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ENVIRONMENTAL HEALTH CRITERIA

DERMAL ABSORPTION

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SUMMARY

This document presents to the newcomer in this field an overview of percutaneous/dermal absorption and its measurement in particular with regard to the risk assessment of chemicals. Further, it presents and discusses current topics of interest in the field of percutaneous penetration.

The skin is a complex organ and is a living membrane. The functions of the skin include protection, regulation of body temperature and pH and water loss, and defence and repair. The skin comprises of an outer region, the epidermis with various cell layers, the outer layer being the stratum corneum or horny layer consisting of corneocytes surrounded by extracellular non-polar lipids; next, the dermis which is the locus of blood vessels, sensory nerves and lymphatics; then subcutaneous fat; and finally skin appendages such as hair follicles, sweat glands and sebaceous glands which originate in the subpapillary dermis.

The skin itself is a source of variability in the measurement of skin permeability. There are differences in permeability due to species variation e.g. differences between human and animal skin. There is little known about variation due to age though the skin structure does change; however, sex and ethnic background do not seem to be a source of variation. Percutaneous absorption does depend on the anatomical site, on the skin condition and hydration of the skin.

The skin is a metabolically active organ and contains enzymes which are able to catalyze not only endogeneous chemicals such as hormones, steroids and inflammatory mediatory but also xenobiotics including drugs, pesticides, industrial and environmental chemicals. Although the metabolism of xenobiotic compounds in the skin is intended to detoxify potentially reactive chemicals, by converting lipophilic compounds into polar, water-soluble compounds, which are readily excreted into the bile and urine, in some cases a compound may be activated leading to enhanced local and/or systemic toxicity.

The transport of chemicals through the skin is a complex process. There are three major mechanisms by which skin absorption may occur 1) transcellular absorption in which the chemical passes through the keratin-packed corneocytes by partitioning in and out of the cell membranes; 2) intercellular absorption in which the chemical passes around the corneocytes in the lipid rich extracellular regions and 3) appendageal absorption where the chemical bypasses the corneocytes by entering the shunts

provided by the hair follicles, sweat glands and sebaceous glands. The stratum corneum is usually the rate-limiting membrane. However diffusion through the epidermis and dermis is possibly the rate controlling process for very lipophilic materials and (or when the stratum cormeum is damaged.

Factors influencing the percutaneous absorption through the skin include 1) physiochemical properties of the test compound, 2) physiochemical and other properties of the vehicle in which the test compound is dissolved, 3) skin properties and metabolism 4) factors inherent to the test sytem used for measurement e.g. dose and volume of test substance, occlusion or non-occlusion of test area, and duration of exposure.

Theoretical equations and models have been developed which describe the transfer of a diffusing substance through the membranes of the skin. Typically the steady state flux (Jss) and the permeability coefficient (*Kp)* are assessed from *in vitro* experiments in which the donor concentration of the penetrant is maintained at constant (infinite) dose conditions.

As the permeability properties of the stratum corneum are unchanged after removal from the body and very good correlation between *in vivo* and *in vitro* experiments with the same chemicals have been observed, *in vitro* experiments are appropriate and offer a number of advantages over whole animal or human volunteer experiments. *In vitro* methods measure the diffusion of chemicals into and across skin to a fluid reservoir and can utilise non-viable skin to measure diffusion only, or fresh, metabolically active skin to simultaneously measure diffusion and skin metabolism. It is only recently that a test guideline has been accepted for *in vitro* dermal penetration (OECD, 2004a,c). However, up till this time, a variety of protocols and database experimental conditions were used leading to the present problems of comparativity of results. Experimental factors affecting dermal absorption *in vitro* include, in addition to those mentioned above, the thickness of skin sample, variations in temperature of the test system, composition of receptor fluid. Static or flow through *in vitro* diffusion cells can be used. Further techniques include tape stripping and the use of artificial, reconstituted skin.

There are a number of advantages in performing an *in vivo* study, as it uses a physiologically and metabolically intact system (OECD, 2004a,b). Within this intact system the vitality of the living skin will determine the metabolism, distribution and

excretion of the compounds. *In vivo* dermal penetration studies are carried out in laboratory animals, usually rodents, but also to a limited extent in human volunteers. It is only recently that a test guideline has been accepted for *in vivo* dermal penetration (OECD, 2004a,b). Up till this time, a variety of protocols were used.

In vitro studies are increasingly being submitted for registration purposes. There are several studies which compare *in vitro* and *in vivo* results, and *in vitro* studies have provided good prediction for *in vivo* dermal absorption. However there is still controversy over the way in which *in vitro* data could be used in risk assessment. Evaluation of available data on *in vitro* dermal absorption was performed under auspices of the OECD and it was found that *in vitro* and *in vivo* studies contained too many variables (e.g. different species, thickness and types of skin, exposure duration, vehicles).

For risk assessment, a value for dermal absorption may be obtained by the use of a tiered approach from a worst case to a more refined estimate. In the first step, 100% can be assumed if no data is available. In the second step, consideration of physicochemical properties could lead to an estimate of dermal absorption (e.g. 10% dermal absorption for those chemicals with a MW> 500 and log P_{ow} smaller than -1 or higher than 4). The third step is consideration of any experimental *in vitro* and *in vivo* dermal absorption data. If at the end of these steps an unacceptable risk is calculated, the risk assessment could be refined by means of actual exposure data.

Recently, there has been much interest in the possibilities available to predict dermal absorption and to avoid unnecessary and costly *in vitro* and *in vivo* testing. This is partly due to ethical difficulties with respect to human and animal experiments and partly due to economic and time considerations due to increasing legislation in the risk assessment of industrial chemicals. Quantitative structure-permeability relationships (QSPeRs) are statistically derived linear relationships between the steady state permeability of a compounds and various physico-chemical descriptors and/or structural properties of the molecule. Quantitative structure-activity relationships (QSARs) are involved at both these levels. Firstly, the octanol-water partition partition coefficient (K_{ow}) has been measured for some chemicals but determined for others from a QSAR, although probably quite well predicted. The skin permeability coefficient can be predicted from the molecular weight (MW) and K_{ow} by a second QSAR (CEFIC, 2004). The statistical nature of such QSARs means that the more data used to derive a

relationship, the more reliable it is likely to be for predictive purposes, assuming that the relationship is acceptable from a statistical point of view, and that the data itself is deemed to be sufficiently varied, and of satisfactory quality. For a QSPeR, the data (observed permeation) should be consistent, produced from standardised experimental procedures, and obtained for a set of chemicals that cover the domain of relevant chemical properties. However, QSPeRs up to now have been based on a heterogeneous data base of skin permeability values, which were measured for purposes other than for a QSAR, and therefore many QSPeRs are inherently subject to a substantial amount of error.

Mathematical models simulate the sequence of partition and transport processes involved in the absorption and can predict the extent and rate of chemical permeation through the skin. Mathematical modelling has a key role in linking the permeability coefficient obtained from tests under idealised, infinite dose conditions (i.e. steady state conditions) to those that will occur under the finite dose conditions more typical of occupational exposure (i.e. non-steady state conditions).

In the last few years there have been several initiatives to accelerate progress in the fields of international harmonization of methodology and protocols culminating in the publication of the OECD Guidelines in 2004. There have been initiatives from the regulatory side in US and in Europe. In Europe there have been two large projects, the Dermal Exposure Network (1997 -1999) leading on to the EDETOX project. A databank has been set up compling data from the majority of *in vitro* and *in vivo* studies publically available on percutaneous absorption. Further projects have been associated with QSPeRs/QSARs linking 1) physicochemical properties to permeation data 2) methods to determine dermal absorption for human risk assessment, so that in the future it may be possible to predict the data for a large number of chemicals rather than undertake expensive testing of chemicals.

[Drafting Note: the final paragraph will contain a summary of the recommendations. Reviewers are referred to Section 13.]

SECTION 1 INTRODUCTION AND DEFINITIONS

1.1 Scope of the document

Toxic substances that are present in workplaces and in the environment can come into contact with the skin in several forms depending on their physicochemical properties such as vapour deposition, liquid contact, and solid contact such as by contaminated soils or metals.

Although interest in percutaneous penetration in environmental medicine is comparatively recent, dermatologists and pharmacologists have been developing methods since the 1960s to study the passage of drugs and cosmetics.

The principle scenarios where percutaneous absorption are taken into account are:

1) The development of transdermal drug delivery systems
2) dermatological formulations
3) safety assessment of cosmetics
4) risk assessment of occupational or environmental hazards

Although these applications all involve the skin, they all have different aims and approaches. For example, for drugs it is important that the substance passes through the skin and into the blood stream. For this purpose it may be necessary to develop enhancers which help the compound to pass the skin barrier. For cosmetics and sunscreen lotions, it may not be necessary or desirable for the product to penetrate the skin but just remain in the upper skin layer. In occupational and consumer scenarios, the skin absorption of chemicals and pesticides, should be avoided. However, this is not always possible, therefore a risk assessment has to be made. For many chemicals there is a lack of information on the absorption properties of the chemical.

The purpose of this document is to present to the newcomer an overview of percutaneous/dermal absorption (Sections 2 to 4) and its measurement (Sections 5-7) in particular with regard to the risk assessment of chemicals. It does not intend to be comprehensive. A further aim is to present and discuss current topics of interest in the field of percutaneous penetration. In the last few years, partly due to regulatory pressures, there have been several initiatives to accelerate progress in the fields of international harmonization of methodology and protocols culminating in the publication of the OECD Guidelines in 2004 (OECD, 2004 a,b,c; see Appendix 1) and

the EDETOX project (Section 11). Further, available data on permeation has been collected into databases (see Section 8) and progress has been made in developing QSAR (quantitative structure-activity relationship) linking physicochemical properties to permeation data so that in the future it may be possible to predict the data for a large number of chemicals rather than undertake expensive testing of chemicals (see Section 9 and 11). Further, projects have been initiated to investigate risk assessment processes (Section 10). In spite of these successes in interdisciplinary international harmonization, there are still points which are topics of discussion (Section 12) and a way forward is proposed (section 13).

Due to the large amount of literature available, only some specific studies are cited but in the respective chapters throughout the document the reader is referred to reviews where more information can be found.

1.2 Definition of percutaneous/dermal absorption

The **percutaneous/dermal absorption** process is a global term which describes the passage of compounds across the skin. This process can be divided into three steps:

- **penetration**, which is the entry of a substance into a particular layer or structure such as the entrance of a compound into the stratum corneum;

- **permeation**, which is the penetration through one layer into another, which is both functionally and structurally different from the first layer;

- **resorption** which is the uptake of a substance into the vascular system (lymph and/or blood vessel), which acts as the central compartment. (Schaefer & Redelmeier, 1996).

1.3 Factors influencing percutaneous absorption measurements and variability in data

There are a number of factors which influence the dermal absorption of a substance. Theses are listed in Table 1.1 and are discussed in the appropriate sections of the document.

Table 1.1 Factors influencing percutaneous absorption measurements and variability in data

		Discussion in
Physiochemical properties of test compound	Physical state Molecular weight Water/lipid partition coefficient Ionisation	Section 3, 9
Vehicle	Solubility Volatility Concentration Distribution in stratum corneum Excipients Penetration enhancer pH	Section 5, 6
Skin	Species, Age, sex, race Anatomical site Temperature Hydration of strateum corneum Damage to SC Metabolism	Section 2, 5, 6
Application	Skin area dose (film thickness, concentration) total skin area in contact with vehicle duration of exposure	Section 5
Other factors	Reservoir effect and its interpretation in risk assessment	Section 5, 11
	Interlaboratory effects	Section 7
	Intralaboratory effects	Section 7

SECTION 2. SKIN STRUCTURE AND FUNCTION

Skin is the largest organ in the body with a surface area of approximately 2 m^2 and a total weight estimated at 4 kg (Pannatier et al., 1978). It receives about one third of the blood circulating through the body (Singh & Singh, 1993) Skin is heterogeneous containing a number of appendages such as sweat glands, hair follicles, sebaceous glands (see Figure 2.1) and the thickness of skin varies according to body region. Until the beginning of the 20th century it was thought to be a relatively inert barrier to chemicals which might enter the body. This is now known to be untrue. The skin has indeed barrier properties, however, many chemicals do penetrate the skin, either intentionally or unintentionally and the skin can metabolize exogenous compounds. Because of its large surface area, the skin may be a major route of entry into the body in some exposure situations.

For reviews on this chapter the reader is referred to e.g. (Schaefer & Redelmeier, 1996; Singh & Singh, 1993; Wiechers, 1989; Walters & Roberts, 2002; Madison, 2003; Montiero-Riviere, 2004).

2.1 Functions of the skin

2.1.1 Barrier function

The skin provides a sturdy, flexible, self-repairing barrier to the exterior, protecting the internal body organs and fluids from external influences. It keeps water and nutrients in (humans are 70 % water) and unwanted toxic substances, viruses, bacteria out. The skin responds to mechanical forces (elasticity and cushioning) and further is a protection from the sun (melanocytes).

2.1.2 Temperature control

The skin is important in temperature control (arteriovenous thermoregulation) and in regulation of pH.

2.1.3 Surveillance and action

The skin has special cells which give it the ability for defense and repair e.g. touch (Merkel cells); immunity (Langerhans cells); wound healing and cutaneous metabolism. Through the continuous turnover of the skin, repair occurs automatically.

2.2 Skin structure

Based on structure and embryonic origin, the cellular layers of the skin are divided into two distinct regions. The outer region the **epidermis** develops from the embryonic ectoderm and covers the connective tissue; the **dermis** is derived from the mesoderm (Maibach & Patrick, 2001).

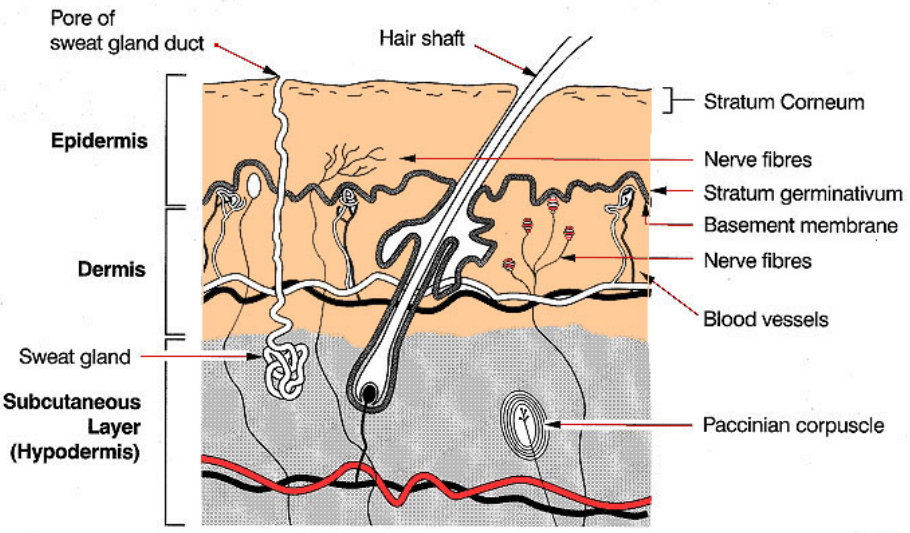

Figure 2.1 Structure of the skin

2.2.1 Epidermis

The epidermis comprises about 5% of full-thickness skin and is divided into five or six layers based on cellular characteristics (see Figure 2.2). The cells from the epidermis are called keratinocytes and are all formed by differentiation from one layer of mitotic basal cells. The number of distinguishable layers depends upon the anatomical site.

Basal layer (stratum germinativum) keratinocytes are metabolically active cells with the capacity to divide. Some of the resulting daughter cells of the basal layer move

upward and differentiate. The cells adjacent to the basal layer produce lamellar granules, intracellular organelles, which later fuse to the cell membrane to release neutral lipids thought to form a barrier to penetration through the epidermis (see paragraph on stratum corneum (SC) below). The desmosomes (adhesive junctions; Hatsell & Cowin, 2001) and bridges connecting the adjacent cells resemble spines giving the name of stratum spinosum to this three to four cell thick layer of cells above the basal layer. The keratinoytes move then upwards to the third division in the epidermis called the stratum granulosum which is characterized by the presence of keratohyaline granules, polyribosomes, large Golgi apparata, and rough endoplasmic reticulum. These are the uppermost viable cells of the epidermis.

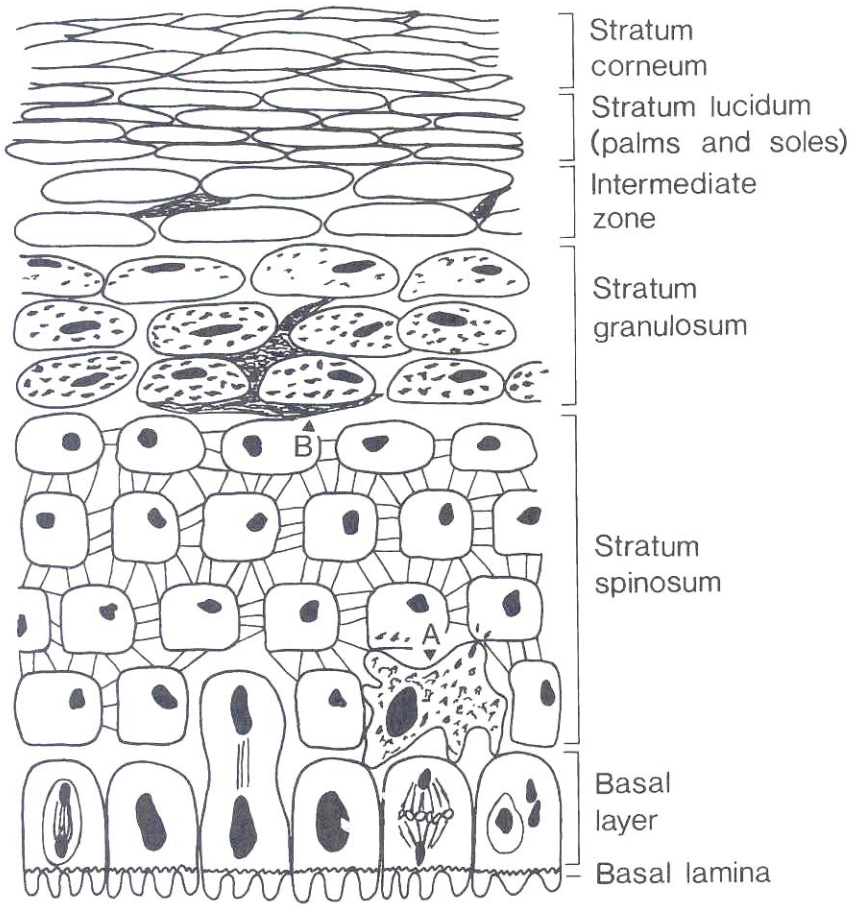

Figure 2.2 The epidermis. All possible cell layers and locations of the two dendritic cell types, melanocytes (A) and Langerhan's cells (B) are shown (from Maibach & Patrick, 2001[permission pending]).

The rate-limiting barrier of the skin is the non-viable stratum corneum (SC), the outermost cornified layer usually 15-20 cells thick, consisting of cells (corneocytes) that have lost their nucleus and all capacity for metabolic activity. The dominant constituent of these cells is keratin, a scleroprotein with chains linked by disulphide and hydrogen bonds. The intracellular attachment gradually breaks and the outermost cells are sloughed from the surface. The turnover rate for keratinocytes has been calculated to be between 17 and 71 days depending upon anatomical site e.g. 32-36 days for the human palm and 58 days for the anterior surface of the forearm (Maibach & Patrick, 2001). Although the thickness of non-hydrated stratum corneum is about 10-50 μm over most of the body, it may be ten times thicker (300-400μm) on friction surfaces such as the hands and soles of the feet (Singh & Singh, 1993). The stratum corneum has a water content of only 20% as compared to 70% in the case of the physiologically active basal layer.

Interconnecting the corneocytes are protein structures, referred to as desmosomes. The corneocytes are surrounded by extracellular non-polar lipids. The SC intracellular barrier lipids originate in the lamellar granules most prominent in the granular cell layer of the epidermis (Madison, 2003).

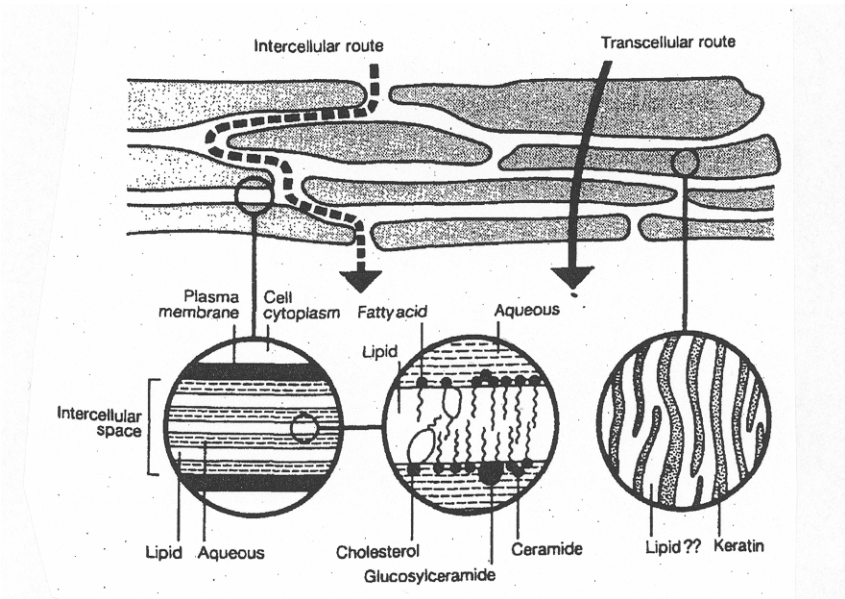

Figure 2.3. Diagram of the stratum corneum membrane showing two possible routes for diffusion (Bouwstra et al., 2003) [permission pending].

Due to the impermeable character of the cornified envelope, the major route of penetration across the SC has been shown to be a tortuous pathway between the corneocytes (see Figure 2.3) implying that SC lipids play a key role in the skin barrier function (Bouwstra et al., 2001; Bouwstra et al., 2003).

The hydrophobic lipids present in the SC are ceramides (CERs), long-chain free fatty acids (FFAs; mostly with chain lengths C22 and C24) and cholesterol (CHOL). Eight subclasses of CERs have been identified in human SC. The CERs consist of a sphingosine or a phytosphingosine base to which a nonhydroxy fatty acid or an alpha-hydroxy fatty acid is chemically linked (see Fig 2.4). The fatty acid chain is mainly C24 and C26. Cholestrol sulphate, although present in only small amounts, has been shown to be involved in the regulation of the desquamation process. The importance of the relationship between lipid organization and composition has been shown from studies with diseased skin in which an impaired barrier function paralled altered lipid composition and organization (Bouwstra et al., 2001; Madison, 2003; Kalinin et al., 2002).

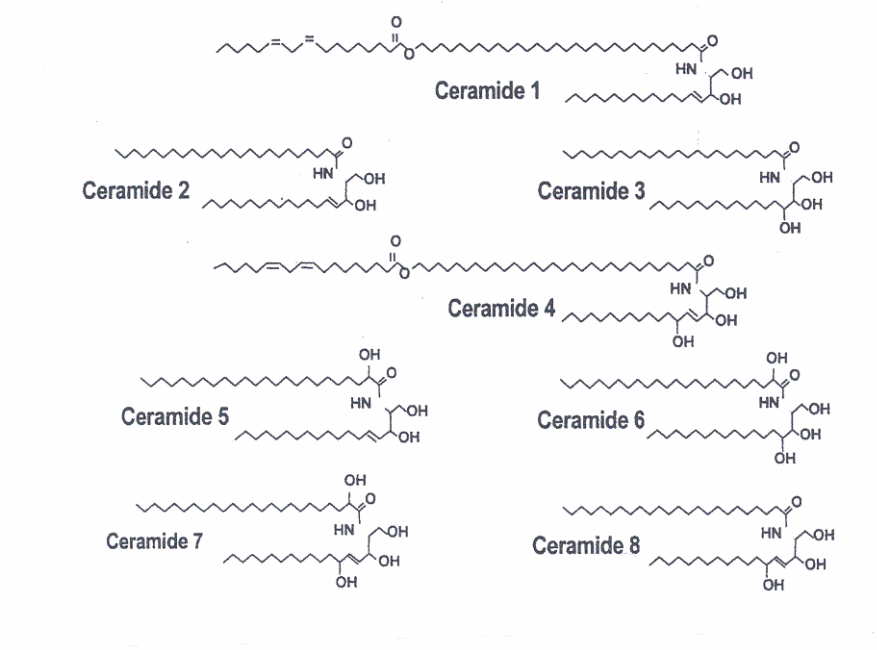

Figure 2.4 Molecular structure of ceramides in human stratum corneum (from Bouwstra et al., 2003) [permission pending].

In addition to keratinocytes, the epidermis contains two dendritic cells types – melanocytes and Langerhans cells. Melanocytes lie directly adjacent to the basal layer

and produce melanin, the principle pigment of human skin, which is then transferred to basal layer keratinocytes.. Langerhans cells express Ia (immune recognition) antigen and receptors of IgG and C3 on their surface. Langerhans cells lie in epidermal layers containing enzymes that can metabolize exogeneous chemicals.

2.2.2 Dermis

The dermis is 0.2-0.3 cm thick and is made of a fibrous protein matrix, mainly collagen, elastin, and reticulum embedded in an amorphous colloidal ground substance. The physical behaviour of the dermis, including elasticity is determined by the fiber bundles and ground substance. The dermis is the locus of blood vessels, sensory nerves (pressure, temperature, and pain) and lymphatics. It contains the inner segments of the sweat glands and pilosebaceous units. The dermis provides flexibility with strength, serves as a barrier to infection, and functions as a water-storage organ (Singh & Singh, 1993).

