1. INTRODUCTION

1.1. SKIN

1.1.1. Structure and function

Skin is the largest organ in the body. Its major function is to protect the body from dehydration and unwanted effects from the external environment. The skin of adults weighs approximately 7 kg, which represents about 10 % of the body weight of an adult and an area of about 1.8 m^2 [1]. Skin morphology and thickness is different at different locations [2]. Thickness reaches up to a few millimetres with palms and soles. Differences in the depth can be caused by differences in the size of single dermal strata, especially a thickening of the stratum corneum (horny layer) [2, 3]. Sex and age also affect the thickness and structure of the skin layers [2].

Skin is made up of two main layers: the epidermis and dermis, below them the subcutis is to be found (Fig. 1).

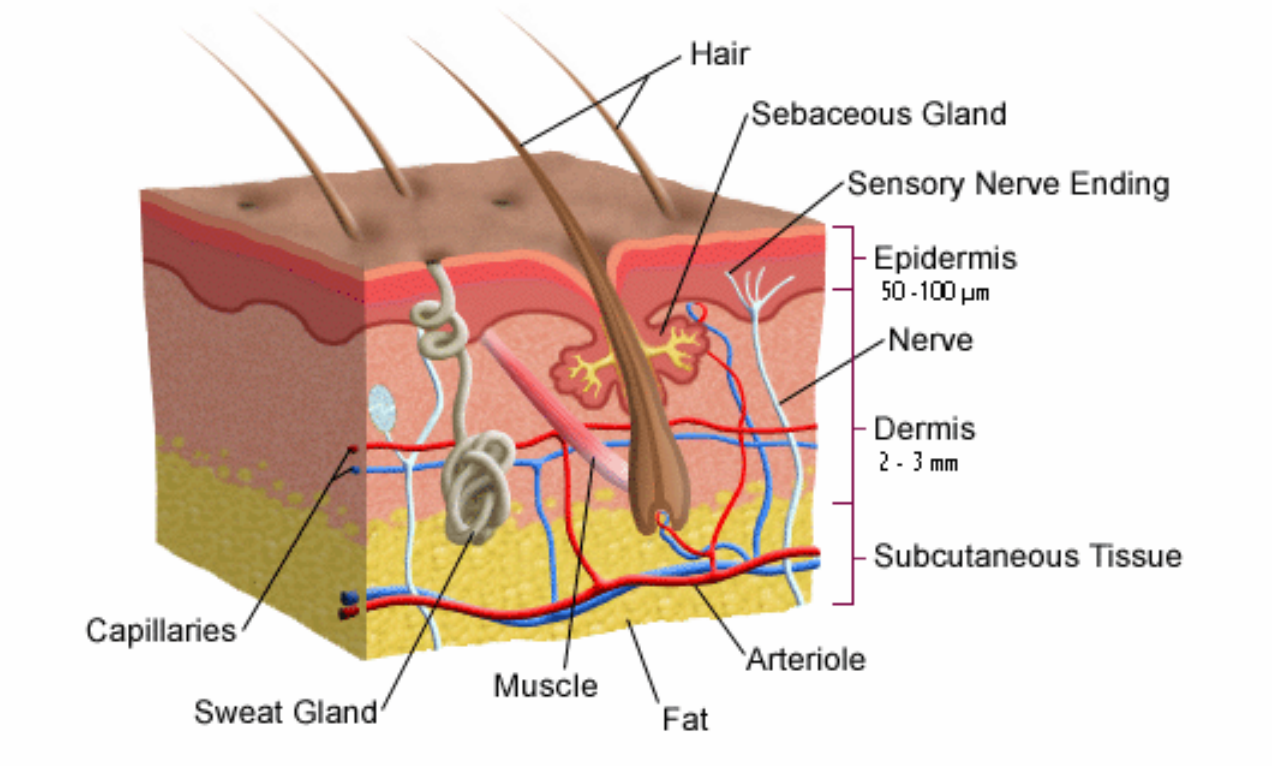

Fig. 1. Structure of the skin. (From Standford Cancer Center, under www.cancer.standfordhospital.com/media/images/greystone.)

Except from palms and soles *the epidermis*, the outermost layer of skin is of about 50-100 µm thickness [1, 2]. It is a protective layer that repels pathogens or noxious agents from the environment and guards against fluid loss. The epidermis is a self-renewing stratified epithelium composed primarily of keratinocytes, healthy epidermis completely renews every

28 days [4]. Therefore the skin looks smoothful for many years and incisions heal quickly. The keratinocytes are responsible for the production of the keratin protein, the basic component of the corneocytes forming the most superficial horny layer (Stratum corneum) but also the nails which belong to the skin appendages [5]. The keratinocytes are organized into various layers that represent different stages of differentiation as illustrated in Fig. 2. These layers are: the basal cell layer, the spinous layer, the granular layer, and the cornified layer which is called 'Stratum corneum'. Between the granular and the cornified layer, which means between the living and dead tissue is the so called 'transition zone' [6]. Keratinocyte differentiation is a genetically programmed process that terminates with a non-viable cell and includes serial events: Synthesis and modification of structural proteins, specifically keratin; appearance of new

