into the extracellular space. Concomitantly, the nucleus and all of the other organelles are broken down as the interior of the cell becomes filled with keratin filaments and associated matrix proteins. A thick band of protein is deposited on the inside surface of the plasma membrane to form the horny cell envelope. The extruded stacks of membranous disks undergo a fusion process to form the broad multilamellar lipid sheets that fill the extracellular spaces between the keratinized cells. With these changes, the transition from the viable basal cell to the keratinized horny cell is complete.

During the course of this differentiation process dramatic changes occur in the amount and compositon of the lipids associated with the keratinocyte. The basal cells contain the usual assortment of phospholipids and cholesterol found in many types of cells. Then as differentiation, the phospholipids are completely catabolized and the sugar units are removed from the glucosylceramides. As a result, the major lipids present in the stratum corneum are ceramides, cholesterol, fatty acids, sterol esters, and cholesteryl sulfate.

D. Desquamation

After a 2 weeks transit through the stratum corneum, corneocytes are sloughed off into the environment through the process, called desquamation. The mechanisms underlying this

process are essentially unknown. Desmosomes, which serve to hold together adjacent cells in the viable epidermis, appear to be few and degenerate in the stratum corneum, and are therefore not thought to be of importance in cohesion or desquamation. The only change in lipid composition known to accompany desquamation is a decrease in the proportion of cholesteryl sulfate. Although this is a relatively minor lipid, it is the most polar component of the stratum corneum, and it has been suggested that cholesteryl sulfate may be necessary to maintain the bilayer structure of the stratum corneum lipids. The loss of cholesteryl sulfate would be accompanied by a loss of bilayer structure, and this physical change could permit desquamation. However, recent studies have demonstrated that desquamation is not accompanied by loss of the intercellular lipid sheets, and that bilayers can be formed in vitro from stratum corneum lipids without cholesteryl sulfate.

Other factors that may be significant determinants of the cohesion – desquamation behavior of the stratum corneum include subtle changes in the physical properties of the lipid bilayers that are not dependent on changes in lipid composition, interdigitaion of stratum corneum cells, and the possible actions of proteases or other hydrolases on the horny cell envelopes or their contents. Additional studies are needed to assess these factors.

Components of the stratum corneum

A. Keratin

Keratins are a family of α-helical polypeptides ranging from 40,000 to 70,000 daltons in size. They are relatively poor in cystine rich in serine and glycine, and contain N-acetylserine at the amino terminus. Keratins accumulate throughout epidermal differentiation and represent the major component of the stratum corneum as well as of epidermal appendages such as hair, nail, and hoof. Early in epidermal differentiation, low molecular weight keratins predominate, whereas higher molecular weight polypeptides seem to be synthesized as pairs of relatively acidic and basic polypeptides. Varying degrees of phosphorylation of serine residues may contribute to charge heterogeneity. The individual keratin molecules aggregate to form superhelices, the detailed structures of which are still under investigation. A histidine-rich protein, called filaggrin that is derived from the keratohyalin granules, facilitates this aggregation. The filaments found in the stratum corneum are 7-10 nm in diameter and many microns in length. They are stabilized by the formation of disulfide bridges and cannot be solubilized in the absence of a reducing agent.

The Keratin filaments, surrounded by an amorphous matrix of sulfur-rich proteins, fill the interior space of the corneocyte. They are probably responsible for maintaining the flat hexagonal shape of the corneocyte, and may contribute to the toughness and flexibility of the stratum corneum.

B. The Corneocyte Envelope

The cornified cells of the stratum corneum are bounded by an envelope produced in the final steps of teminal differentiation. In

transmission electron microscopy, this envelope appears as a uniform 12 nm thick electron dense band that has replaced or been added to the electron dense polar region of the inner leaflet of the granular cell plasma membrane. The lucent hydrophobic interior of the plasma membrane and the outer polar region appear to remain intact, and early workers often referred to this aspect of the horny cell envelope as the horny cell plasma membrane.

The thickened inner portion of the envelope consists of cross-linked proteins, predominantly involucrin. Involucrin becomes cross-linked through γ-glutamyl-ε-aminolysyl isopeptide bonds introduced by the action of γ-glutamyltranspeptidase. This enzyme is apparently activated by an influx of calcium resulting from a change in the permeability of the plasma membrane late in the differentiation process. In addition to involucrin, at least six other soluble and membrane associated proteins become incorporated into the cross-linked protein envelope. Several of these are specific keratinocyte proteins, whereas several others are nonspecifically incorporated into the envelope superstructure.

While the band of material lying immediately outside the protein envelope has the appearance under the electron microscope of a residual plasma membrane, it is actually quite different from plasma membranes found in the viable epidermis. Unlike other membranous structures, it persists after extensive extraction with chloroform methanol mixtures, and whereas typical plasma membranes consist mainly of phospholipids and cholesterol, with varying amounts of peripherally associated or integral membrane proteins, the lipid

portion of the corneocyte envelope consists almost entirely of ester-linked ω-hydroxyacylsphingosines. These hydroxyceramide molecules contain mainly 30- through 34- carbon ω-hydroxyacyl chains and represent 2% of the dry weight of the stratum corneum.

Radiotracer studies with C14-acetate have indicated that the hydroxyceramide of the envelope is derived from a structurally related acylglucosylceramide found in the living cells of the epidermis. Evidence has been presented indication that at least half of the hydroxyceramide molecules are linked to the protein envelope through the ω-hydroxy terminus, and it has been suggested that this would allow the sphingosine moieties to interdigitate into the adjacent bilayer in the intercellular space. This latter arrangement could account for the observation that lipid extraction causes collapse of the intercellular space, but does not result in disintegration of the extracted stratum corneum. The lipid envelope hydroxyceramides would normally anchor the corneocyte to the intercellular lipid lamellae. On removal of the intercellular lipids, the covalently bound hydroxyceramides would interdigitate in a ziperlike manner to close the intercellular space and hold together the stratum corneum. Although additional work is needed to better define the nature and properties of the lipid envelope, this recently identified anatomical feature would seem to be of potential significance in the interaction between the corneocytes and the surrounding lipids, the permeability of the individual cells, and desquamation. It may also account for the unusual resistance of the stratum corneum to proteolytic enzymes.