2.2.3 Subcutaneous fat

The subcutaneous fat layer cushions the epidermis and dermis. It acts as a heat insulator and a shock absorber. As it lies below the vascular system it has no effect on percutaneous absorption (Singh & Singh, 1993).

2.2.4 Skin appendages

The skin appendages originate in the subpapillary dermis, eccrine sweat glands, apocrine sweat glands, sebaceous glands, and hair follicles with their erector muscles are found in most anatomical sites though the number of each varies significantly by site. An average human skin surface is known to contain, on average 40-70 hair follicles and 200-250 sweat ducts/square cm. However they occupy only 0.1% of the total human surface (Singh & Singh, 1993). Sebaceous glands are most numerous and largest on the face, forehead, in the ear, on the midline of the back and on anogenital surfaces. They secrete sebum having the principal components glycerides, free fatty acids, cholesterol, cholesterol esters, wax esters, and sequalene. It acts as a skin lubricant and a source of stratum corneum plasticizing lipid and maintains an acidic condition on the skin's outer surface (pH 5) (Singh & Singh, 1993).

The eccrine glands are epidermal structures which are simple, coiled tubes arising from a coiled ball of approximately 100μm in diameter located in the lower dermis. It

secretes a dilute salt solution with a pH of about 5. This secretion is stimulated by temperature controlling determinants such as exercise and high environmental temperature as well as emotional stress through the autonomic (sympathic) nervous system. The apocrine glands are limited to specific body regions (e.g. armpit, the breast areola, and the perianal region) and are about 10 times the size of eccrine ducts. The apoeccrine glands, present after puberty, are sweat ducts in axillary skin (Singh & Singh, 1993).

2.3 The transport of chemicals through the skin

The transport of chemicals through the skin is a complex process. The skin is a complex organ and is further, a living membrane. The skin and the environment are in constant interaction.

There are three major mechanisms, by which skin absorption may occur (Hotchkiss, 1995; see also Figures 2.1 and 2.3)

1) **Transcellular absorption**: The chemical passes through the keratin-packed corneocytes by partitioning in and out of the cell membranes
2) **Intercellular absorption**: The chemical passes around the corneocytes in the lipid rich extracellular regions
3) **Appendageal absorption**: The chemical bypasses the corneocytes by entering the shunts provided by the hair follicles, sweat glands and sebaceous glands

Because of the relative surface area of these shunts (appendages), 0.1-1.0% of the total area, they do not play a decisive role in absorption. However, they may be important initially after application of the penetrant and sebaceous glands may act as a drug reservoir for some materials (Maibach & Patrick, 2001).

2.4 Factors causing variability in skin permeability

Passive diffusion through the cell is affected by the physicochemical properties of the chemical (see Section 3) and the physiologic and pathophysiologic conditions of the skin.

2.4.1 Species variation

The differences in permeability observed between human and animal skin depend on differences in their physiological and biochemical structures (Wiechert. 1989). Animal skin has a much higher number of appendageal openings per unit area (Barry, 1983), however this does not appear to be the reason for the differences in permeability. Differences in composition of lipid content of SC seem to be an important factor. The skin of weanling pigs and monkeys appears to be the most predictive model for human percutaneous penetration (Wester & Maibach 1985); see also Section 6.

2.4.2 Age, gender and race

Premature, low-birth weight babies have a poorly developed stratum corneum, however they develop a competent barrier within 4 weeks after birth (Kalia et al., 1996).

Changes that occur in aging skin include 1) increased SC dryness, 2) reduction in sebaceous gland activity resulting in a decrease in the amount of skin surface lipids 3) flattening of the dermal-epidermal junction and 4) atrophy of the skin capillary network resulting in a gradual attenuation of blood supply to the viable epidermis. Some studies have shown that the barrier function of the skin *in vivo* increases with increasing chronological age. It seems that relatively hydrophilic compounds are particularly sensitive. However relatively little is known about the influence of such age-related changes on percutaneous absorption (Roskos et al., 1989).

In general, regardless of environmental conditions, sex and ethnic background, the barrier properties of the skin are surprisingly constant. No statistical differences were found in PA of benzoic acid, caffeine or acetylsalicylic acid between Asian, black and Caucasian subjects (Lotte et al., 1993).

2.4.3 Anatomical site

The thickness of the eyelid is approximately 0.02 in and of the palm and sole about 0.16 in (Maibach & Patrick, 2001). Percutaneous absorption varies depending on the site of the body (Feldman & Maibach, 1967; Wester & Maibach, 1999a); see also Section 6.

2.4.4 Skin condition

Skin condition can have a significant impact on the rate of penetration of chemicals when the barrier function is disrupted. The permeability of the skin can be increased by physical (e.g. weather, sunlight, occlusion), chemical (e.g. solvents detergents, acids and alkalies) and pathological factors (e.g mechanical damge, pathological factors) (Wiechers, 1989). Mixtures of polar and non-polar solvents delipidize the skin resulting in a substantial reduction of the barrier function of the skin.

Some types of the most common skin diseases, psoriasis and eczema, are characterized by a virtual absence of the granular layer in the viable epidermis. In psoriasis, the proliferation is excessive whereas the keratinisation is incomplete (Barry, 1983). However, in diseased skin the degree of barrier efficiency may vary widely and depends on the precise pathological conditions of the SC.

There are several genetic skin diseases with known defects in lipid metabolism that have scaly or ichthyotic skin as part of the clinical picture(for details see Williams & Elias, 2000; Madison, 2003)

2.4.5 Temperature and blood flow rate

Skin temperature can have an impact on the rate of penetration of chemicals in two different ways (Bunge & McDougal, 1999). First, increasing the temperature of the skin has been shown to increase the rate of penetration by a direct effect in the skin that follows an Arrhenius relationship (Scheuplain & Blank, 1971). Secondly, temperature may affect the blood flow to the skin and therefore affect the amount of chemical absorbed.

2.4.6 Effect of hydration

The SC normally contains 5 to 15 % water under normal conditions, but can contain up to 50 % when hydrated (Blank & Scheuplein, 1964). The level of hydration can affect the permeability of the skin to chemicals. Idson (1971) claimed that increasing hydration increases the absorption of all substances that penetrate the skin. However, there is no reliable information about the effect of bathing and showering for 10 to 15 minutes on the hydration of the skin (Bunge & McDougal, 1999).

SECTION 3. SKIN TRANSPORT MECHANISMS AND THEORETICAL CONCEPTS

3.1 Transport through the skin

Percutaneous absorption includes penetration through the SC, diffusion through each layer of skin, and uptake by the capillary network at the dermo-epidermal junction. Percutaneous absorption occur mainly transepidermally (across SC intracellularly and intercellularly) (see Section 2)

Permeation of a chemical through the various layers of skin is basically a diffusion process in which active transport plays no role. The barrier for percutaneous absorption is the lipophilic SC in most cases.

Permeation involves penetration of the chemical (or drug) 1) from the vehicle into the SC, 2) diffusion through the SC and subsequently 3) penetration into the hydrophilic viable epidermis. Then after 4) diffusion in the aqueous environment (living epidermis and dermis) it can be washed away by the blood system. This is equivalent to a ‘sink’ condition. However, the blood flow is not always sufficient for uptaking molecules that can accumulate on the dermis and deeper tissues. The clearance from the skin is a limiting factor for substances that are poorly water soluble products.

The two limiting steps are 1) where no partitioning of substance between ‘vehicle’ and horny layer (SC) means no penetration and no diffusion in the horny layer and 2) where no partitioning induces storage in the SC.

The skin structure with the highest resistance to diffusion is the rate limiting membrane. This is usually the SC. However, diffusion through the epidermis and dermis is possibly a rate controlling process for very lipophilic materials and/or when the horny layer is damaged (dermatological conditions).

3.2 Concepts of passive diffusion

In contrast to the situation in physics where diffusion is a passive process that leads to the equalization of disparate concentrations in a **closed** system, diffusion

through the **skin** is a passive process that leads to an attempt of equalization of disparate concentrations in an **open** system.

1) The concept of **flux**: when a system is not in equilibrium, it moves towards equilibrium

2) The concept of **rate**; the further a system from equlibirium, the faster it moves towards equilibrium.

3.3 Theoretical Aspects of Diffusion

Diffusion of uncharged compounds across a membrane or any homogeneous barrier is described by Fick's first and second laws. Fick's first law can be applied to describe the diffusion processes in the individual layers of the skin. However this law may be an oversimplification of the more complex processes taking place, as it does not account for phenomena such as binding or metabolism.

Fick's first law describes the transfer of a diffusing substance through a particular material. It states that the **steady state flux** [rate of transfer per unit area] of a compound (J, mol/cm s) per unit path length (δx cm) is proportional to the concentration gradient (δC) and the diffusion coefficient or diffusivity (D, cm^2/s)

$$J = -D \cdot \delta C/\delta x \qquad \textbf{[Equation 1]}$$

*Where J is the rate of transfer per unit area of the surface (i.e. the **Flux,**)*
C is the concentration of the diffusing substance
x is the spatial co-ordinate measured normal to the section
D is the diffusion coefficient, or diffusivity

The negative sign indicates that the net flux is in the direction of the lower concentration. This equation holds for diffusion-mediated processes in isotropic solutions under steady state conditions (Schaefer & Redelmeier, 1996).

The **dermal permeability coefficient**, K_p, is defined by the equations (Singh & Singh, 1993)

$J_{ss} = K_p \cdot C_v$ or

$$\boldsymbol{K_p = J_{ss}/C_v} \qquad \textbf{[Equation 2]}$$

Combining equations 1 and 2 gives:

$$\boldsymbol{K_p = K_m \cdot D/h} \qquad \textbf{[Equation 3]}$$

Where K_p is the permeability coefficient (cm/s or cm/h),

C_v = concentration of the penetrant in the vehicle when sink conditions apply

J_{ss} = the steady-state flux of the solute

D is the average diffusion coefficient (cm^2/s or cm^2/h)

K_m is the partition, or distribution, coefficient between the stratum corneum and the vehicle

h is the thickness of the skin.

Typically, the steady-state flux and K_p are assessed from an *in vitro* experiment in which the donor concentration of the penetrant is maintained (more or less) constant [infinite dose conditions] while the receiver phase provides 'sink' conditions. Over time the flux increases to reach a steady-state value (J_{SS}). K_p is simply calculated from the slope of the linear portion of the graph of the cumulative amount penetrated as a function of time (Geinoz et al., 2004). Back-extrapolation of the latter plot to the x (time) axis yields the so-called lag-time (T_{lag}). The time taken to reach steady state is approximately equal to 2.7 times the lag time (Barry, 1983;). However, inaccurate estimations of the lag time will lead to a significant underestimation of permeability coefficients (Shah, 1993; Schaefer & Redelmeier, 1996; Geinoz, 2004).

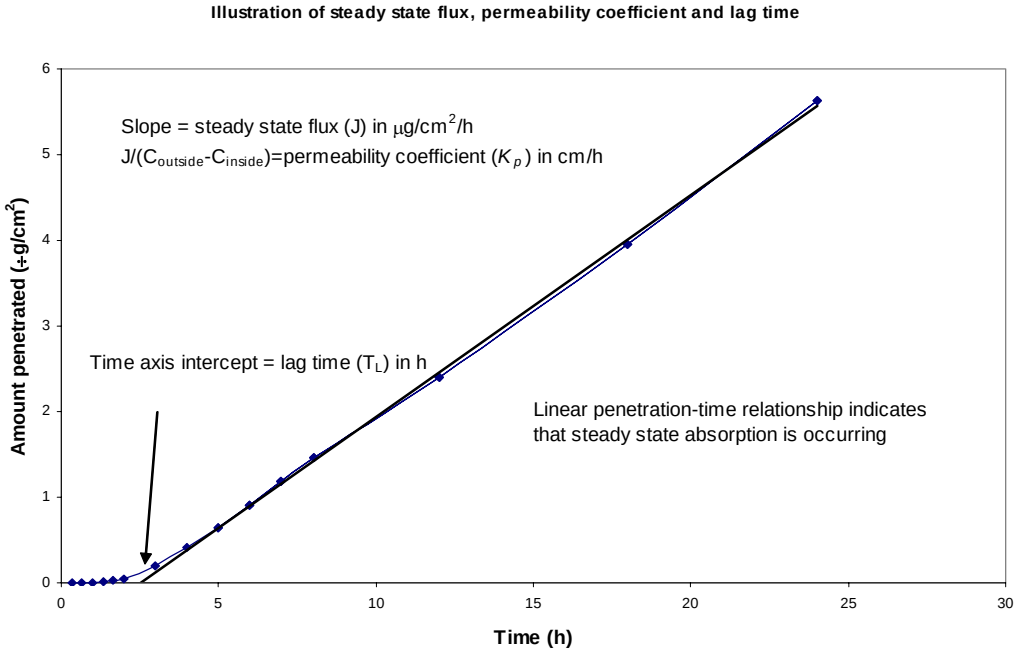

Figure 3.1 Illustration of steady state flux, permeability coefficient and lag time [infinite dose conditions] (from CEFIC, 2004 [permission pending])

Because the measurement of the membrane/water partition coefficient (K_m) is difficult, the more readily available octanol/water partition coeffcient (K_{ow}) is often used instead (Potts & Guy, 1992). The use of K_{ow} in Equation 3 ($K_p = K_{ow} \cdot D/h$) predicts that a plot of log *Kp* versus log K_{ow} should be linear with a slope of unity and intercept equal to log (D/h). Potts & Guy (1992) plotted the data from Scheuplein & Blank (1973) for the transport of water and *n*-alkanols through the human epidermis and found that although the larger more lipophilic permeants increased linearly with increasing K_{ow}, the small, polar molecules deviate from this line (see Figure 3.2).

Membrane transport as described above assumes that *D* remains constant for all permeants. However, even the simplest descriptions of diffusion predict an inverse relationship between *D* and permeant size . For SC (and other lipid membranes), it has been suggested that the functional dependence of *D* on molecular volume (*V*) is expontential (Kasting et al., 1987)

$$D = D^0 \cdot e^{-B(V)} \qquad \text{[Equation 4]}$$

where D_0 is the diffusivity of a hypothetical molecule having zero molecular volume, and *B* is a constant, *V* is the molecular volume.

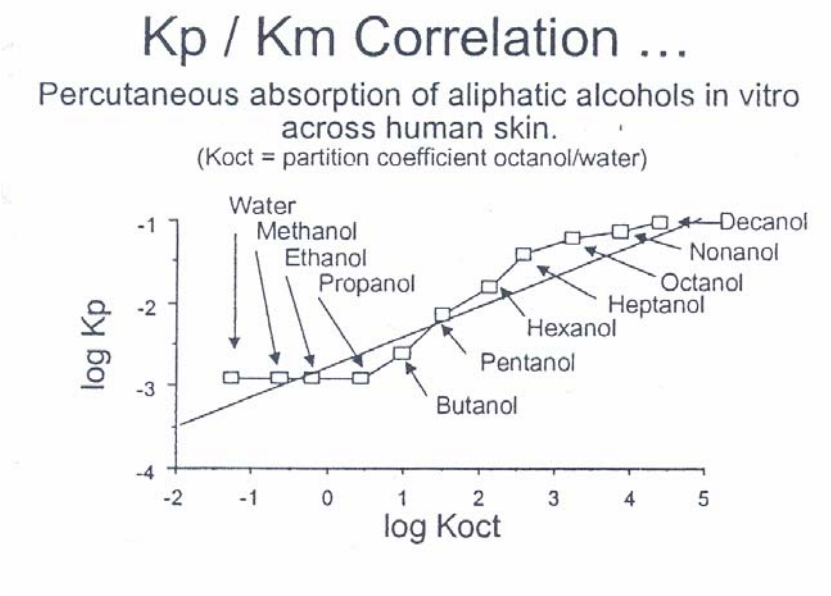

Figure 3.2 (from PPP course permission pending)

Combining Equations 3 and 4 yields

Log (Kp/Kow) = log (D^0/h) - B (V) [Equation 5]

If the data on alcohols is replotted according to Equation 5 using molecular volume values calculated by the method of Bondi (), the linear correlation is excellent (see Figure 3.3). Therefore the apparently anomalously high *Kp* for the smaller, more hydrophilic penetrants in Figure 3.2 can be explained by their relatively high diffusivities, due to their small molecular volume (Potts & Guy, 1992).

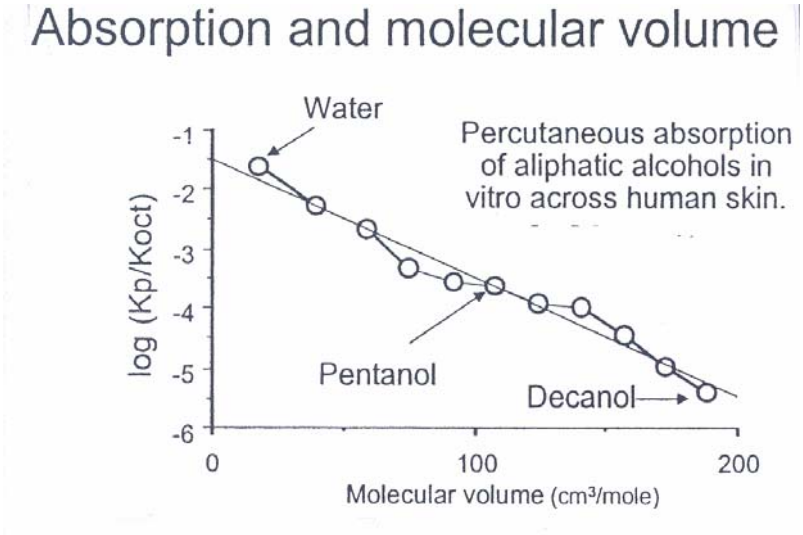

Figure 3.3 (from PPP course permission pending)

The implied equality between *Km* and *Kow* is only an approximation which assumes 1) that the lipophilic environment of the SC is the same as that of octanol and 2) it ignores the anisotrophic nature of the SC lipid alkyl domains (Potts & Guy, 1992). The fact that *Kp* depends on both *Km* and D means that the solute's volume contributes both positively and negatively to its permeation (Geinoz et al. 2004).

3.4 Physicochemical factors affecting skin permeation

The physical and chemical properties of a compound have a decisive influence on its penetration through the skin.

Physical state

Liquids and substances in solution are taken up more readily than dry particulates. Dry particulates will have to dissolve into the surface moisture of the skin before uptake can begin. Absorption of volatile liquids across the skin may be limited by the rate at which the liquid evaporates off the skin surface (Pryde and Payne, 1999).

Molecular size/Molecular weight

Molecular size is believed to play a distinct role in membrane permeation (Bunge & Cleek, 1995).

It is disputed whether molecular weight (MW) or molecular volume (V) is a better predictor of flux (J max) and or permeation coefficient (*Kp*) but recently there has been some preference for the simpler and therefore less error-proned MW (Patel et al., 2002; Magnusson et al., 2004).

With a MW increasing over 500 Dalton, absorption of molecules through normal human skin rapidly declines. A MW of less than 100 favours dermal uptake (Bos & Meinardi, 2000).

Water solubility

The substance must be sufficiently soluble in water to partition from the stratum corneum into the epidermis. Therefore if the water solubility is below 1mg/l, dermal

uptake is likely to be low. Between 1-100 mg/l, absorption is anticipated to be low to moderate, and between 10-10,000 mg/l, moderate to high. However, if water solubility is above 10,000mg/l and the Log P value below 0 the substance may be too hydrophilic to cross the lipid rich environment of the stratum corneum. Dermal uptake for these substances will be low (EC, 2003).

Liposolubility [Log P (octanol/water)]

For substances with Log P_{ow} values below 0, poor lipophilicity will limit penetration into the stratum corneum and hence dermal absorption. Values below –1 suggest that a substance is not likely to be sufficiently lipophilic to cross the stratum corneum, therefore dermal absorption is likely to be low. Log P_{ow} values between 1 and 4 favour dermal absorption (values between 2 and 3 are optimal) particulary if water solubility is high. Above 4, the rate of penetration may be limited by the rate of transfer between the stratum corneum and the epidermis, but uptake into the stratum corneum will be high. For substances with a Log P_{ow} above 6, the rate of transfer between the stratum corneum and the epidermis will be slow and will limit absorption across the skin. Uptake into the stratum corneum itself may be slow (EC, 2003).

Lipophilicity is markedly influenced by intramolecular interactions; i.e. electronic conjugation, interactions between polar groups, and steric and hydrophobic effects (Geinoz et al. 2004).

Ionisation

Highly ionised products do not penetrate very well. This certainly seems to be the case for proton acids, where *Kp* for the neutral form [*Kp*(N)] is very much larger than *Kp* [*Kp*(I)] for the ionized form, factors of around 10,000 being found for a number of chromone carboxylic acids. For proton bases, the situation is not so clear as it seems that not only the neutral forms of the bases can permeate human skin. For four different bases, studied by two different sets of workers, the *Kp*(N)/ *Kp*(I) ratios averaged 17.5 (Abraham & Martins, 2004).

The nature of the vehicle and the dilution factor of the substance is decisive (polar or non-polar) : non-polar carriers increase penetration (EC, 2003).

(Hadgraft & Valenta, 2000) have shown that there is significant permeation of ionized drugs through a lipophilic pathway, possibly as a result of ion pairing. Since the aqueous solubility of the ionized material is significantly higher than the unionized, the maximum flux through the skin may occur at a pH where ionization is high.

Chemical structure: binding properties

The uptake of some chemicals is slowed down due to binding to skin components e.g.: certain metal ions, particulary Ag^{+}, Cd^{2+}, Be^{2+}, Hg^{2+} ; acrylates; quaternary ammonium ions; heterocyclic ammonium ions; sulphonium salts.

A slight reduction in the dermal uptake of chemicals with the following groups could also be anticipated for the same reason: quinines; ialkyl sulphides; acid chlorides; halotriazines; dintro or trinitro benzenes (EC, 2003).

3.5 Concepts of finite and infinite dose

An **infinite dose** is defined as the amount of test preparation applied to the skin being such that a maximum rate of absorption of the test substance (per unit area of skin) is achieved and maintained (OECD, 2004a). It is the maximum rate for whatever chosen concentration of test substance is applied. Therefore, the volume of the donor fluid is large enough that the donor fluid concentration of the chemical is not depleted. This does nor reflect the real occupational scenario.

Under the conditions of a **finite dose**, the maximum absorption rate may be reached for some of the time, but is not maintained or may not be achieved (OECD, 2004a). The concentration of the chemical in the donor fluid changes due to uptake of chemical into the skin and may change due to evaporation of donor fluid. This situation happens in the *in vitro* cell when it is not occluded and also relates more to occupational exposure.

These concepts are shown graphical in Figure 3.4.

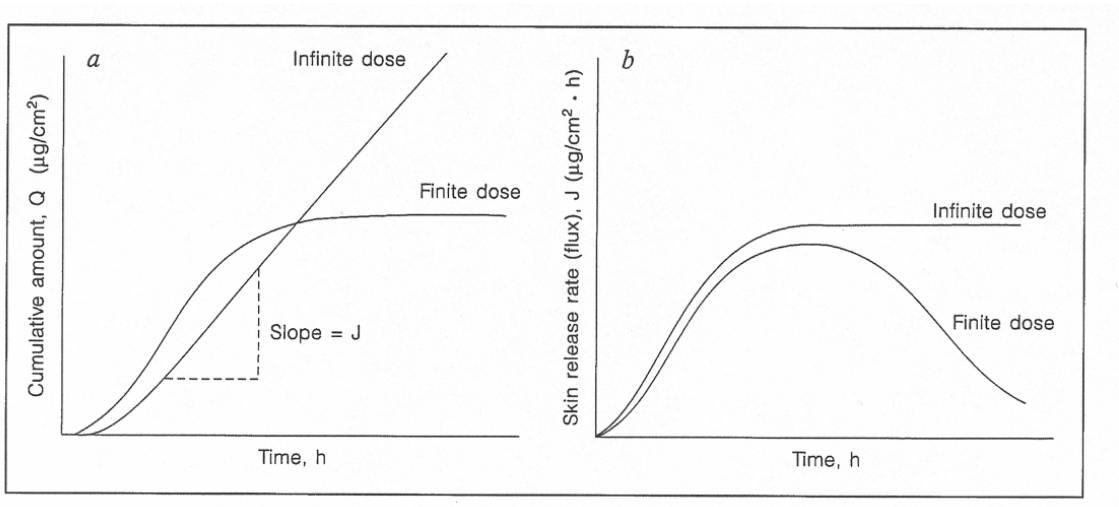

Figure 3.4 a) The cumulative dose absorbed as a function of time under 'infinite' and 'finite' conditions. b) The skin flux as a function of time (from Schaefer & Redelmeier, 1996) [permission pending].

SECTION 4 METABOLISM IN THE SKIN

The skin is a metabolically active organ and contains enzymes which are able to catalyze not only endogeneous chemicals such as hormones, steroids and inflammatory mediatory but also xenobiotics including drugs, pesticides, industrial and environmental chemicals. Although the metabolism of xenobiotic compounds in the skin is intended to detoxify potentially reactive chemicals, by converting lipophilic compounds into polar, water-soluble compounds, which are readily excreted into the bile and urine, in some cases a compound may be activated leading to enhanced local and/or systemic toxicity (Hotchkiss, 1998). The balance between cutaneous activation and detoxification is therefore a critical determinant of systemic exposure in humans.

