CHAPTER II

LITERATURE REVIEW

Whatever class of drug is considered, a number of formulation guidelines can be established to optimize topical performance. It is important to address the problem of facilitating the transport of the pharmacologically active substance to a local site of action. The biological activity of the NSAID formulation will depend on its ability to reach the target site in order to inhibit the synthesis of the inflammation mediators. These mediators are located within the viable region of the skin or the immediate by underlying tissues. Thus, the drug will have to partition into and out of the stratum corneum to get to this region. The overall biological activity of a topical preparation depends on a number of factors. For example, the physicochemical properties will determine the release rate from the chosen formulation and also how fast the drug can reach its target site. It may, therefore be possible to choose an anti-inflammatory agent which has a low inherent pharmacological activity but is easily released from the formulation and transported to its site of action. On the other hand, it would also be very easy to choose an extremely potent anti-inflammatory agent which is poorly absorbed and, therefore, exhibits minimal activity.

The different physicochemical properties relevant to release from the formulation and subsequent absorption, are illustrated schematically in Figure 1. The dominant processes are diffusion and partition and a knowledge of

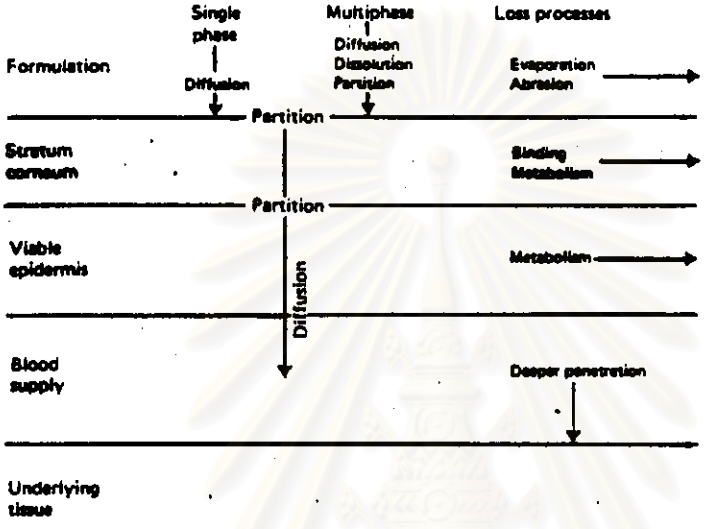


Fig. 1 Schematic representation of the physiochemical processes involved in drug release and topical absorption.

these is vital in the rational optimization of any topical delivery system (Hadgraft, 1989).

Percutaneous Absorption of Anti-Inflammatory Agents

It is apparent from Figure 1 that the first step in the absorption process is partitioning of the drug into the outer layers of the stratum corneum. This region of the skin is largely lipophilic in nature, and the first step in absorption is facilitated for drugs which have a high lipid-water partition coefficient.

After the drug has partitioned into the skin, it will diffuse through the stratum corneum as a result of the concentration gradient that exists. The route of penetration has been the subject of considerable debate. But current opinion suggests that the major pathway is through the intercellular channels (Hadgraft, 1989). These are filled with a structured array of neutral lipids, and it is through these that the drug must diffuse. Most non-steroidal anti-inflammatory drugs (NSAIDs) have a molecular weight between 200 and 500 daltons and within this range it is not anticipated that the diffusion coefficient will vary considerably. Specific groups on the drug may interact with components of the skin and may lead to slow diffusion but quantification of this is not possible. Steroids with -OH groups diffuse more slowly than their congeners without -OH group. It is also feasible for the drug to contain functional groups which can disrupt the structured lipids in the intercellular channels making them more fluid. In this case, the drug may act as its own penetration enhancer (Hadgraft, 1989).

At the interface between the stratum corneum and the viable tissue, the drug must undergo a further partitioning step in order to reach its target. The viable tissue is largely aqueous in nature, and transfer from the stratum corneum will be favored for NSAIDs which have hydrophilic properties. These attributes are obviously in direct competition to those required for entry into the stratum corneum and it is not surprising that optimum penetration is achieved for drugs which are neither too hydrophilic nor too lipophilic. Absorption of a range of NSAIDs in humans has been investigated, and it was possible to conduct limited quantitative structure activity relationships with these data.

Once the drug has partitioned into the viable epidermis it will diffuse deeper into the skin, and eventually be transported away by the blood supply which is located at the dermal-epidermal junction. Diffusion through the viable tissue will be relatively rapid compared to that of the stratum corneum. The resistance experiences has been likened to that of an aqueous protein gel, and typical diffusion coefficients are in the region of 10^{-7} $cm^2 s^{-1}$ (Scheuplein, 1967). Again, the value is unlikely to be strongly dependent on the molecular weight of the diffusant.

During both diffusion processes and perhaps more significantly in the latter, the NSAIDs will encounter various enzymes. The exact location of enzyme systems within the epidermis has not been identified but many metabolic processes have been observed. Perhaps the most widespread are non-specific esterases, and they may either deactivate the drug or activate a

prodrug which has ideal characteristics for partitioning into the stratum corneum.

As it diffuses through the intercellular channels, it can be metabolized into the active more hydrophilic free acid which will partition into the viable tissue more readily. Currently, it is not known how easily this process occurs nor at which point the enzymes become saturated. The rate of metabolism is dependent on the structural characteristics of the molecule. The overall utility of the prodrug approach will depend on a careful balance between the physicochemical properties of the parent drug, the prodrug, the location of metabolism and its rate. N,N-dialkyl hydroxylamine derivatives of indomethacin have been produced as prodrugs and enhanced absorption observed through mouse skin. However, in an *in vivo* human assessment it was found only to be as effective as the parent drug (Hadgraft, 1989).

Drug Release from Formulations

Given the constraints determined by the barrier of the skin, drug release from the formulation should be maximized. The optimization of this will also be dependent on the physicochemical properties of the NSAID. At the same time, it should be noted that the constituents of the base may themselves modify the stratum corneum. Often the interdependence of the different factors involved make it difficult to separate the influence that the formulation is having on release. However, some indicators can be established.

The choice of the final formulation will depend on the pharmaceutical/cosmetic acceptability and the stability of the drug in the solvent chosen. NSAIDs have been formulated in ointments, creams and gels and fundamental physicochemical concepts common to all may be identified.

In order to maximize the rate of release, the drug should be at its maximum thermodynamic activity. It should also be realized that enhancers appear to act predominantly on more polar compounds. If the NSAIDs chosen are extremely lipophilic, it may prove difficult to promote their absorption using conventional enhancers. If the topical base contains occlusive agents or ones which hydrate the stratum corneum, the absorption of the drug may be increased. Although many compounds do penetrate hydrated skin more readily, the effect was seen to be more pronounced for more polar materials.