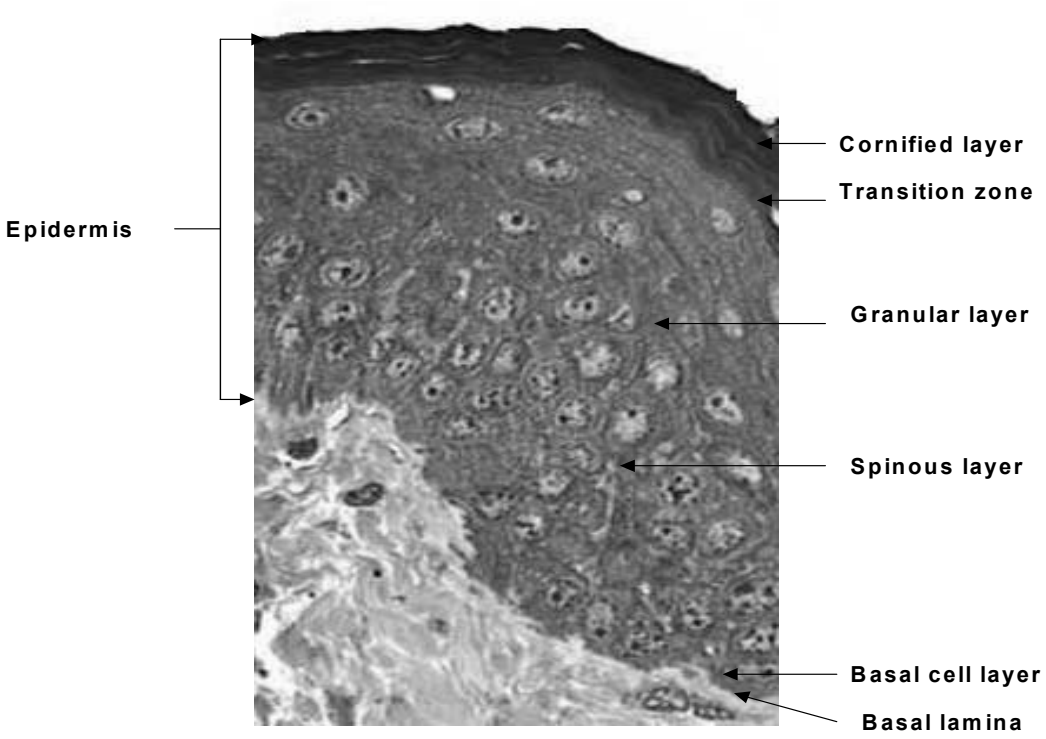

Fig. 2. Structure of the epidermis

organelles, reorganization of existing organelles, and loss of organelles; change in cell size and shape; specialization of cellular metabolism; changes in the properties of cell membranes; and finally the dehydration of the cell [5, 6]. Besides keratinocytes, two more special cell types are located in the epidermis: melanocytes, which produce melanin, the pigment that gives skin its colour [7] and the Langerhans cells, specific macrophages which protect the body against infections [8].

Underneath, *the dermis* nourishes the epidermis. It has about 2-3 mm thickness, and is made up of connective tissue and contains blood vessels and nerve endings. The dermis is fitted to

epidermis through the papillae. Although the stratum corneum is the main permeability barrier to percutaneous absorption and the viable epidermis is primarily responsible for the generation of this barrier, it is the underlying dermis which forms the bulk of the skin. The main cell types of the dermis are fibroblasts, macrophages, and mast cells. Major synthetic products of the dermis are collagen and elastin protein fibres which form the matrix of the dermis and are responsible for the strength and elasticity of the skin [1, 8]. Both, the cells and the matrix have an influence on the rate and extent of differentiation of the keratinocytes and thus the formation of the epidermal layer. Conversely fibroblasts themselves are influenced by the epidermis [1, 5].

The subcutis is composed of a network of adipocytes (fat cells) arranged in lobules, connective tissue and blood vessels. This tissue is important for energy storage and metabolism as well as protection against hypothermia and injury [1, 6, 8]. Because of the interaction between collagen fibres and subcutaneous fat, it is very difficult to remove all of the subcutaneous fat from excised skin. The intercellular fat droplets may also act as reservoirs for hydrophobic compounds, i.e. as depot for many compounds which permeate the stratum corneum [1]. This is of special concern in in-vitro models of percutaneous absorption in which the vascular system is not properly maintained.

Appendages: A variety of appendages which in part penetrate the Stratum corneum and the epidermis are derived from the dermis or subcutis and provide various functions including thermal control and a protective covering. The Stratum corneum penetrating appendages are potential sites of discontinuity in the integrity of stratum corneum. Appendages represent less than 1% of the total skin surface [1] and include: *Sweat glands*; eccrine glands (two thirds) and apocrine glands (one third) are distributed over the entire body at a density of 400 glands /cm^2 [1]. The function of these glands is to respond to the elevated body temperature by secreting a nearly isotonic solution composed of water, salts, urea, ammonium, and amino acids; *Hair follicles* may be an important site for percutaneous absorption (shunt pathway) although their surface area accounts for only 0.1-1% of the total surface area of the skin [9-12]. This will be important during the early stages of topical drug absorption especially for the compounds which exhibit relatively slow rates of transepidermal permeation [13]. *Sebaceous glands* are holocrine glands. They are found in all regions covering the body at a density of 1-2 glands per hair follicle and the activity of these glands varies according to site and age [14]. Under hormonal control these glands secret sebum which is composed mainly

of triglycerides, free fatty acids with lesser amounts of squalene and waxes. The activity of these glands may have a significant impact on the composition of the lipid in the Stratum corneum because of the possible mixing of the secreted sebum with topically applied formulations [1].

1.1.2. Barrier function of the skin

The most important role for the skin is its barrier function against water loss and uptake of environmental substances making contact with the skin surface [1, 6, 15]. Yet, the skin can be used for topical application of pharmacologically active compounds. To enhance the percutaneous absorption of drugs, we have to know the native of this barrier. Most of all the epidermis and dermis contribute to the barrier function and also to the overall resistance of the skin. Resistance of the epidermis includes resistance of the viable epidermis and - most importantly - that of Stratum corneum.

The Stratum corneum is a matrix of keratinised cells named ‘corneocytes’, which are highly ordered in about 15-20 layers of about 20 μm thickness [16-17]. They are flattened, elongated in shape and held together by corneosomes [17, 18]. The corneocytes contain two major components: keratin bundles protein and the cornified envelope. The cornified envelope is a covalently cross-linked sheet of proteins [19-21]. The corneocytes are surrounded by hydrophobic lipid bilayers (15-20 layers surrounding each single cell) forming multilamellar arrays which are aligned approximately parallel to the surface of corneocytes. These hydrophobic lipid bilayers form the only continuous medium from top to bottom of the Stratum corneum matrix [22]. The Stratum corneum lipids are most abundant in the intercellular spaces and primarily composed of cholesterol, ceramides, long-chain free fatty acids and cholesterol sulphate. Phospholipids are not present [15, 23].

The characteristic morphology of the horny layer inspired Elias [24] to formulate the brick-wall model of the Stratum corneum. This model describes the keratinocytes as the bricks (with high intracellular hydrophilicity) and the unique intercellular lipid domain as the mortar (with high extracellular lipophilicity). This so called ‘brick wall’ forms the barrier against the external environment and protects the body from dehydration. Moreover, because of the highly tortuous appearance of the intercellular lipid domain (the diffusion path length is about 15-25 times longer than the actual thickness of 20 μm), the Stratum corneum is most important in the prevention of permeation of especially lipophilic compounds [25].