4.1 The drug metabolizing systems of the skin

The skin contains enzymes which catalyze Phase 1 (e.g. oxidation, reduction, hydrolysis) and Phase 2 (conjugation) reactions. (see Table 4.1). All of the major enzymes important for systemic metabolism in the liver and other tissues have been identified in skin (Pannatier et al., 1978). The specific activities of cutaneous xenobiotic metabolizing enzymes in the skin appear to be lower than their counterparts in the liver (generally 0.1–28% for Phase 1; 0.6-50% for Phase 2; see review Hotchkiss, 1998; Kao & Carver, 1990, Hewitt et al., 2000). However, although the basal activities may be relatively low, the surface area of the skin is very large so that xenobiotic metabolism is likely to make a significant contribution to the overall metabolism of compounds following dermal exposure.

The extent to which topically applied chemicals will be metabolized depends on the chemical and the enzymes involved. Some chemical groups such as esters, primary amines, alcohols and acids are particularly susceptible to metabolism in skin (Bronaugh, 2004b).

Esterases are particularly active in the skin so that esters may be extensively, if not completely hydrolyzed to their parent alcohol and acid molecules during skin penetration. Examples of this are the fragrance chemicals such as benzyl acetate as well as the phthalate esters dimethyl-, diethyl- and dibutylphthalates (Hotchkiss, 1998; Boehnlein et al., 1994). Primary amines are frequently acetylated during percutaneous

absorption through the skin (e.g. Nathan et al., 1990; Kraeling et al., 1996, Yourick & Bronaugh, 2000). Oxidation/reduction and conjugation of alcohols and acids also commonly occurs in the skin (Nathan et al., 1990; Boehnlein et al., 1994).

Table 4.1. Some cutaneous xenobiotic metabolizing pathways (from Hotchkiss in Sartorelli et al., 2000).

Phase 1 reactions	**Phase 2 reactions**
Oxidation	Glucoronidation
Hydroxylation	Sulfation
Deamination	Glutathione conjugation
Dealkylation	Acetylation
Epoxidation	Amino acid conjugation
Aldehyde oxidation	Methylation
Alcohol oxidation	
Reduction	
Azo reduction	
Nitroxide reduction	
Quinone reduction	
Hydrolysis	
Carboxylester hydrolysis	
Sulfate ester hydrolysis	
Phosphate ester hydrolysis	
Peptide hydrolysis	
Epoxide hydrolation	

Localisation of cutaneous metabolizing enzymes

Studies in human and rodent skin have shown that cytochrome P450 (CYP1A1/A2) and Phase II metabolising enzymes (e.g. glutathione S-transferase) in the skin are be localised within specific cell types, particularly in the epidermis and epidermal derived tissues-namely sebaceous glands and hair follicles (Pendlington et

al., 1994; Hotchkiss, 1998). Lipase, protease, phosphatase, sulphatase and glycosidase activities have been identified in SC; these activities have been linked with the maturation and desquamation processes (Howes et al., 1996). Alcohol dehydrogenase and aldehyde dehydrogenase have also been shown to be present in epidermal basal layers (Haselbeck et al., 1997; Lockley et al., 2004a).

4.2 Methodology for evaluating skin metabolism

The extent of cutaneous metabolism is difficult to differentiate from systemic metabolism *in vivo* (Lockley et al., 2002). *In vitro* studies isolate the skin from the metabolic activity in the rest of the body. The use of viable skin is essential. An *in vitro* flow-through diffusion cell system (Bronaugh & Steward, 1985; Bronaugh, 2000a; see Section 5) using a HEPES-buffered Hanks' balanced salt solution (HHBSS) as receptor fluid has proved to be the method of choice for studying the effect of metabolism on percutaneous absorption. Additional information can be obtained from studies on the cytosolic fraction of whole and dermatomed skin e.g. studies on glycol ethers (Lockley et al., 2004).

4.3 Effects of skin metabolism

Cutaneous metabolism may result in

1) activation of inert compounds to toxicologically active species e.g. polycyclic aromatic hydrocarbons (benzo[a]pyrene [Ng et al., 1992] and 3-methylcholanthrene)

2) detoxification of toxicologically active chemicals to inactive metabolites e.g. organophosphorus pesticides (diisopropylfluorophosphat, paraoxon) and/or

3) conversion of active chemicals to active metabolites, e.g drugs (testosterone and oestradiol [Collier et al., 1989])

An overview of skin metabolism of compounds is given in Hotchkiss, 1995 and compounds where the metabolism has been studied during *in vitro* absorption studies is given Bronaugh, 2004a. Examples of these studies include azo dyes (Collier et al., 1993); 2-nitro-*p*-phenylenediamine (2NPPD), which is a dye used in semipermanent

and permanent hair dye formulations where extensive metabolism was found on absorption (Yourick & Bronaugh, 2000) and 2-ethoxyethanol where no metabolism was observed (Lockley et al., 2002).

4.4 Importance of metabolism for percutaneous absorption

That skin can metabolise compounds before they enter the blood stream is important for risk assessment purposes and in terms of drug delivery (Howes et al, 1996). However, there are conflicting views as to the importance of metabolism for measurement of percutaneous absorption. For materials which are stable in the SC and where the metabolism occurs after this step, metabolism will not be rate-limiting. Only for those chemicals which undergo biotransformation during its passage through the stratum corneum to metabolites which penetrate the skin to a greater extent than the parent compound, will metabolism be a critical determinant. Above all, the importance of using viable skin for *in vitro* determinations must be emphasized (Kao et al., 1985; Bronaugh, 2000a, see above).

Although skin contains enzymes that have the capacity to metabolise glycol ethers localised in the basal layer of the epidermis, the physicochemical properties of the penetrants resulting in rapid penetration significantly reduce the potential for first-pass dermal metabolism during percutaneous penetration (Lockley et al., 2004a).

However, for compounds that either bind in the skin or due to their physicochemical properties stay longer in the skin, the metabolism may be of importance. This may be the case for example for PAHs. 7% phenanthrene was biotransformed to the three diol metabolites (*trans*-9,10-dihydrodiol, *trans*-1,2-dihydrodiol, *trans*-3,4-dihydrodiol) in the skin (Ng et al., 1991). These metabolites are not carcinogenic which correlates with lack of tumouricity of phenanthrene in rodents. In contrast, in skin metabolism studies with benzo[*a*]pyrene (BaP), the metabolite identified in the receptor fluid was benzo[*a*]pyrene 7,8,9,10 tetrahydrotetrol which is the hydrolysis product of the ultimate carcinogen, 7,8-dihydroxy.9,10-epoxy-7,8,9,10 tetrahydrotetrol (Ng et al., 1992). These results agree with the formation of skin tumours following topical administration of BaP. Studies in mouse skin had previously shown that induction of cutaneous drug-metabolizing enzymes can result in a two to threefold increase in the *in vitro* permeation of topical BaP (Kao et al., 1985).

2-nitro-*p*-phenylenediamine (2NPPD), which is a dye used in semipermanent and permanent hair dye formulations, rapidly penetrates both human and rat skin. Under conditions that simulate normal consumer use conditions, approximately 5-10% of the 2NPPD that contacts the skin would be expected to be absorbed. There was extensive metabolism of 2NPPD upon absorption. The extent of metabolism and the metabolic profile depended on species (man, rat) and dosing vehicle (ethanol or formulation) and also tissue (when compared to results with intestinal tissue) (Yourick & Bronaugh, 2000)

SECTION 5 *IN VITRO* TESTS FOR DERMAL ABSORPTION

5.1 Introduction

In vitro methods measure the diffusion of chemicals into and across skin to a fluid reservoir and can utilise non-viable skin to measure diffusion only, or fresh, metabolically active skin to simultaneously measure diffusion and skin metabolism (OECD, 2004a). Diffusion across the nonliving outer layer of skin, the stratum corneum, is normally the rate-limiting step for percutaneous absorption (Dugard & Scott, 1984, Dugard et al., 1984). The absorption, i.e., diffusion, of a chemical through the stratum corneum is believed to depend on chemical specific factors such as molecular weight, water and lipid solubility, polarity and state of ionization (see section 3). As the permeability properties of the stratum corneum are unchanged after removal from the body and very good correlation between *in vivo* and *in vitro* experiments with the same chemicals were observed (Feldmann & Maibach, 1974; Franz, 1975), respective *in vitro* experiments are appropriate and offer a number of advantages over whole animal or human volunteer experiments (van Ravenzwaay & Leibold, 2004).

5.2 Test Guidelines

It is only recently that a test guideline has been accepted for *in vitro* dermal penetration (OECD, 2004a, c). The following descriptions are based on the OECD protocol. However, up till this time, a variety of protocols and database experimental conditions were used (see EDETOX database, Diembeck et al., 1999, US-EPA, 2004, cosmetic testing – SCCNFP, 2003b) leading to the present problems of comparativity of results (see also Appendix 1).

5.3 Principle of the standard *in vitro* tests using skin samples

The test substance, which may be radiolabelled, is applied to the surface of a skin sample separating the two chambers of a diffusion cell (OECD, 2004a). Skin from many mammalian species, including humans can be used (OECD, 2004c). The chemical remains on the skin for a specified time under specified conditions, before removal by an appropriate cleansing procedure. The receptor fluid is sampled at time points

throughout the experiment and analysed for the test chemical and/or metabolites (OECD, 2004a).

5.3.1 Test chambers

There are two basic designs of one-chambered diffusion cells – the static cell (Franz, 1975) and the flow-through cell (Bronaugh & Stewart, 1985). *In vitro* protocols generally allow the use of either the flow-through or static diffusion cell (US EPA, 2004, OECD, 2004a). The one-chambered cells have a chamber (receptor) beneath the skin, and open to the environment above the skin to simulate many exposure conditions (Bronaugh 2004b). The one-chambered cells may be used for finite and infinite dosing experiments.

Two-chambered cells have two chambers of equal volume, separated by the skin membrane. Infinite doses are added to one side of the membrane, and its rate of diffusion across a concentration gradient into a solution on the opposite side is determined. Permeation through this type of cell may be measured by collecting receptor fluid using static or continuous (flow-through) collection, depending on the solubility of the test compound in the receptor fluid (Schäfer & Redelmeier, 1996). Mechanisms of diffusion through the skin may be studied with the two-chambered cells (Bronaugh 2004). To evaluate topical products intended for human use standard infinite dose cells in a side by side configuration have been used by several authors (or side by side diffusion cells for skin delivery experiments) Feldmann & Maibach 1969; Michaels et al., 1975 in Moss et al., 2002, Bronaugh and Maibach, 1985) (Sartorelli et al., 2000).

5.3.1.1 Static diffusion cells (Franz diffusion cell and Saarbruecken penetration model)

The Franz diffusion cell is one of the most widely used systems for *in vitro* skin permeation studies (Friend, 1992). The Franz diffusion cell systems are relatively simple in design, the receptor fluid beneath the skin is manually sampled by removing aliquots periodically for analysis (Bronaugh 2004b) and may be run as static or as stirred cells (ECETOC, 1993). With this type of apparatus any type or any amount of vehicle, may be applied to the skin. Usually, 5-10 µl of a liquid vehicle containing the test compound is applied per cm^2 of skin, using a micropipette otherwise, 2-5 mg of a non-liquid vehicle containing the test compound is applied per cm^2, using a small stirring rod (Franz, 1978). The Franz diffusion cell is shown in Figure 5.1.

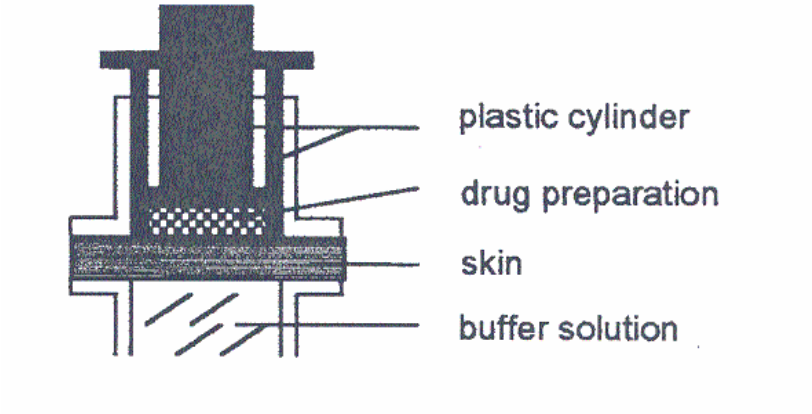

Figure 5.1: Franz diffusion cell – upper part (from Wagner et al., 2000) [permission pending]

In the Saarbruecken Penetration Model the skin is put onto a filter paper soaked with Ringer solution and placed into the cavity of a Teflon bloc. The test compound is placed into a cavity of a Teflon punch, which is applied on the surface of the skin. A weight of 0.5 kg is placed on the top of the punch for 2 minutes and afterwards the whole apparatus – after putting it into a plastic box - transferred into a water bath at 32 +/- 1 °C (Wagner et al., 2000). In the Saarbruecken penetration model no receptor fluid is used – the penetrated test compound meets directly the filter paper. Compared to the Franz Diffusion Cell experiments, nonphysiological hydration of the skin is avoided due to the absence of the liquid acceptor medium (Wagner et al., 2000). The Saarbruecken Penetration model is shown in Figure 5.2 (Wagner et al., 2000).

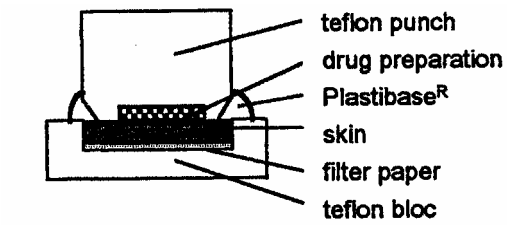

Figure 5.2: The Saarbruecken penetration model (from Wagner et al., 2000) [permission pending]**.**

5.3.1.2 Flow-through cells

The flow-through cells are characterised by continuously replacing the receptor fluid, which represent more or less *in vivo* conditions. A flow-through diffusion cell system has been developed by Bronaugh & Stewart, 1985. The cells were constructed from Teflon and contained a glass window in the bottom for viewing the receptor

contents. A flow rate of at least 5 ml/h is required through the receptor (volume: 0.4 ml) (Bronaugh & Stewart, 1985). For volatile compounds, a flow-through diffusion cell, has been designed with Teflon and a septum and a septum cap that insures nothing is lost through evaporation (Sartorelli et al., 2000).

A schematic picture of the flow-through principle is given in Figure 5.3.

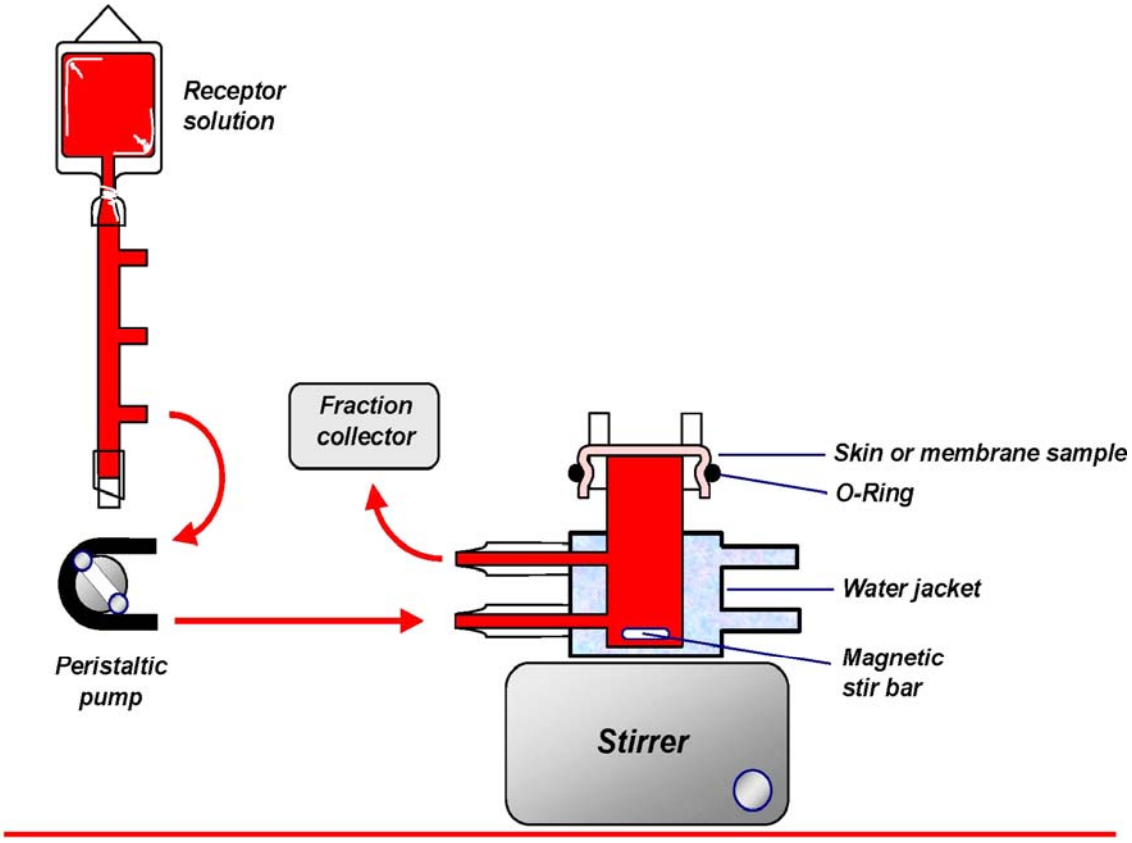

Fig 5.3: Principle of a flow-through diffusion cell (from Steiling, 2004) [permission pending]

5.3.1.3 Comparison of different in vitro cell systems

The static cells are simpler in design, the costs are lower and they are available in a wide range of larger openings for skin (Bronaugh 2004b). Comparing the static Franz cell and the static Saarbruecken penetration model differences in the amount of drug penetration into deeper skin layers were observed. The amount of detected test

substances in the deeper skin layers increased with the incubation time in the Saarbruecken penetration model, while in the Franz diffusion cell, only very small drug amounts were observed after incubation times of up to 1 hour (Wagner et al., 2000, Jacobi, et al., 2004).

The flow-through cell provides – in contrast to the static cell types - the continuous replacement of a nutrient medium necessary to maintain physiological conditions and is recommended for metabolism studies (Bronaugh et al., 1999b, Bronaugh 2004b). An advantage of a flow-through diffusion cell is in receptor fluid sampling, which can be done automatically using a fraction collector. Use of flow-through cell helps prevent high concentrations of test compound in the receptor fluid that can reduce absorption and the cell may facilitate partitioning of water insoluble chemicals from skin. (Bronaugh 2004).

Bronaugh & Maibach (1985a) and Hughes et al. (1993) found no differences in flux from static and flow-through cells (Moss et al., 2002). No significant differences in the percentages of applied doses were found for tritiated water, cortisone and benzoic acid in the flow-through and the static cell (Bronaugh and Stewart, 1985).

5.3.2 Finite/Infinite dosing

Two dosing regimes are under consideration: infinite and finite dose (see section 3). In the finite dose regime the dose solution is applied in a volume sufficient to cover the skin and normally remains unoccluded. Finite dose experiments may be performed with all types of one-chambered cells. In the infinite dose procedure the dose solution is applied in excess and is occluded for the duration of the study (Sartorelli et al., 2000, OECD, 2004c).

Table 5.1 gives an overview over suitable test chambers for finite/infinite dosing experiments and on the results, which are obtained after the respective experiments.

Table 5.1: Suitable chambers for finite and infinite dosing experiments

	Finite dosing	**Infinite dosing**
Static	Yes	Yes
Flow-through	Yes	Yes
One-chambered	Yes	Yes
Two-chambered	No	yes
Parameters evaluated	% absorbed/cm^2 skin surface; absolute amount penetrated/cm^2 skin surface	Steady-state flux or rate of absorption; Permeability constant (Kp)

5.3.3 Skin preparations

5.3.3.1 Choice of skin

The choice of skin depends on the purpose of the test and the availability of skin samples. Skin from human and animal sources can be used. Although the use of human skin samples gives data more approximate to human *in vivo* conditions, it is not always readily available. Further, the use of human skin is subject to national and international ethical considerations and conditions (ECETOC, 1993). Typical human *in vitro* experiments involve the use of female abdominal and/or breast skin obtained at autopsy or from cosmetic surgery (Dressler, 1999).

A comparison of the human and monkey *in vitro* data revealed, that monkey skin is a good model of human skin (Bronaugh and Maibach, 1985). However monkeys are relatively difficult and expensive to hold and there are specific ethical and animal protection issues to consider; therefore skin samples are difficult to obtain and comparable *in vivo* data are rare (Klain & Reifenrath, 1991).

Rat skin is often preferred where *in vivo* toxicological studies in rat have been performed. Pig skin is often used – especially for cosmetic testing - because it shares essential permeation characteristics with human skin (SCCNFP 2003b, Steiling et al., 2001). According to the SCCNFP human skin (abdomen or breast) or pig skin (abdomen, breast, back, flanks and ears) are recommended for cosmetic testing (SCCNFP 2003b).

Although viable skin (Bronaugh, 2004b) is preferred especially for metabolism studies, non-viable skin can also be used for certain tests provided that the integrity of the skin can be demonstrated (OECD, 2004a).

5.3.3.2 Preparation of tissue samples

Skin samples that may be used are split-thickness (200 – 400/500 µm) (OECD, 2004c, US-EPA, 2004) or full-thickness (500 – 1000 µm) skin preparations (OECD, 2004c). Skin has to be shaved and the sub-cutaneous fat and - in the case of split-thickness skin samples - parts of the dermis are removed.

Preparation of an epidermal layer by separation of the epidermis and dermis using heat is effective for nonhairy skin (Full-thickness skin is submerged in 60°C water for approximately 45 seconds up to 1 minute and the epidermal and dermal layers can be pulled apart with forceps, but viability of skin is destroyed (Bronaugh et al., 1999b, US-Epa, 2004). According to Bronaugh, chemical separation techniques and enzyme methods have only limited usage (Bronaugh 2004b in Zhai and Maibach). But according to OECD, 2004c they may also be used in skin preparation.

Full-thickness and split-thickness skin is often trimmed with a dermatome (kind of a wooden roll), to obtain skin samples of uniform shape and thickness (Steiling et al., 2001). The trimming and thickness reduction of pig skin is done from the dermis side, in order to generate skin samples with intact stratum corneum and epidermis (Steiling et al., 2001). Skin thickness (usually 0.8 to 1.0 mm) should be measured by an appropriate method (using a micrometer gauge (Kenyon et al., 2004)). The skin samples should be prepared to fit the experimental cell (SCCNFP 2003b).

Animal and human skin can be stored for several months (OECD, 2004c) up to one year for dermatomed pig skin samples (Steiling et al., 2001) at -20°C, but not for metabolism studies.

5.3.3.3 Checking of barrier integrity

Before and after the experiment, the barrier integrity of the skin must be checked by physical methods like TEWL (Transepidermal Water Loss) or TER (Transcutaneous Electrical Resistance) (OECD, 2004c, US-Epa, 2004). The viability of the skin samples may also be checked using the Tritium method, where the movement of [^{3}H] water through the skin is observed and compared with standard values (OECD, 2004a c, US-EPA, 2004, Ursin et al., 1995).

5.3.4 Application of test substance

5.3.4.1 Test substance

For practical reasons, the test substance ideally should be radio-labelled (preferably ^{14}C at a metabolically stable position). However, if radio labelling is not possible, suitable validated assay procedures must exist for the respective chemicals and metabolites (OECD, 2004c). Before the test substance can be applied to the skin sample, some factors, that may influence the penetration results have to be considered: vehicle, dermal area dose (which depends on the concentration of the test substance in the vehicle and the applied amount/cm^2 of skin), occlusion, finite or infinite dosage regime.

5.3.4.2 Vehicle

The choice of the vehicle may have an influence on the obtained results. The vehicle effect is described by the vehicle/stratum corneum partition coefficient (Km), which is an important factor in determining the rate of penetration of a chemical (Scheuplein & Blank, 1971). This coefficient describes the relative affinity of a chemical for the vehicle in which it is applied and the stratum corneum (Suskind, 1977). The more soluble the penetrant in the vehicle, the more likely it is to be retained within the vehicle (Baker, 1986). A greater solubility in the stratum corneum than in the vehicle promotes penetration (Nater and de Groot, 1985).

The test substance preparation should be the same as that to which humans or other target species may be exposed and may be applied neat, diluted (ideally with water) or otherwise formulated (OECD, 2004a, c). US-EPA recommends a relatively strict scheme to prepare the test substance: If the test substance is a liquid at room temperature and does not damage the skin during the determination of Kp, it must be applied neat. If this is not possible, it must be dissolved in water. If the solubility in water is not high enough so that a steady-state absorption can be obtained, the test substance must be dissolved in isopropyl myristate (US-EPA, 2004).

5.3.4.3 Applied doses/cm^2 of skin

For finite dose experiments an appropriate quantity (up to 10 mg/cm^2 or 10 µl/cm^2) of the test preparation should be spread on the skin surface (OECD, 2004c). It was noted, that this volume may not adequately cover the skin (CEFIC-QSAR, 2004).

For infinite dose experiments, typical doses of >100 µl/cm^2 or >10 mg/cm^2 may be appropriate to obtain steady-state conditions from which the steady-state flux or absorption rate and the permeability constant (Kp value) can be calculated (OECD, 2004c).

According to US-EPA a sufficient volume of liquid must be used to completely cover the skin (US-EPA, 2004).