Assessment of Topical Drug Release

In the absence of skin, monitoring the release of drug from a base is relatively straightforward. A simple apparatus as shown in Figure 2 can be used. The formulation is separated from the receptor compartment by an inert membrane which must be carefully selected. Since release from the base is being studied, the separating membrane must in no way limit the rate of transfer out of the base. Various membranes have been used successfully, e.g. Visking, silicone rubber, polypropylene impregnated with isopropyl myristate. Visking has the disadvantage that it allows free passage of water

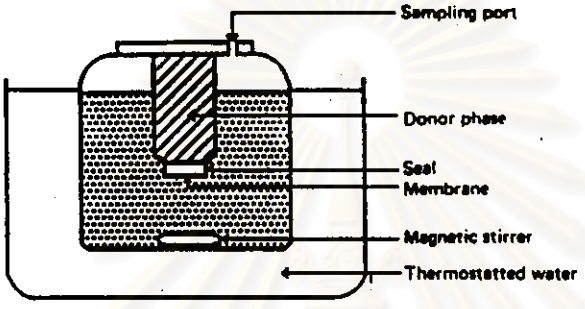


Fig. 2 Equipment for evaluating drug release from semi-solid formulation.

and osmotic flow as a result of an electrolyte concentration gradient. This can influence the results. Silicone rubber is relatively impermeable and, therefore, it may act as a rate-controlling membrane. In the case of impregnated membranes, surfactants present within the formulation may interact with the solvent and destroy the membrane integrity throughout the course of the experiment. These problems can be avoided with judicious choice of membrane. The receptor phase is usually water, isotonic saline or pH 7.4 buffer. Other solvents can be used if the solubility of the drug is a problem. The concentration of drug in the receptor phase should not exceed 10% of its saturated solubility. If the drug is dissolved in the formulation, the amount released per unit area at time (t) is given by :

$$Q_t = 2C_o(D_{app}t / \pi)^{1/2} \qquad (1)$$

Where C_o is the initial concentration of drug, and D_{app} is the diffusion coefficient with in the base (equation 1 only holds if the total amount released does not exceed 60% of the total amount of drug in the donor phase). Equation 1 shows that there is a linear relationship between the amount of drug released and the square root of time.

If the drug is not in solution, a different equation derived from Fick 's second law is applicable. In this case:

$$Q_t = (2D_{app}C_sC_ot)^{1/2} \qquad (2)$$

Where C_o is the initial concentration of drug, C_s is the solubility of the drug in the topical base and D_{app} is the diffusion coefficient with in the base. Many examples have been cited verifying equation (2). One example

consists of a dispersion of salicylic acid in Plastibase® (Al-Khamis et al, 1987).

1. *In Vitro* Models for the Evaluation of Anti-Inflammatory Drug Action in the Skin.

The general advantage of using, for example, an excised skin technique is that the investigator may control the laboratory environment and so elucidate individual factors which modify drug penetration. Thus, *in vitro* methods are valuable for screening procedures and for deducing physicochemical parameters such as fluxes, partition coefficients, and diffusion coefficients. A theoretical disadvantage of such a technique is that the method does not exactly duplicate the behavior of living tissue in situ, particularly with respect to a capricious blood supply and metabolism. A minor problem with topical steroids is that they constrict the capillaries *in vivo* and may decrease their own clearances. I*n vitro* this would not happen. However, provided it can be established that the rate-limiting step in the percutaneous absorption of a compound *in vivo* is diffusion through the dead horny layer, then a well-designed *in vitro* methodology should produce results which correlate reasonably well with *in vivo* studies.

When human skin is available, the biological diversity in its permeability between specimens and with in samples from one specimen or individual poses a problem. Even with close control of laboratory conditions and experimental technique, the variability for replicate experiments may exceed that for a well-designed *in vivo* procedure. If possible, the

experimental design should make each skin sample function as its own control.

Any suitable assay technique may be used to measure the penetrant concentration, but scintillation counting, gas chromatography (GC), or high-performance liquid chromatography (HPLC) methods predominate because of the chemical into the receptor solution or its rate of loss from the donor phase. A few workers determine the radioactive drug concentrations within discrete skin layers by serial removal of the stratum corneum with adhesive tape and by sectioning the remaining epidermis and dermis with a microtome The tapes with adherent horny layers and the sections are analyzed by scintillation counting. However, the technique provides only a "snapshot in time" of the tissue distribution of the drug, with the danger also that material may dislodge during manipulation (Barry, 1983).

In vitro experiments should be conducted using appropriate diffusion cells in which the formulation is in contact with excised skin. Ideally, human skin should be used which has been cut with a dermatome such that it is such that it is approximately 250 μm thick. The surface temperature of the skin should be 32 °C. Diffusion cells should be constructed of inert materials such that they do not absorb or adsorb significant amounts of the drug. The receptor phase should be chosen carefully so that it does not influence the barrier properties of the skin. It should also be capable of acting as a "sink" for the drug (the concentration of the diffusant should not exceed 10% of its saturated solubility in the receptor fluid). The dose of

drug applied to the skin surface should be in the same range as that applied clinically (Hadgraft, 1989).

1.1 Release Methods without a Rate-limiting Membrane

These procedures record the kinetics of drug release from a formulation to a simple immersible phase which is supposed to correspond in properties with human skin. The limitations of this assumption are evident in light of the biological and physicochemical complexity of mammalian skin. Such technique measure drug-vehicle interactions and the release characteristics of the formulation, and as such they are valuable but have little direct relevance to the process of percutaneous absorption. Solvents which have been used include simple aqueous media such as water, agar, gelatin and isopropyl myristate, and organic solvent with a blend of polar and nonpolar characteristics which make it more like skin (Barry, 1983).

1.2 Diffusion Methods with a Rate-Controlling Membrane

1.2.1 Simulated skin membrane

Because human skin may be difficult to obtain and varies in its permeability, many workers use other materials to simulates it. Thus, a cellulose acetate membrane can be sandwiched in a diffusion cell. However in most circumstances the membrane simply hinders the penetrants as it diffuses through its channels and the transport process correlates at best with molecular permeation across porous capillary endothelium; the transfer mechanism is dialysis or passage through macroscopic ducts filled with solvent (Barry, 1983).

Dimethylpolysiloxane (silicone rubber or silastic) has been used in many studies to investigate the diffusion of drugs. It acts as a non-porous, partitioning membrane and, because of its hydrophobic nature, has a very much higher permeability to un-ionized species. Because skin appears to act as a lipophilic membrane, it can be assumed that some basic physicochemical concepts can be examined using a model membrane such as silastic. However, it will not be possible to model enhancer effects because these will be specific to the complex nature of the lipids in the biomembrane. The permeation rates of homologous series of compounds have been studied through silastic. Alkyl p-amino benzoates, hydrocortisone-21, alkyl esters and alkanols have been studied. In these structure-activity studies, it was seen that, as expected, the permeability coefficient increased exponentially with increasing alkyl chain length. As the carbon chain length exceeded about 5, there was a tendency for the permeability increase to level out. At this point, the membrane resistance is becoming comparable to that from the stagnant diffusion layers and transfer is becoming limited by diffusion across the aqueous unstirred water layer. This can be compared to the skin where the silicone represents the stratum corneum, and the unstirred layers the viable tissue (Hadgraft and Guy, 1992).

An interesting concept is to use, as a model membrane, synthetic zeolites (aluminosilicates with a rigid three-dimensional structure) incorporated into a polystyrene metrix. The composite is claimed to be useful for examining effect such as the dependence of permeation on pH and the action of penetration enhancer (Barry, 1983).