C. Intercellular Lamellae

The intercellular spaces of the stratum corneum are completely filled with broad, multiple lipid lamellae. In osmium-postfixed thin sections, these lamellae are rarely evident and the intercellular spaces appear empty, but recent use of ruthenium tetroxide as postfixative has permitted routine visualization of the intercellular membranes. Use of this technique has revealed that the lamellae are found throughout the stratum corneum, and even persist after desquamation. Although no quantitative studies have yet been performed, the number of lamellae between each pair of cells seems to vary considerably, ranging from four to 20 or more.

These extracellular membranes in the stratum appear to be produced by edge-to-edge fusion of the flattened lipid vesicles that are extruded from the lamellar granules. Before extrusion, the stacks of disks in the lamellar granules appear to have alternation major and minor electron-dense bands with electron-lucent material in between. Each minor dense band is thought to represent the apposition of two polar regions on the interior of a flattened bilayer vesicle, whereas the major dense bands represent the polar regions between adjacent vesicles. This appearance is essentially preserved in the lipid sheets of the stratum corneum, for which the center-to-center distance between adjacent major dense bands has been estimated to range from 9.7 to 12.8 nm. This distance corresponds to the thickness of two bilayer membranes.

The epidermal sphingolipids are structurally heterogeneous, consisting of seven series of ceramides and a corresponding series of glucosylated ceramides. Representative structures of the ceramides

from human stratum corneum are summarized in Figure 2. As can be seen, the ceramides include both sphingosines (ceramides 1,2,4, and 5) and phytosphingosines (ceramides 3 and 6). Also, the amide-linked fatty acids include non-hydroxy acids (ceramide 2 and 3), α-hydroxyacids (ceramides 4, 5 and 6), and ω-hydroxyacids are the same 30- through 34- carbon species found in the lipid envelope, and a high proportion of the ester-linked fatty acid is normally linoleate. The acrylceramide appears to be mostly synthesized in parallel with a related acylglucosylceramide in the viable epidermis rather than being generated by deglycosylation of the analogous glycolipid in the final stages of differentiation. Unlike the acylglucosylceramide, which is confined to the living portion of the epidermis, the acylceramide is found throughout the stratum corneum and even persists after desquamation. Both of these molecules are associated with the lamellar granules and are thought to serve as molecular rivets in formation of the stacked and flattened lipid vesicles of the lamellar granules. In addition, the acylceramide may serve in the formation and stabilization of the intercellular lamellae in the stratum corneum. (Wertz and Downing, 1989:1-16)

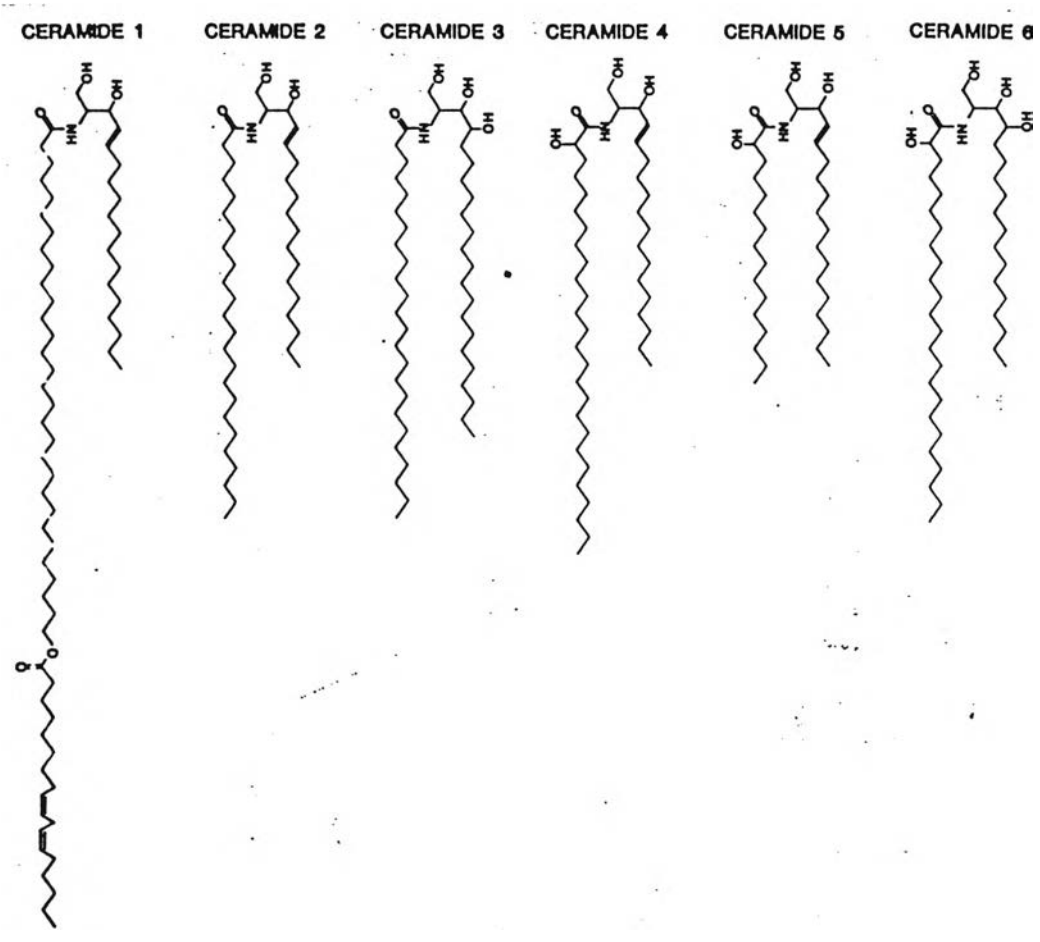

Figure 2 Representative structures of the stratum corneum ceramides. (Wertz and Downing 1989:11)