5.3.4.4 Duration of exposure and sampling time

The exposure time should reflect in-use conditions. The exposure time may therefore vary between a few minutes for a rinse-off product, 24 hours or longer for a leave-on product and 8 hours for industrial products (OECD, 2004c). For finite dose experiments the wash-off from the skin – usually performed with an aqueous soap - determines the exposure time (OECD, 2004c). It is important to sample the receptor fluid for at least a 24-hour period. Increased exposure times are only appropriate in case of long lag phases or for infinite applications in order to achieve a steady-state flux (OECD, 2004c). Usually the exposure times for test substances in finite dosing experiments are above 24 hours (see EDETOX database).

5.3.5. Evaluation of the results

The terminal procedures of an *in vitro* dermal absorption study are slightly different after having performed an infinite or a finite-dosing experiment. After finite dosing a mean maximum amount of dermally absorbed material is determined, which requires nearly complete recovery of the test substance. For infinite dose applications, the steady-state flux or the permeability coefficient (Kp) are determined; the recovery determination is not relevant, because the only important end-point is the appearance of the test substance in the receptor fluid (OECD, 2004c)

5.3.5.1 Dermal absorption results after finite dosing

The quantity of the test compound or its metabolites must be determined in (OECD, 2004c, Steiling et al., 2001):

- Dislodgeable dose from the skin surface (washing water),
- the stratum corneum (adhesive tape strips)
    - 15-25 strips are recommended fur human skin (OECD, 2004c)

- the epidermis without stratum corneum
- the dermis
- the receptor fluid
- Check also: substance adsorbed in the equipment (applicator, donor and receptor chamber)

For radiolabeled test substances scintillation counting has to be performed. For non-radiolabeled substances HPLC or GC analysis may be appropriate. In general, radio-labeled methods are the most sensitive analyzing methods (SCCNFP 2003b).

The mass balance analysis / recovery must be determined and should be within the range of 85 – 115%. Lower recovery rates are expected for volatile substances (Bronaugh et al., 1999b). According to the OECD an adequate mean recovery is in the range of 100 +/- 10% (for volatile substances 100 +/- 20% may be acceptable); explanations in the report should be given in case of lower recovery rates (OECD, 2004c).

Dermal absorption should be expressed as an absolute amount (µg/cm^2 of skin surface) and as a percentage of the amount of test substance contained in the intended dose applied per square centimetre of skin surface (SCCNFP 2003b, Bronaugh et al., 1999b)

Absorbed substance amounts are summarised as follows:

- Amount determined in the receptor fluid
- Amounts found in the epidermis (except for the stratum corneum) and dermis[1]
- According to the SCCNFP, for cosmetic tests, amounts that are retained by the stratum corneum at the time of sampling are not considered to be dermally absorbed, and thus they do not contribute to the systemic dose (SCCNFP 2003b). However, for other risk assessments this amount is considered as a possible reservoir for systemic dose.

<u>Typical results after penetration studies with hair dyes</u>

[1] It has been demonstrated that lipophilic test substances may be artificially retained in skin (Bronaugh et al., 1994)

A typical summary of analysed test chemicals found in relevant compartments, related to the terms: rinsing, adsorption, absorption and penetration (see chapter definitions) was found for three different hair dyes (Steiling et al., 2001). The results are shown in Table 5.2.

Table 5.2: Results of percutanous absorption studies related to the terms: rinsing, adsorption, absorption and penetration (from Steiling et al., 2001)

	Hair dye I without developer	Hair dye I with developer	Hair dye II with developer
Rinsings (skin, equipment)	89.5	85.2	88.4
Adsorption (on tape strips)	3.3	2.6	3.7
Absorption (in residual skin)	0.91	0.39	0.63
Penetration (in the receptor)	0.11	0.01	0.30
Bioavailability	1.02	0.40	0.93
Percent recovery*	94.7	89.1	93.0

* Values probably include amounts not included in this table

Catechol has been used as a coupler in hair dye formulation. As it has been assumed that some amounts of catechol remain on the skin after hair dying, the *in vitro* percutanous absorption of catechol – which has stomach carcinogenic activity in animal studies – has been studied. The results of the investigation in human and fuzzy rat skin after a 30 min and a 24 h application of 0.6% catechol (in a consumer permanent hair dye) are summarized in Table 5.3 (Jung et al., 2003). Dosing was based on the assumption that 10% of the mixed product will reach the scalp. Assuming the scalp has an area of 650 cm^2, approximately 0.0112 g of hair dye product was applied per diffusion cell using a positive displacement pipette.

5.3.5.2 Dermal absorption results after infinite dosing

The permeability constant (*Kp*) must be calculated by dividing the steady-state rate of absorption (measured in $\mu g/h/cm^2$ by the concentration of the test substance (measured in $\mu g/cm^3$) applied to the skin (US-EPA, 2004, Bronaugh et al., 1999b).

Table 5.3: *In vitro* skin penetration of catechol (0.6/4%) over 24 h and 72 h (from Jung et al., 2003)

Application % of applied dose found in	Human skin 30 min application	Fuzzy rat skin 30 min application	Fuzzy rat skin 24 h application 24 h sampling	Fuzzy rat skin 24 h application 72 h sampling
	0.6% catechol		4% catechol	
Skin - Stratum corneum - Viable epidermis & dermis	3.5 +/- 0.7 - 2.0 +/- 0.0 - 1.5 +/- 0.6	1.6 +/- 0.2 - 1.2 +/- 0.0 - 0.5 +/- 0.2	8.6 +/- 0.7 - 5.7 +/- 1.2 - 2.9 +/- 0.5	8.3 +/- 1.0 - 3.9 +/- 0.4 - 4.4 +/- 0.7
Receptor fluid	0.4 +/- 0.1	0.2 +/- 0.0	80.9 +/- 0.8	81.1 +/- 3.4
Total penetration	4.0 +/- 0.7	1.8 +/- 0.2	89.5 +/- 1.5	89.4 +/- 3.8
Wash at 24 h	74.4 +/- 1.4	76.9 +/- 2.8	6.1 +/- 0.1	7.2 +/- 1.0
Percent recovery*	79.2 +/- 2.0	79.4 +/- 2.6	95.6 +/- 1.7	96.6 +/- 2.9

*Percent recoveries include amounts not reported in this table (patch designating dosing site or on protective screen

5.4 Other test systems

5.4.1 Artificial skin

Several researchers have developed artificial skin equivalents. Such materials generally attempt to produce membranes which exhibit both hydrophilic and hydrophobic regions and therefore mimic the stratum corneum adequately. Living skin equivalent models have also been employed to assess percutaneous absorption. They consist of skin membranes including, for example, reconstituted epidermis, grown in tissue culture and employed as alternatives to animal tissues, e.g EpiDerm (Moss et al., 2002; Wagner et al., 2001). The use of artificial skin is still under development and not yet advised for *in vitro* testing because of differences in barrier function (Coquette et al., 2000). Epidermal membranes are quite fragile and mass balance techniques often cannot be applied in this case. They sometimes overestimate human *in vivo* skin absorption (Van de Sandt et al., 2000; SCCNFP 2003b).

5.4.2 Tape-stripping technique in vitro

The *in vitro* tape-stripping experiment represents a potential alternative to *in vitro* dermal absorption measurement with diffusion cells. This technique is based on a linear correlation existing between the amount of substance penetrating the skin within an exposure period and the amount remaining in the stratum corneum within a defined time after application (Dupuis et al., 1984). With the tape-stripping technique the amount of

substance absorbable through the skin may be assessed (see also section 6) (Rougier et al., 1999, Surber et al., 1999). The results of the tape-stripping experiment should be expressed as amount of test compound per square centimetre area of the adhesive tape (e.g. ng/cm^2) or the another adequate means (e.g., ng/protein content) (Surber et al., 1999).

The amount of uptake into the stratum corneum is highly dependant on the vehicle, as both the adhesive properties of the tape as well as the cohesion of the corneocytes may be influenced by the vehicle (Surber et al., 1999).

5.5 Examination of reservoir characteristics

Reservoir effects are well-documented for steroids (Miselnicky et al., 1988). The reservoir can exist in the stratum corneum, in the viable avascular tissue (viable epidermis and supracapillary dermis) and in the dermis (Roberts et al., 2004).

In order to investigate the potential for the skin to act as a storage depot for a specific substance a flow-through diffusion cell with either animal or human skin full- or split-thickness skin samples may be used. The radiolabeled substance is applied to the skin surface for 5 h. After this time, any remaining test substance is removed by wiping and the experiment was then left to run for another 15 h, during which time any radioactivity already absorbed into the skin would be able to continue to diffuse across into the receptor fluid. This receptor fluid was collected every hour (2 ml) for a total of 20 consecutive hours until the end of the experiment. Radioactivity still remaining in the upper stratum corneum may be estimated by the method of “tape stripping” (Kenyon et al., 2004).

In one study – investigating the fate of dihydroxyacetone, 7-(2H-naphtho[1,2-d]triazol-2-yl)-3-phenylcoumarin, and disperse blue 1 – the skin penetration and absorption through human and fuzzy rat skin was determined over 24 or 72 h in flow through diffusion cells. The skin penetration of these chemicals resulted in relatively low receptor fluid levels but high skin levels, demonstrating the importance of determining the fate of chemicals remaining in skin, which could significantly affect the estimates of systemically available material to be used in exposure estimates (Yourick et al., 2004).

5.6 Experimental factors affecting dermal absorption *in vitro*

5.6.1 Species, strain, gender, age of skin source

One of the most important factors influencing the results of *in vitro* dermal penetration studies is the choice of the tissue sample. Rat, mouse and rabbit skin are more permeable than human skin; thus their use results in a conservative estimate of skin penetration for safety assessments (Bronaugh et al., 1999b, Bartek et al., 1972, Feldman & Maibach 1969, 1970, Scott et al., 1986, ECETOC 1993, van Ravenzwaay & Leibold, 2004). Rat skin was more permeable to all tested substances (organic compounds, molecular weight: 231 – 466 g/mol; aqueous solubility: 0.057 – 600000 mg/l; log POW: 0.7 – 4.5) than human skin with a mean difference of 10.9-fold (van Ravenzwaay & Leibold, 2004). Lipophilic compounds showed the highest penetration rates through rat skin *in vitro* (van Ravenzwaay & Leibold, 2004).

The passage of trimethylamine across rat and human skin samples was compared, applying each 0.1, 1.0 and 10 mg per skin membrane 0.32 cm^2. The apparent dermal flux was calculated as 3.40 +/- 1.60, 58.3 +/- 30.6 and 265.0 +/- 155.0 $\mu g/cm^2/h$ for rat and 0.98 +/- 0.75, 9.21 +/- 3.06 and 92.7 +/- 31.9 $\mu g/cm^2/h$ for human skin, respectively (Kenyon et al., 2004). Cross-species *in vitro* dermal absorption tests were conducted with 14C-labelled benzo[a]pyrene dissolved in acetone and applied to dermatomed skin (0.5 mm thickness) at comparable dose rates (8-13 $\mu g/cm^2$). The %age dermal absorption included the % ^{14}C-activity detected persisting in the skin added to that detected in the receiver solution. No tape stripping of the stratum corneum was performed prior to radioactive analysis of the skin sample. Table 5.4 shows the results of the total % *in vitro* dermal absorption obtained by 48 h postexposure.

Strain, gender, anatomical site and the age of the skin source are also potential sources of variability in the obtained *in vitro* absorption results; these are probably expressed in inter- and intralaboratory variations (see Section 7).

Table 5.4: % *in vitro* dermal absorption of benzo[a]pyrene after 48h exposure (from Moody et al., 1995)

Skin sample	% dermal absorption
Rat	95 +/- 9.6%
Hairless guinea pig	51 +/- 3.0%
Human, 50 years old	43 +/- 8.7%
Testskin	34 +/- 12.4%
Human, 32 years old	23 +/- 5.3%

5.6.2 Temperature

During the *in vitro* testing, the skin must be maintained at a physiological temperature of 32°C during the test (US-Epa, 2004, OECD, 2004c). Since diffusion is a temperature-dependant process, alteration in the skin temperature may affect the absorption process (OECD, 2004c). It has been recently shown, that temperature variations are a potential factor for inter-laboratory variations in dermal absorption results (see above). Differences in the temperature between flow-through and static cells have been determined as well as temperature differences within the flow through cell, due to the position of a flow cell on the warmer (Romunchuk & Bunge, 2004).

5.6.3 Occlusion

There are some arguments pro and against occlusion of the skin. First of all the normal exposure situation is best simulated by an open unoccluded atmosphere, as the human skin during exposure situations may be protected but not occluded. Unoccluded or semi-occluded conditions can avoid skin integrity damage caused by excessive hydration which increases the penetration rate (Bronaugh & Stewart, 1985, Kligman, 1983, Hurley, 1985 cited in ECETOC, 1993 Baker, 1986). A five- to ten-fold increase of permeability of the stratum corneum was observed during occluded infinite dosing, as the hydration of the stratum corneum drastically altered their function (Sartorelli et al., 2000). However volatile substances may evaporate during unoccluded testing, and infinite dosing experiments are only realizable under occluded conditions (Bronaugh 2004b, OECD, 2004c).This was observed in a dermal absorption study with catechol in ethanol. Diffusion cells that were occluded with Teflon caps to prevent evaporation showed approximately 78% of the applied dose absorbed into the receptor fluid. About 55% of the applied dose was absorbed into the receptor fluid of skin samples that were not occluded (Jung et al., 2003).

5.6.4 Thickness of skin

The thickness of the skin sample has an influence on the diffusion properties of the test chemical. Bronaugh recommends not using full-thickness skin for absorption studies in general, unless the animal skin used is already very thin, such as occurs in the mouse (400 µm). According to Bronaugh all or most of the dermis should be removed to simulate the *in vivo* diffusional barrier layer (Bronaugh 2004b), as full-thickness skin can artificially retain absorbed compounds that bind or diffuse poorly through it. In contrast Cnubben et al found, that viable full-thickness skin membranes correlated well with respective *in vivo* experiments (see Section 7, Cnubben et al., 2002). In this investigation they studied the *in vitro* skin penetration of ^{14}C-ortho-phenylphenol through human and rat viable skin, human and rat epidermal membranes and perfused pig ears. Human and rat epidermis showed a marked higher cumulative penetration (µg/cm^2) than human and rat full thickness viable skin (see Figure 5.4).

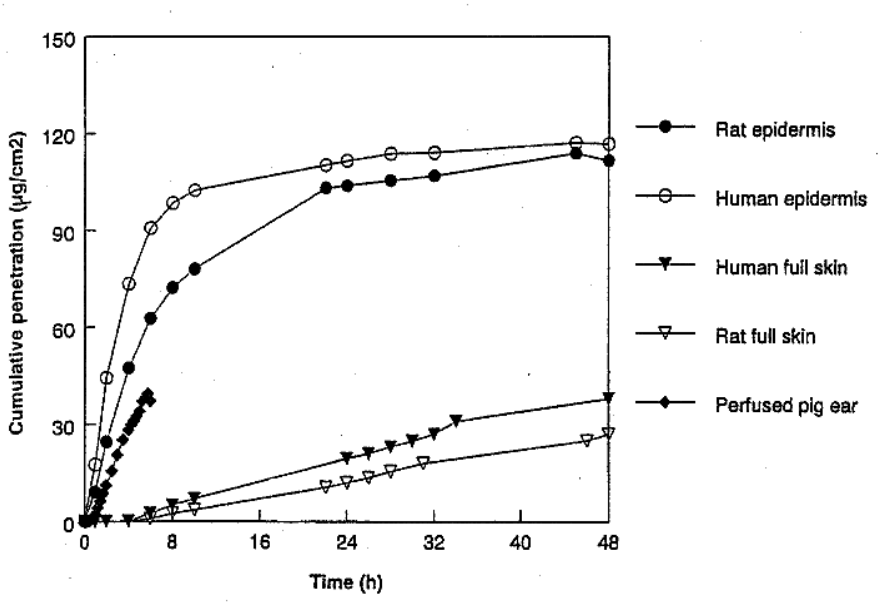

Figure 5.4: *In vitro* skin penetration expressed as cumulative amount reaching the receptor fluid (Cnubben et al., 2002) [permission pending].

The influence of the skin thickness (0.5 – 1.3 mm) on percutaneous penetration has been studied using caffeine, testosterone, butoxyethanol and propoxur by Wilkinson et al., 2004. Some changes in the maximum flux and the culumative doses in receptor fluid dependant on the skin thickness were seen, but no clear effects on the extent and rate of the penetration was observed, as the relationship between skin thickness and

physicochemical properties is complex (Wilkinson et al., 2004). No specific investigations have been found on differences between viable and non-viable skin samples.

5.6.5 Composition of receptor fluid

The receptor fluid should not act as barrier to absorption (due to poor solubility of the test compound) and not affect skin preparation integrity (OECD, 2004a). For water-soluble compounds, the use of normal saline or an isotonic buffer saline solution may be sufficient. A physiological buffer is needed to maintain viability of the skin for at least 24 h. When fresh skin is obtained, the viability of skin can be be maintained with either a tissue culture medium or a HEPES-buffered Hanks´ balanced salt solution. Bovine serum albumin (4%) or PEG 20 (6%) can be added to the receptor fluid to facilitate partitioning of lipophilic compounds into the receptor fluid (Bronaugh 2004b, Sartorelli et al., 2000). Care must be taken with surfactants and organic solvents, as the skin barrier can be damaged, particularly when split-thickness skin preparations are used (Bronaugh et al., 1999b). It is reported that one percent aqueous solutions of sodium laurate, sodium lauryl sulphate and other surfactants were found to increase the permeability of water through human epidermis (Sartorelli et al., 2000).

To ensure that an increase in concentration of the test substance in the receptor fluid does not alter penetration rate, the concentration of the test substance in the receptor fluid has to be less than 10% of the initial concentration the donor chamber (US-EPA, 2004). When hydrophobic chemicals are tested polyethoxyoleate must be added to the receptor fluid at a concentration of 6% (US-EPA, 2004).

5.6.6 Vehicle

The *in vitro* percutaneous absorption in terms of permeability constants (*Kp*) of both caffeine and testosterone have been compared in petrolatum (petroleum jelly), ethylene glycol gel and water gel. Similar Kp values were seen for caffeine with petrolatum and water vehicle and slower absorption rate in ethylene glycol. Testosterone was found to be highly permeable in water and low *Kp*-values were determined in petrolatum and ethylene glycol. The solubility of testosterone is low following in high *Km* values (Bronaugh & Franz, 1986).

An additional vehicle effect, which may carry the substances through human skin, was observed in a recent study performed with caffeine. For the non-lipophilic vehicles

water, butoxyethanol and water/butoxyethanol an increase in the permeability constant (*Kp*) for the vehicle reflects an increase in the *Kp* for caffeine. However different results have been found for more lipophilic vehicles (Dick et al., 2004).

In a recent investigation with a flow-through system the permeability of ^{3}H-ricinoleic acid – a machine cutting fluid ingredient – through silastic membranes and porcine skin was significantly reduced in polyethylenglycol ethers with several additives (2% triazine, 5% triethanolamine, 5% linear alkylbenzene sulphate) compared to several mineral oil mixtures. The presence of one or more additives (2% triazine, 5% triethanolamine, 5% linear alkylbenzene sulphate) in mineral oil mixtures resulted in significantly greater ricinoleic acid partitioning into the stratum corneum compared with polyethylene glycol-200 mixtures (Baynes & Riviere, 2004).

However the cumulative percutaneous penetration of ^{14}C-labeled sunscreens in two different formulations (hydroalcoholic lotion and oil/water emulsion) was very similar in each case (1.58 µg/cm^2 over 48 h), although the amount of applied material remaining in the epidermal membranes at 48 h was slightly higher for the hydroalcoholic solution (32.77%) than for the oil/water emulsions (Walters et al., 1999).

5.6.7 Substance concentration

When applying a test chemical in increasing concentrations to the skin surface for a constant exposure time, the penetration rate does not necessarily increase linearly with dose (ECETOC, 1993). An inverse relation between concentration (area dose) and percentage of absorption is given. At low concentrations, the absorbed test substance expressed as percent of applied dose per time interval is in general higher than the percentage absorption at high concentrations.

5.6.8 Dosing volume

The dosing volume has an influence on the obtained results of dermal penetration. Wester and Maibach studied the effects of finite and infinite dosing on the dermal absorption. Mercury was dosed on human skin *in vitro* in volumes of 10, 100 and 1000 µl/cm^2. The absorption of the infinite dose 1000 µl/cm^2 was enhanced compared to the lower volumes, which showed equal penetration rates (Wester & Maibach, 1999b).

SECTION 6 *IN VIVO* TESTS FOR DERMAL ABSORPTION

6.1 Introduction

There are a number of advantages in performing an *in vivo* study, as it uses a physiologically and metabolically intact system (OECD, 2004a,b). Within this intact system the vitality of the living skin will determine the metabolism, distribution and excretion of the compounds e.g. through the specific functions of the stratum corneum, which is capable of absorbing a quantity of material, limited only by the solubility of the chemical in sebaceous and epidermal lipids (Wester & Maibach, 1999b). *In vivo* dermal penetration studies are carried out in laboratory animals, usually rodents, but also to a limited extent in human volunteers.

6.2 Animal studies

6.2.1 Test guidelines for animal studies

It is only recently that a test guideline has been accepted for *in vivo* dermal penetration (OECD, 2004a,b). The following descriptions are based on the OECD protocol. Up till this time, a variety of protocols were used.

6.2.2 Principle of the standard in vivo tests

The test chemical is applied to a designated area of skin in solvent or formulation or to a patch of material which is then placed at a predetermined site in direct contact with skin for a defined period. Body fluids, tissue or excreta are collected at predefined intervals and the quantity of chemical and/or metabolite in the samples is measured by a suitable analytical procedure. The analytical method of choice must be appropriately sensitive since percutaneous absorption is often low. (ECETOC, 1993). The rat is the most commonly used species for the *in vivo* test (OECD, 2004c, US-EPA, 1998).

6.2.2.1 Skin preparation

At least 16 hours before treatment, the application site should be prepared. In rat for example, the hair on the shoulders and the back is removed with animal hair

clippers, shaving of the application site should be avoided, in order to avoid abrasions, which will artificially increase the dermal penetration (OECD, 2004a, c). A further relevant point is the size of skin area; it must be large enough to allow reliable calculation of the absorbed amount of test substance per cm^2 skin, preferably at least 10 cm^2 (OECD, 2004c). If the skin is washed prior to application (with water or a mild detergent) the possibility of skin or barrier property modifications has to be carefully considered (OECD, 2004c).

In vivo studies were conducted in monkeys according to the protocol of Feldmann & Maibach, 1969 for human volunteers. Monkey skin was lightly shaved with electric clippers, using care to prevent damage to skin. The compounds were applied to skin in an acetone vehicle at a concentration of 4 $\mu g/cm^2$ to a defined circular area (13 cm^2) demarcated by petrolatum (Bronaugh & Maibach, 1985).

6.2.2.1 Application of the test substance to the skin

The test preparation, which ideally is radiolabelled in a metabolic stable position, is applied to the 5-10% of the surface of skin (for rats with body weight 200 – 250g: 10 cm^2). 1-5 mg/cm^2 or up to 10 $\mu l/cm^2$ of the test substance may be used. A suitable application system must be used, that prevents any spreading of the chemical outside the defined area and prevents the animal from interfering with the application site. A common procedure uses a ring of an inert material (rubber, PTFE), which is glued to the skin over the clipped area, before applying the test formulation using cyanoacrylate adhesive to confine the treated area (OECD, 2004c). The application system must be covered with e.g. a nylon gauze, which may be glued over the ring to protect the test preparation against unintentional removal or spreading. A collar or a rubber tubing is placed behind the forelegs or neck of the animals (Franz, 1975). The ring should be deep enough to account for non-occlusive conditions and to prevent the cover rubbing on the treated skin (OECD, 2004c).

6.2.2.2 Evaluation of results

After the exposure, which should represent in-vivo conditions (for duration of exposure see section 5), the test preparation is removed by an appropriate cleansing procedure. During and after exposure the animals are housed individually in metabolism cages from which excreta (urine and feces) are collected (if appropriate, exhaled volatile metabolites are also collected) (OECD, 2004c).

It is recommended to perform skin fractionation, in order to further define the localisation of the test substance within the skin: Tape stripping is suitable for the removal of the stratum corneum; freezing or otherwise fixing allows the usage of a microtome to cut the skin sample in horizontal slices (OECD, 2004c, Schaefer & Redelmeier, 1996).

The quantity of test compound or its metabolites must be determined in (OECD, 2004c):

- Dislodgeable dose from the skin surface (washing water),
- Skin from the treated site (stratum corneum, epidermis and dermis)
- Desquamated skin (from protective dressings post-exposure)
- Urine, faeces, and cage washing
- Expired gas, if applicable (> 5% volatile radioactive metabolites of applied dose)
- Blood, and remaining carcass
- Solvent washing of contaminated material and application system

An adequate mean recovery is in the range of 100 +/- 10% (for details see chapter 5.).

The dermal absorption is usually given as % of dose applied and is obtained by addition of amounts recovered from urine, faeces and cage washing, expired gas, blood and remaining carcass. An example for the data obtained is given in Table 6.1.