Membranes may also be obtained from biological materials, such as collagen. A promising approach uses egg shell membranes. It is interesting that IPM has also been used in combination with eggshell membranes. Washitake et al. (1980) removed the shell using hydrochloric acid which left a membrane primarily rich in keratin. The permeability of salicylic acid as a function of pH was determined through eggshell membranes (with and without IPM impregnation), cellulose acetate and polyamide. The eggshell membrane alone gave permeability coefficients independent of pH, whereas the membrane impregnanted with IPM behaved as a classic partitioning type membrane with the permeability decreasing with pH up to pH 6 where the transfer rate became pH-independent. As with the rotating diffusion cell, permeability through this membrane was significantly greater than that for human stratum corneum (Barry, 1983).

1.2.2 Natural skin membrane

In vitro skin permeation experiments are performed at the beginning of most studies involving percutaneous absorption. Ideally, drug permeation through skin under *in vitro* conditions can be used to predict percutaneous absorption in human. Fresh human skin should be used in percutaneous absorption studies when research will ultimately lead to clinical applications. While it has been difficult to obtain human skin on regular basis. It is possible that skin obtained from commercial organ suppliers may be treated with a variety solvents, particularly if the skin is prepared for cryogenic freezing. Such treatments can lead to extraction of lipids and proteinaceous materials as well as alter phase or conformational structures in the skin.

Skin thickness can vary considerably and the investigator should measure the thickness of the skin prior to performing permeation experiments (Friend, 1992).

There are a host of physiologic factors that must be considered when evaluating the permeability of human skin under *in vitro* conditions. One issue is the storage of the skin prior to use in the *in vitro* permeability experiment. It is common to freeze human skin for storage until needed for experimental purposes. In one case, it was found that there was no significant difference between the permeability of human skin stored frozen for over 1 year and the same skin stored without freezing (fresh). Franz has also concluded that freezing for upto 3 months does not damage the barrier properties of excised skin. In contrast, Swarbrick found that skin (stratum corneum plus epidermis) gave consistently higher permeability when frozen at -17 °C and subsequently thawed than the same skin samples examined prior to storage. The studies mentioned above used a single model permeate and skin from only one site. It has also been recommended that glycerol be used in the storage of frozen skin to inhibit the formation of ice crystals which can disrupt cell developed. There is considerable variation in permeability observed within and between specimens. Interestingly, it was noted that variability *in vivo* was lower on average than that observed in *in vitro* experiments (Friend, 1992).

For most compounds, the stratum corneum is the primary diffusional barrier toward drugs, particularly hydrophilic compounds. There may be

instances when partitioning of hydrophobic drugs from the stratum corneum into the viable tissues may lead to low transdermal fluxes. Most drugs are normally cleared by the microvasculature, which lie close to the stratum corneum at a depth of 150-200 μm from the skin surface. The microcirculation begins close to the dermal/epidermal junction (the average thickness of the epidermis is approximately 40 to 50 μm. Under *in vitro* conditions, the microcirculation system is absent; therefore, an important aspect of proper design of *in vitro* permeability experiments is the ability to ensure skin conditions similar to those that exist *in vivo* (Friend, 1992).

The complex structure of skin with its multiple diffusion pathways and (*in vitro*) its varying thickness and degree of damage leads to a distribution of experimental penetration rates that is a function of exposure time and of the physicochemical properties of the test compound. Freshly excised tissue yields less variable penetration data than does frozen tissue and the observation to neutral but poorly lipid-soluble compounds that might be expected to penetrate skin via polar pathways. These facts alone lead us to associate the right-skewed nature of the permeability distributions for these materials with microscopic damage incurred during tissue collection and storage (Liu et al, 1994).

Nearly all investigators clamp such membranes in a diffusion cell and measure the passage of a compound from the stratum corneum side through to a fluid bath. However, it is important that the tissue be equilibrated with receptor solution before it is fastened in the cell. If it is

mounted dry and then donor and receptor solutions are applied, the stratum corneum hydrates and swells and the shunt route may constrict. This closure would lead to erroneous results for experiments which measure the permeation of drugs entering via this pathway.

Excised skin from a variety of animals, including rats, mice (normal and hairless), rabbits, guinea pigs, hamsters, pig, hairless dogs, and monkeys, has been used in diffusion cells. Mammalian skin varies widely in characteristics such as stratum corneum thickness, and the number density of sweat glands and hair follicles. In screening situations it is not always possible to use human skin and animal substitutes are often used. These, at best, can only be regarded as an approximation. Animal skin is, in general, more permeable than human skin and formulation effects will therefore be more apparent (Barry, 1983).

The *in vitro* penetration of indomethacin through excised rat skin (Barry, 1983). Simple formulations were assessed with various penetration enhancers. They were good *in vitro-in vivo* correlations established. The utility of the *in vitro* experimentation is seen, but the results have to be interpreted carefully before extrapolation to a human application.

In an attempt to find a model membrane representing of skin, Walkow and Mc Ginty (1987) have examined the absorption of methyl salicylate across silicone rubber, cellulose, polyethylene and pig skin. A number of solutions and emulsions were examined, but the general

conclusion was that the transfer of methyl salicylate across the model membranes did not parallel the diffusion across pig skin. It is difficult to produce model membranes which do simulate the properties of skin and it is probable that the future "model membrane" of choice will be a skin sheet reproducibly produced as a result of tissue culture (Hadgraft, 1989).

1.3. Diffusion Cells for Measuring *In Vitro* Permeation

A myriad of cell designs have been used over a past 30 years. However, most designs fall into one of two general categories: side-by-side diffusion cells and *in vivo* mimic diffusion cells (Friend, 1992).

1.3.1 Side-by-side diffusion cells

Side-by-side diffusion cells usually comprise two chambers wherein one chamber contains the permeate in solution and the other contains the receptor solution. These two chambers are separated by a membrane (skin in percutaneous permeation experiments). The contents of one or both chambers can be agitated to ensure adequate dispersion of the drug molecules and to minimize the static diffusion boundary layers. Configurations of side-by-side diffusion cells include T-shapes (Washitake et al, 1980) and identical L-shapes (Chien and Valia, 1984). Most cells are composed of glass as illustrated by the modified conical flask design of Wurster et al (1979). This cell has several drawbacks, most notably the inability to agitate the solutions internally although the authors propose gently shaking the entire apparatus in the plane of the membrane. The apparatus is suspended in a water bath for

temperature control. While this design was shown to be adequate for rapidly diffusing compound, its broader applicability is unknown (Friend, 1992).

A considerably more complex design is that of Flynn and Smith (1971). As designed, the device requires relatively large amounts of membrane, which limits its usefulness to skin available in large amounts. While many of the features of this side-by-side are well-suited for assessing skin permeation under *in vitro* conditions, the complexity of the design (motors, shafts, cogs) make the widespread use of this system limited despite its advantages over many other *in vitro* diffusion cells.