Franz Diffusion Cell

The aim of in vitro experimentation in transdermal delivery is to understand and/or predict the delivery and penetration of a molecule from the skin surface into the body via the skin of a living animal. Typically, this is achieved using a variety of skin diffusion cells and various experimental protocols. However, if the results of such experiments are to be of value, then one should question to what extent an in vitro experiment should reproduce the exact in vivo situation. Should it be so accurate as to take into account all of those variables that could possibly affect transdermal delivery, or should the experiment be representative, allowing space for calculated interpretation of the results? The ultimate goal of all workers in the field of transdermal delivery is to produce, in vitro, an experimental design that will predict, exactly, and the penetration of the candidate molecule into the human body in vivo. When one considers the vast amount of transdermal delivery information generated in vitro, it is somewhat surprising to find so few publications that deal directly with the quantitation of the in vitro experiment. Many, it appears, have come from experimental hindsight. It is, therefore, left to the transdermal delivery investigator to glean from existing experiments the methods, interpretation, and control of in vitro experiments in transdermal delivery.

Theoretically, the in vitro experiment lends itself to easy control, i.e., one is not confounded by the idiosyncrasies and habits of the living animal. However, it is rare that a single variable can be controlled without affecting other aspects of the experimental

procedure. Alternatively, the trade-off between should we control it and how difficult will it be to control often means that assumptions are made and interpretation of the experiment is stretched to unacceptable limits. As there appears to be so little data on the affects of varying in vitro experimental design, it is the responsibility of the investigator to attempt to design the most stable experiment in order to avoid chance variables; e.g., prevailing ambient conditions. Without doubt, every new worker to the field of transdermal delivery will add to the design of diffusion cells. New cells should, however, be designed with two factors in mind. Ease of operation and/or a quantifiable design improvement. Without such changes one would simply be adding to the plethora of diffusion cells and experimental procedures that already exist.

As previously stated, the aim of the in vitro experiment is to understand the kinetics of a molecule traveling from the body surface, via the skin, into the body mass. For the in vitro investigator transdermal delivery can be des cribbed in three principal stages, each posing its own problems of experimental design:

1. Delivery of the molecule to the skin surface
2. Passage of the molecule through the skin
3. Delivery of the molecule into the body in vivo = recovery of the molecule in vitro

In vivo all of the above are under the control of the host animal. Even though a delivery system may be tailored to optimize release, the environment in which it finds itself is at the mercy of the host. However, in vitro all of the above can be controlled to a greater or

lesser extent. It is important to appreciate the need for control, as the apparent kinetics of penetration of a molecule can be affected at any of the above stages and may in turn lead to erroneous in vivo predictions.

In general, in vitro transdermal delivery experiments are conducted on either vertically or horizontally arranged diffusion cells. This nomenclature is immediately confusing, as the barrier membrane, usually skin, is oriented at right angles to the diffusion cell. All references in this text to diffusion cells will be based on the orientation of the apparatus, not the barrier.

Delivery of the molecule to the skin surface

In order to optimize delivery of a molecule, it is desirable to present it in molecular form and in a vehicle that favors release of the molecule to the skin. In vivo, once placed on the skin the formulation is immediately subjected to a variety of environmental conditions; e.g., temperature (~37°C), a surface covering of sebum and/or sweat, desquamation of corneocytes, and a complex microbial flora. Few, if any of these variables are controlled or reproduced in vitro. Is there a need to reproduce such a complex series of variables, and will they affect the final result?

A. Temperature

The rate of diffusion will increase with increasing temperature. Similarly, the thickness of the applied vehicle would affect diffusion of the molecule through the vehicle to the skin. Typically with viscous formulations as the temperature increases the viscosity decreases there

by aiding diffusion through the vehicle. It is desirable to at least control and preferably regulates the temperature to 37°C ± 1°C to avoid additional extrapolations from fluctuating ambient conditions.

B. Skin surface coverings

The skin surface has a covering of sebum and /or sweat. Depending on the eventual sight of in vivo delivery it could indeed be important to reflect the in vivo conditions. Both sebum and sweat, under normal circumstances would not be expected to change greatly between in vitro and baseline in vivo situations. However, if the proposed action of the candidate molecule is in a dermatosis such as hyperhydrosis, i.e., high sweat levels creating essentially an aqueous environment or seborrheic dermatitis, essentially a lipophilic environment, then in vitro experimentation on normal skin cannot be expected to give reliable in vivo predictions.

C. Stirring the vehicle formulation

As mentioned, the diffusion of the molecule under study through the vehicle may act as a rate-limiting step. There is little doubt that stirring of the vehicle would enhance homogeneity of the formulation and hence presentation of the candidate molecule to the skin surface. However, this has to be weighed against the practicalities of stirring the vehicle. Horizontal diffusion cells lend themselves will to control of this parameter owing to a comparatively large vehicle volume, total containment of the vehicle, and the fact that the delivery chamber can be placed in direct contact with a magnetic stirrer. However, in choosing to use the horizontal cell the investigator has

imposed two conditions on the skin that are known to affect transdermal delivery:

1. Increased hydration of the delivery site
2. Occlusion of the delivery site

Although allowing for easy temperature control and stirring of the donor or vehicle phase, use of the horizontal cell necessitates a volume of donor phase sufficient to contact the whole of the skin surface (vertically oriented); i.e., the larger the delivery area, the greater the volume required. In so doing the skin is continually bathed with the vehicle formulation. Therefore, with aqueous formulations, including emulsions, the comparatively dry stratum corneum becomes totally hydrated, therefore enhancing penetration. Similarly, occlusion of the delivery site may enhance penetration by either causing hydration of the stratum corneum with water diffusing from the viable epidermis, or by preventing loss of the formulation from the skin surface. It was considered that enhancement was due to two factors: (a) preventing evaporation and thereby keeping the molecule at the delivery site, and (b) increasing hydration of the membrane. It was also noted that liquid in excess of the delivered dose accumulated under the occlusive material and was considered to originate from the receptor phase by diffusion; i.e., diffusion will occur in both directions. When emulsion-type formulations are applied to the skin surface the aqueous component rapidly hydrates the stratum corneum, causing a measurable change in viscoelastic properties. These rapidly disperse after only a few minutes owing to water evaporation and are then followed by longer-term changes (several hours) imparted by the

oil phase of the emulsion. It is evident, therefore, that containment of the vehicle will change both the physical nature of the skin and the expected thermodynamic status of the candidate molecule in the formulation; i.e., it is no longer a true representation of the in vivo situation.

In contrast, the horizontal cell may be useful in the development of delivery devices; e.g., patches. In such circumstances, there can be little difference in the occlusive or hydrative conditions that exist under an established patch delivery system and a horizontal in vitro cell. Initial predictions could however be erroneous, as hydration in the cell is almost certainly more rapid than under the newly applied patch; hence, lag times must be interpreted with care.

Other factors, which would be expected to affect transdermal delivery in vivo and should be considered in vitro are as follows:

1. Duration of contact with the skin
2. Removal of the vehicle and washing procedures
3. Donor compartment design

It is common practice to run in vitro experiments for at least 24 h, although it is rare in vivo that an appreciable amount of the applied dose remains in situ for extended lengths of time unless protected. Removal of the dose occurs either by rubbing or washing off. Even washing with plain tap water should be considered as having the potential to enhance penetration and as such should be duplicated in vitro. Washing procedures that involve surfactants are known to damage the barrier properties of the skin. Although damage to the barrier in vivo may be short lived, it is capable of enhancing

penetration. A large increase in water permeability after treating the skin surface with 1% sodium laurate has been observed. It has also been found that increasing the concentration increased both the degree and rapidity of damage. Following removal of the surfactant, the skin showed varying degrees of barrier function recovery, though with higher surfactant concentrations recovery was not complete. In vitro it must be considered that the regenerative properties of the skin have been compromised and as such the barrier, once damaged, cannot be repaired.

D. Donor compartment design

Design of the donor compartment normally receives little attention. Orientation of the horizontal diffusion cell lends itself to stirring and temperature control. The vertical cell additionally lends itself to ease of dose application, varying dose size and various degrees of occlusivity. However, care should be taken in sealing the skin between the two halves of any the diffusion cell. Typically, the forces used to clamp the cell halves together are sufficient to cause a tight seal. The addition of rubber or Teflon O rings may help. In cells using large surface areas it may be difficult to maintain equal pressure around the whole of the junction. In this case, erroneous data may be generated where the applied dose is allowed to contact the cell joint. One wonders exactly what damage is caused to the stratum corneum immediately adjacent to the seal and whether this affects penetration kinetics.

Passage of the molecule through the skin

The vast majority of transdermal delivery experiments are conducted with human in vivo studies in mind. However, there exists a continual, and as yet unresolved, debate as to whether alternative "membranes" can be substituted for human skin. In general, the decision to use human skin rests on availability and not the search for scientific excellence. If one assumes that the in vitro experimental design should reflect exactly the in vivo situation then only human skin can be used.

A. Availability of human skin

Human skin is for many investigators not readily available. Those samples obtained are taken from a variety of body sites, a factor known to influence penetration and under a variety of conditions; e.g., from tummy reductions, mastectomies, amputations, and cadavers. The latter has generated the most cause for concern, as there appears to be no definitive study relating post mortem changes to barrier function. One may well expect autolytic processes within the epidermis following death to have a direct effect on the integrity of the overlying barrier.

B. Alternatives to human skin

The vast majority of in vitro experiments are conducted on animal skin, in particular the hairless mouse; although the hairless and fuzzy rat, guinea pig, snake and rabbit also are used. Throughout the history of transdermal delivery investigators have sought to find a predictive correlation between the penetration of molecules through animal and human skin. Although there exists a number of

similarities, there is as yet no animal skin the completely mimics the penetration characteristics of human skin. It is recognized that in such fields as product development, the large number of experimental replicates necessitates the use of animal skin. However, such experiments should be used to assess the release characteristics of the vehicle and/or delivery device and not the penetration kinetics of the molecule through skin.

As a third alternative, artificial membranes, designed to mimic the barrier properties of the skin, have been used with some success.

C. Removal and preparation of skin

Skin is usually removed from the animal by blunt dissection, although dermatome sectioning may be used. In vitro experiments differ widely in the thickness of skin used, though virtually all hinge on the now accepted dogma that the principal barrier to penetration is the stratum corneum. Therefore, some investigators argue that only intact stratum corneum need be used. However, the skin, from the desquamating stratum corneum through to the viable dermis is a carefully organized, interrelated structure. In particular, the epidermis, originating from the stratum basale, is a stratified system of continually maturing cells. The delineation of distinct layers within the skin is one of histological convenience, labeled by man and not nature. It should be considered, therefore, that each layer has the potential to play an integral role in the functions of the skin, including the penetration of exogenous molecules.

Human skin in its entirely tends to be thick with large amounts of subcutaneous fat. Owing to the constraints of in vitro apparatus and

on the assumption that the lower skin layers have little effect on penetration, it is often desirable to reduce skin thickness without disturbing the barrier properties. This can be achieved in a number of ways.