Table 6.1: *In vivo* percutaneous absorption and distribution of catechol (4%) in fuzzy rat (Jung et al., 2003)

% of applied dose	24 h sampling	72 h sampling
Skin	7.7 +/- 0.4	9.4 +/- 1.3
Total systemic absorption	44.9 +/- 0.8	53.1 +/- 4.8
Urine	35.8 +/- 1.3	46.3 +/- 6.2
Feces	0.09 +/- 0.02	0.12 +/- 0.04
Carcass/Organs/ Blood	9.0 +/- 9.9	6.7 +/- 1.7
Wash at 24 h	3.5 +/- 0.5	2.3 +/- 0.6
Percent recovery*	67.5 +/- 1.5**	75.6 +/- 4.1**

*Percent recoveries include amounts not reported in this table (patch designating dosing site or on protective screen)

** It is assumed, that the recovery values are so low, because the application site was not occluded and the concentration in the gas phase was not determined. In respective *in vitro* investigations, marked differences in the recovery values were seen in occluded and not-occluded diffusion cell experiments.

6.3 Studies with human volunteers

6.3.1 Protocols and applications

Studies with human volunteers provide definitive data for the assessment of the absorption of chemicals through human skin but for technical and ethical reasons their use is limited and their conduct is closely regulated (ECETOC, 1993). A study protocol with supporting toxicological data is submitted to an ethical committee and approval is obtained (EC, 2004). The use of radiolabelled compounds for human studies is subject to further regulation (ECETOC, 1993).

For studies with human volunteers the amount retained in the tissues cannot be determined. Therefore the so called “indirect method” for the determination of levels absorbed has to be followed, which was developed by Feldmann and Maibach (1965, 1970). The dermal penetration of the test compound is determined from the extent of excretion from urine or feces. As the compound may be retained in the tissues, a correction must be made by administration of a single parenteral dose and a determination of the extent of excretion of the dose, according to the following formula:

% dermal absorption = Total % excreted radioactivity after topical dose / total % excreted radioactivity after parenteral dose x 100 % (Klain & Reifenrath, 1991).

6.3.2 Dermal uptake from liquid application

Usually liquid formulations are applied to a defined area usually of the forearms, in a few investigations also of the abdomen (just above the umbilicus, Bronaugh Franz, 1986) or the back (Hawkins et al., 2002). The skin is usually not protected, and the volunteers are asked not to wash for 24 hours (Feldman & Maibach, 1969, Feldmann & Maibach, 1974). Either the parent compound or the (main) metabolites are determined in the excreta. In some studies blood or plasma levels (area under the plasma concentration-time curves) are determined. A good correlation has been found in bioavailability of topical nitroglycerin determined by the urinary excretion measurements and by the plama concentration-time curve (Wester & Maibach, 1983). Human volunteer studies are widely used for example for the determination of the absorption of hormones (e.g. Feldmann & Maibach, 1969), drugs (e.g. Dehghanyar et al., 2004; Martin et al., 2004), hair dyes (e.g. Dressler et al., 1999; Wolfram & Maibach, 1985), disinfectants (e.g. Turner et al., 2004), fragrances (e.g. Ford et al., 2001; Hawkins et al., 2002), industrial chemicals (e.g. Feldmann & Maibach, 1970; Nomiyama et al., 2000), pesticides and herbicides (e.g. Feldmann & Maibach, 1974; Meuling et al., 2004; Ross et al., 2005). Some of these studies used special protocols.

For hair dyes the exposure of the scalp decreases with increasing exposure duration due to binding of the dye to the hair. Furthermore the dye is removed after the dyeing process with detergents. Therefore, for absorption studies, exactly the conditions of hair dying were mimicked. The dye mixture was applied to dry hair, worked gently int the hair mass over a certain period, the left on the hair for 20 to 30 minutes. After the dying process the the hair was rinsed, towel blotted and dried (Dressler et al., 1999; Wolfram & Maibach, 1985) .

Similarly the absorption of diclofenac, a nonsteroidal anti-inflammatory drug, was analysed under use conditions: the compound was applied to the thigh at a therapeutic dose 3 times daily for 4 days with subsequent occlusion with a plastic foil for 4 h (Dehghanyar et al., 2004).

6.3.3 Dermal uptake from vapours

Usually human volunteer investigations have studied dermal absorption by liquid application. However a few studies have tested vapours and demonstrated that uptake of vapours can be considerable.

To study the uptake of vapours via the skin, human volunteers are exposed under conditions allowing good access of the vapours to the whole skin of the body. For example in the study of Brooke et al. (1998) the volunteers were wearing T-shirts and shorts to optimise the dermal surface available for vapour contact. To prevent uptake via inhalation of the vapours, the volunteers wore air-fed breathing masks. Exposure without the masks accounts for the overall uptake (dermal and inhalation). It was shown that for some substances, such as the glycol ethers, skin uptake from vapours may be an important contributor to the total uptake with a 5-10% contribution to the total body burden seen for 1-methoxypropan-2-ol (Brooke et al., 1998). Also other studies demonstrate good dermal absorption of various glycol ether vapours (Kezic et al., 1997, Johanson & Boman, 1991) or xylene (Kezic et al., 2004).

In the latter studies, the volunteer was situated in a clean air cabin with a slightly increased pressure to avoid additional inhalation exposure. The arm of the subject was the only part of the body outside the cabin. During the vapour exposure experiments, the subject placed the lower arm into the exposure cylinder into which the m-xylene vapour was led. The m-xylene exposure, and temperature and humidity conditions were controlled (Kezic et al., 2004). The skin area of the forearm and hand exposed to the vapour was measured.

6.4 Other test systems

6.4.1 Microdialysis

Microdialysis is an *in vivo* sampling technique used for the measurement of endogenous and exogenous substances in the extracellular space (El Marbou et al., 2000; Schnetz & Fartasch, 2001). The microdialysis system consists of microinjection pumps and microdialysis probes with polyurethane (semipermeable) membranes (El Marbou et al., 2000), which may be implanted into blood vessels, the dermis or the subcutaneous tissue (see Fig 6.1). The blood-flow underneath the skin surface is mimicked by continuously passing a receptor fluid through the microdialysis tubing, which is collected in a refrigerated collector. Using this method it is possible to measure

the local concentrations of a test compound in the dermis and to monitor percutanous absorption of various substances (El Marbouh et al., 2000).

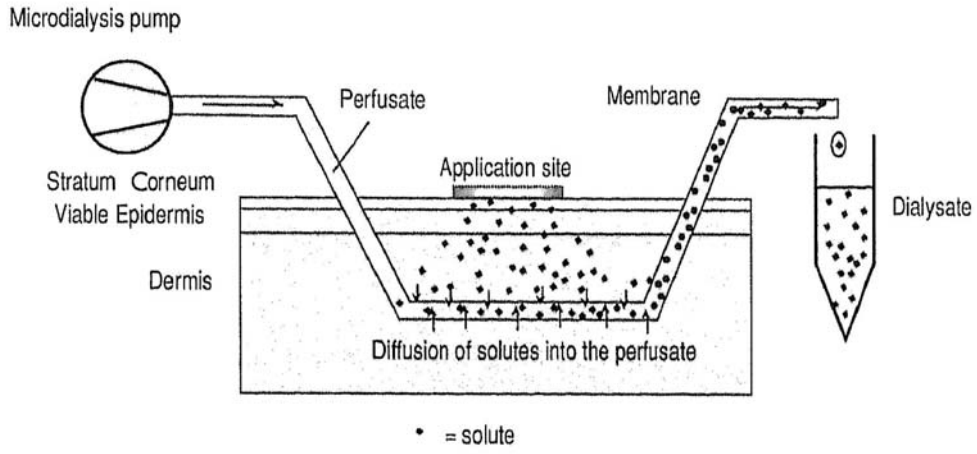

Fig 6.1: Scheme of the microdialysis system (Schnetz & Fartasch, 1991)

The penetration process, the dermal absorption kinetics and the dermal metabolism may be studied in animals (e.g. Mathy et al., 2004) and human volunteers (e.g. Korinth et al., 2004,).using this technique (Sartorelli et al., 2000). The results are usually expressed in terms of relative recovery (Leveque et al., 2004) as the quantity of a compound recovered by microdialysis is only a fraction of the quantity present in the tissue.

Microdialysis has been used to investigate the influence of penetration enhancers, vehicles or iontophoresis on percutaneous absorption *in vivo* in rats. In human volunteers, most of the experiments have been performd to study the kinetics of fast penetrating substances. However the main problem of microdialysis remains the calibration of the technique e.g. the assessment of the reliable recovery. This can be determined using a parallel *in vitro* approach. But this method has not yet found its place in standard research, due to lack of a standardized test procedure and evaluation of reproducibility (Schnetz & Fartasch, 2001).

6.4.2 Tape stripping

Animals

Independently of the physicochemical nature of the chemical and whatever dose was administered, there is a highly significant linear correlation between the total amount of substance applied to animals that penetrated over a 4-day period through the skin and the amount recovered in the stratum corneum at the end of application time ($r=0.98$, $p<0.001$) (Rougier et al., 1999, Dupuis et al., 1984). This was confirmed in studies with theophylline, nicotinic acid, acetylsalicylic acid and benzoic acid (each radiolabeled) applied to the skin of hairless rat for 30 min (Rougier et al., 1999).

At the end of application and washing, the stratum corneum of the treated area of the animals was removed by six strippings, using 3M adhesive tape. The amount of stratum corneum removed from the skin is determined via weighing or – as weighing is often subject to artefacts – also via a colorimetric method (Dreher et al., 1998).

The radioactivity on each strip was measured using liquid scintillation counting. The capacity of the stratum corneum reservoir for each compound has been defined as the sum of the amounts found in the first six strippings (Rougier et al., 1999).

Human volunteers

The tape-stripping method is also suitable for studies with human volunteers. Recently Jakasa et al., 2004 studied the percutaneous absorption of polyethylene glycols of different MW by tape stripping in volunteers. A marked difference was seen between intact and impaired skin (Jakasa et al., 2004a). In a further investigation with jet fuel (containing naphthalene), it was seen that the tape stripping method, if used within 20 min of the initial exposure, can be used to measure reliably the amount of naphthalene initially in the stratum corneum due to a single exposure to jet fuel (Mattorano et al., 2004)

In a study with human volunteers it was shown that neither the type of tape (3 tapes were used: D-squame®, Transpore®, Micropore®) nor the site stripped significantly influenced the mass of stratum corneum removed (Bashir et al., 2001).

Results from tape stripping experiments can be affected significantly by chemical diffusion into the stratum corneum during the time required to apply and remove all of the tape strips. In human studies it was shown that if the time used for the tape stripping

(t_{TS}) < 0.2 lag time for a chemical to cross the stratum corneum (t_{lag}), and the exposure time is > 0.3 t_{lag}, the results from the tape strip experiments are not significantly affected by the t_{TS} (Reddy et al., 2002).

6.5 Factors affecting dermal absorption *in vivo*

6.5.1 Species, strain, gender

Due to the differences between human and animal skin, human studies are the "gold standard" against which all methods measuring percutaneous absorption of substances through human skin should be judged (Howes et al., 1996 cited in Schnetz & Fartasch et al., 2001). The skin of rats and rabbits is more permeable than that of humans, whereas the skin permeability of guinea pigs, pigs and monkeys is more similar to that of humans (OECD, 2004b,). The results of several studies indicate that the pig (Klain & Reifenrath, 1991) and the monkey (Klain & Reifenrath, 1991, Ross et al., 2005) are the animals of choice for dermal absorption studies. Disadvantages are the relatively high maintenance cost and the possible handling difficulties of big laboratory animals (Klain & Reifenrath, 1991). Further, with monkeys there are also ethical and animal protection issues, which have to be considered. Other possible animal models include athymic (nude) rat skin flap model, hairless rats, hairless mice and fuzzy rats (Klain & Reifenrath, 1991). Radiolabeled haloprogin, N-acetylcysteine, cortisone, testosterone, caffeine and butter yellow dissolved in acetone was applied to the skin of rats, rabbits, minipigs and man (only haloprogin and n-acetylcysteine). The dose applied was 4 μg/cm^2 skin surface with a non-occlusive foam pad). The amount of radioactivity excreted in urine for 5 days following application of the test compound was employed as the index for quantifying skin penetration. The results obtained in this study indicated that skin permeability decreases in the following order: rabbit, rat, pig and man. Overall, skin of miniature swine has the closest permeability characteristics to that of human skin with this series of compounds (Bartek et al., 1972). The total absorption properties for haloprogin, n-acetylcysteine, testosterone, cortisone, caffeine and butter yellow in rat, rabbit, pig and man are shown in Figure 6.2 (Bartek et al., 1972).

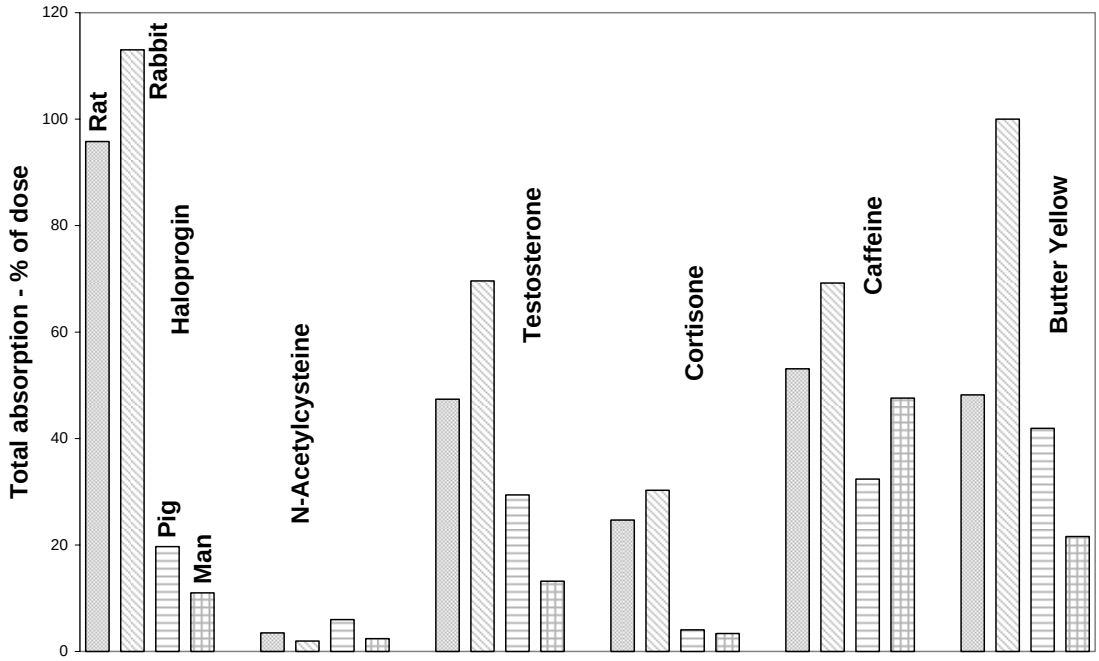

Figure 6.2: Total absorption of different compounds in different species (adapted from Bartek et al., 1972)

6.5.2 Age

In a comparative study with human volunteers Roskos et al. (1989) have demonstrated that age can affect dermal absorption. Permeation of hydrocortisone, benzoic acid, acetylsalicylic acid and caffeine was significantly lower in aged subjects, whereas the absorption of testosterone and estradiol was similar in young and aged subjects. An explanation might be that the diminished surface lipid content and reduced hydration of "old" skin implies a diminished dissolution medium for compounds administered topically, which reduces especially the uptake of somewhat hydrophilic compounds. In contrast, highly lipid-soluble chemicals such as testosterone and estrogen may still be able to dissolve readily into the stratum corneum, even when the available lipid medium is reduced.

6.5.3 Anatomical site

Humans

The percutaneous absorption of acetylsalicylic acid, benzoic acid, caffeine, benzoic acid sodium salt (radiolabeled) was measured in humans on four body sites (arm, abdomen, postauricular, forehead), using the tape-stripping method. Six to eight male Caucasian 28 +/- 2 years old volunteers were applied 1000 nmols of each

compound (1 cm^2, ethylene glycol/water/Triton X-100, 30 min) in two strictly identical applications (left- and right-hand side of the body), which were performed in an 48 hours interval. The stratum corneum of the treated area was removed by 15 successive stripping, and the radioactivity present in the horny layer was measured. Skin permeability appears to be as follows: arm ≤ abdomen ≤ postauricular ≤ forehead. It is noteworthy that whatever the compound applied, the forehead is about twice as permeable as the arm or the abdomen (Rougier et al., 1999, Rougier et al., 1987).

Great differences were observed in absorption of radiolabelled cortisol through various anatomical sites, shown after topical application of hydrocortisone in acetone to normal male volunteers (13 cm^2 skin surface) and analyzing of the urine for a total of 5 days. The forearm value of each volunteer served as control. The absorption increased in the following order: foot arch (plantar) < ankle (lateral) < palm < forearm (ventral, factor 1) < forearm (dorsal) < back (factor 1.7) < scalp (factor 3.5) < axilla (factor 3.6) < forehead (factor 6.0) < jaw angle (factor 13) < scrotum (factor 42). These results are shown in Figure 6.3. It was suggested, that the absorption is higher, with increasing size or number of the follicles, thickness of the stratum corneum. But these generalizations are not consistent with the observation after palm and scrotum application of hydrocortisone (Feldman & Maibach, 1967).

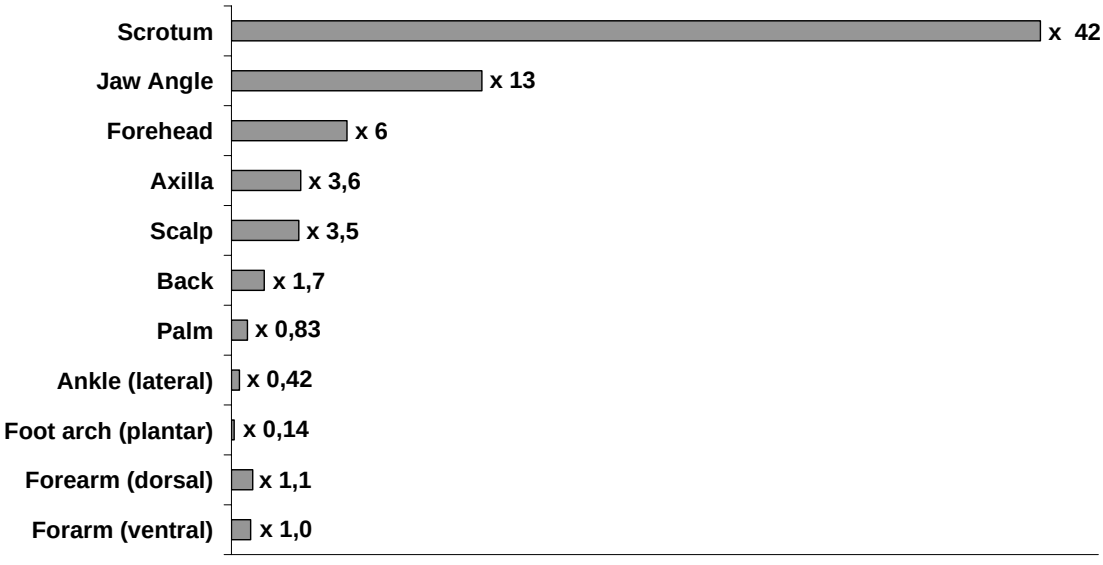

Figure 6.3: Hydrocortisone absorption – effect of anatomic region (adapted from Feldman & Maibach, 1967)

These and similar data on hydrocortisone and pesticides were combined to construct penetration indices for five anatomical sites, as shown in Table 6.2 (Wester and Maibach, 1999a).

Table 6.2: Penetration indices for different anatomical sites

Site	Penetration index based on	
	Hydrocortisone data	Pesticide data
Genitals	40	12
Arms	1	1
Legs	0.5	1
Trunk	2.5	3
Head	5	4

Although all authors agree on the importance of anatomical location in percutaneous absorption, the reviews on this topic give contradictory explanations, for example, the different thicknesses and diffusivities of the horny layers, size and number of follicles etc. (Barry, 1983, Idson, 1975, Scheuplein, 1971).

Postulated reasons for differences in percutanous absorption in different parts of the body are possible variations between **sebum and follicular pathways of absorption.** In the past, the sebum was believed to reduce absorption of hydrophilic compounds. If this were the case, in the parts of the body where the skin contains more sebaceous glands, this effect should be more pronounced. However, the ratio between number of sebaceous glands in forehead to that in the arm is 50, but the ratio in terms of dermal penetration forehead/arm is often lower (2 for benzoic acid, 6 for hydrocortisol). Furthermore, the factor 2 between forehead and arm is the same for benzoic acid and benzoic acid sodium which are very different in terms of lipophilicity. A further explanation may be the **closeness of capillaries to the surface** of the skin in certain regions of the body. This effect may promote resorption and may explain high percutanous penetration in postauricular area found for benzoic acid, benzoic acid sodium salt and acetyl salicylic acid (Rougier et al., 1999).

6.5.4 Amount and area of application

An increased concentration of an applied chemical on the skin increases percutaneous penetration (Maibach and Feldmann, 1969, Wester and Maibach, 1976) as does increasing the surface area treated or the application time. For example the penetration rate of tested compounds theophylline, nicotinic acid, acetylsalicylic acid, benzoic acid (radiolabeled) is strictly proportional to the duration of application, as shown in hairless rats and also in human volunteers (Rougier et al., 1983, Rougier et al., 1987). One thousand nanomoles of each compound was applied onto 1 cm^2 of dorsal skin during 0.5, 2, 4, and 6 hours ($r=0.98$, $p<0.001$) (Rougier et al., 1983, Rougier et al., 1999)

For radiolabeled theophylline, nicotinic acid, acetylsalicylic acid and benzoic acid 125 – 1000 nmol, 1 cm^2 dorsal skin, 30 min), total percutaneous absorption within 4 days and the stratum corneum reservoir at the end of application time were assessed (stripping method). Within the limits of the concentration used, there exists a linear dose-penetration relationship ($r=0.98$, $p<0.001$). However Skog and Wahlberg have shown, that when the applied concentration was increased, penetration was increased up to a certain point, at which a plateau was reached (Rougier et al., 1999, Skog and Wahlberg, 1964). As the physicochemical properties of the tested compounds are different, there might exist - at least for a range of concentration, a linear relationship between dose applied and percutaneous absorption level.

The influences of amount and area of dermal exposure to kerosene upon the levels of kerosene components in rats were examined *in vivo*. The results suggest, that the absorption of trimethylbenzentes – which are easily absorbed kerosene components – is influenced by the total amount of kerosene rather than area of exposure (Tsujino et al., 2003).

6.5.5 Type of application and influence of vehicle

Animals

The influence of nine vehicles on the *in vivo* percutaneous absorption of radiolabeled benzoic acid was studied in the hairless rat, by assessing total percutaneous absorption and stratum corneum reservoir (stripping method). Although the vehicles

used were simple in composition, the total amount of benzoic acid that penetrated over 4 days varied by a factor of 50. Applied vehicles have the potential to either increase or decrease the quantity of water in the horny layer and, thereby, to increase or decrease penetration.

The penetration of benzoic acid is enhanced by increasing the water content of the vehicles whatever the organic phase (Rougier et al., 1999). With chloroform, which is a solvent likely to denature the stratum corneum and/or soften its lipoidal phases, a contrary result was obtained in hairless rat, showing increased dermal penetration after application of neat chloroforme compared with exposure to aqueous solutions containing chloroforme (Islam et al., 1999).

However no marked difference was reported in the amount of dermally penetrated substance after *in vivo* application of three aromatic compounds to the skin either as a solid, aqueous paste, suspension or in the volatile vehicle ethanol of rats (Hughes et al., 1992).

Humans

This influence of the presence of water on the dermal absorption was also studied in volunteers using 2-butoxyethanol. The percutaneous absorption of the test substance from aqueous solution increased markedly when compared with neat 2-butoxyethanol. Even a water content as low as 10% led to an approximate fourfold increase in the permeation rates (Jakasa et al., 2004b).

6.5.6 Effect of temperature and humidity conditions

Increased percutaneous absorption rates were seen for 2-butoxyethanol vapours with raised temperature and humidity conditions (Jones et al., 2003, Johanson & Boman, 1991). At 30°C and 65% relative humidity the dermal absorption of 2-butoxyethanol increased from 11% of the total absorbed dose (20°C, 60% relative humidity) up to 39% of the total absorbed dose (Jones et al., 2003).

As shown for nicotine, the absorption of transdermally delivered nicotin to human volunteers increased after staying in a sauna bath (mean temperature 82°C, 28% humidity) for three 10-minute periods separated by two 5-minute breaks (Vanakoski J et al., 1996).

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SECTION 7 COMPARATIVE STUDIES

7.1 Comparison between *in vitro* and *in vivo* skin absorption results

Although the literature on *in vitro* percutaneous absorption is extensive, only a few studies have been designed for *in vitro- in vivo* comparison. In general, they verify the premise that properly conducted *in vitro* measurements can be used to predict *in vivo* absorption.

For radiolabeled 2-nitro-p-phenylene-diamine, 4-amino-2-nitrophenol, nitrobenzene, p-nitroaniline, and 2,4-dinitrochlorobenzene (4 µg/cm2, acetone, 24 hours) the absorption in monkeys was slightly less *in vitro* compared to the *in vivo* results, the values were not significantly different (Bronaugh and Maibach, 1985). However, human data indicate higher percutaneous absorption *in vivo* compared to respective *in vitro* data for 2,4-dinitrochlorobenzene and nitrobenzene (Bronaugh and Maibach, 1985). A comparison of the *in vitro* and *in vivo* percutaneous absorption of caffeine and testosterone in three vehicles (petrolatum, ethylene glycol gel and water gel) revealed no significant differences between most values. However there was a trend toward lower penetration in the *in vitro* system (Bronaugh and Franz, 1986). *In vitro* absorption utilizing flow-through diffusion methodology with human cadaver skin and human plasma receptor fluid gave the similar % dose absorbed to *in vivo* human volunteer studies for the pesticide, isofenphos (Wester et al., 1992).