1.1.3. Percutaneous absorption

The transport of molecules across the skin occurs by passive diffusion which means that the solute flux is linearly dependent on the solute concentration gradient [25, 26]. So far no data exist, which support the theory that active transport plays a role in the uptake of substances by the skin [26].

Considering the skin as a single membrane with two aqueous phases on both sides; one serves as a solute reservoir and the other as a sink. Data obtained from a variety of diffusion experiments can therefore be fitted using Fick's 1st law of diffusion [27-29] despite the heterogeneity of the skin barrier. At steady state, the solute flux is directly proportional to the concentration gradient and to the diffusional area.

$$J = K_p * \Delta C \qquad (1)$$

Where J stands for the flux of the solute [moles x cm^{-2} x s^{-1}], K_p for the permeability coefficient of the solute through skin [cm x s^{-1}], and ΔC for the concentration gradient across the skin (the concentration difference between the donor and acceptor compartment [moles x cm^{-3}]). K_p is the inverse of the resistance of the skin against the solute transport and is defined by

$$K_p = K * D / h \qquad (2)$$

where K stands for the partition coefficient of the solute which is directly proportional to the lipophilicity of the solute, and D for the diffusion coefficient of the solute in skin [cm^2 x s^{-1}] which is inversely proportional to the size of the solute. h represents the diffusion path length through the skin [cm]. The solubility of the permeated substance in the Stratum corneum lipids should exceed its solubility in the formulation in order to obtain good permeation through the skin.

Assuming that the concentration gradient of the solute, the driving force for passive diffusion, and the path length through skin (h) are constant, the permeability of a substance through the skin is directly related to its lipophilicity and inversely related to its molecular size. In general, there is a correlation between the permeability of a substance and its

lipophilicity (log P). This correlation can be direct and represented by linear relationship for closely related compounds. However, the results of permeation experiments for other classes of compounds of different lipophilicity (log P) such as alkanoic acids [30-31], p-substituted phenols [32], hydrocortisone esters [33], substituted p-cresols [34] and series of steroidal anti-inflammatory agents [35] demonstrated a direct relationship in each of these classes at low to moderate lipophilicity and an apparent maximum permeability at high lipophilicity (high value of log P). These results led to a characteristic parabolic shape for this relationship with an apparent minimum and maximum permeability [36-37]. It was found that the horny layer presented a 1000-fold larger resistance than that of the viable epidermis and dermis [38-39]. Although the resistance of the Stratum corneum for more lipophilic substances is smaller, the Stratum corneum maintains the rate-limiting role for penetration. This results from the fact that a lipophilic molecule does not favourably partition out of the Stratum corneum into the more aqueous viable epidermis [40].

Percutaneous penetration involves: partitioning of the molecule into the Stratum corneum from the applied vehicle, diffusion through the Stratum corneum, partitioning from the horny layer into the viable epidermis, diffusion through the viable epidermis and upper dermis, and finally the capillary uptake [41]. Several permeation experiments using tape-stripped skin which is deprived of its Stratum corneum have strongly ensured that the Stratum corneum is the primary rate-limiting barrier to the penetration across the skin [40]. Thus, one can expect that the diffusing substance can take one or more of the following penetration routes across the horny layer (Fig. 3):
(1) the transcellular path through the corneocytes and the unique lipid bilayers; (2) the intercellular path through the continuous intercellular lipids; and (3) the transappendagel path through hair follicles [42].

Although the transport through the appendagel route is significant during the initial state of percutaneous absorption, its contribution to the total transport at a steady state is insignificant [9-13, 40]. Several evidences support the intercellular pathway via the highly tortuous lipid domain is of highest relevance [43-44].

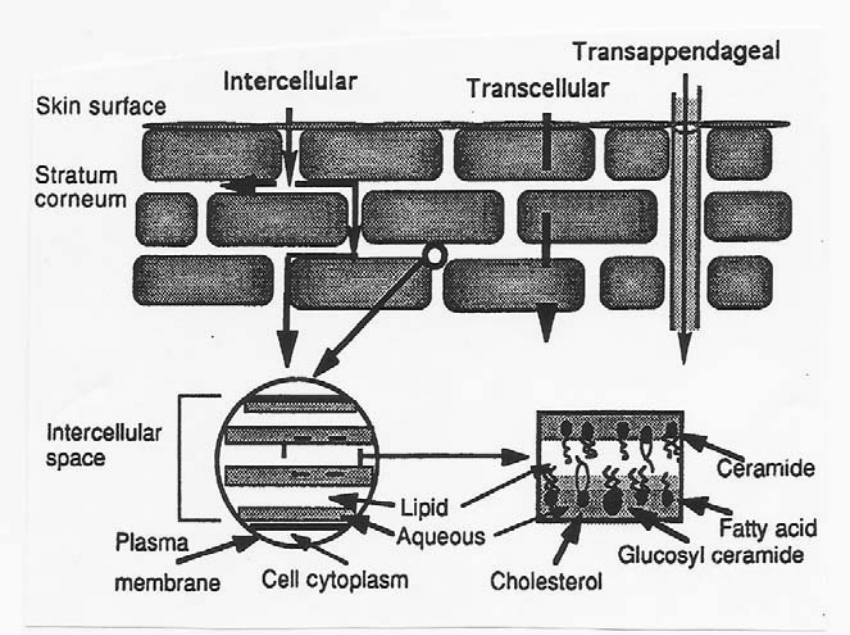

Fig. 3 Diagrammatic representation of the absorption of substances through the skin through the main route for transport in the stratum corneum, the intercellular space (Ref. 42)

It is well known from the literature that the binding of a substance to skin constituents can retard its penetration across skin [40]. If a substance exhibits strong preference for the Stratum corneum, its uptake will be impeded. This may necessitate the use of a higher dose of the drug in order to increase the number of free diffusing molecules. Because of this reversible binding of the drug, the Stratum corneum acts not only as barrier, but also as reservoir for the topically applied drugs leading to a steady drug release [45-47].