Despite the dissimilarity between side-by-side designs and assessing skin permeation under *in vivo* conditions, a large amount of the skin permeation data has been collected skin using the side-by-side chambers. Side-by-side designs expose the membrane (skin) to solvent on both sides throughout the experiment leading to potential solvation effects. Measurement of permeation rates under conditions similar to those encountered *in vivo* requires a different cell design as explained below.

1.3.2 Diffusion cells designed to mimic *in vivo* conditions

Systems that parallel conditions found *in vivo* are normally vertical with the bottom chamber designed to hold a receptor fluid. The bottom chamber is agitated or recycled in an attempt to maintain sink conditions throughout the experiment. An advantage of the vertical cell design is the ability to vary the nature of the donor vehicle. A film of material can be

applied by solvent evaporation; ointments, pastes, synthetic membranes in series with skin, and entire transdermal devices can also be studied. Atmospheric conditions (e.g., humidity) can be controlled in these cells as well. Sequential treatments, such as pretreatment with an enhancer followed by deposition of a drug, are easily accomplished using vertical cells. It is also possible to conduct infinite and finite dose experiments. The actual experimental design will vary depending on the type of formulation under investigation. For instance, testing of drug permeation from a topical vehicle to deliver corticosteroids is accomplished through semiinfinite dose or finite dose techniques.

There have been a number of vertical cells designed and tested over the past 25-30 years. One of the earlier cells is shown in Figure 3. This static cell is composed primarily of glass with a side arm for sampling. A Teflon-coated stirring bar is attached to a polyethylene sail to provide mixing of the receptor solution. The skin is held in place by a Teflon disk on a flat ground glass surface at the top of the receptor chamber. The exposed surface area of this cell as 0.30 cm^2 and the receptor chamber volume was 10 ml, a portion of which is located in the side-arm (mixing may not be adequate in the side-arm). Fluid mixing and mass transfer characteristics of this diffusion cell have not been fully investigated (Friend, 1992).

The Franz diffusion cell is one of the most widely used systems for *in vitro* skin permeation studies. First disclosed in 1978 and subsequently marketed, this cell as a small donor compartment and a dumbbell-shaped

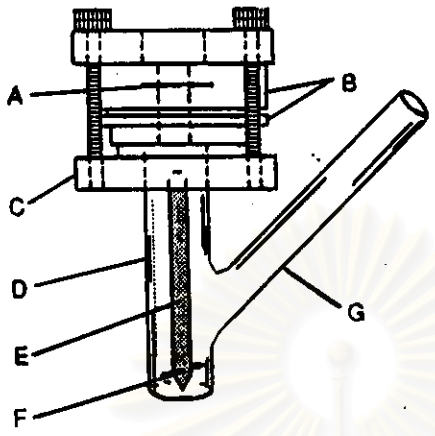


Fig. 3 Glass diffusion cell consisting of a lower chamber with a side arm for sampling of receptor phase.

A = Skin specimen, B = Teflon pieces holding skin, C = Clamp, D = Receptor chamber, E = Polyethylene sail, F = Telflon coated magnetic bar

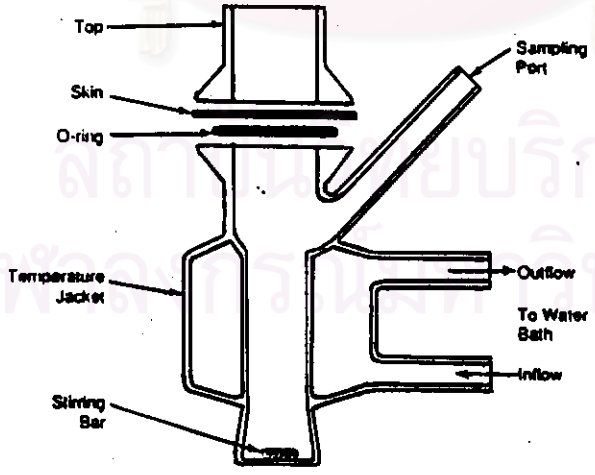


Fig. 4 The Franz diffusion cell.

receptor chamber (Figure 4). The central part of the receptor chamber is enclosed in a water-jacket for temperature control. Portions of the receptor chamber and the entire donor compartment are open to ambient conditions. As with most vertical systems, the receptor chamber is agitated with a teflon-coated magnetic stir bar.

A number of modifications have been introduced into the original design by Franz. For example, O-Ring flanges have been added. While the Franz cell is widely used, it has several potential drawbacks, most notably relatively poor mixing hydrodynamics. Poor mixing results from the fact that agitation in the lower bulb must be transmitted through the narrow cylinder (Friend, 1992).

The Keshary-Chien cell was carefully calibrated. The hydrodynamic conditions of the new cell were found to be much improved over the original Franz diffusion apparatus. (Figure 5). These improvements, result in a thinner hydrodynamic boundary layer, more efficient solution mixing, and better temperature control in the cell. So Keshary-Chien cell is more suitable for studying the fundamentals and mechanisms of drug release and skin permeation (Huang, 1987).

The poor mixing properties in the receptor chamber of Franz cells have been studied. It was found that the time to complete mixing, as measured by homogeneous dye dispersion, was inadequate in the side-arm and the upper portion of the dumbbell-shaped receptor cell in that