The *in vitro* and *in vivo* absorption and metabolism of pyrene, benzo[a]pyrene, and di(2-ethylhexyl) phthalate (DEHP) were investigated in hairless guinea pig. The *in vitro* method, which involved the use of flow-through diffusion cells and Hepes-buffered Hanks'balanced salt solution containing 4% bovine serum albumin as perfusate, was shown to be a suitable system for predicting *in vivo* absorption of these lipophilic compounds (Ng et al., 1992).

The dermal absorption of benzo[a]pyrene was studied *in vitro* in different species (rat, hairless guinea pig, human 50 and 32 year-old; see chapter 5) and *in vivo* in rat and hairless guinea pig. The total percentage of benzo[a]pyrene *in vitro* was consistent with that of *in vivo* data in demonstrating that ^{14}C-benzo[a]pyrene was well absorbed through skin (Moody et al., 1995).

Beck et al. (1994) compared *in vivo* and *in vitro* data of hair dyes with rat and pig from data available from two laboratories performed under similar conditions but in

general spaced by several years, performed by different persons and with different chamber systems and found that despite these differences, the comparisons were good between *in vivo* and *in vitro* results. Dick et al. (1997a,b) investigated the dermal absorption of lindane in *in vitro* and *in vivo* studies.

Jung et al. 2003 carried out *in vitro* and *in vivo* percutaneous studies with catechol (see Sections 5 and 6). Total *in vitro* and *in vivo* skin penetration differed by a factor of 1.4. However, there were differences in conditions (e.g the dorsal skin temperature of fuzzy rat was 35°C and in the *in vitro* studies were carried out at 32°C. There were low recovery rates in the *in vivo* study compared to the *in vitro* (see Table 6.1).

Van de Sandt et al. (2000) pointed out that in most comparative studies up to then, *in vitro* and *in vivo* data were obtained under different experimental circumstances. Moreover, the comparison is based on the basis of the cumulative amount of the test substance excreted (*in vivo*) or reaching the receptor fluid (*in vitro*) providing only a partial insight into the more complex kinetic events of skin absorption. In their study on the pesticide propoxur, the experimental conditions were standardized with respect to dose, vehicle, and exposure duration. *In vivo* studies were performed in rats and human volunteers, while *in vitro* experiments were carried out in static diffusion cells using viable skin membranes (rat and human), non-viable epidermal membranes (rat and human), and a perfused pig ear-model. *In vivo* and *in vitro* absorption was compared on the basis of the absorbed dose after 4 and 24h, the maximum flux, the lag time, and the potential absorbed dose. In human volunteers, it was found that approximately 6% of the applied dose was excreted via the urine (as the metabolite 2-isopropoxyphenol) after 24h, while the potentially absorbed dose (amount applied minus amount washed off) was 23 µg/cm^2. In rats these values were 21% and 88% µg/cm^2 respectively. Data obtained *in vitro* were almost always higher than those obtained in human volunteers. The potentially absorbed dose seemed to give the best prediction of human *in vivo* percutaneous absorption. The absorbed dose and the maximal flux in viable full-thickness skin membranes corresponded well with the human *in vivo* situation (maximal overestimation x3); epidermal membranes overestimated the human *in vivo* data up to a factor of 8.

The same group carried out a second study (Cnubben et al., 2002) assessing the percutaneous penetration of the fungicide, *ortho*-phenylphenol, using different *in vitro* approaches: static diffusion cells with viable full-thickness skin membranes of rats and

humans, nonviable epidermal membranes of rats and humans and a perfused pig ear model and compared to respective rat *in vivo* and human volunteer data using standardised conditions.

In viable full-thickness skin membranes, the amount systemically available and the potentially absorbed dose correlated reasonably well with the human *in vivo* situation. In contrast, the *Kp*/maximal flux considerably underestimated the human *in vivo* situation. The results obtained with epidermal membranes overestimated human *in vivo* absorption data (Cnubben et al., 2002).

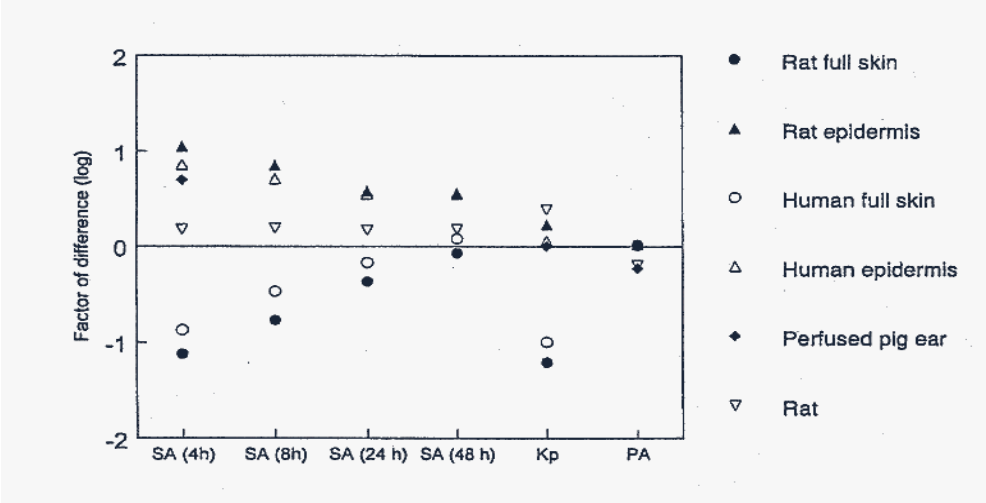

Figure 7.1 Factor of difference (FOD); compared to human volunteer data) between *in vitro* and *in vivo* skin absorption of ^{14}C- *ortho*-phenylphenol based on the systemically available amount at 4, 8, 24, and 48 h after a 4-h exposure period of 120 µg/cm^2, the permeability coefficient (*Kp*), and the potentially absorbed dose (PA) (Cnubben et al., 2002) [permission pending].

In the EDETOX project, for a range of chemicals of different physicochemical properties (2-butoxyethanol (aqueous solutions of different composition), trichloroethylene, m-xylene vapour, and caffeine), the percutaneous absorption was determined in parallel in human volunteers and *in vitro* with human skin using the same dose, vehicle and application time (Kezic, 2004). Furthermore, parallel *in vivo-in vitro* measurements were performed in rats for 2-butoxyethanol, pyrene and benzo(a)pyrene. In volunteer studies, dermal absorption was determined using two different methods, microdialysis and biomonitoring. The absorption flux determined for 50% aqueous

solution of 2-butoxyethanol of 1.3 mg/cm^2/h in rat was in good agreement with the flux obtained from volunteer experiments (0.9 to 2.4 mg/cm^2/h). Also the correlation of *in vivo* and *in vitro* data of 2-butoxyethanol in rat skin was good (2.0 and 1.3 mg/cm^2/h, respectively).

7.2 Intra- and interlaboratory variation in *in vitro* percutaneous absorption methodology

As *in vitro* measurements of skin absorption are an increasingly important aspect of regulatory studies, potential sources of inter- and intralaboratory variations have to be investigated. Much effort has been placed into the validation of different diffusion cells used to measure percutaneous penetration and it is generally accepted that most diffusion cell systems provide comparable measurements of skin absorption rates for a range of penetrants. It is well documented that there is a large (natural) variation in skin permeability (Chilcott et al., 2005). However, in addition to the known factors influencing percutaneous penetration (see Table 1.1) which have been discussed in other parts of this document, there is a likelihood of intra- and interlaboratory variation. There are a few studies which have investigated this.

The *in vitro* absorption of benzoic acid, caffeine, and testosterone – representing a range of different physico-chemical properties – through human skin (9 laboratories) and rat skin (1 laboratory) was determined (van de Sandt et al., 2004). All laboratories performed their studies according to detailed protocols (dose, exposure time, vehicle, receptor fluid, preparation of membranes, analysis) and each laboratory performed at least three independent experiments for each test chemical. The ranking of dermal penetration of all chemicals was the same for all participating laboratories. There was some variability between the results due to a large extent to inter-individual variability in absorption between samples of human skin and skin source. Skin thickness only slightly influenced the absorption of benzoic acid and caffeine, however, the maximum absorption rate of the most lipophilic compound, testosterone, was clearly higher in the laboratories using thin, dermatomed skin membranes (van de Sandt et al., 2004).

In an international multicenter study involving 18 laboratories, interlaboratory and intralaboratory variation in diffusion cell measurements was determined excluding the

known factor of skin variability (Chilcott et al., 2005). The study was performed using artificial rate limiting membranes (silicon rubber) membranes and the provision of materials including a standard penetrant, methyl paraben (MP), and a minimally prescriptive protocol. 'Standardized' calculations of MP flux were determined from the data submitted by each laboratory by applying a predefined mathematical model. The coefficient of variation between laboratories was approximately 35%. There was a fourfold difference between the lowest and highest average flux values and a six-fold difference between the lowest and highest individual flux values. Intralaboratory variation was lower averaging 10% for five individuals using the same equipment within a single laboratory.

Temperature variation in diffusion cells were identified as a potential factor contributing to inter-laboratory variation of dermal absorption (Romonchuk & Bunge, 2004).

In the EDETOX project, there were interlaboratory and intralaboratory studies e.g The comparison of 2-butoxyethanol data between the laboratories performing the *in vivo* experiments in volunteers showed good agreement; the apparent steady state flux differed by a factor of two (Kezic, 2004).

SECTION 8 DATA COLLECTIONS

Over the decades, a large amount of data has been generated on the percutaneous penetration of a wide range of chemicals, pesticides, cosmetics and pharmaceuticals. Studies have included work on human volunteers and *in vivo* studies using animal models (see Section 6) and *in vitro* studies on excised human, rodent, pig, guinea pig etc, and more recently synthetic skin (see Section 5). There has up till recently (OECD, 2004a,b,c) been no agreed standard procedure for measuring dermal absorption. There are numerous factors that can influence the dermal penetration values such as species variation, application site, dosing regime, occlusion, sex and age etc as well as interlaboratory and intralaboratory variations (see Table 1.1).

Many of the studies are unpublished being company or governmental property, however there are many studies in the open press. Until recently there were individual publications of datasets but they were of varying quality. Under auspices of the EDETOX project, a database has been compiled giving details of all of the evaluated studies on percutaneous penetration. This database is freely available on the EDETOX website. Therefore in the present document it was not attempted to mention every study, as these can be found easily in this EDETOX database, but to concentrate on the key studies in the respective areas.

8.1 Data sets

As well as studies on single compounds, several investigators determined permeability data from homologous or closely related series of molecules (see Table 7.1). These data have been used for modelling the skin permeability in these series (Idson, 1975, Wester & Maibach, 1985; Idson & Behl, 1987; Ridout & Guy, 1988; Ridout et al., 1992) in the compilation of subsequent databases. For an overview of datasets see also Vecchia & Bunge (2003a, c).

Table 8.1 Permeability data from some homologous or closely related series of molecules

n-alkanols	Scheuplein & Blank, 1971, 1973; Flynn & Yalkowsky, 1972; Wiechers, 1989
Ethyl ether, 2-butanone, 1-butanol, 2-ethoxyethanl, 2,3-butanediol	Blank et al., 1967
Steroids	Scheuplein et al., 1969; Feldman & Maibach, 1969
hydrocortisone-21 esters (and 5`-vidarabine esters	Flynn, 1985
Phenolic compounds	Roberts et al., 1977
Glycol ethers	Dugard et al., 1984
Polycyclic aromatic hydrocarbons (PAHs)	Sartorelli et al., 1998
Organophosphorous insecticides	Sartorelli et al., 1998
8 salicylates and 10 non-steroidal anti-inflammatory drugs in humans	Yano et al., 1986
Hair dyes	Bronaugh & Congdon, 1984
metals	Hostyneck, 2003
aromatic amines	Levillain et al., 1998
para-substituted phenols	Hinz et al., 1991

8.1.1 Flynn dataset

A milestone in the development of percutaneous absorption prediction was the publication of what is known to be the Flynn database, which is a publication of 97 permeability coefficients for 94 compounds *in vitro* through human skin and *in vivo* with toluene, ethyl benzene and styrene (Flynn, 1990) and was for over a decade the largest database of skin permeability values. However, this was a compilation of 15 different literature sources (some of these are given in Table 8.1) with the inherent disadvantage of having a high degree of variability due to interlaboratory and intralaboratory error as well a variation due to skin from different sources and location in the body (Moss et al., 2002). Based on this dataset Flynn proposed a number of algorithms to predict skin permeability (see Section 9 on QSARs).

8.1.2 Expanded datasets

For modelling studies, a number of datasets were compiled from various earlier publications. For example in the study by Wilschut et al. (1995), data on 123 measured permeation coefficients of 99 different chemicals; Vecchia & Bunge (2003a) presented a sizeable and diverse dataset of 170 measurements for 127 compounds covering MW 18 to 584 and log K_{ow} from -3.1 to 4.6); Patel et al. (2002) collected a comprehensive dataset containing 186 permeability coefficients for some 158 structurally diverse compounds (from human *in vitro* skin data (see aslso Section 9).

Further there are repositories of data kept by industry and regulatory bodies e.g. pesticide data sets with almost 300 dermal absorption studies of more than 160 different pesticides (Reddy & Bunge, 2002; CEFIC, 2004).

8.2 Databases

8.2.1 EDETOX database

The EDETOX database was generated for the EDETOX project, a multipartner EU project funded under the 5th Framework Program www.ncl.ac.uk/edetox; see also Section 12). The purpose of the database was to bring together *in vivo* and *in vitro* percutaneous absorption and distribution data from all available sources and procedures together with the physicochemical data for each chemical of interest (Soyei & Williams, 2004). There are two different versions, an MS Access application, and a web-based version (www.ncl.ac.uk/edetox/theedetoxdatabase.html). The database contains over 180 skin permeability data (including 21 new data).

Studies entering the database had to meet the EDETOX criteria. All of the following must be clearly stated:

- Chemical concentration (of chemical applied)
- Dose Volume (Volume of chemical applied to skin)
- Loading (Amount of chemical added per unit area)
- Area (Area of skin to which the chemical was applied)
- Vehicle (Application medium)
- Species (Species of animal used in the study)
- Exposure Time (Length of time the chemical was left on the skin)

- Analytical Method (Method by which the results were determined)
- Receptor Fluid (medium that bathes the underside of the skin)
- Temperature (temperature of the receptor fluid/skin/water bath during *in vitro* experiments)

Initially it was intended to only include papers that satisfied the EDETOX criteria. However, very few did. Therefore all papers were entered but those not fitting the criteria (well over 50%) were highlighted as such (personal communication, FM Williiams, 20th Dec, 2004).

The database is user friendly and data can be filtered in different ways e.g. sorted into the different methodologies, by chemical properties, by author of reference etc either as an overview or with a click into the details of the study itself. The data can be extracted in printed reports or exported to excel spreadsheets.

The EDETOX database is also intended as a basis for information that can be used for mathematical modelling e.g. to predict percutaneous penetration and has been used as the basis for some QSAR studies (see Section 9).

Since the end of the EDETOX Project there has been no funding and this data base is not being further supported.

SECTION 9 ESTIMATION/PREDICTION OF DERMAL PENETRATION

Recently there has been much interest in the possibilities available to predict dermal absorption and to avoid unnecessary and costly *in vitro* and *in vivo* testing. This is partly due to ethical difficulties with respect to human and animal experiments and partly due to economic and time considerations due to increasing legislation in the risk assessment of industrial chemicals e.g. proposed new European chemicals strategy: **R**egistration, **E**valuation, **A**uthorisation and Restriction of **Ch**emicals (REACH) (see also CEFIC, 2004).

Quantitative structure-activity relationships (**QSARs**) are generally used to relate properties of chemicals to biological effects or transport properties and is an observation of the association between an outcome and the properties likely to affect that outcome. A QSAR provides predictions of coefficients needed to estimate absorption for untested chemicals. It is not an expression of a theoretical relationship and is therefore complementary to mathematical models which express theoretical relationships. Mathematical models simulate the sequence of partition and transport processes involved in the absorption (see Section 3) and can predict the extent and rate of chemical permeation through the skin (Fitzpatrick et al., 2004). Mathematical modelling has a key role in linking the permeability coefficient obtained from tests under idealised, infinite dose conditions (i.e. steady state conditions) to those that will occur under the finite dose conditions more typical of occupational exposure (i.e. non-steady state conditions) (CEFIC, 2004).

9.1 QSARs (quantitative structure-permeability relationships QSPeR) analysis

QSARs, when applied to estimating dermal permeation are sometimes known as quantitative structure-permeability relationships (QSPRs or QSPeRs). Recent overviews are given by Moss et al., 2002; Vecchia & Bunge, 2003a,b, Geinoz et al., 2004; and Fitzpatrick et al., 2004.

Prerequisites for QSPeR analysis

Ideally the QSPeR must be related directly to the mechanism of action of dermal penetration which depends on the structure of the skin (See section 2). Considering the

passage of a chemical through the skin, the stratum corneum is essentially a lipidic layer, which interfaces with an aqueous medium beneath it. Therefore descriptors of hydrophobicity, molecular size and possibly hydrogen bonding (which may describe non-covalent interactions with skin proteins) are of importance for the development of QSARs (Moss et al., 2002).

QSPeRs are statistically derived linear relationships between the steady state permeability of a compounds and various physico-chemical descriptors and/or structural properties of the molecule. QSARs are involved at both these levels. Firstly, the octanol-water partition partition coefficient (K_{ow}) has been measured for some chemicals but determined for others from a QSAR, although probably quite well predicted. The skin permeability coefficient is predicted from Molecular Weight (MW) and K_{ow} by a second QSAR (CEFIC, 2004).

The statistical nature of such QSARs means that the more data used to derive a relationship, the more reliable it is likely to be for predictive purposes, assuming that the relationship is acceptable from a statistical point of view, and that the data itself is deemed to be sufficiently varied, and of satisfactory quality. For a QSPeR, the data (observed permeation) should be consistent, produced from standardised experimental procedures, and obtained for a set of chemicals that cover the domain of relevant chemical properties (CEFIC, 2004)

The measure of skin permeability that has been most widely used for QSPeR modelling is the permeability coefficient (*Kp*) because it characterises the intrinsic steady state properties of the chemical and the membrane. Although *Kp* is not directly suitable for application in risk assessment, it can be used in conjunction with measured (or estimated) solubility to predict a maximum flux through the skin (CEFIC)

The permeability (K_p) of a substance through the *Stratum corneum*

$K_p = K_m \cdot D/h$ [Equation 3 in section 3]

Where *h* is the thickness of the *S. corneum*, *D* is the permeant diffusivity in the membrane and K_m is the partition coefficient between the *S. corneum* and the vehicle. In calculations, K_m is often substituted for by the octanol-water partition coefficient, K_{ow}.(see Section 3).

Typically, such assessment is made following *in vitro* exposure of skin to the permeant. Ideally, to develop predictive models for skin penetration, all data should be

measured using the same protocol, with skin for the same animal (or human), probably also the same sex, from the same location in the body and measured in the same laboratory by the same workers. However, this has been up to now unrealistic though thoughts have been directed at such a study (CEFIC, 2004). However, QSPeRs up to now have been based of a heterogeneous data base of skin permeability values, which were measured for purposes other than for a QSAR, and therefore many QSPeRs are inherently subject to a substantial amount of error (Moss et al., 2002).

Historical Overview

QSPeRs for skin permeability prior to 1990s

The majority of these older studies were based on the analysis of homologous, or closely related, series of molecules and often only a relatively small number of compounds were assessed (see Section 8n). Many of these studies revealed a linear relationship with hydrophobicity where increasing hydrophobicity is associated with increasing skin permeation (where skin permeation may be described as flux or permeability coefficient, K_p) (Roberts et al., 1977; Scheuplein & Blank, 1971). Some studies also reported a parabolic relationship with hydrophobicity, particularly if there was a selection of compounds that included those that were highly hydrophobic (Scheuplein & Blank, 1971). However, each model existed in isolation for a particular class, or series of compounds. Further, as a consequence, there was co-linearity between the descriptors for a congeneric series e.g. hydrophobicity and molecular size so that it is not possible to discriminate the effect of these two factors on the permeability of large hydrophobic molecules (Moss et al., 2002). Reviews of modelling the skin permeability of homologous, or closely related compounds are given by Idson, 1975; Wester & Maibach, 1985; Idson & Behl, 1987; Ridout & Guy, 1988; Ridout et al., 1992).

The Flynn (1990) data set and subsequent analyses

The first large, and until recently the largest, global database of skin permeability values in a single species was that by Flynn (1990) of 97 permeability coefficients for

94 compounds for human skin, mostly *in vitro* experiments but a few *in vivo*. However, as this was a compilation from 15 different literature sources taken from 10 different research groups, these data have the disadvantage of inter-laboratory variation, from the skin from different sources and location on the body and for lack of a single protocol for skin permeability determination though there are few variations in the techniques employed (Moss et al., 2002). Flynn (1990) proposed a number of algorithms to predict skin permeability which stated that very hydrophilic and hydrophobic compounds had low and high skin permeability, respectively, and that different K_{ow}-dependent QSARs could be used to predict skin permeability for high and low molecular weight compounds. Potts and Guys (1992) demonstrated the use of log K_{ow} in combination with either molecular weight (MW) or molecular volume to predict the skin permeabilitiesy(units cm/s) collated by Flynn (1990).

$$\text{Log } K_p = 0.71 \log Kow - 0.0061\text{MW} - 6.3 \qquad \text{[Equation 4]}$$

$n = 93$ …$r^2 = 0.67$ …s not reported …F not reported
n… is the number of observations;
r …is the correlation coefficient;
s …is the standard error of the estimate
F …is Fisher's statistic

Potts and Guy (1992) did not perform a full statistical analysis on the data set. Though both descriptors used in the above equation are statistically significant, the statistical fit to Equation 4 is comparatively poor (Moss et al., 2002). Potts and Guy did observe that up to a 30% variability in the experimental data was to be expected, however they did not investigate the relationship for outliers or other statistical anomalies.

The publication of Flynn's large heterogeneous database was a significant milestone and was subsequently the basis for several analyses and publications (see Table 9.1 and detailed discussion in Moss et al., 2002).

A larger database of 114 skin permeability values was prepared by Kirchner et al., 1997 from the Flynn (1990) data set together with additional data from regulatory reports from Health Canada. This larger database was reanalysed by Cronin et al., 1999 who identified seven significant outliers (large compounds such as estriol, atropine, hydrocortisone, etorphine and digitoxin, as well as compounds with more than 10 sites

to accept or donate a hydrogen bond). Removal of these outlers and reanalysis against a wide variety of QSAR parmeters, including those for hydrogen bonding and other molecular properties, revealed the following equation which in spite of the larger data set is very similar to that proposed by Potts and Guy (1992)

$$\text{Log } K_p = 0.77 \log \text{Kow} - 0.0103\text{MW} - 2.33 \qquad \text{[Equation 5]}$$

$$n = 107 \ldots r^2 = 0.86 \ldots s = 0.39 \ldots F = 317$$

The problem of identifying and dealing with outliers is a controversial issue. From the various studies (Pugh & Hadgraft, 1994, Barratt, 1995 Cronin et al., 1999, some compounds are found consistently as outliers. Reinvestigation of some of the values e.g. of steroids (Johnson et al., 1995); naproxen, atrophine and nicotine (Degim et al., 1998), showed that at least some of the dataset of Flynn (1990) may have considerable error associated with them and justified the exclusion of these compounds e.g. by Cronin et al., 1999 to produce a statistically valid model. This issue, in particular concerning steroid permeabilities, is extensively discussed in Moss et al. (2002).

Patel et al. (2002) collected a comprehensive data set containing 186 permeability coefficients for some 158 structurally diverse compounds from human *in vitro* skin data from Flynn (1990) and Wilschut et al. (1995). They removed some compounds (atropine, diclofenac, naproxen, nicotine) which were considered as outliers from the dataset and developed a QSPeR that gave a value of $r^2 = 0.090$ for the remaining 143 compounds:

$$\text{Log } K_p = 0.652 \log Kow - 0.00603\text{MW} - 6.23\text{ABSQon} - 0.313\text{SsssCH} - 2.30 \qquad \text{[Equation 6]}$$

where ABSQon is the sum of absolute charges on oxygen and nitrogen atoms and SsssCH is the sum of E-state indices for all methyl groups.

The authors also fitted these data with a QSPeR of the general form:

$$\text{Log } K_p = a(\text{hydrophobicity}) - b(\text{molecular size}) + c \qquad \text{[Equation 7]}$$

Although calculated estimates of molecular vaolume have previously been shown to be better than molecular weight for the prediction of skin permeation (Barratt,

1995, Potts & Guy, 1995, Patel et al., 2002) found molecular weight to be a better predective model and has the advantage that it an easier descriptor to obtain and apply.

Fitzpatrick et al. (2004) reanalysed the same data set as that of (Patel et al., 2002) and discusses the variability of the database and points out that the partition coefficients (*Kow*) used to calculate permeability coefficients (*Kp*) in QSPeRs, in particular those from ionisable compounds, are determined using guidelines (e.g. OECD 117) which require pH adjustment to conditions that are outside the normal physiological range encountered in transdermal transport (see also O'Neill & Fitzpatrick, 2004). This topic of ionisable compounds is discussed further in Section 3.

Details of all the recent QSPeR models is beyond the scope of this document. Table 9.1 gives an overview of some of the studies and the source of data used. A more comprehensive listing is given in Vecchia & Bunge, 2003b.