The percutaneous absorption is affected by the application site. Not only the thickness of the horny layer, but also the size of the corneocytes (which can significantly control the absolute value of h) and the dermal microcirculation at this site are of relevance [48]. Moreover, aged skin appeared less permeable to relatively polar permeants than young skin. However, an age-effect for the permeation of lipophilic substances was not detected [49]. Apart from that, the permeability of damaged or diseased skin (damaged Stratum corneum) is enhanced [50]. Moreover, temperature of the epidermis strongly affects the skin permeability. The penetration of corticosteroid through human epidermis was enhanced 4.5-fold by raising the temperature from 25 °C to 37 °C [51] and skin hydration promotes the penetration of many substances through the skin, too [52].

1.1.4. Cutaneous metabolism

Recently, it has been indicated that the metabolising enzymes and their activities in the skin must be taken into consideration in topical drug therapy [53]. Most metabolising enzyme activities in the skin are as much as one or two orders of magnitude lower than in the liver, if normalised for g tissue [54]. Yet, one must consider that the skin involves a body surface area of man of about 1.8 m^2 and that the total weight of skin exceeds that of the liver about 3 fold [55]. Therefore, the skin may contribute to drug metabolism and in the protection of the organism from the effects of environmental contaminants as a first line of defence. It is also possible that the activity of some enzymes may equal or even exceed their activity in the liver [56].

Drug metabolism consists of two distinct phases; phase I and phase II. In the skin both phase I and phase II enzymes are to be found [55]. Phase I involves oxidation, reduction, hydrolysis, dehalogenation, dealkylation, deamination and decarboxylation reactions. In phase I most of the cytochrome (P-450)-dependent monooxygenases convert pharmacologically active drug to pharmacologically inactive, chemically more reactive, intermediates can bind covalently to cellular macromolecules such as DNA, leading to the initiation of mutagenic or carcinogenic processes [57-59]. Not always the phase I metabolic products are pharmacologically inactive. Phase I enzymes e.g. esterase may form pharmacologically active metabolite from e.g. prednicarbat and even activate other glucocorticoid 21-esters [60].

The metabolic products, which are formed by introducing polar groups such as a free hydroxyl, carboxyl, thiol or amino groups into the molecule in phase I, may underlay conjugation with e.g. active glucuronide, sulphate, acetyl, methyl, glycyl, glutamyl or glutathione. These reactions are called ‘phase II reactions’ and the metabolic products (conjugates) are almost always pharmacologically inactive. A rare exception is for example the sulphate conjugate of minoxidil [61-62]. In general, the hydrophilic conjugates are readily excreted from the body.

The glucuronide formation, the most common conjugation reaction, is catalysed by the glucuronosyl transferase (GT) enzyme which has low substrate specificity. Therefore, the reaction occurs with a wide variety of compounds. A high-energy phosphate donor, uridine diphosphate (UDP)-glucuronic acid, is formed which transfers the glucuronic acid to an electron reach atom (N, O or S) of the substrate which includes steroid hormones [63].

Although it is widely accepted that the liver is the major site of glucuronidation, it is now clear that extrahepatic tissues such as the skin are also involved [64-65]. The cutaneous GT activity was quantified and found to exceed the cytochrome P-450-dependent mono oxygenase activities in the skin [66]. The enzyme appears to be present in the epidermis but not in the dermis [55]. Cutaneous glucuronide formation in the skin of ginea pig, mouse and man was detected using benzo[a]pyrene and o-aminophenol as substrates [55, 67].

The sulphation reaction catalysed by the sulfotransferase enzyme (ST) [66] was detected in human skin in vitro for dehydroepiandrosterone and Δ^5-androstene-3β, 17β-diol [68] and for minoxidil in rat skin, keratinocytes, and hair follicles [61-62]. Formation of a sulphate conjugate involves synthesis of 3´-phosphoadenosine-5´-phosphosulphate from inorganic sulphate, from which the sulphate is transferred to an electron reach atom on the compounds [61-62]. Sulphate conjugation is a minor metabolic pathway in cutaneous tissue [67, 69].
Hydrolase activities including β-glucuronidase, sulphatase, phosphatase, and esterase are responsible for conjugate or ester degradation in skin [70-73]. 17β-Hydroxysteroid dehydrogenase type II (17β-HSD), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 5α-reductase are active in the skin [55, 74-76].
With transdermal therapeutic systems (TTS), enzyme activities in the skin may influence the proportion of systemically available drugs, e.g. steroids, if cutaneous metabolism is significant [53]. Pronounced cutaneous metabolism is reported for testosterone, cortisol, 17β-estradiol (E2), estrone (E1), and estriol (E3) [57, 64, 71, 74-81].
Differences in cutaneous enzyme activity at different anatomical sites of the skin may be explained by differences in the gene expression and numbers of viable dermal and epidermal cells [55]. For example, with respect to hydrocortisone both the dermis and the epidermis contribute to the drug metabolism, while the epidermis is more active than the dermis in transforming estradiol [78-79]. The weight ratio of the epidermis to the dermis varies from 1:3 for human foreskin to 1:11 with abdominal skin which may explain E2 oxidation in foreskin to exceed that in abdominal skin about four fold [78]. Hydrocortisone 5α-reductase has been detected in human foreskin only, while testosterone 5α-reductase has also been detected in the skin of other origins such as abdominal skin [80-81]. With respect to experimental procedures it has to be kept in mind that the separation of the epidermis from the dermis by warming for 2 min at 57°C can destroy about 40% of the enzymatic activity [78].

1.2. SEX-STEROID HORMONES

Estrogens, progesterone and testosterone are of major relevance in the maturation of the reproductive organs of female and male. In both men and women sex hormones account for secondary sexual characteristics and physical differences. Sex hormones are involved in the menstrual cycle, progesterone is most important for the maintenance of the pregnancy. Here, two sex steroid hormones were investigated: testosterone and estrogens.

Only recently also effects of estrogens on wound healing were detected. Sex-steroid hormones, in particular estrogens, cause thickening of the epidermis and increase collagen production by fibroblasts and so thicken the overall skin thickness [82-85]. This way, sex-steroid hormones may also play a pivotal role in the healing process of the skin after an injury by enhancing the inflammatory response and then repair [86-87], promotion of angiogenesis and stimulation of wound contraction [88-91]. Estrogens also increase the level of collagen deposition which is possibly mediated by an increased secretion of transforming growth factor (TGF)-β_1 by fibroblasts. This way, estrogens contribute to the matrix formation and tissue remodelling [92]. This thesis focuses on sex-steroid hormones which are described with respect pharmacology in detail. Moreover, some experiments have been run with hydrocortisone. Since this serves as a more hydrophilic control, effects and metabolism will not be described here.