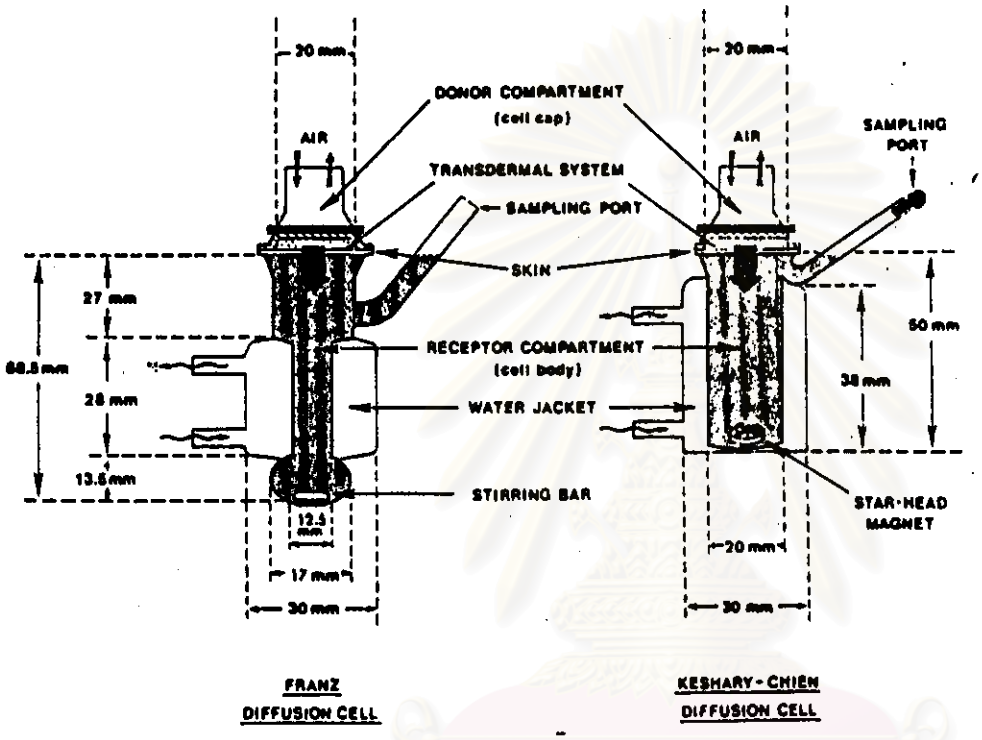
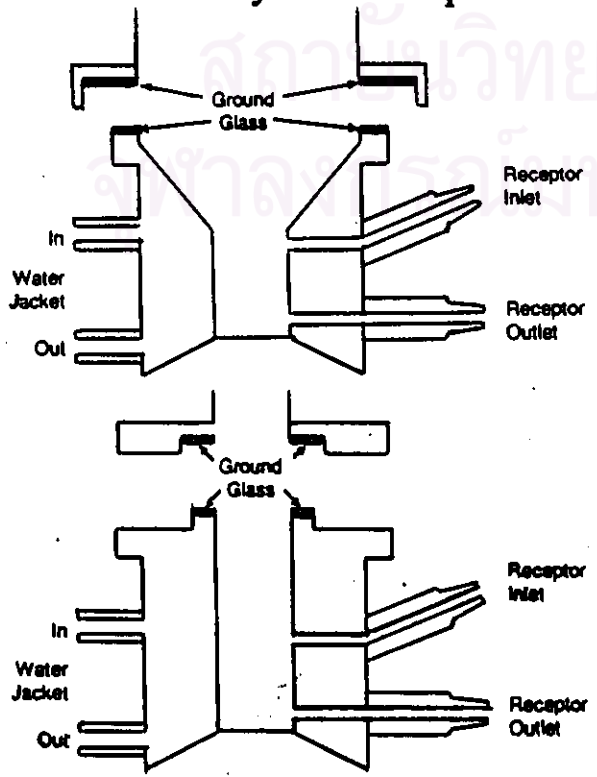


Fig. 5 Diagrammatic illustration and comparison of the Franz diffusion cell and the Keshary-Chien skin permeation cell



homogeneity was not reached until 30 min had passed in some cases (Gummer et al, 1987). Based on the data collected with the Franz cell, two flow-through *in vitro* penetration cells were designed to obviate the problem of poor mixing (Figure 6). Two types of cells were prepared to accommodate two different surface areas. The central design feature of these cells is the receptor chamber. Its diameter is wider than that of the Franz cell to achieve rapid and even stirring. As can be seen in Figure 6, these cells feature a flow-through receptor chamber. O-Rings are absent in this design: the skin is sandwiched between two areas of ground glass. No leakage of material was observed under any experimental conditions used. Using the time for homogeneous dispersion, both cell designs gave nearly instantaneous mixing (< 30 sec) (Gummer et al, 1987).

Flow-through systems offer an alternative to sampling ports; by replacing the entire contents of the receptor chamber on a continuous basis, sink conditions are more easily maintained. As a result, flow-through cell design coupled with a vertical chamber represents conditions similar to those encountered *in vivo*. A relatively complicated permeation cell for assessing penetration-evaporation was designed with a flow-through system and a magnetic stir bar (Figure 7). The receptor chamber (thermostatically jacketed) is stirred with a magnetic stir bar. The donor chamber was designed to control evaporation from the surface of the skin by forced, warm air ventilation (Friend, 1992).

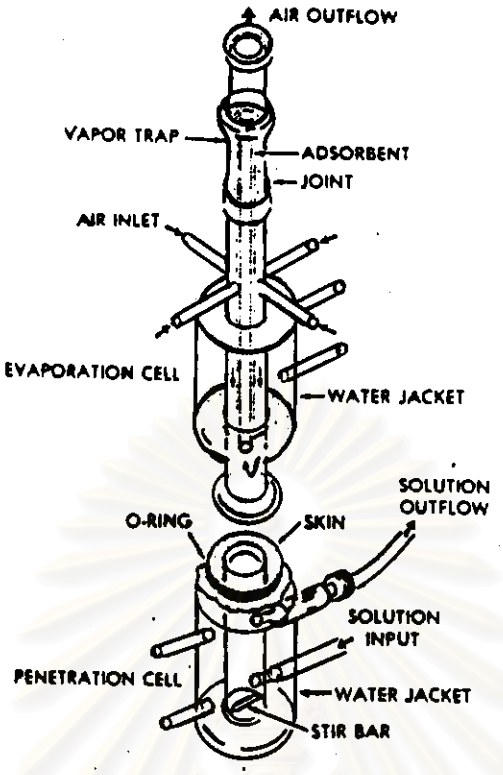


Fig. 7 In vitro skin penetration-evaporation cell.

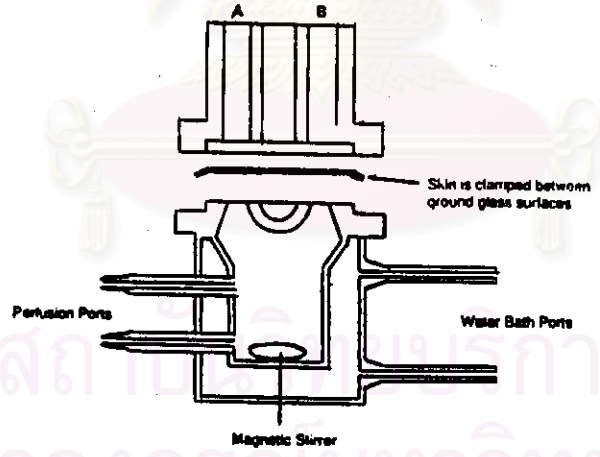


Fig. 8 Iontophoretic diffusioncell constructed of glass. Effective surface area is 0.8 cm^2 with a donor phase volume of about 0.5 cm^3. Receptor phase volume is approximately 7 cm^3. A and B are the electrode chambers.

Often, biological membranes are fragile and therefore difficult to handle. In particular, measuring the permeability of sheets of stratum corneum can be a formidable task. Maintaining a specific water content in the stratum corneum can also be a demanding prospect. While support structures have been used to maintain the integrity of the stratum corneum, a new technique has been developed recently to control the integrity and water content of stratum corneum sheets. It is possible to control the humidity such that the stratum corneum inside the sandwich adopts a water content which is in thermodynamic equilibrium with salt solutions outside the membrane. A minor drawback of this system is the need to determine not only the permeability of stratum corneum, but also that of the silicone sandwich.

1.3.3 Diffusion cells for iontophoresis and phonophoresis

The previous diffusion cells were designed to measure the passive diffusion of drugs. Iontophoresis and phonophoresis are techniques used to increase the transcutaneous flux of drugs. Iontophoresis is defined as the increase in permeation rate of a molecule induced by an applied current through the skin. While the concept is relatively old, it has received considerable attention recently because it appears possible to use iontophoresis to deliver certain macromolecules, viz., peptides and proteins, through skin. Phonophoresis is defined as the increase in rate of a solute through skin observed under the influence of a ultrasonic perturbation.