1. Heat separation

Although sometimes difficult to perform, the epidermis can be removed in large sheets by immersing the whole skin in water at 60°C for 2 min. Typically, the separated stratum corneum or epidermis is placed directly onto diffusion cells, as once dry the sample is more difficult to handle and prone to cracking.

2. Chemical separation

The skin can be split at approximately the level of the stratum lucidum by means of a number of chemical agents; e.g., trypsin, sodium hydroxide, formic acid, and sodium bromide. Although the reports of such techniques carry data to support the unchanged status of the barrier properties, agents such as sodium hydroxide and formic acid will not only affect different animal skin types to differing degrees, but also will with time cause total breakdown of the stratum corneum. Such techniques should, therefore, be used with great care and suitable controls conducted.

3. Physical separation

Physical separation of the skin is best undertaken with an electric dermatome offering controllable thickness of section. The lack of sample width, ~2-3 cm, does not create a problem as more emphasis is placed upon small diffusion cells. Of principal concern is the abundance of skin appendages, e.g., hair follicles and sweat

glands, which reach from surface level deep into the dermis. During sectioning all of these are severed, in essence creating a series of pores into the skin. It is, however, intriguing to note that such sections show identical barrier properties to full-thickness skin; i.e., as if the holes were not there.

Following any physical or chemical treatment of skin samples it is recommended that the status of the barrier should be assessed using a test molecule of known penetration kinetics; e.g., water.

Recovery of the molecule in vitro

Once the candidate molecule has penetrated through the stratum corneum, it will, with luck, be transported to its proposed sight of action. Although termed transdermal delivery, a number of molecules are active or may be metabolized at the level of the epidermis. Alternatively, the goal may be to traverse the skin in total and deliver the molecule to either a specific organ or to the body as a whole. Typically, in vitro the body mass is represented by physiological saline, with or without a preservative. Other mediums such as blood plasma or cell culture medium have been used, but have not found favor. They are expensive and are no more, ore less, representative of the in vivo situation than saline. Cell culture medium may be of value where it is considered that viability of the epidermis must be maintained. Saline alone has the principal disadvantage of being a simple aqueous medium and as such may hinder the release of lipophilic molecules. The addition of surfactants to the receptor phase must be viewed with caution because surfactants have the ability to

disrupt barrier function and diffusion can occur in both directions through the skin. Therefore, it is possible that increased recovery of the molecule was a function of increased penetration due to barrier damage. In general, although not usually practiced, if there is doubt about the applicability of the receptor phase, then simple partitioning experiments should be conducted to establish the potential limiting effects of the receptor formulation.

A. Receptor compartment

The receptor compartment has achieved the bulk of attention in the design of skin diffusion cells. In either horizontal or vertical cells it is easily stirred and temperature controlled. Typically, the receptor compartment is made as small as possible while maintaining infinite sink conditions. Great play is made of the hydrodynamics that exist within the receptor compartment. Stagnent or boundary layers are known to have a predictable effect in controlled partitioning experiments. However, assuming the diffusion cell has been designed to optimize receptor stirring, and then such hydrodynamic problems are unlikely to affect results. The underside of the skin, whether full or split thickness, is quite simply very bumpy. Such a rough surface would be expected to create sufficient turbulence so as to dispel any concerns over limiting boundary layers. However, some diffusion cells have been shown to provide inadequate stirring with the formation of stirred and non-stirred layers within the receptor phase.