1.2.1. Biosynthesis and chemistry of testosterone and estrogens

Testosterone is the main physiological androgen. It is mainly formed by the testis in males, and in small amounts by the ovary in females and the adrenal cortex in both sexes. This organ, however, predominantly forms the precursors, dehydroepiandrosterone and androstenedione (Fig. 4).

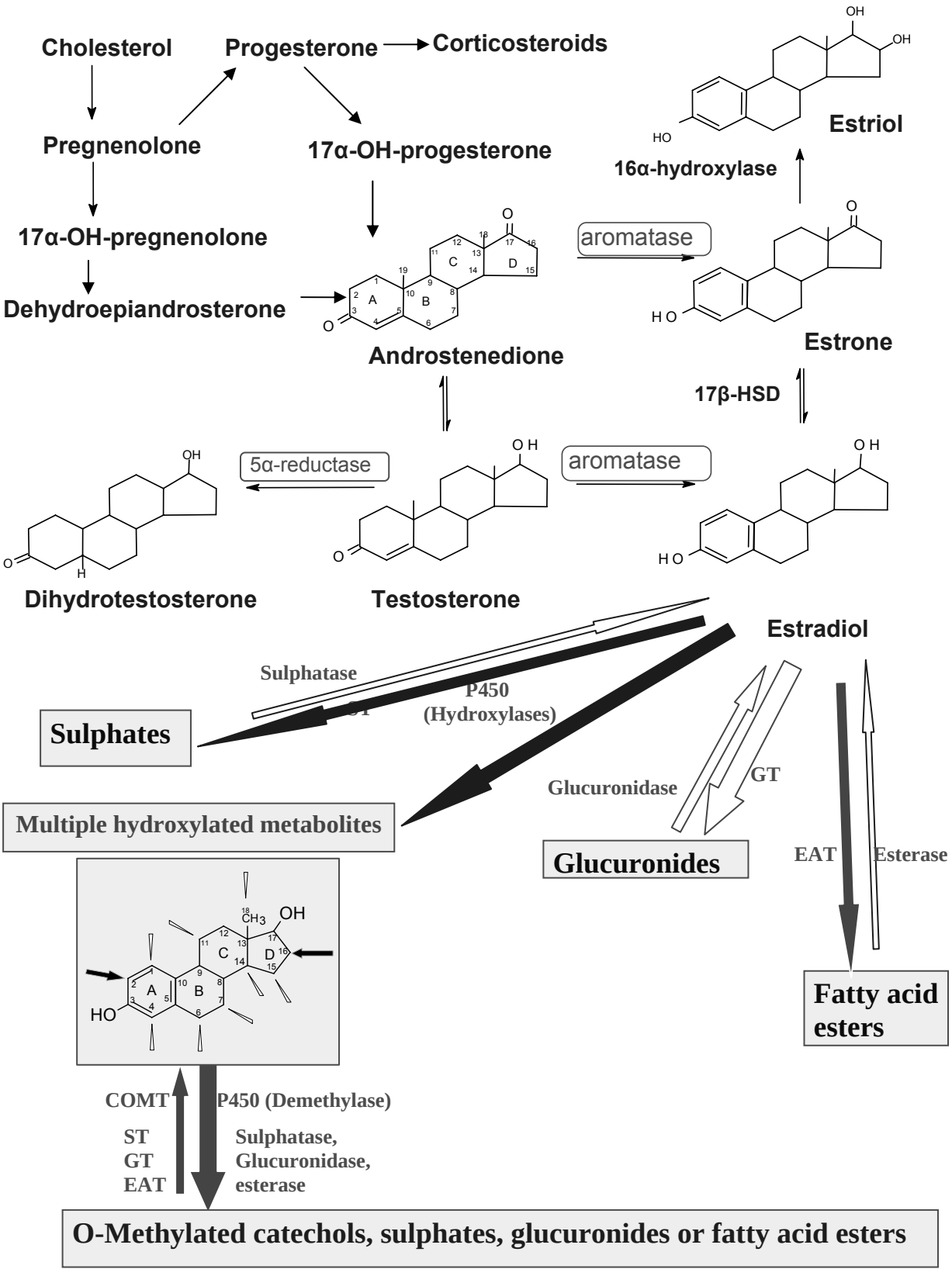

Fig. 4 The biosynthetic pathway for androgens and estrogens. EAT estrogen acyltransferase (for fatty acid ester formation); ST sulfotransferase; GT glucuronosyltransferase; COMT catechol-O-methyltransferase; 17β-HSD 17β-hydroxysteroid dehydrogenase.

Estrogens 17 β-estradiol (E2), estrone (E1), and estriol (E3) (Fig. 4) are mainly formed by the ovaries. During pregnancy, the placenta, which expresses aromatase in high amounts, forms large amounts. Small amounts are synthesized by the testis in males and by the adrenal cortex in both sexes. The immediate precursors of the estrogens are testosterone or androstenedione. Aromatase activity is responsible for the conversion of these androgenic precursors (C19-steroids) into estrogens (C18-steroids), e.g. E2 and E1 [93]. E2 is most potent and is the principal estrogen, while E3 is the least potent estrogen. There is also aromatase expression and estrogen formation, especially E2 and E1, in many other tissues such as adipose tissue, skin fibroblasts, bone, smooth and skeletal muscles and the brain. Estrogen production by these organs contributes significantly to the circulating pool of estrogens, especially in men and postmenopause women [93-98]. But also cleavage of estrogen sulphates, especially E1-sulphate, by the sulphatase in the target cells contributes substantially to estrogens formation and activity [99]. Extra ovaries estrogens are of paramount importance in slowing the rate of postmenopausal bone loss [93].

1.2.2. Receptor binding and mechanism of action

Receptor binding and mechanism of action of sex steroids are rather similar, too. Testosterone and estrogens enter the cell and bind to the specific receptors in the nucleus (Fig. 5.) After interaction with the steroid, the receptor becomes activated and undergoes a conformational change which results in the exposure of a DNA-binding domain. The activated steroid-receptor complex binds to the promoter regions of sex steroid genes and either induces or represses the transcription of these particular genes [100]. The induction of transcription involves the formation of a specific mRNA, which is transcribed into specific proteins, whereas the repression of transcription inhibits the production of specific proteins. One of the most important effects of the estrogens on DNA is the induction of progesterone receptor formation in target tissues such as uterus and vagina. Both, estrogen and androgen receptors have been identified in the epidermal keratinocytes, in the dermal fibroblasts, macrophages, and in vascular endothelium and aortic smooth muscle cells [101-106].