Diffusion cells used in iontophoresis experiments are similar to those used to assess passive diffusion except electrodes have been added, one in the donor chamber and one in the receptor compartment (Tyle and Agarwala, 1989). While this type of apparatus works well experimentally, electrodes can not be placed on opposite sides of the skin *in vivo*. Glikfeld et al (1988) designed an iontophoretic cell for *in vitro* studies to account for the *in vivo* situation (Figure 8). In this design, both electrodes are applied to the same side of the membrane (skin). Such a design should permit better approximation of drug delivery using iontophoresis under *in vivo* conditions. The cell used is based on that of Gummer et al (1987) and hence should exhibit properties similar to those claimed for the diffusion cell shown in Figure 6 (Friend, 1992).

Phonophoresis is rarely studied under *in vitro* conditions. Most experiments involve the use of existing commercially available ultrasonic equipment. These systems are to large to use under *in vitro* conditions, particularly with human skin. Design of an *in vitro* diffusion cell capable of varying experimental parameters important in phonophoretic drug delivery would probably help accelerate research (Friend, 1992).

2. *In Vivo* Models for the Evaluation of Anti-Inflammatory Drug Action in the Skin

The development of new drugs for the treatment of chronic inflammatory diseases of the skin relies on the existence of suitable animal models for the assessment of these agents. Although the most severe and debilitating inflammatory disease states are generally chronic in nature, the

available animal models are generally acute/sub-acute. This is for three main reasons :

1. The acute models are simple and reproducible.

2. They are useful for studies of the mechanisms involved in the transition of acute to chronic inflammation.

3. They permit the investigation of both the vascular and cellular components of inflammation as a function of time and to evaluate the action of known and potential anti-inflammatory agents.

To gain a full in sight into the percutaneous absorption process of a drug in a living animal we must determine the permeation in many species. However, particularly in man, this approach is often fraught with experimental and ethical difficulties. Many *in vitro* procedures monitor steady state fluxes across the intact stratum corneum, whereas *in vivo* methods often employ physiological and points. These may be sensitive to minute amounts of the penetrant entering the skin during the transient phase of diffusion and particularly down the shunt routes. A further complication is that many drug molecules probably penetrate the living skin mainly through the shunt routes, and an *in vitro* procedure which neglects this aspect is potentially misleading. Another severe difficulty with human subjects or living experimental animals is that the techniques determine the extent of absorption indirectly. In a typical experiment, the investigator applies a radioactive drug in an ointment to the animal's skin and assesses the kinetics of skin permeation from the rate of excretion in the urine. However, the

drug may be partially metabolized while crossing the viable tissues of the skin (Barry, 1983).

Most studies involve the use of an animal model and usually, rabbits are chosen. Drug penetration is measure of either by determining the amount lost from the formulation, its appearance in the plasma, or its excretion in the urine and faces. Often radiolabeled materials are employed, but it should always be realized that this will give concentrations which are the sum of the parent drug plus its metabolites. If the drug concentration alone is required, more specific assays should be employed. A less quantitative means of assessing percutaneous absorption of NSAIDs is to observe their effect in reducing a previously induced inflamed state. This may be produced by, for example, UV irradiation, carrageenan-induced paw edema or adjuvant arthritis (Hadgraft, 1989).

The cardinal signs of inflammation (rubor, calor, tumor, dolor and loss of function) have been long used to establish methods for the detection and definition of anti-inflammatory drug action in models of cutaneous inflammation induced either chemically, physically or mechanically. The end point of the induction of inflammation is characterized by one or more of the following symptoms: erythema (rubor), hyperthermia (calor), edema (tumor) or exudation of circulating leucocytes which can be observed, scored and analyzed (Hadgraft, 1989).

2.1 Animals Models

A persistent theme in work on percutaneous absorption is the development of suitable animal models which correlate adequately with man. However most experimental animal differ significantly from man in the features which affect percutaneous absorption: the thickness and nature of the stratum corneum, the density of hair follicles and sweat glands, the papillary blood supply, and subtle biochemical aspects (Barry, 1983). An additional limitation, arises when the investigation aims primarily to assess the therapeutic activity of a drug as well as its bioavailability from a formulation; few techniques produce disease states in animals which are similar to human afflictions. Even when simulated disease states can be developed their response to drugs may mislead in an extrapolation to man.

Animal models are in valuable for the more detailed study of the anatomy, physiology, and biochemistry of the skin, for screening topical agents, and for detecting possible toxic hazards. We can add that they are also useful in biopharmaceutical studies. However, experience gained with experimental animals is not a substitute for detailed, careful studies in man.

2.2 Technique

2.2.1 Observation of a physiological of pharmacological response

If the penetrant stimulates a biological reaction when it reaches the viable tissues, then this response may provide the basis for determining the penetration kinetics. At least in theory, local allergic, toxic or physiological

reactions may be used and various topical agents affect such skin functions as sweat gland secretion, pigmentation, sebaceous gland activity, vasodilatation, vasoconstriction, vascular permeability, epidermal proliferation, and keratinization. The most productive technique in terms of biopharmaceutical application is the vasoconstrictor or blanching response to topical steroids (Barry, 1983).

2.2.2 Physical properties of the skin

A list of just some of the methods includes the measurement of trans-epidermal water loss, thermal determinations, mechanical analysis, use of ultrasound, spectral analysis and use of photoacoustic and electrical properties. Many of these procedures reveal information which may be relevant to a specific aspect of percutaneous absorption (Barry, 1983).

2.2.3 Analysis of body tissues or fluids

Urinary analysis is often used to study percutaneous absorption. However, as emphasized earlier, all the drug which penetrates the skin should be accounted for by a "calibration" of the subject with a slow intravenous injection and a simultaneous determination of blood levels. The aim is to allow for all the pharmacokinetic factors inherent in drug absorption, distribution, storage, metabolism, and excretion. This combined procedure is a good method for *in vivo* assessment of topical bioavailability.

Analysis of the penetrant in the circulating blood can present difficulties with dilution, extraction, and detection, although modern analytical techniques are developing so rapidly.

A combination of blood, urine, and feces analysis was used with rats, monkeys, and human volunteers to examine the percutaneous absorption and excretion of tritium-labeled diflorasone diacetate, a novel topical steroid (Barry, 1983).

2.2.4 Surface loss

The measurements of the rate of loss of a penetrant from an applied vehicle should lead to a determination of the flux of the material into the skin. Thus, chambers could be filled with the formulation, attach to the skin, and the absorption rate calculated from loss measurements. However, because of the general impermeability of the skin, the concentration decrease would generally be small and analytical techniques would have to be very accurate to measure tiny differences. Such concentration differences as could be detected would most likely arise from the vehicle composition changing by evaporation or by dilution with sweat or tranepidermal water. Alternatively, any drug decrease may only reflect deposition on the skin surface or combination with the stratum corneum, rather than penetration to the systemic circulation (Barry, 1983).