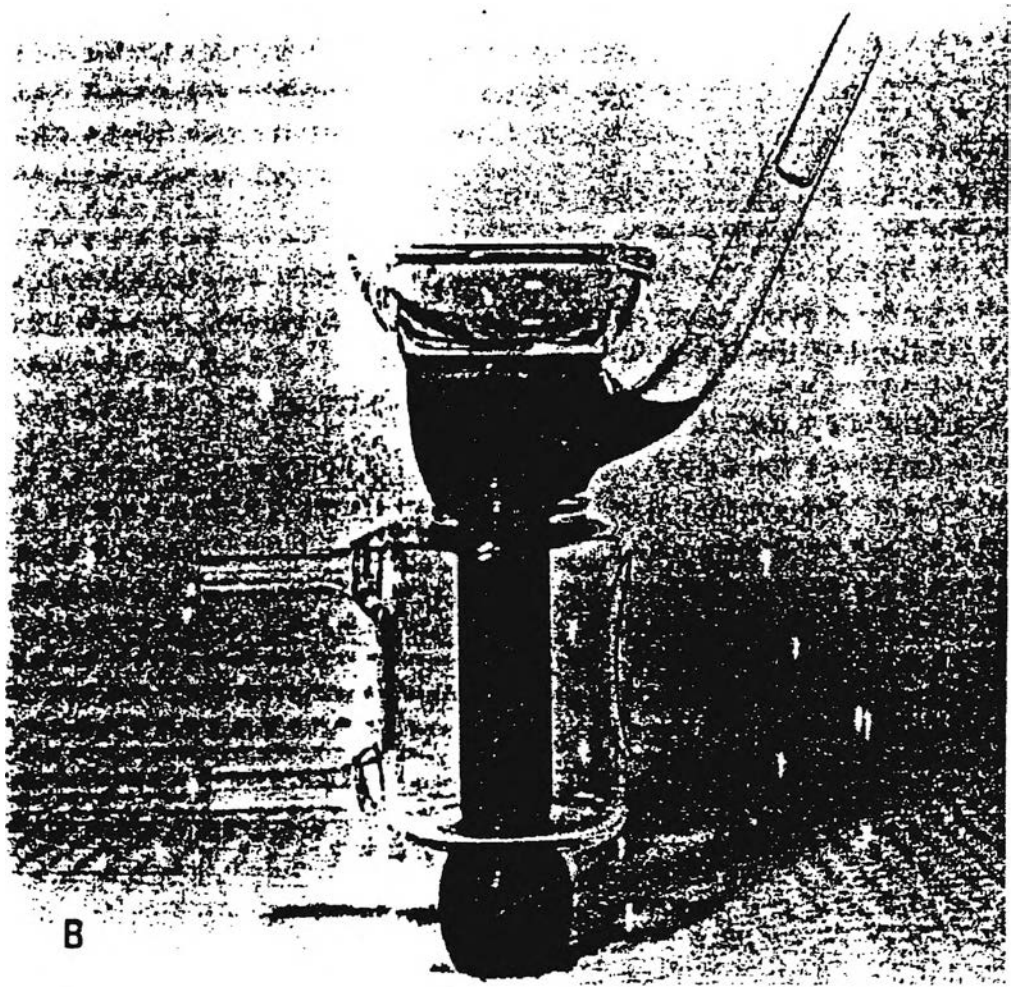

Figure 3 Typical diffusion cells with incomplete stirring of the receptor compartment (Gummer 1989:189)

Temperature control of the receptor compartment, as with the donor compartment, is essential in order to avoid changes in ambient conditions. Typically, skin diffusion experiments are conducted at 35-37°C in order to mimic in vivo core temperatures. The whole of the receptor compartment should be controlled, normally by use of a flow-through water jacket. While it is expected that temperature would directly affect diffusion in such in vitro transdermal delivery experiments, it is difficult to find the definitive publication that addresses the issue of at what temperature should such experiments be conducted.

B. Collecting the molecule

Assuming the molecule under study has penetrated the skin it has to be collected for assay. The problems pertaining to the receptor phase and compartment design have been discussed. In general, two sampling systems are used:

1. Aliquot sampling: typical of static diffusion cells. Inherent problems are high labor input, odd interval sampling, and disturbance of the experimental apparatus; the latter is cause for the greatest concern. Ideally, aliquot size is kept to a minimum (100 μl) and the volume of the receptor compartment replenished after sampling. However, with the trend for small receptor compartments (< 5 ml) larger aliquots (500 μl) even when replenished must be considered in the final kinetic calculations as replenishment results in continual dilution of the receptor. In addition, in those cells that do not show adequate stirring the initial aliquots may not be representative of the total receptor phase.

2. Continuous sampling: typical of flow-through cell designs. The main attraction of this system is that once established it can be left unattended to run its course. Disadvantages of the system are comparatively large overall receptor volumes (including collection tubing), establishing a suitable flow rate to maintain sink conditions, and handling of the collected samples. It has been shown that samples left uncovered overnight, lost appreciable amounts (~ 30%) of the collected penetrant. Great care should, therefore, be exercised at all stages of the experiment.

3. Maintaining infinite sink conditions: where small doses of penetrant are used this usually does not present a problem. However, in infinite dose experiments it must be ensured that the amount of applied penetrant does not exceed its solubility in the receptor phase assuming 100% penetration. With flow-through cell designs placing the emphasis on small receptor compartments it is important to maintain a flow rate sufficient to provide sink conditions. The comparatively high barrier properties of the skin to many molecules suggest that for poorly penetrating substances this should not present a problem. It has been shown that varying the flow rate between 2 and 7.5 ml/h has no significant effect on the penetration. It should not; however, be accepted carte blanche that flow rate does not affect recovery of the molecule.

Ideally, all of the components and materials used in the experiment should be assessed for their ability to absorb or adsorb the penetrant. Materials such as rubber and Plexiglas may show a high affinity for the test molecule affecting initial assessments of

penetration as the system comes to equilibrium. Care should also be taken in cleaning of the equipment to avoid contamination of future experiments, especially when radiolabeling is the method of choices for penetrant assay.

C. Recommendations for diffusion cell design (Gummer, 1989:177-194)

1. All materials should be assessed for their ability to absorb or adsorb the test penetrant.
2. Donor compartment.
    A. Easy access to deliver the penetrant to the skin.
    B. Stirred where possible
    C. Temperature controlled
    D. Control of evaporation for volatile vehicles and penetrants.
3. Membrane.
    A. For the study of penetration kinetics only human skin should be used.
    B. For vehicle/device release studies other barrier may be used.
    C. The skin sample should contain both stratum corneum and viable epidermis
    D. A molecule of known penetration kinetics should be used prior to the test molecule to assess barrier function.
    E. Where applicable, metabolic viability of the epidermis must be assessed.
4. Receptor compartment

A. Either flow-through or static
B. Temperature controlled
C. Sufficient volume to maintain infinite sink conditions.
D. Stirred without obvious formation of boundary layers.

5. Receptor fluid
   A. Should not compromise barrier function
   B. Of favorable partitioning characteristics to receive the penetrant.
   C. Capable of maintaining epidermal viability where necessary.
   D. Must be contained once collected.

Franz diffusion cell, the diffusion cell used in this experiment, a finite-dosing upright type, one of the most frequently used *in-vitro* techniques for skin permeation studies, was designed and developed by Franz in 1975. Franz diffusion cell, a commercial model, has been marketed and extensively used for skin permeation studies, over the years, to assist the development and the evaluation of a controlled-release transdermal therapeutic system as seen in Figure 4. Each of the diffusion cells consist of two compartments; a donor compartment, which is exposed to an ambient condition, and a receptor compartment which is maintained at 37°C by circulating magnetic bar. (Chien 1987:127-157)