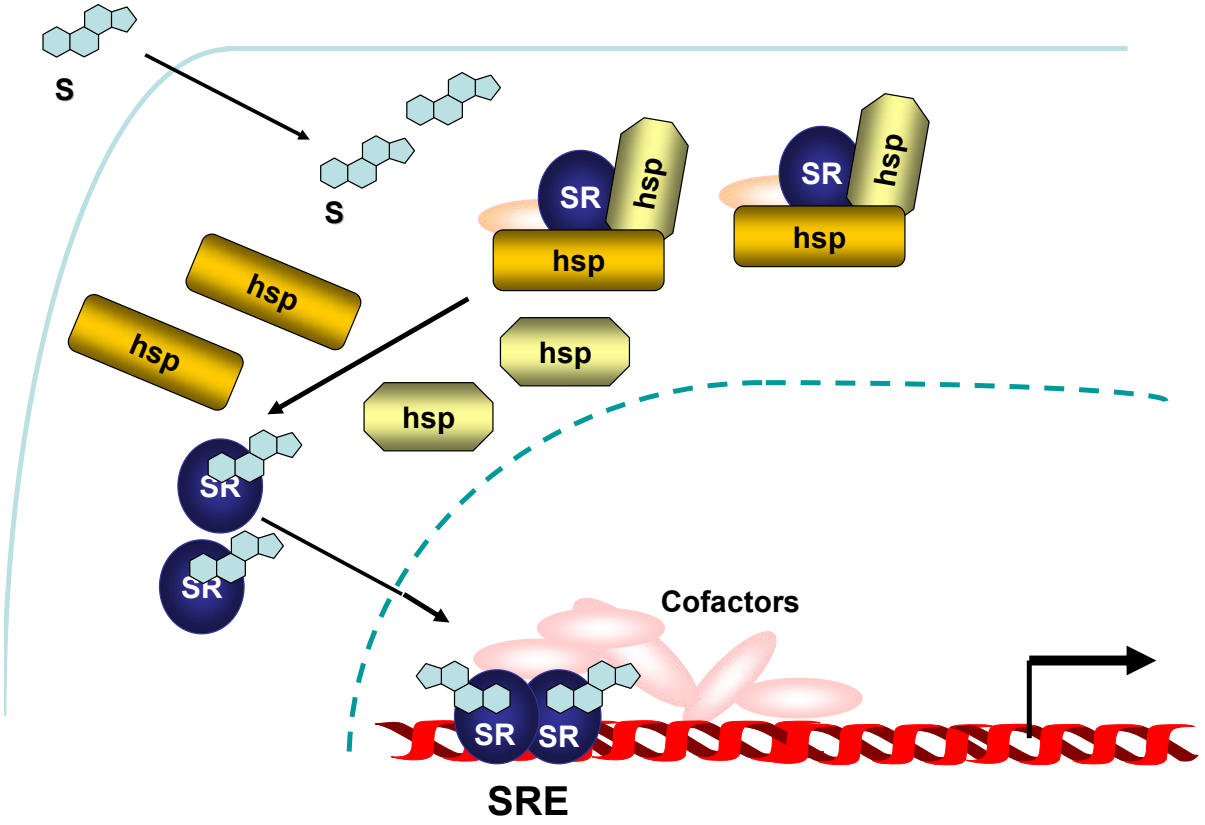

Fig. 5 Receptor binding and mechanism of action of sex steroid hormones. hsp= heat shock protein, S= Steroid, and R= Receptor.

1.2.3. Pharmacokinetics of testosterone

Testosterone is well absorbed in the gastrointestinal tract, but it is rapidly metabolised in the liver. Testosterone is readily absorbed from the skin, buccal mucosa, and rectum. So, it can be given transdermally. It is bound to plasma proteins, mainly to the sex-steroid-binding globulin. The half-life of testosterone is 10-20 min [100].

Testosterone is rapidly converted in the liver to 5α- and 5β-dihydrotestosterone (DHT). DHT is known to be the active androgen in the tissues expressing 5α-reductase [107]. The primary route for the metabolic inactivation of testosterone and DHT is the oxidation of 17-hydroxy group resulting in ketone formation (androstanedione and androstenedione). Moreover, the C3-keto group is reduced by 3α-HSD forming androstanediol and androsterone. Androstenedione and androsterone are the two main metabolites for testosterone and DHT which have week androgenic activity [74, 77, 100]. The conjugation with glucuronides and sulphates are also possible. About 90% of the metabolites are excreted in the urine.

Testosterone metabolism in the skin

In the skin, especially in the fibroblasts [108-110], testosterone is stereospecifically reduced to 5α-DHT by 5α-reductase which is in contrast to the liver which produces both 5α- and 5β-DHT [111] and to keratinocytes which deactivate testosterone forming androstenedione, which is also a precursor for estrogen formation [112]. Besides activation, skin fibroblasts may also deactivate testosterone. At low concentrations, the activation pathway (DHT formation) predominates, whereas at higher testosterone concentrations as to be expected for transdermal testosterone applications the inactivation pathway (androstenedione formation) predominates [74]. Metabolic inactivation of testosterone and DHT occurs also by the oxidation of the 17-hydroxy group to the corresponding ketone, but to a lesser extent. The skin has a minor potential to form testosterone and DHT from androgen precursors.

1.2.4. Pharmacokinetics of estrogens

Estrogens are well absorbed in the gastrointestinal tract, but they are rapidly metabolised in the liver. They are bound to plasma proteins such as albumin and steroid binding globulin.

E2 is converted to the less active hormone, E1 by the widely distributed 17β-HSD in the liver and to lesser extent in many other tissues, e.g. adipose tissue, skin and red blood cells [55, 57, 75, 78, 93]. Because of the rapid interconversion, E1 is considered as a storage form of E2. This interconversion has long been recognised as an important regulatory mechanism for the modulation of estrogen action [57]. Uterine 17β-HSD predominantly catalyzes the conversion of E2 to E1. Progesterone induces this enzyme resulting in a significant loss in the estrogenic activity [55].