2.3 Bioassay for Topical Steroids

Topical corticosteroid bioassays are the most sophisticated and refined of all bioassays which we use to develop and to assess dermatological formulation. In particular, we may not only employ the vasoconstrictor assay to evaluate the intrinsic activity of a topical steroid for correlation with possible clinical anti-inflammatory action, but we may use it also as a test in fundamental biopharmaceutical studies. Many other types of bioassay used to screen various topical formulations prior to clinical trial.

2.3.1 Vascular-type Acute Inflammation

The majority of the models used are related to vascular-type acute inflammatory responses and have edema or erythema as their end point. Inflammation was induced by a chemical such as croton oil, arachidonic acid, carrageenan, or by physical methods such as heat and UV irradiation (Bouclier et al, 1989).

A blend of 20% pyridine, 5% water, 74% ether, and 1% croton oil is applied to one ear, and a solution of the test steroid in this irritant solvent vehicle is applied to the other. After 6 hr, the ears are removed and weighed or small biopsies are weighed. The anti-inflammatory effect of the test compounds may be determined from the relative suppression of the croton-oil-induced inflammation. This bioassay has several advantages as a preliminary screening process. It is simple, non occlusive, and it provides dose-response data; the test is highly sensitive and reasonably specific for anti-inflammatory activity. The procedure yields clues regarding the

potential dissociation of topical and systemic drug action, and it is one of the few topical animal screening models (Barry, 1983).

Intradermal dextran and ovoniucoid increase capillary permeability in rats by a mechanism involving substances other than histamine and 5-HT, and that this mechanism is absent in rats which do not respond to intraperitoneal dextran (Bonaccorsi and West, 1963). While prostaglandin E_1 caused a dose-dependent weal-and-flare inflammatory response when injected intradermally. The clinical appearance of the cutaneous responses induced by PGE, and histamine differ, since the flare due to PGE, is darker red, and of longer duration than that due to histamine (Sondergaard and Greares, 1971).

The thermal injury induces a biphasic permeability response- immediate and delayed- in the unanaesthetized guinea-pig, rat and rabbit (Wilhelm and Mason, 1960). The study of the permeability responses to the minimal injury effective in unanaesthetized animals has establish that the pattern and time-course of the diphasic response is similar in the guinea-pig and rat, as well as in the rabbit; that, in terms of duration and intensity, the delayed response is the major one; and it appears to resolve the apparent discrepancies in the role of histamine as a principal permeability factor in thermal injury in man.

Pretreatment of animals with non-steroidal anti-rheumatic drugs modify the UV-induced erythema and permeability response of guinea-pig

skin. In dose comparable to those used in clinics, these drugs delay the onset of reaction and slow down the time-course of erythema development. Studies with aspirin reveal a similar effect on the permeability response. In presence of aspirin, phenylbutazone and indomethacin, administered prior to UV exposure, the inflammatory reaction is partially suppressed, depending upon the dose. The drugs are ineffective in aborting or minimizing the response when given after the inflammation is established. Corticosteroid fail to influence the UV inflammation in this test (Gupta and Levy, 1973).

The production of erythema in the skin of guinea-pigs exposed to ultraviolet (UV) light has been used as a model of inflammation for the assessment of new anti-inflammatory drugs (Law and Lewis, 1977). Furthermore, a good correlation between the erythema delaying action of systemically administered non-steroidal agents and their anti-rheumatic properties. However, steroidal anti-inflammatory drugs were ineffective in altering this erythema response when administered systemically or topically. A similar erythema response in rats has not previously been described although Logan & Wilhelm demonstrated biphasic vascular permeability change in the skin of UV irradiated rats in the absence of a significant hyperemia. The studies were undertaken to establish whether UV-induced erythema could be elicited in the rat using longer exposure time in an attempt to develop a test for assessment of topically applied steroid and nonsteroidal anti-inflammatory drug.

Steroidal anti-inflammatories have been reported to be in-active in the guinea-pig model when administered either topically or systemically. However, applied topically some were as effective as non-steroided agents in suppressing erythema in the rat. The relevance for man of the effects in the rat UV model of the steroidal anti-inflammatories is uncertain but topically applied anti-inflammatory corticosteroids can suppress UV erythema induced in man. There is mounting evidence that prostaglandins may play a role in the mediation of UV-induced erythema. After UV irradiation prostaglandin formation occurs in human, rat and guinea-pig skin. Moreover, non-steroidal anti-inflammatory drugs are able to inhibit prostaglandin formation in the skin and has recently demonstrated that topical application of indomethacin, reduces both the erythema and prostaglandin levels present in the UV irradiated guinea-pig skin (Law and Lewis, 1977).

2.3.2 Cellular-type Inflammation

With increasing recognition of the role of leucocytes in both the acute and chronic inflammatory reactions and with the discovery of agents which can modify cell movement, particular attention has been focused on acute models of inflammation which allow quantitative estimates of cell accumulation to be made (cellular-type inflammation).

The subcutaneously implanted sponge and the air pouch models are used by a number of laboratories to investigate cell migration. In these models the inflammatory response is evaluated as a function of time by measuring exudate volume, leucocytes number and types, other cell types

(i.e. red blood cells, platelets, etc), putative mediator concentrations, enzyme activities (Bouclier et al, 1989).

The standard procedure implants subcutaneous control cotton pellet and pellet impregnated with steroid into acrenalectomized rats the day after their operations. The implants are removed after 6 days and dried to constant weight. From log dose-log response curves, the relative inhibition of granuloma formation assesses the relative potencies of the test compounds. This bioassay measures local activity rather than topical action (Barry, 1983).

2.3.3 Immune-type Acute Inflammation

Recent studies to produce models of allergic contact hypersensitivity have been successful, both form the standpoint of lesion induction and treatment. The animals are sensitized by painting the abdomen with the sensitive (oxazolone or other) (Bouclier et al, 1989).

Evans et al 1971 sensitized mice by painting the abdomen with oxazolone in olive oil. Seven days later the animals were challenged on one ear with oxazolone in acetone. Graded dosed of Steroids (hydrocortisone, betamethasone 17-valerate, triamcinolone acetonide, and fluocinolone acetonide) dissolved in the oxazolone solution were applied to the opposite ear. Activity of the corticosteroids was expressed as percentage of inhibition of ear weight gain in treated versus control ears, or as relative ear weight among the ears treated with various steroids. The data revealed a

disproportionately low activity for betamethasone 17-valerate relative to the acetonide compounds.

A phenyl phosphonate derivative, indomethacin and triamcinolone acetonide tested reduced the inflammatory response to oxazolone contact dermatitis as measured by inhibition of edema (weight increase) and erythema. It has been found that prostaglandins (mainly E series) are increased in the skin in human contact dermatitis. The maximum prostaglandin activity occurred at 72 hr after the elicitation antigen application. The cutaneous erythema in the mice ears began within 2-6 hr after oxazolone elicitation dose, i.e. much earlier than in human contact dermatitis. Any release of inflammatory mediators is therefore likely to occur earlier in mice than in human contact dermatitis. There was a difference of erythema and weight between drug treated and control ears, signifying a topical anti-inflammatory action on the drug treated ears. The three topical agents show anti-inflammatory properties in this animal model of contact allergic dermatitis (Lowe et al, 1977).