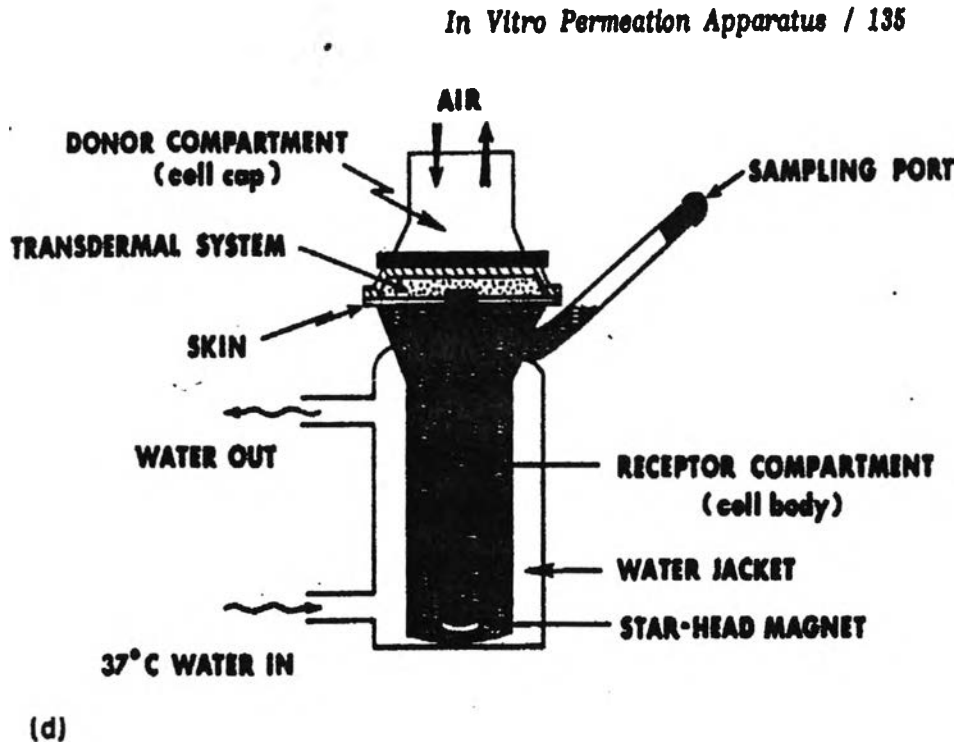

Figure 4 Franz diffusion cell : In vitro membrane permeation systems. (Chien 1987:135)

The permeation rate of the component, calculate from franz diffusion cell, is given by Fick's law, which can be written:

$$dM/dt = PS(C_D - C_R) \quad (1)$$

Where M is the amount of diffused component, t the time of diffusion, P the permeability coefficient, S the cross-sectional area of the membrane, and C_D and C_R are, respectively, the concentrations of the component in the donor and receiver sides of the membrane.

The concentration of the component in the donor side of the membrane is equal to its concentration in the continuous phase. This is difficult to measure in disperse systems, so usually the component concentration in the whole donor compartment is used. In this case an apparent permeability coefficient, P_a, is calculated instead of the permeability coefficient, P.

Because the amount of diffused component is the difference between the mass of the component in the receiver compartment and the initial amount of the component in the donor compartment:

$$M = M_{D(0)} - M_R \quad (2)$$

and the volume of the receiver compartment, V_R, was three times that of the donor compartment.

$$C_D = C_{D(0)} - 3C_R \quad (3)$$

where $C_{D(0)}$, is the initial concentration of the component in the donor compartment.

Integration of equation 1, after the above substitutions, gives

$$-\ln [1 - (4 C_R / C_{D(0)})] = (4P_aS/V_R)t \quad (4)$$

P_a can then be obtained from the slope of a linear plot of $-\ln [1-(4 C_R/ C_{D(0)})]$ against t. (Ktistis and Niopas 1998:413-418)

Snake skin and human skin

A variety of model membranes have been used for transdermal research, such as human cadaver skin, hairless mouse skin, and synthetic membranes. Although human skin is the best model membrane, the cost and limited availability put a limitation on the use of human skin. Also, the permeability through human skin varies up to 10 fold depending on the body site. On the other hand, it is easy to obtain animal skins of the same species with the same line and age. However, the time for experimental use of some animal skins in in vitro penetration studies is limited because of deterioration of membrane integrity after prolonged use. Moreover, most animal skins are more permeable than human skin partly because of a larger number of hair follicles. The use of artificial membranes in transdermal research is limited because they lack keratinized proteins and lipids, which are primary components in the stratum corneum of mammalian skins.

Shed snake skin is a nonliving pure stratum corneum with no hair follicles. Snake shed their skins periodically, leaving their old stratum corneum behind, which makes it possible to obtain multiple shed skins from the same individual snake. Unlike human stratum corneum, which consists of 10-20 layers of an alpha-keratin-rich intracellular layer and a lipid-rich intercellular layer. Shed snake skin consists of three distinctive layers, these are the beta-keratin-rich outermost beta layer, keratin- and lipid-rich intermediate mesos layer, and keratin-rich innermost alpha layer. Furthermore, mesos layer shows three to five layers of multilayer structure with cornified cells

surrounded by intercellular lipids, is similar to human stratum corneum. This layer is also a major depot of lipids, and the mesos layer and alpha layer are considered to be the main barrier to penetration through the skin. Further, water permeability has been compared between shed snake skins normal snakes and scaleless snakes, and the water permeability was found to be similar between normal and scaleless skins, indicates that the existence of scales may not affect significantly the permeability of compounds through shed snake skin.

There are similarities between the shed snake skin and the human stratum corneum in terms of thickness and lipid content summarized in Table 1. Lipids are important components to the skin in controlling the permeability of compounds. Delipidization has been shown to increase water permeability through both shed snake skin and human skin. Lipid compositions of shed snake skin and human stratum corneum are also similar, that is, neutral lipids are a main lipid component in both skins and fatty acids, with carbon chain lengths of C16 and C18 predominant.

Reported water evaporation rates are also compared between human skin and shed snake skin in Table 1. Although the water permeability through snake skin varies among species and is especially dependent on habitat conditions, water permeability through shed snake skin is very similar to that through human skin, suggesting that shed skin may offer a good model membrane for transdermal research.