Other data sets

A few studies have been performed on smaller data sets which were not a part of the Flynn (1990) data set. Several of these are based on congeneric series of compounds, typically drugs, and assesses their permeation rates As the emphasis of this document is on chemical risk assessment these smaller studies are not described in detail here.

Several studies have investigated the percutaneous absorption of polycyclic aromatic hydrocarbons (PAHs). That of van Rooij et al. (1995) was based on 10 PAHs. A more extensive study included 60 PAHs all of which were extremely hydrophobic (lowest K_{ow} being 4.00) (Roy et al., 1998). A negative correlation was found between the percentage of applied dose that penetrated the rat skin *in vitro* after 24h (PADA) and hydrophobicity. The data was reanalysed by Gute et al. (1999) who modelled molecular weight rather than K_{ow} against percutaneous absorption of PAHs but had difficulty showing a correlation due to the high hydrophobic selection of chemicals.

Table 9.1 A selection of recent QSPeR models illustrating sample size (*n*), correlation coefficient (r^2) and the source of the data used[a)]

Model	*n*	r^2	Experimental data source
Flynn (1990)	95	-	Flynn
Potts and Guy (1992)	93	0.67	Flynn
Lien and Gao (1995)	22	0.96	Flynn
Barrett (1995)	60	0.90	Flynn
Potts and Guy (1995)	37	0.94	Flynn
Abraham et al. (1995)	46	0.96	Flynn
Kirchner et al. (1997)	114	0.32	Flynn + Health Canada
Hostynek and Magee (1997)	20	0.80	Miscellaneous
Roy et al. (1998)	60	0.64	Roy et al.
Abraham et al. (1999)	53	0.96	Flynn
Gute et al. (1999)	60	0.67	Roy et al.
Cronin et al. (1999)	107	0.86	Flynn + Health Canada
Dearden et al. (2000)	91	0.83	Flynn
Patel et al. (2002)	143	0.90	Flynn, Wilschut et al.

a) from Fitzpatrick et al. (2004)

Other approaches to QSPeR

Hostynek & Magee (1997) suggested that for the human *in vivo* absorption of chemicals both the vehicle in which the material is delivered and the degree of occlusion of the device was important. Using data from various sources, a significant relationship was derived for the *in vivo* absorption data [penetration constant at maximum flux (Kpmax)] of 20 miscellaneous compounds with an indicator variable (VEH) for the different vehicles used (i.e. a value of 1 for acetone and 2 for ethanol).

Variability of data and its relevance for QSPeRs.

Taking the values for estradiol reviewed by Johnson et al., 1995 (excluding that from Scheuplein et al., 1969 which seems to be incorrect), there is a variability of about 25%

in permeability coefficients which due to the inherent variability of the tissue and interlaboratory differences (and temperature differences) is tolerated by those working in the field. But for deriving QSPeRs such variance is detrimental not just to the development of a robust and biologically relevant model, but also to its credibility and value in the question of predicitability of skin permeability for other compounds (Moss et al., 2002). Walters & Brain (2000) argue further that the nature of drug/vehicle interactions, solvent choice and other physicochemical factors are not considered by such models. Any conclusions can only relate to the flux of a penetrant from the same formulation system from which the model was derived. Permeability coefficients, being fluxes normalised by solution concentrations, will differ from one solvent to another, depending upon the solubility of the penetrant and the effect of the solvent on the skin.

Statistical analysis (linear vs non linear) methods

For many QSARs including QSPeRs, regression analysis is the statistical method of choice being simple, transparent and highly portable (Cronin & Schultz, 2001). However, there are a number of disadvantages to using this method: in particular, firstly, the linear technique and secondly, that it is adversely affected by collinearity between independent variables (e.g. log Pow and MW). It not clear whether regression analysis is a suitable technique for the development of QSPeRs; nor is it clear wheter linearity is appropriate for modelling of highly hydrophilic and hydrophobic molecules. Other possibilities would be partial least squares and neural networks.

Selection of chemicals for further tests on dermal penetration

The process of selection of chemicals should include a chemometric analysis to ensure that those compounds provide the maximum possible information. If the physico-chemical descriptors for the QSPeR are restricted to log Pow and MW, then selection of chemicals is easier than for a mutlivariant system. The current database must be critically analysed to decide how representative it is and what deficiencies it has (CEFIC, 2004). The CEFIC Working group noted that there may not be a linear relationship between permeability coefficient and hydrophobicity for the complete range of log Kow. Highly hydrophobic compounds may not be modelled well by a linear QSAR [see EC 2002 where a reduced default absorption is assumed for risk assessment if log K_{ow} is either <-1 or >4).

Applicability domain for QSPeR

The applicability domain for QSAR is defined as 'the physico-chemical, structural, or biological space, knowledge or information on which the training set of the model has been developed, and for which it is applicable to make predictions for new compounds' (Jaworska et al., 2003). At present, no formal methods exist to define such an applicability domain. However, it is accepted practice that a QSAR should not be used to make predictions outside of its applicability domain (Cronin & Schultz, 2003). If a global QSPeR can be based on log Pow and MW, then an applicability domain may be defined relatively easily and may be shown graphically on a 2-dimensional plot and will probably be elliptical in shape due to the paucity of low molecular weight molecules that are hydrophobic. The CEFIC Work Group recommended that the applicability domain should be defined for any QSPeR developed; and all predictions should only be for chemicals within the applicability domain (CEFIC, 2004)

9.2 Mathematical Modelling

There are several models which have been used for calculating skin permeation. The simplest treats the model as a single barrier with the permeation calculated from an equation of the same form as Fick's Law of Diffusion (see Section 3). Once the steady state conditions are reached, the cumulative amount absorbed increases linearly with time (see Figure 3.1) .

A more detailed model as used by Kruse and Kesic (2004) addresses the effects of differences in the solubility of the chemical in the different media (the water, the stratum corneum, and the epidermis). These differences are expressed as partition coefficients (the ratio of the concentration in one medium compared to that in the next medium at equilibrium) for the aqueous solution /stratum corneum boundary and for the stratum corneum /epidermis boundary. The model is based on Fick's Law of diffusion within each layer (and based on a diffusion coefficient for the chemical in each layer). The model has more parameters (to be fitted), but it describes the time course of permeation more thoroughly, and is in principle more appropriate to predicting the consequences of non-steady state doses. It has been fitted to data describing the time course of permeation of chemical into the skin, *in vitro.*

The rate limiting step of permeation is usually diffusion through the stratum corneum. However, for some compounds (highly lipophilic compounds), that have a much higher solubility in the skin than in water (e.g. by a factor of 1000), the diffusion within the aqueous solution towards the skin surface may become a rate limiting factor (see also Sec 3).

Mathematical modelling has a key role in linking the permeability coefficient obtained from tests under idealised, infinite dose conditions (i.e. steady state conditions) to those that will occur under the finite dose conditions more typical of occupational exposure (i.e. non-steady state conditions).

Mathematical pharmacokinetic models of percutaneous penetration

Recent reviews on mathematical pharmacokinetic models of percutaneous penetration are given by Roberts et al. (1999) and McCarley & Bunge (1998a, b; 2001). In such models the algebraic equations that accurately represent the partition processes and diffusion migration of a molecule through the different layers of the *S. corneum*, are written down and solved within certain chosen simplifying assumptions (Fitzpatrick et al., 2004). Such a set of equations have been solved analytically for both steady-and non-steady-state transport through a two-membrane composite representing the lipophilic s.coreum and the hydrophilic viable epidermis layers (Cleek & Bunge, 1993). Another model has been developed by Kruse & Verberk (2001) in which the skin is treated as a two-layer membrane with the possibility for a parallel route to circumvent this barrier. After its passage through the skin the clearance of the diffusant by blood perfusion can also be simulated. This model is very versatile and can be used for vapours, liquid solutions and solids in contact with the skin with the appropriate differential equations being numerically integrated using the ACSL-advanced continuous simulation language (www.aclsim.com) software package. There is considerable current interest in occupational risk assessment and there is a need to make reliable estimates of the risks ensuing from dermal exposure (e.g. van Hemmen et al., 2003; Marquart et al., 2003).

Estimates of dermal absorption are used in exposure assessment to calculate the internal dose of persons contacting e.g. pesticides and are a critical part of risk assessments. An exponential saturation model with lag time was validated against a

classic dermal absorption study of 12 pesticides administered to human volunteers. The model gave dermal absorption estimates consistent with reported values in the literature (Thongsinthusak et al., 1999).

In occupational exposure to toxic chemicals, dermal absorption may be in multiple short-term exposures as well as by long-term exposures. Corish et al. (2004) describe the application of two diffusion type numerical models of percutaneous penetration to non-steady state time course data generated by participants in the EDETOX project. The first is a new numerical model developed by J Krüse (Krüse & Kezic, 2004 and implemented using the Berkeley Madonna package (http://www.berkeleymadonna.com). The second is an implementation using Mathematica (http://www.wolfram.com) of an existing pair of models developed by Anissimov & Roberts, 1999; 2000; 2001 (see also Roberts et al., 1999). Both models allow finite and 'infinite' doses to be modelled.

SECTION 10 USE OF DERMAL PENETRATION STUDIES IN RISK ASSESSMENT

10.1 Introduction

Dermal absorption studies are complex kinetic studies which themselves provide no information on the toxicity of a compound. Dermal absorption studies may be required for compounds having a serious toxic effect identified by oral or inhalation studies and for which a significant route of human exposure is dermal [and for which the assumption of 100 percent dermal absorption does not produce an adequate margin of exposure (MOE)]. Here a risk assessment must be performed to determine the need of a dermal absorption study. Dermal absorption studies cannot replace general dermal toxicity studies (US EPA, 1998).

Risk Assessment procedures (for chemicals and pesticides) consist of 4 steps

1) Hazard identification
2) Dose-response assessment (and determination of the NOAEL if possible)
3) Exposure assessment and
4) Risk characterisation

The risk characterisation is a comparison of the exposure level with the NOAEL (EHC 170). For a risk assessment of dermal uptake, percutaneous absorption data, contamination levels, and toxicokinetics of occupational toxicants would be required (Benford et al., 1999).

When no literature is available, a value of 100% (worst-case scenario) is assumed for the dermal absorption for the hazard assessment process. A less conservative approach could be used when penetration rates through human skin are known or can be estimated.

10.2 The decision-making processes for setting absorption percentages

10.2.1 Dermal absorption based on default values

Estimates of dermal absorption can be made by considering data on physico-chemical properties of the substance (molecular weight (MW), log P_{ow}). If an initial assessment ends up with a prediction of risk, information on actual exposure conditions can be used to refine the conditions under which the dermal absorption studies are conducted (EC, 2003).

10.2.1.1 Default values

However, at least theoretically, it would be expected that there should be an optimum in log P_{ow} and a maximum in MW for facilitating percutaneous absorption. Criteria to distinguish between chemicals with high and low dermal absorption have fbeen proposed (De Heer, 1999). Assume

- **10% dermal absorption** for those chemicals with a MW> 500 and log P_{ow} smaller than -1 or higher than 4,
- otherwise assume **100% dermal absorption**.

The reason for assuming 10% as the lower limit was that the data presented in the literature indicated the occurrence of dermal absorption for tested compounds even beyond the extremes of log P_{ow} and/or MW values (De Heer, 1999; EC, 2004).

However, by expert judgement, a deviation from these 10% or 100% values can be chosen on a case by case basis taking into account all the data available (e.g. water solubility, ionogenic state, 'molecular volume', oral absorption/ADME and dermal area dose in exposure situations in practice).

10.2.1.2 Use of mathematical skin permeation models (e.g. QSARs/QSPeRs)

The use of mathematical skin permeation models for quantitative risk assessment purposes is limited by the fact that they have generally been validated by *in vitro* data from various sources ignoring the fate of the skin residue levels (OECD, 2000; EC,

2004). However the use of QSARs may prove to be a useful screening tool and may prove useful within a group of closely related substances (EC, 2003; CEFIC, 2004) for prediction purposes.

10.2.2 Dermal absorption based on in vitro human and rat studies

In vitro studies are increasingly being submitted for registration purposes. There are several studies which compare *in vitro* and *in vivo* results (see section 7), and *in vitro* studies have provided good prediction for *in vivo* dermal absorption. However there is still controversy over the way in which *in vitro* data could be used in risk assessment (EC, 2003). Evaluation of available data on *in vitro* dermal absorption was performed under auspices of the OECD (OECD, 2000). The available studies, comparing *in vitro* and *in vivo* data contained too many variables (e.g. different species, thickness and types of skin, exposure duration, vehicles; see Table 1.1). There is now an OECD Guideline 427 (OECD, 2004b) to encourage harmonization of methodology.

Water-soluble substances can be tested more accurately *in vitro* because they more readily diffuse into the receptor fluid (OECD, 2000, 2004a).

A major issue of concern was the presence of test substance in the various skin layers i.e. absorbed into the skin but not passed into the receptor fluid. By including the amount retained in the skin *in vitro*, a more acceptable estimation of skin absorption is obtained. At present, provided that skin levels are included as absorbed, results from *in vitro* methods seem to adequately reflect those from *in vivo* experiments supporting their use as a replacement test to measure percutaneous absorption. This gives a conservative estimate. However, if it can be shown that the skin dose does not become absorbed at a later stage, refinement can be made (EC, 2004).

It is desirable that studies reflect the anticipated exposure situation. If this is not the case, the validity of the *in vitro* data may be questioned.

Preferably human *in vitro* dermal absorption data should be carried out. However if such data is not available, rat *in vitro* studies can be used. This leads to a more conservative approach as usually absorption by human skin is lower than that in rat skin (EC, 2004).

10.2.3 Dermal absorption based on in vivo data

There is now an OECD Guideline 428 (OECD, 2004c) to encourage harmonization of methodology. The skin bound dose is also critical for the calculation of the percentage dermal absorption in *in vivo* studies (Chu et al., 1996; EC, 2004). The decision about the quantity that remains bound in the skin can be based on the excretion curve – a decline of radioactivity in the excreta at the end of the experiment indicates that the dose at the dosed skin site may not become completely systemically available (Thonginthusak et al., 1999, De Heer et al., 1999).

As with *in vitro* studies, experimental absorption percentages used in risk assessment should be determined on in use preparation (EC, 2004).

When only rat *in vivo* dermal absorption studies are available, the most conservative approach would be to assume that human skin absorption would be equal to rat *in vivo* dermal absorption (EC, 2004).

The most reliable data for determining absorption through human skin are obtained from *in vivo* human volunteer studies performed under occupationally relevant test conditions (Ross et al., 2005). For technical and ethical reasons the conduct of these studies is limited and closely regulated.

10.2.4 Dermal absorption based on in vivo rat studies in combination with in vitro data

If appropriate dermal absorption data are available for rats *in vivo* and for rat and human skin *in vitro*, the *in vivo* human can be given by adjusting the *in vivo* rat absorption in relation to the comparison of rat and human *in vitro* studies (van Ravenzwaay & Leibold, 2004; EC, 2004).

$$\textit{In vivo}\ \text{human absorption} = \textit{in vivo}\ \text{rat absorption} \times \frac{\textit{in vitro}\ \text{human absorption}}{\textit{in vitro}\ \text{rat absorption}}$$

10.2.5 A tiered approach to risk assessment for chemical exposure, using default dermal absorption percentage or dermal absorption percentage determined experimentally.

A value for dermal absorption may be obtained by the use of a tiered approach from a worst case to a more refined estimate (De Heer, 1999; EC, 2004). In the first step, 100% can be assumed if no data is available. In the second step, consideration of physicochemical properties could lead to an estimate of dermal absorption. The third step is consideration of any experimental *in vitro* and *in vivo* dermal absorption data. If at the end of these steps an unacceptable risk is calculated, the risk assessment could be refined by means of actual exposure data (EC, 2004).

SECTION 11 INITIATIVES PAST AND PRESENT

In the last few years there have been several initiatives to accelerate progress in the fields of international harmonization of methodology and protocols culminating in the publication of the OECD Guidelines in 2004 (OECD, 2004 a,b,c; see Appendix 1). There have been initiatives from the regulatory side in US (US EPA, 1999; 2004) and in Europe. In Europe there have been two large projects, the Dermal Exposure Network (1997 -1999) leading on to the EDETOX project. From the industrial side the European Chemical Industry Council (CEFIC) has supported two projects associated with QSARs (quantitative structure-activity relationships) linking 1) physicochemical properties to permeation data 2) methods to determine dermal absorption for human risk assessment, for so that in the future it may be possible to predict the data for a large number of chemicals rather than undertake expensive testing of chemicals. Here an overview is given of these projects, the results of which are also cited in other parts of the document.

11.1 OECD Guidelines

The most important recent international initiative was the effort to harmonise the protocols for *in vitro* and *in vivo* testing (see also Sections 5, 6 and Appendix 1). The need for a Guidance document became apparent in 1997 when national experts from a number of member countries could not agree on the possibilities and limitations of the invitro test. The draft outlines were presented in 2000 and the final guidelines were published in 2004 (OECD, 2004a,b,c). The Guidance document (OECD, 2004a) provides additional technical background to both the *in vivo* and *in vitro* methods for skin absorption as described in Test Guidelines 427 (OECD, 2004b) and 428 (OECD, 2004c) respectively.

11.2 Dermal Exposure Network (1997-1999)

At an international meeting of experts from European countries and US in June, 1994, it was decided to form an European network on dermal exposure to encourage development of projects in the area and pool research capabilities, to harmonize techniques, expertise and knowledge and to guide the European Commission in developing a comprehensive and harmonized risk assessment strategy in the workplace. Meetings of the Dermal Exposure Network of EC (DEN) started in 1997 and five

subgroups were organized: Risk Assessment, Biological Monitoring, Percutaneous Penetration, Skin and Surface Contamination, Contribution of different Sources.

The work of ***Percutaneous Penetration Subgroup*** focussed on the standardization and validation of *in vitro* experiments necessary to obtain internationally accepted penetration rates for regulatory purposes. The key items discussed were:

- The use of PP data in risk assessment
- Ín *in vitro* studies the factors influencing
    - the choice of cell characteristics,
    - the choice of donor phase,
    - receptor fluids
- The presentation of *in vitro* PP results
- Existing guidelines on PP *in vitro* studies
- Prediction of plasma levels from penetration data
- The influence of cutaneous metabolism on skin absorption
- Criteria for the selection of reference compounds for *in vitro* PP
- The use of microdialysis for the determination of PP of hazardous substances *in vivo*
- Correlation between *in vitro* and *in vivo* experiments

The members of the Subgroup analyzed the guidelines on percutaneous penetration (PP) *in vitro* studies presented by various organisations and suggested a standardization of *in vitro* models for PP taking into account their individual experiences, literature data and guidelines already in existence. The Subgroup also presented a number of short papers of up to date information on the key issues to focus the existing knowledge and gaps in the knowledge in the field of percutaneous penetration. The publication (Sartorelli et al., 2000) is an outcome of work of this subgroup during the Dermal Exposure Network Project (1997-1999).

11.3 EDETOX. Evaluations and predictions of DErmal absorption of TOXic chemicals

EDETOX was a 3-year multipartner EU project (2001-2003/4) funded under the 5th Framework program (www.ncl.ac.uk/edetox) to generate new data on dermal absorption of chemicals. The consortium comprised 12 participants from seven EU member states. EDETOX member laboratories were all participants in the Percutaneous Penetration Sub-group of the Dermal Exposure Network (1996-1999)(see Section 666 and Sartorelli et al., 2000). The aims of the EDETOX project were to create new data and know-how that would standardize *in vitro* systems for predicting percutaneous penetration and compare these with relevant *in vivo* studies (Williams, 2004a, b). The available literature data was assessed as to whether it fitted certain criteria (see Section 7) and from these data, together with data generated during the EDETOX project, a databank was produced (EDETOX database) which was used for Quantitative Structure Activity Relationship (QSAR) simulations. A further aim was to develop strategies for predictive testing and the evaluation of dermal toxicity that would reduce the need for animal testing. The results of the EDETOX project were presented at the 2004 PPP conference in the form of presentations and posters and subsequent publications (Perspectives in Percutaneous Penetration, 2004 and below). The full report of the EDETOX project is not yet available.

Intra- and interlaboratory variation in in vitro percutaneous absorption methodology

One large part of the EDETOX project was a study into the **intra- and interlaboratory variation** in *in vitro* percutaneous absorption methodology (Van de Sandt, 2004; van de Sandt et al., 2004). The *in vitro* absorption of benzoic acid, caffeine and testosterone were tested according to detailed protocols using human skin (9 laboratories) and rat skin (1 laboratory). There was some variability between the results due to a large extent to inter-individual variability in absorption between samples of human skin. The results are discussed in Section 7.

Effects of experimental conditions on percutaneous absorption

Further studies were presented on particular aspects of optimisation of experimental conditions e.g using caffeine, testosterone, butoxyethanol and propoxur, the **influence of skin thickness** on percutaneous penetration *in vitro* was further investigated (Wilkinson et al., 2004). It was found that the relationship between skin thickness and physicochemical properties is complex and has implications for risk assessment and for the validation of mathematical models (see Section 5 & 12).

In vivo and *in vitro* absorption data were generated with rodent and human skin, for five pesticides of varying lipophilicity in a range of formulations to investigate whether the **amount recovered from the stratum corneum** should be included or excluded from risk estimations. Inclusion of the material remaining in the SC leads to an overprediction of the level of absorption by comparison to the currently recommended *in vivo*/*in vitro* calculation. However, this is considered to be more acceptable from a risk assessment perspective (O'Connor & Cage, 2004a, b) (see Section 5 &12).

Human in vivo studies of dermal penetration

Determination of the percutaneous absorption of polyethylene glycols of different MW by **tape stripping in volunteers**. A marked difference was seen between intact and impaired skin (Jakasa et al., 2004a; see Section 6).

Human in vivo studies of dermal penetration: their relation to in vitro prediction

In the EDETOX project, *in vivo* studies have served as the golden standard to evaluate *in vitro* results generated using standardised protocols. For a range of chemicals of different physicochemical properties (2-butoxyethanol (aqueous solutions of different composition), trichloroethylene, m-xylene vapour, and caffeine), the percutaneous absorption was determined in parallel in human volunteers and *in vitro* with human skin using the same dose, vehicle and application time (Kezic, 2004. Furthermore, parallel *in vivo-in vitro* measurements were performed in rats for 2-butoxyethanol, pyrene and benzo(a)pyrene. In volunteer studies, dermal absorption was determined using two different methods, microdialysis and biomonitoring. The

comparison of 2-butoxyethanol data **between the laboratories** performing the *in vivo* experiments in volunteers showed good agreement; the apparent steady state flux differed by a factor of two. The correlation between ***in vivo* and *in vitro* data** was also good (see Section 5,6 & 7).

Percutaneous penetration of polyethylene glycols of different molecular weights were determined in volunteers by tape stripping (Jakasa et al., 2004a). The amount penetrated was significantly higher in impaired skin. In another study, the percutaneous absorption of the 2-butoxyethanol from aqueous solution increased markedly when compared with neat 2-butoxyethanol (Jakasa et al., 2004b).

New techniques

The **microdialysis technique** is mostly used *in vivo* e.g. 2-butoxyethanol by microdialysis in volunteers (Korinth et al., 2004). The technique has been modified for *in vitro* tests e.g. studying the absorption of 3 low molecular (radiolabelled) test compounds (toluene, 50% aqueous butoxyethanol (2-BE) amd propoxur using glass diffusion cells (Franz cells) (Maas et al., 2004) see Sections 5 and 6). In a further experiment, an infinite dose of non-radiolabelled pyrene in ethanol was investigated using *in vitro* microdialysis (Wellner & Korinth, 2004).

In vitro percutaneous absorption of metals

Studies into the *in vitro* percutaneous absorption of metal powders [Ni, Co and Cr] (Venier et al., 2004) showed that metallic ions can easily permeate the skin and using the Franz cell it was possible to measure a flux of ions through the skin for Co and Ni but not for Cr [Filon et al., 2004]. A similar study was described by Sartorelli et al., (2004)).

Percutaneous penetration of pesticides

In an *in vitro* percutaneous penetration study of five pesticides covering a range of solubilities and MW it was shown that MW as well as solubility affects dermal penetration. After short-term occupational exposures, an exposure assessment based on

penetrated pesticide at the end of the work shift may underestimate the exposure. Suggest that data used by the regulators agencies should include maximal flux (or *Kp*), lag-time as well as an estimation of the potential importance of the skin reservoir (Nielsen et al., 2004a,b).

EDETOX Database

Under auspices of the EDETOX project, a database has been compiled giving details of all of the evaluated studies on percutaneous penetration. This database is freely available on the EDETOX website. The purpose of the database was to bring together *in vivo* and *in vitro* percutaneous absorption and distribution data from all available sources and procedures together with the physicochemical data for each chemical of interest (Soyei & Williams, 2004). There are two different versions, an MS Access application, and a web-based version (www.ncl.ac.uk/edetox/theedetoxdatabase.html) (Soyei & Williams, 2004) see also section 7.

QSARs for percutaneous penetration

Another aspect of the EDETOX Project was the evaluation and development of statistical Quantitative Structure-Activity Relationships (QSARs) for percutaneous penetration using e.g data from the EDETOX database (Golden et al., 2004).