Various concentrations of mustard oil in liquid petrolatum and nitric acid in water (usually 15%) were kept in contact with the normal skin of volunteers. Corticosteroid ointments were rubbed into the insulted sites at time intervals ranging from 24 hr before to 24 hr after the application of the two primary irritants. The time of appearance of the inflammatory reaction, its rate of progress, and the final degree of response were noted. The inflammatory reaction was graded as erythema, erythema plus obvious

edema, additional formation of papules or vesicles, or necrosis. The major factors which influenced the results were the relationship between the time of steroid application and the induction of inflammation, the duration of steroid contact with the skin site, the concentration of the corticosteroid, the intensity of the inflammatory stimulus, and the thickness of the epidermis (Barry, 1983).

2.3.4 Tape stripping

Stripping the epidermis with adhesive tape damage local tissue. This technique could be used to study the anti-inflammatory activity of topical steroids. Hydrocortisone inhibited the vasodilatation normally produced by 30 stripping of the stratum corneum. Although tape stripping essentially removes the barrier to steroid penetration through the skin, with all that this implies with respect to biopharmaceutical studies (Barry, 1983).

2.3.5 Vasoconstrictor test

It has been known for more than 30 years that certain, steroids make human tissue pale. The vasoconstriction assay may be employed both to screen new steroids for clinical efficacy and to determine the bioavailability of steroids from topical vehicles. The term " bioavailability is taken here to mean the relative absorption efficiency for a medicament as determined by the release of the steroid from the formulation and its penetration through the stratum corneum and viable epidermis into the dermis to produce the characteristic vasoconstrictor effect. Thus we may measure the intensity and duration of the steroid-induced pallor to assess both the activity of a

corticosteroid and its bioavailability from different vehicles, as determined by a pharmacological response.

To design a standard procedure using the arms of volunteers, many features must be considered. For example, blanching may be inconsistent for people with very short or very narrow forearms, for application sites nearer to the pulse or elbow than 6 cm, and for typists, i.e., persons exercising the forearm muscles during the period of steroid application. Because of the difference between sites on the same forearm, preparations should be applied according to randomization charts. Lower blanching scores and more inter- and intrasubject variation arise with non-occlusion. A short steroid application time of 6 hr provides better differentiation between products than dose 12 hr of occlusion. Variations in the applied amount of a cream or ointment between 3 and 8 mg applied over 50 mm^2 of skin do not significantly affect the degree of pallor produced over 96 hr. After the application period of 6 hr, skin sites may be gently washed with soap and water (30 to 40 ^{o}C) and patted dry. Vigorous drying may produce erythema which obscures the immediate blanching response. Washing with 70% aqueous ethanol confers no advantage and may produce occasional transient pallor. Blanching reading should be taken under standard lighting conditions with the arms held horizontally or slightly upward; the blood vessels in some volunteers enlarge when their arms hang downward, and this swelling may obscure the pallor (Barry, 1983).

An *in vivo* technique has been developed with simultaneously compares a skin blanching bioassay with drug content in human stratum corneum following topical application of betamethasone dipropionate formulations. Bioavailability of drug from commercial cream and ointment formulations was assessed by quantification of drug content in tape-stripped stratum corneum and skin blanching in the treated skin site under occluded conditions. Tape-stripping removed stratum corneum to a varying degree between individuals but was consistent (35%) within an individual with all formulations. A correlation ($r = 0.9935$) between the amount of drug in the treated stratum corneum normalized for surface area and the corresponding skin blanching score was observed with betamethasone dipropionate formulations. Increasing the amount of drug in the tape-stripping stratum corneum correlated with an increased skin blanching score. Ointment formulations delivered more drug to the skin and produced greater blanching scores than the cream formulations. Topical corticosteroid content in the treated skin site can therefore be quantified and correlates well with the resulting pharmacodynamic activity (Pershing et al, 1992).

A relationship between stratum corneum reservoir function and percutaneous absorption has been established in the hairless rat. Two hundred nanomoles of 10 substances that have a wide range of chemical structures were topically applied for 30 min, and the total body distribution was measured after 96 hr. The quantity of substance present in the stratum corneum reservoir after 30-min application was measured by liquid scintillation counting after tape-stripping the treated area. The quantity of

substance penetrating through intact rat skin can be predicted by measuring the horny layer concentration. This animal data reported, should be verified in humans (Rougier et al, 1983).

Choice of animal species and strains

It is essential that the selected animal species gives the same desired pharmacological response as that observed in man. For example, UV-induced erythema can be easily developed both in the guinea pig and man but not in the rat. A most suitable non-primate animal for the evaluation of the anti-inflammatory activity for potential human therapy, is the minipig. This is because in this animal species, the skin presents a structural aspect very similar to human skin. In addition, the minipig also demonstrates a very close similarity in its pharmacological responsiveness to both pro- and anti-inflammatory agents, to that observed in man (Bouclier et al, 1989).

In skin pharmacology research today, extensive use is made of rats and mice. Thus, in view of the marked morphological differences observed in the skin of the two species, the extrapolation of the results obtained from one to the other and also to man must be carefully evaluated.

Standardization-Reproducibility-Validation-Calibration

Studying an animal model before it is used for routine screening, need to carefully. Each pharmacologist who is working with animal models knows that results fluctuate from one experiment to another and with certain models it can be difficult to obtain an acceptable reproducibility of the

results, even when the experiments are performed in the same laboratory. The problem is much greater when trying to correlate and reproduce results obtained between laboratories. Often, this fluctuation or "biological variation" could be due to the poor control of variables that ultimately influence the end result (Bouclier et al, 1989).

: Animal Skin

Researchers have for many years used skin excised from rodents and other animals. Skin from animals is much easier to obtain, the age and sex of the animals can be controlled, and large numbers of samples can easily be obtained. The primary problem with using rodent skin as a model for human skin is that it can over estimate permeation relative to that in human skin. This problem is partly associated with the effects of hydration wherein prolonged (genarally 24 hr or longer) exposure of rodent skin (hairless mouse skin is the most notorious) to aqueous donor and receptor phases brings about a marked diminution in the barrier properties of the skin. The primary difference between human skin and rodent skin is the lipid composition and organization in the stratum corneum. Some species of rodent skin may be useful in studying permeation of compounds if the total exposure time is 12 hr or less. It has been suggested that hairless mouse skin can, where using limited amounts of acetone, be used to provide relevant guide lines for risk assessment calculations and bioavailability determinations although this conclusion is limited to using acetone in small volumes to deposit a penetrant onto skin (Bouclier et al, 1989).

: Other Models

A reliable model for human skin has been a highly desirable goal for a number of years. The goal remains elusive; nonetheless, significant advances are being made in the area of tissue culture. For example, human keratinocyte cultures grown at the air-liquid interface have been found to develop substantial barrier properties to water diffusion. However, preparation of a reconstituted membrane using a surfactant to disaggregate stratum corneum into cells requires intact human skin, which could be used directly in permeability experiments. At present, the most common means to evaluate the *in vitro* permeability of skin involves the use of excised human skin obtained during plastic surgery or cadavers and from common laboratory animals such as rodents (Bouclier et al, 1989).