Estrogens, E2 and E1, can be also hydroxylated at multiple positions by NADPH-dependent cytochrome P450 enzymes [57]. E2 and E1 may be also converted to E3 through 16α-hydroxylation [57]. The 2-hydroxylation of E2 and E1 (represented by solid arrows in Fig 4) is a major metabolic hydroxylation pathway in the liver, whereas the 4-hydroxylation is a minor pathway. 6- and 7-Hydroxylation is also possible at α and β-position, especially for E1 in the uterus [57].

Quantitatively more important is the estrogen conjugation. The glucuronides and sulphates are the main metabolites excreted in the urine [113-115]. Estrogen metabolism also includes

the formation of the highly lipophilic products such as 2-methoxy-E1 and 2-methoxy-E2 by O-methylation, which is catalyzed by catechol-O-methyl transferase (COMT) in the liver, kidney, red blood cells and mammary glands. 2-Methoxy-E1 is one of the most abundant estrogen metabolites in human plasma and urine [57, 116]. E2 (at C17 position only) can form very lipophilic estrogen fatty acid esters by fatty acid acyl-Co-enzyme-A present in liver, uterus, breast and placenta [57, 117]. These products have long half-lives and serve as a reservoir and direct precursors for E2 formation, storage and activation may occur in the mammary glands [57].

Metabolism of estrogens in the skin

Despite of the wide-use of transdermal E2 replacement, however, there are limited data about cutaneous estrogen metabolism (Fig. 6). E2 and E1 are also interconverted within the skin which possesses 17β-HSD [57, 75, 78, 93, 99]. The reductive pathway is predominant in vaginal mucosa, another target tissue for E2, while oxidative pathway to E1 occurs in non-target tissues such as foreskin and abdominal skin [55]. The epidermis is more active than the dermis with respect to the oxidative pathway of E2 [78]. However, relatively little is known about the steroid glucuronidation in skin.

1.2.5. Clinical uses of testosterone and estrogens

Testosterone has two important activities: androgenic (male-sex-characteristic-promoting) and anabolic effects (muscle-building). Testosterone is of major importance in male behaviour, increases the protein formation and muscle mass, and decreases subcutaneous fat.

The principal use of testosterone in adult men is for hormone replacement therapy (HRT) in hypogonadism [118]. Testosterone is also used for prophylaxis of osteoporosis in the men and sometimes for treatment of breast carcinoma [97, 100].

In adult women, the principal use of estrogens is for contraception. Since the negative outcome of the heart estrogen/progestin replacement study (HERS) and women's health initiative (WHI) studies [119-120], estrogen replacement therapy [121] for the prophylaxis of postmenopausal osteoporosis has been seriously questioned. More rarely estrogens are used for treatment of prostatic cancer and -locally- for vaginitis [100]. Recently, also a stimulation of wound healing has been described [86-87].

1.2.6. Estrogen hormone replacement therapy: Benefits and risks

Estrogen HRT has been and -to a lower extent- still widely used for treatment of the postmenopausal symptoms [121]. The proposed benefits of HRT include the relief of climactric symptoms with improving women's quality of life, prevention of bone loss and fractures, preservation of cognitive function, and protection against urogenital atrophy as well as reducing the risk of tooth loss, cancer of the colon, and coronary heart disease [121]. These advantages of estrogen HRT have to be weighed up against its reported possible risks including endometrial and breast cancer as well as the increased risk of venous thrombosis, myocardial infarction and ischemic stroke [121].

With respect to endometrial and breast cancer, the estrogen-sensitive target tissues breast and endometrium have a high capacity to metabolize E1-sulphate to E2 [122]. E1-sulphate is the most abundant estrogen in the circulation of postmenopausal women exceeding E2 and E1 levels 10-25 fold [123]. Due to a long plasma half-life and slow clearance rate, E1-sulphate acts as a reservoir for local E1 and E2 formation [124]. In fact, E1 and E2 concentration in breast cancer tissue exceed plasma level several fold [122].

With respect to the risks of cardiovascular diseases, the results of WHI study in 8506 postmenopausal women subjected to oral conjugated estrogens plus medroxyprogesterone acetate in a double-blind, placebo-controlled, randomized clinical trial, showed an increased risk of ischemic stroke [120] and WHI stopped 3 years early this clinical trial. Recent results from HERS, a secondary prevention trial, verified that there is no overall benefit from estrogen HRT, while once more cardiovascular events were detected in the verum group (conjugated equine estrogen plus progestin) more frequently than in the placebo group [119]. Therefore, a recent report stated that these possible risks of oral estrogen HRT outweigh the potentially beneficial effects [125].

In postmenopausal women, oral estrogen HRT increases C-reactive protein due to a first-pass hepatic metabolism which can be avoided by transdermal application [126]. This increase in the C-reactive protein may explain the initial increase of cardiovascular disease [126]. Moreover, oral estrogen HRT also increases SHBG to a greater extent than transdermal estrogens. To minimize the adverse effects of estrogen HRT the transdermal route of administration may be an important consideration.

1.2.7. Transdermal application of testosterone and estrogens

Most estrogens are readily absorbed from the skin and mucous membranes. So, they can be given transdermally or topically. Transdermal application of estrogens for systemic therapy has several advantages over oral route [126-129]. Avoidance of first pass metabolism may not only reduce the daily dose considerably, avoid peaking of plasma levels and overcome the short half life but may also decrease risks of estrogen HRT [126, 130-132]. In contrast to the oral administration transdermal application maintains the E2/E1 ratio at 1.0 as in the menopause women [131-133]. Yet, TTS can provoke skin reactions such as irritation or contact sensitization.

1.3. IN-VITRO AND EX-VIVO SKIN MODELS

Topical formulations, which are designed for treatment of skin diseases, still involve animal testing. Moreover, the use of animals in safety testing of drugs, pesticides and industrial chemicals has increased in the last decades. Recently, however, the use of animals in safety testing has been questioned [134] which stimulated the development of in vitro models such as the 2D-model (fibroblast and keratinocyte monolayer) and 3D-models, such as reconstructed epidermis as well as reconstructed whole skin. Currently more frequently, however, excised skin of animal or of human origin is used for in vitro testing which has resulted in a substantial reduction in animal testing. Animal skin is used because of the limited availability of excised human skin. Test procedures recently have been approved by the OECD despite of no formal validation study [135]. Morphology of reconstructed human skin is similar to native human skin and therefore this tissue which is not restricted in availability due to the biotechnological generation may be good alternative for animal testing [136-138]. In fact the 3D-model may be even more predictive for uptake and metabolism by the human skin.