Table 1 Comparison of thickness, lipid content, and water evaporation rate between human stratum corneum and shed snake skin. (Itoh et al. 1990:1043)

	Human stratum corneum	Shed snake skin (*Elaphe obsoleta*)
Thickness	13–15 μm[a] 10 μm[b]	10–20 μm[c]
Lipid content	2.0–6.5%[d] 3.0–6.8%[e]	ca. 6%[f]
Water evaporation rate	0.1–0.8 mg/cm^2 hr[g] 0.34 mg/cm^2 hr[h] ca. 0.2 mg/cm^2 hr[i]	0.15–0.22 mg/cm^2 hr[j]

The potential usefulness of shed snake skin as a model membrane for transdermal research was examined. There are similarities between shed snake skin and human stratum corneum in terms of structure, composition, lipid content, water permeability, etc. The permeability of various compounds and the contribution of several functional groups to the permeability were also found to be similar between shed snake skin and human skin. Considering the similarities between shed snake skin and human skin, ease of storage and handling, and low cost, shed snake skin may offer a good model membrane for transdermal research. (Itoh et al., 1990:1042-1047) In-vitro permeability to salicylic acid of human, rodent, and shed snake skin has been examined for the purpose of selecting model membranes for human skin corresponding to different anatomic sites, since a marked regional variation is suggested among the different sites. The greatest permeability to salicylic acid was observed in the scrotum, whereas negligible permeability of the sole to salicylic acid was apparent. The cheek, neck and inguinal skin seem more permeable than the breast, back, thigh, lower leg and foot. Shed snake skin and hairless rat skin were found to show similar permeability to human breast, back, thigh, lower leg and foot skin, while wistar rat skin and nude mouse skin showed similar permeability to human cheek, neck and inguinal skin. Moreover, the stratum corneum of human breast, back, thigh, lower leg and foot skin has barrier function similar to that of shed snake skin and hairless rat skin. Shed snake skin has been reported to be similar to human stratum corneum in its structure and lipid content, although their lipid components are different. The main

polar lipids in shed snake skin are phospholipids but are ceramides in man. This study suggested that total lipid content is more important than total lipid composition in barrier functions of the stratum corneum. (Harada et al., 1993:414-418)

Gel Dosage Form

For optimum consumer appeal, the gel should have good optical clarity and sparkle. A high viscosity and a high-yield value are essential, but how high is primarily a matter of intended product application. To preserve product integrity, the gel should maintain its viscosity at all temperatures that may be encountered during shipment and storage. During formulation, there is frequently a trade-off between these optimum characteristics and the chemical requirements of active ingredients.

Carbomer is a commonly used gelling agent that produces gels having a number of these desirable characteristics. The grade, carbomer 934 P NF, is most commonly used in the pharmaceutical industry and has been selected as the exemplary polymer for formulation discussion. Chemically, carbomer 934 P is a cross-linked acrylic acid polymer having a molecular weight of approximately $3x10^6$. The gelation mechanism depends on neutralization of the carboxylic acid moiety to form a soluble salt. The polymer is hydrophilic and produces sparkling clear gels when neutralized. Although carbomer tolerates large amounts of alcohol, it does so with decreased viscosity and clarity. Gel viscosity is strongly dependent on pH and the presence of electrolytes. A maximum of approximately 3% electrolyte can be tolerated before precipitation occurs as a rubbery mass. Carbomer gels possess good thermal stability in that gel viscosity and yield value are essentially unaffected by temperature. As a topical product, carbomer gels possess optimum rheological properties. The inherent pseudoplastic flow permits immediate

recovery of viscosity when shear is terminated and the high-yield value and quick break make it ideal for dispensing.

The viscosity building effects of carbomer are readily apparent when examining the gelation mechanism in association with the colloidal network structure. Before neutralization, carbomer in water exists in its unionized form and yields a thin opalescent dispersion of approximately pH 3. At this pH, the polymer is very flexible and behaves like a random coil. Addition of sodium hydroxide or a neutralizing amine to the dispersion shifts the ionic equilibrium in favor of the soluble salt form. This results in ionic repulsion of the carboxylate groups and the polymer becomes stiff and rigid, thereby increasing the viscosity of the water. Overneutralization and excess salts reduce the viscosity of carbomer gels or cause precipitation by the counterion effect.

pH has dramatically effect on the viscosity development of carbomer gels. As pH increases and the carboxylic acid moieties of the polymer are neutralized, viscosity and clarity increase. Acceptable gel clarity and viscosity occur at approximately pH 4.5 to 5.0, but optimum viscosity and clarity are at pH 7. Overneutralization results in a decrease in viscosity that cannot be reversed by addition of acid to lower the pH because an electrolyte is formed.

Although carbomer can be used in gel formulations with a large proportion of alcohol, the dehydration effects of the alcohol on the polymer are still substantial. The viscosity responses of the gels to alcohol may be interpreted by its action as a non-solvent. Because the solvent affinity is reduced, the polymer contracts, with a consequential

increase in the interparticle distance and subsequent decrease in the number of entanglements and cross-links. To decrease the interparticle distances and restore the integrity of the gel network structure, a greater concentration of polymer must be used. The reduction in solvent affinity and change in the polymer conformation results in increased haziness of the gel as the alcohol content increases.

To manufacture clear, uniform, air-free gels, certain key processing characteristics must be provided. The nature of carbomer requires initial high-shear mixing to form a uniform smooth dispersion, followed by low-shear planetary mixing during the neutralization-gelling process. Air entrainment during the neutralization process can be minimized by subsurface addition of liquids in conjunction with low-shear mixing. In addition, mixing under vacuum, if available, will withdraw entrapped air from the dispersion during manufacture and prevent further air entrainment by incidental surface breaks. Minimization of air entrainment is necessary from the aesthetic standpoint and, most importantly, from the aspect of controlling fills weights during packaging operations. (Pena 1990:381-388)