Further Modelling of dermal absorption data

Another approach has been the modelling of the macroscopic behaviour of the absorption process using diffusion equations or compartmental models that represent the vehicle, the sink and the various layers of the skin (Corish, 2004). A comparative analysis of non-steady state data using two diffusion models was presented (Corish et al., 2004). Further, a mechanistically based mathematical model (Krüse & Kezic, 2004)

Skin Notations

At present there are large discrepancies between countries giving skin notations (Nielsen et al., 2004c). A skin notation should relate to the potential for toxicity following relevant dermal exposure and may include the degree of toxicity and the dermal penetration rate. It should refer to the product and not to the chemical itself because, as shown by the results of the EDETOX project, butoxyethanol has very different penetration characteristics depending upon whether it was in an aqueous solution or not. The risk assessment which includes an assessment of the exposure and will vary over time as well as between scenarios.

11.4 European Chemical Industry Council (CEFIC) Initiatives

11.4.1 Determination of the optimal physico-chemical parameters to use in a QSAR-approach to predict skin permeation rate

The main results of this study were published in the open press (Patel & Cronin, 2001; Patel et al. 2002a; Moss et al., 2002; Patel et al. 2002b) and have been discussed in Sec. 9 of this document.

11.4 2 CEFIC Workshop on methods to determine dermal permeation for human risk assessment (Utrecht 13-15th June 2004) (taken from Report to CEFIC; Jones et al., 2004)

Introduction

The proposed new European chemicals strategy (REACH) involving the **R**egistration, **E**valuation, **A**uthorisation and Restriction of **Ch**emicals is likely to involve 10,000 industrial chemicals and many more mixtures. Data on the potential for dermal uptake is only available for a small number of these chemicals and these were often obtained not using a standardized protocol. Obtaining human *in vivo* or *in vitro* data on all (REACH) chemicals is not feasible. The European Chemical Industry (through CEFIC [European Chemical Industry Council]) recognised the need to

establish a systematic tiered approach to predict dermal permeation of chemicals for risk assessment.

With this in mind, a CEFIC-LRI sponsored meeting was convened to reach a consensus on methods to determine dermal absorption *in vitro* and it was intended that data produced from a limited number of these studies would be used to develop a QSAR (quantitative structure-activity relationship) linking physicochemical properties to permeation data so that ideally it would be possible to predict the data for a large number of chemicals rather than undertake expensive testing.

Approximately 20 experts in skin permeation, risk assessment and QSAR were invited from academia, contract testing laboratories, industry, regulatory agencies, EC. At the meeting, presentations and discussions spanned the assessment of dermal exposure, permeation measurements in the laboratory, and the application of permeation data to risk assessment and brought together the views from two preceeding conferences: PPP (Predicting Percutaneous Penetration) Conference, April 2004 and a QSAR, 2004 meeting.

Previously there had been concerns that the QSAR predictions that had previously been made might be unreliable as that has been based on *in vitro* tests conducted using a variety of methods over more than a decade. However, discussions at the meeting led to the conclusion that the existing databases for (*Kp*), despite some inherent variability due to methodological differences, were acceptable for derivation of the existing QSARs. Further, it was not expected that further data would produce any significant revision of the QSARs. However, it was recognised that the existing database was primarily from chemicals from specialised sectors or selected on the basis of physicochemical properties and the meeting recommended that generation of *in vitro* data and *Kp* on a range of relevant industrial chemicals would be valuable in reassuring all stakeholders of the validity and relevance of QSARs within the broad application area of REACH. Chemicals should be selected as being of greatest relevance to high volume production chemical manufacture and should provide a good coverage of the range of physicochemical properties needed to produce data that will support the development of QSAR models.

Further to this was the discussion of the relevance of infinite dose studies to realistic risk assessment scenarios which usually correspond to finite dose conditions. Data used for QSARs have up to now been those from infinite *in vitro* assays which are

most suitable for establishing a stable maximum flux and calculating a permeability coefficient (*Kp*), and which can be related by QSARs to physicochemical properties.

Therefore a link must be established between finite and infinite dose experiments, thus linking the QSAR-derived information with the inputs required for risk assessment. This link relies on mathematical modelling which requires that a sound theoretical basis is used in the interpretation of data from each experiment and should improve the reliability od parameters calculated from experimental data. The model also enable extrapolation to predict absorption under different dosing conditions.

Proposed Finite and Infinite Dose Protocols (Appendix 2 and 3 of the CEFIC Protocol)

These protocols have been suggested as the most effective and timely way of obtaining data robust enough to construct an QSAR model that could be acceptable to all interest groups. [It should be discussed at the Task Group Meeting if these should be in the final document]; further whether this project is in progress].

Calculation of the steady state permeability coefficient (Kp)

The conditions of the infinite assay allow the permeation rate to reach a steady state (see Section 3).

Relating Finite dose and Infinite dose results

Mathematical models can use the permeability coefficient (determined with an infinite dose and in an *in vitro* test see Sec and Figure 1) to calculate the flux and the dose received in a finite dose situation. Krüse's model can also use finite dose test data to obtain values for *Kp*. Using this numerical model implemented using the Berkley Madonna Package, Krüse analysed data from the EDETOX project (Wilkinson et al. 2004) using two different weightings (Krüse & Kezic, 2004; Golden et al., 2004). Although only a limited number of substances have been modelled so far, the model seems to be able to link finite and infinite experiments. If relaiable parameters have been derived for a substance, Krüse's model allows for the modelling of a range of pertinent absorption regimes including those relevant to real exposure scenarios such as finite doses at multiple exposures.

The analysis of Frasch & Barbero, 2003 was also mentioned at the meeting. This models the diffusion through a more complex and realistic representation of the strateum corneum structure.

In a further contribution, data for a range of pesticides were presented which showed how predictions of absorption (based on QSAR predicted *Kp*) compare with systemic absorption *in vivo* measured in rats (Zendzian, 2000; Reddy & Bunge, 2002;). Such data could be used to predict a safety factor such that the safety factor times the predicted systemic absorption would encompass the majority of the set of measured values for the *in vivo* systemic absoption.

Chemicals to be selected for testing

The following recommendations were made to CEFIC by the meeting members for the selection of chemicals for developing the acceptance of QSARs as a valid and reliable method of predicting the permeability for industrial chemicals:

- span the physical chemical space of the parameters log $P_{o\text{-}w}$ from -3 to 7 and molecular weight from 30 to 1000 (an elliptical space on a two dimensional plot of chemicals by these two parameters);
- be chosen from chemicals which are produced in high volume in industry;
- be chosen in conjunction with industry;
- if possible, be radiolabelled versions of the production chemicals (by selecting chemicals for which radioactive versions are available);
- if some non-radiolabelled chemicals are used (to obtain the range of physical/chemical characteristics), then the chosen chemical needs to be suitable for sensitive chemical analysis.

Strategy Recommendations

The CEFIC workshop recommended a two-stage strategy:

Stage 1 Build on an already extensive data set for aqueous soluble chemicals and the QSARs that have been made for these chemicals

1) To promote the recognition of the likely reliability of QSAR predictions of dermal permeation for industrial chemicals. The CEFIC workshop suggested a project measuring the permeation of a selection of about 50 chemicals using the proposed *infinite* dose protocol.

2) To establish the validity of using the mathematical models to extrapolate from infinite dose to finite dose. The model should be used to extrapolate from infinite dose to predict the results of a finite dose experiment before the finite dose experiment results are available.

It is important, especially for QSAR, that variability should be minimised. Recent studies concerning robustness and variability (van de Sandt, 2004; van de Sandt et al., 2004; Chilcott et al., 2005) have highlighted the problems that still exist in the field of *in vitro* dermal absorption measurements.

The CEFIC Workshop recommended that the first stage would use a wide range of chemicals with the standard aqueous solution protocol. The data from this project would be directly comparable with that of the existing data set already used for the development of QSARs and could be extrapolated using mathematical models for comparisons with finite dose data.

Stage 2 of the CEFIC Workshop recommendations involve more detailed testing of those chemicals in subset 1 together with more lipophilic chemicals that cannot be tested in the aqueous solution protocol. Further, there would be investigations into the effects of various donor fluids, receptor fluids, and mixtures.

The aim is to facilitate the development of models bridging the results from Stage 1 (standard aqueous solution test conditions) to Stage 2 (more complex and more realistic exposure conditions for an extended range of chemicals). A further objective would be a standardised protocol for chemicals in solution in other liquids.

Recommendations of CEFIC Workshop for use of their proposed new data in risk assessments.

- Assume either 100% absorption, or 10% default assumption for high molecular weight and log Pow <-1 or >4; and then, if necessary,
- Use saturated water concentration and *Kp* to calculate an estimate of maximum flux, allowing for any effects from the vehicle; then if necessary,

- Use the more complete mathematical model with diffusion coefficients and partition coefficients to obtain a best estimate of the flux and dose for the likely occupational exposure concentration (i.e. finite dose)

Dermal exposure assessment

Future developments in determining dermal permeation should be conducted in close liaison with developments in dermal exposure assessment (Semple et al., 2004; Kromhout et al, 2004).

SECTION 12 TOPICS UNDER DISCUSSION

Discussion points for the IPCS EHC Task Group that will be convened to finalize this document: Problems that still have not been addressed or which are still under discussion.

This section is rudimentary as it intended to be discussion topics for the Task Group. Some points are described at length, others are suggestions for topics to be discussed. It is intended to encourage reviewers to add suggestions of difficulties of which the authors of the document may not be aware.

12.1 OECD Guidelines

What is the applicability domain for these guidelines? (e.g. are there circumstances where deviation may be indicated?) Will use of the guidelines lead to a database of results from which reliable QSPeRs can be made?

12.2 Variation in experiments conditions

Intralaboratory variation / Interlaboratory variation. How can this be reduced?

There is a still a debate going on about how *in vitro* data could or should be used in risk assessment. An evaluation of available data on *in vitro* dermal absorption was performed under the auspices of the OECD (OECD, 2000d in EC, 2004). Because the available studies, comparing the *in vitro* and *in vivo* test results contained too many variables (different species, thickness and types of skin, exposure duration, vehicles, etc) evaluation/consensus is difficult. Table 1.1 lists some of these points and refers the reader to the discussion in the respective sections. Some of these topics have been addressed in recent projects e.g. EDETOX (see Section 12) and publications therefrom.

e.g. Skin thickness -Is dermatomed skin or full skin thickness more appropriate for dermal absorption measurements (e.g. Cnubben et al., 2002; Wilkinson et al., 2004).

Problems with ***ionic species /ionisation***

12.3 Reservoir effects and risk assessment

Reservoir effects

It has long been understood that a substance, instead of passing entirely through the skin, can remain partly in the skin and can act as a reservoir, being released (or not) at a later time (Vickers, 1972; Roberts et al., 2004). This effect has been used in the topical application of medications e.g. salicylic acid was found to be excreted in the urine more slowly when applied topically than when injected intradermally (Guillot, 1954). This is applicable for the more slowly diffusing drugs, i.e. those with long lag times. The reservoir function of the skin can also act as a depot for drugs (or chemicals). The release of the substance can be rapid on appropriate provocation of the skin some time later e.g. this has been shown with steroids where an occlusive dressing was applied to the original steroid application site several weeks after the original application, or alternatively using chemical enhancement (Roberts et al., 2004). The duration of the reservoir depends on the nature of the drug (or chemical) the vehicle used, the temperature of the skin and the relative humidity to which the skin is exposed (Vickers, 1972).

Although most studies have emphasized the stratum corneum as a reservoir, viable epidermis, dermis and underlying tissues may themselves act as reservoirs (Roberts et al., 2004).

Reservoir effects are well-documented for steroids (Miselnicky et al., 1988) e.g. the water insoluble fragrance musk xylol showed rapid and significant diffusion from the skin within 72h after applying the dermal dose (Hood et al., 1996). However, the formation of a skin reservoir for a chemical during percutaneous absorption is not limited to lipophilic chemicals but also applies to polar and nonpolar chemicals that bind to the skin during diffusion through the skin (Yourick et al., 2004). Nicotine, caffeine, cationic ß-blocking drugs, surfactants and testosterone, malathione, hairdyes and vitamin E, have all been reported as forming a skin reservoir, glycolic and lactic acids (Roberts et al., 2004). Amounts of phenanthrene, BaP and di(2-ethylhexyl)phthalate, remaining in hairless guinea pig skin after 24h eventually became available for systemic absorption (Chu et al., 1996). The catechol reservoir formed in skin during a 24h study with catechol (having good water and liposolubility) did not

decrease (*in vivo*) or only partially decreased (*in vitro*) in a 72-h extended study (Jung et al., 2003).

The potential for a chemical to form a skin reservoir can be at least partially predicted by the extent of protein binding, rate of penetration through skin, and the chemical's solubility properties (Miselnicky et al., 1988).

Consequences of reservoir effect for risk assessment

A major issue of concern was the presence of test substance in the various skin layers i.e. absorbed into the skin but not passed into the receptor fluid. In particular, very lipophilic compounds are difficult to investigate *in vitro* due to their low solubility in most receptor fluids. If the amount retained in the skin is also counted as being absorbed, a more acceptable but conservative estimate can be made. Water soluble substances can be tested more accurately *in vitro* because they more readily diffuse into the receptor fluid. If skin levels are included in the overall percentage absorption figure, results from *in vitro* methods seem to adequately reflect those from *in vivo* experiments and support their use as a replacement of *in vivo* testing (EC, 2003).

Different approaches are taken by different bodies. ECETOC (1993) base their measurements of percutaneous absorption on receptor fluids only. In the cosmetic guidelines issued by COLIPA (European Cosmetic Toiletry and Perfumery Association) (Diembeck et al., 1999) and Scientific Committee on Cosmetic Products and Non-food products intended for Consumers (SCCNFP 2003b), the material remaining in the epidermis and dermis in addition to that in the receptor fluid is considered as being systemically available, but not the test substance remaining in the stratum corneum (SC) at the end of the study. In OECD # 428 (2004c) skin absorption may sometimes be expressed using receptor fluid data alone. However, when the test substance remains in the skin at the end of the study e.g. by lipophilic test substances, it may need to be included in the total amount absorbed. The OECD Guidance document (2004a) notes that skin fractionation, e.g by tape stripping, may be performed to further define the localisation of the test substances within the skin as required by the objectives of the study. Alternatively, distribution within the skin can be determined by taking vertical sections and using autoradiography or other analytical techniques to visualise the test substance.

A recent publication has discussed this topic and concludes that when the movement of chemicals from a skin reservoir to the receptor fluid is shown to occur, it is appropriate to add skin levels to receptor fluid values to obtain a more realistic estimate of dermal absorption (Yourick et al., 2004).

12.4 Relevance of percutaneous measurements to data required by risk assessors

Finite and infinite exposures

– discrepency between percutaneous measurements and data required by risk assessors. [These estimates must be as close as possible to real exposure conditions. To achieve this, experiments should be conducted under finite dose conditions, using vehicles, concentrations of chemicals, and periods of exposure which reflect in-use conditions (Benford et al., 1999; EC, 2004]

Use of single exposure regimes

Another drawback in dermal absorption studies is that most use single exposure regimes. Data on the effects of repeated exposure is scarce and conflicting. Some data show that repeated exposures may increase dermal absorption (e.g. Wester et al., 1996) or may have no effect (e.g. Tauber & Matthes, 1992 in EC, 2003)

Barrier integrity test for skin barrier function of human skin in skin penetration tests.

The OECD guidance document 428 recommends the use of a barrier integrity test when performimg skin penetration studies for regulatory submission. Typically, skin samples exhibiting a permeability coefficient (Kp) above 2.5 x 10^{-3} cm/h are rejected as being 'damaged'. Kp values from 1110 human skin samples followed a non-Gaussian distribution (Roper et al., 2004). A rejection criterion of (Kp) above 2.5 x 10^{-3} cm/h resulted in rejection of 230 (21%) of these samples. It is likely that many of these rejected samples were atypical rather than damaged resulting in an underestimation of absorption in such an individual.

12.5 Methodology – future perspectives

e.g Microdialysis.

12.6 QSARs/QSPeRs – is this the answer?

Pro – We need a way to manage more efficiently chemical risk assessment

Contra - Variability of data; metabolism not considered; nature of drug/vehicle interactions, solvent choice and other physicochemical factors are not considered.

SECTION 13 WAY FORWARD: RECOMMENDATIONS BY THE TASK GROUP MEMBERS

(Suggestions to be discussed and supplemented by the IPCS **EHC Task Group that will be convened to finalize this document**).

- EDETOX database should be maintained and updated
- Encourage the acceptance of QSARs for dermal penetration (QSPeRs).
- Further efforts should be made to minimise variability in *in vitro* dermal absorption measurements
- Collection of more data on more lipophilic effects thereby investigating the effects of various donor fluids, receptor fluids and mixtures
- The use of modelling and other methods should be encouraged to bridge the gap between finite and infinite dosing

APPENDIX 1 GUIDELINES AND PROTOCOLS

Historical perspectives

Although for several decades methodology and assessment of percutaneous absorption has been advancing, it is only comparatively recently that the interest in this field has expanded from the drugs and cosmetic branches to the fields of chemical risk assessment. In the last decade, a number of documents have been prepared, reviewed internationally and published for various associations or regulatory bodies. The following is intended only as a short guide to the available publications, which have, in part, been stepping stones in the preparation of the OECD Guidelines (2004a, 2004b, 2004c) . Details are to be found in the original documents.

ECETOX, 1993

This document (ECETOX, 1993) was prepared and reviewed by experts in the field of percutaneous absorption on behalf of European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC). With emphasis on industrial chemicals rather than on drugs or cosmetics, this publication describes methodology of percutaneous absorption, discusses the relevance of results when forming interspecies comparisons, and reviews the assessment of dermal exposure.

ECVAM (Howes et al, 1996)

This publication is the Report and Recommendations of the European Centre for the Validation of Alternative Methods (ECVAM) Workshop which was held in May/June 1994. The participants comprised of scientists from both academia and industry. The aim of the workshop was to review the status of *in vivo* and *in vitro* methods for studying dermal absorption at that time with particular emphasis in recommending and optimising *in vitro* methods to reduce the number of animals needed for testing.

The group noted that the regulatory requirements for submission of relevant data on dermal penetration varied world-wide, from the very demanding and precise *in vivo* (rat) protocols of the US EPA for dermal absorption of pesticides (e.g. US EPA, 1998)

to absence of guidelines. In Europe the requirement for percutaneous absorption data was usually fulfilled as part of the absorption, distribution, metabolism and excretion (ADME) studies (pharmacokinetics/toxicokinetics) undertaken for new chemicals, drugs, and pesticides as well as experimental data on the delivery of drugs. There was/is increasing pressure in industry to reduce the number of animals used in safety testing.

The ECVAM Report discusses mechanisms and modelling of skin penetration, *in vitro* and in vivio methods for measuring percutaneous absorption and skin metabolism re

COLIPA (European Cosmetic Toiletry and Perfumery Association)

Due to potential ban on the use of animals in the testing of cosmetic producets and their ingredients (6^{th} amendment to the European Cosmetics Directive (93/35/EEC), a Task Force of COLIPA met in 1995 to develop test guidelines for *in vitro* assessment of dermal absorption and percutaneous penetration of cosmetic ingredients (Diembeck et al., 1999). This publication was the basis for further activities by the cosmetic industry (see also Steiling et al., 2001).

SCCNFP (Scientific Committee on Cosmetic Products and Non-food products intended for Consumers)

SCCNFP (2003a) contains notes of guidance for testing of cosmetic ingredients for their safety evaluation. Only one sub.chapter (3-4.4 is related specifically to dermal/percutaneous absorption.

The document points out the wide variety of terms used in definitions. For cosmetics, the SCCNFP makes a clear distinction between

SC or dermal adsorption which is defined as the amount of topically applied test substance present in or sticking to the SC. It is considered not to be systemically available and is excluded from the risk assessment [see Diembeck et al., 1999; also Steiling et al., 2001.

Dermal / percutaneous absorption is defined as the amount of dermally applied substance remaining in the residual skin (excluding SC) plus the amount of dermally applied substance which has transpassed the skin and is detected in the receptor fluid.

The sum is considered to be systemically available (= dermal bioavailability) [see Diembeck et al., 1999).

Test formulations and concentrations tested should be an adequate representation of the final cosmetic products. In case dermal absorption studies are not available, a default value for dermal absorption of 100% is applied in the calculation of MoS.

Details of SCCNFP basic criteria for *in vitro* testing of cosmetic ingredients is given in SCCNFP/0750/03 (SCCNFP, 2003b) which revises an update SCCNFP/0167/99 (SCCNFP, 1999).

US EPA, 1998

This guideline document is intended to meet testing requirements of US Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA) (US EPA, 1998. The guideline has been designed and validated using the laboratory rat and this is the required species for testing. Other animal species were considered but were rejected. It is recommended to use the same strain of rat as those used for the metabolism and toxicology studies. For risk assessment, the absorption rates determined in the rat can be used as a 'modest overestimate' of human dermal absorption or to perform a kinetic evaluation [see section risk?].

US EPA, 2004

In their Final Test Rule (US EPA, 2004 update of US EPA, 1999) under section 4(a) of the Toxic Substances Control Act (TSCA), manufacturers, importers, and processors of 34 (formerly 47) chemical substances of interest to the Occupational Safety and Health Administration (OSHA) will be required to conduct *in vitro* dermal absorption rate testing. These dermal absorption rate data are to be used to support OSHA's development of 'skin designations' for the chemical substances. For measuring the permeability constant (Kp), the test standard specifies the use of static or flow-through diffusion cells and non-viable human cadaver skin. It also requires the use of radiolabelled test substances unless the analytical methods used have an equivalent sensitivity. For compounds that may damage the skin with prolonged contact, a short-term absorption rate measurement is more appropriate. The six parameters (choice of

membrane, preparation of membrane, diffusion cell design, temperature, testing hydrophobic chemicals, and vehicle) are similar for the determination of either of the two percutaneous absorption rate values. In contrast, the remaining two parameters (dose and study duration) are different for the two percutaneous absorption rate values.

EC Technical Guidance Document (TGD) on Risk Assessment (2003)

In Appendix IVB of this document (EC, 2003), the contribution dermal exposure may make to systemic body and its estimate is discussed. Although guidelines give a general description of the experimental design, it is important for risk assessment that the anticipated exposure conditions should be taken into account. (Benford et al., 1999).

The duration and frequency of exposure as well as the level of exposure may vary tremendously – it may be incidental or continuous. Studies addressing more than one relevant exposure per unit area are recommended as well as the use of various exposure times and vehicles. For risk assessment, the percentage absorption is the most useful parameter. Ideally, 1) the exposure duration of the study should be as long or longer than the anticipated exposure duration and 2) the concentrations tested should include the lowest concentration anticipated.

The TGD presents the problems encountered when making risk assessments 1) when studies are not available (default values) and 2) from *in vitro* and in *vivo* data. Some of this discussion is given in the chapter on Risk Assessment (Section 10).

EC, 2004

This guidance document on dermal absorption has been prepared for the European Commission Directorate E1 – Plant health (EC, 2004 update of EC, 2002). Therefore the emphasis is on guidance for notifiers and EU Member States on the setting of dermal absorption values to be used in risk assessment for users of plant protection products reviewed for inclusion in Annex I of Directive 91/414/EEC. Inclusion of active substances in this Annex is only possible if the products containing them can be used with acceptable risk to humans (i.e. operators, workers, bystanders). The dermal route is the main exposure route for most pesticides for operators applying them as well as for workers and bystanders

The EC 2004 document contains an overview of dermal absorption and the methodologies used in measuring dermal absorption. It discusses the decision-making processes for setting absorption percentages and includes a proposal for a tiered approach to risk assessment for operator exposure, using default dermal absorption percentage or dermal absorption percentage determined experimentally (see also Section 10, the use of dermal penetration data in risk assessment.

In the absence of experimental data, the occupational exposure is based on models e.g. UK Predictive Operator Exposure Model (POEM) and BBA (in future EURO POEM) [www.pesticides.gov] each calculating external dermal and inhalation exposure. For risk assessment, these external exposure data are compared toxicity data [AOEL (Acceptable Operator Exposure Level) defined as an internal value and expressed in mg/kg bw/day (EC draft Guidance Document, 2001). In order to compare the external exposure with the internal AOEL, external exposure data have to be turned into internal levels. This is where knowledge of dermal absorption is essential. [Directive 91/414/EEC indicates where dermal absorption studies are required (for pesticides?)]. The studies should be performed in accordance with OECD guidelines 427 (OECD, 2004b) and 428 (OECD, 2004c) and the associated guidance document Nr 28 (OECD, 2004a.

OECD Publications

OECD Guidance document for the conduct of skin absorption studies (OECD, 2004a)

OECD Environmental Health and Safety Publications Series on Testing and Assessment No. 28 (March 2004)

This guidance document was written to guide scientists unfamiliar with the procedures of skin absorption studies and to support technical aspects of the OECD skin absorption test guidelines (OECD, 2004b, OECD, 2004c).

OECD Guideline for the testing of chemicals No. 427 Skin Absorption: in vivo Method (OECD, 2004b) [Adopted 13 April 2004]

This guideline describes the details of the *in vivo* studies commonly on rats but also on hairless species as well as other species having skin absorption rates more similar to those of human. [See also Section 6] It replaces the draft document of the same name (OECD, 2000).

OECD Guideline for the testing of chemicals No. 428 Skin Absorption: in vitro Method (OECD, 2004c) [Adopted 13 April 2004]

Skin from humans or animal sources can be used. It is recognised that the use of human skin is subject to national and international ethical considerations and conditions. Both static and flow-through diffusion cells are acceptable. Normally, donor chambers are left unoccluded during exposure to a finite dose of a test preparation. However, for some studies infinite applications are necessary when donor chambers may be occluded [See also Section 5]. It replaces the draft document of the same name (OECD, 2000).

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