1.3.1. Excised human and animal skin

For percutaneous absorption, the OECD [135] favors freshly excised human skin (abdominal or breast). Since the availability of human skin is less than the need by the pharmaceutical, chemical and cosmetic industries, animal skin is used, too. For regulatory toxicology, testing is performed using rats or pig skin. Dorsal or ventral rat skin may be used, however, dorsal rat skin is preferred [135]. While in vitro experiments are run in rat skin since these species are also used for toxicity testing, pig skin is used because of its similarity to human skin in

terms of its morphology and permeability characteristics [139]. Flank and the ear are the most commonly used sites of removal, although skin from back or limb is also used. Animal skin is generally more permeable and therefore may overestimate human percutaneous absorption [14-141]. For determination of percutaneous absorption testing, excised skin can be used immediately (fresh) or stored frozen prior to use [142-143]. Human and animal skin can be stored for several months at -20 °C [144]. The viable skin is prefered, however, for reasons of comparability of several experiments, non viable skin may have advantages. After excision and removal of the subcutaneous fat, the skin must be kept cool and transported into the laboratory as soon as possible. Under well controlled experimental conditions (composition of the acceptor medium 37 °C, pH 7.4) skin viability and metabolic activity of the skin is given for 6-12 h [145], which allows studying drug penetration and cutaneous metabolism.

1.3.2. Perfusion model (isolated perfused porcine forelimb)

More complex are organ perfusion models. Nowadays, many isolated organs of pigs may be used such as the liver [146], heart [147], ear [148], kidney [149], and skin [150-151]. Recently, an isolated skin perfusion model has been developed using the porcine forelimb [152] which aims to be as close as possible to the physiology of the intact organ. The isolated perfused porcine forelimb model was already successfully applied for the investigation of the transdermal absorption of nitroglycerine and estradiol [153-154].

1.3.3. Fibroblast and keratinocyte mono-layer culture

In-vitro cell monolayer cultures offer an interesting approach to test e.g. cellular toxicity and drug metabolism under standard experimental conditions [155]. For cell cultures keratinocytes and fibroblasts are isolated from foreskin obtained from surgical operations by the incubation with proteolytic enzymes. Plated on plastic dishes, keratinocytes and fibroblasts are grown in selective culture media such as keratinocyte culture medium and Dulbecco's modified Eagle medium, DMEM, respectively [156]. The culture is supplemented with keratinocyte and fibroblast growth factors to stimulate proliferation and increase the life span of the culture [157]. The proliferation and differentiation of keratinocytes is affected by the concentration of calcium in the medium. At concentrations of < 0.1 mM, Ca^{2+} keratinocytes proliferate readily but differentiate slowly and remain as mono - layer cultures. Calcium concentrations above 1.0 mM lead to the formation of a multi - layer system [158]. Fibroblasts in a mono - layer

culture differ from the fibroblasts in the dermis with respect to membrane permeability, enzyme activity, and collagen synthesis. Keratinocytes can be sub-cultured for at least three passages and fibroblasts for 20-30 passages [159].

1.3.4. Three-dimensional organotypic models

Recent progress in cell culture led to the development of **reconstructed human epidermis** which exhibits morphological and biochemical differentiation features close to those of native human epidermis [136-138]. The reconstructed epidermis is a three-dimensional tissue, obtained by seeding human foreskin keratinocytes on an appropriate substrate, e.g. artificial membrane. After attachment of the cells, the culture is kept submerged for several days to stimulate the proliferation [160]. Subsequently, the supporting membrane is raised at the air-medium interface to expose the keratinocyte multilayer (20-25 layers) to the air, which is crucial for their differentiation (cornification) forming a coherent Stratum corneum [161]. Nowadays, several types of reconstructed epidermis are commercially available. Keratinocytes are seeded on a cellulose acetate (EpiDerm®) or polycarbonate membrane (SkinEthic®), or collagen gel (Episkin®). Rosdy et al. showed that the architecture of the epidermis obtained in-vitro on a cellulose acetate or polycarbonate membrane was quite similar to that of normal human epidermis [162]. The barrier function, however, is less well developed as compared to healthy human skin [163-166] since reconstructed epidermis does not synthesize epidermal barrier lipids in the physiological proportion and epidermal lipids are improperly organized [167].

The equivalence in the structure of reconstructed epidermis to native epidermis alone is not the only criterion of the quality of the reconstructed tissue, the expression of differentiation-specific markers, the lipid composition, and the barrier function are other important criteria. With respect to protein differentiation markers, compared with native tissue, involucrin, filagrin, keratin, and transglutaminase are also expressed in the reconstructed tissue. Keratin 1 and 10 are expressed in the correct locations, while keratin 6 which is absent in the native tissue, is expressed in the reconstructed epidermis [167]. While keratinocytes in the reconstructed tissue reproduce to a high degree the lipids of the native epidermis, differences in the lipid composition are to be found. The reconstructed epidermis contains more triglycerides and less glycosphingolipid [168]. The most important difference, however, is the low content of linoleic acid, which as a component of acylceramides is crucial for the epidermal barrier function [169]. These deviations in the lipid composition may explain

imperfections in the intercorneocyte lipids and may also be the cause of the less well developed barrier function of the reconstructed epidermis [163, 167].

Another **organotypic skin model**, recombines the epidermal and dermal layers as described by Freeman et al. [170] using reticular structures of dead pig dermis. More recently, keratinocyte seeding to dermal equivalents obtained by mixing fibroblasts, type I collagen, tissue culture medium, and reconstitution buffer was described [171-172]. The culture is kept submerged for several days and then is raised to the air-medium interface. The functional permeability barrier of this new organotypic skin model was determined measuring the permeation of substances with different lipophilicities and molecular weights [173]. The functional permeability was found to be close to those measured in intact human skin.

Currently, a commercially available **in vitro-airway tissue (EpiAirway) model** is developed. EpiAirway is a three-dimensional tissue originating from normal, human derived tracheal or bronchial epithelial cells [174-176]. The cells are grown on a collagen coated membrane to form a ciliated, pseudo-stratified, highly differentiated model closely resembling the epithelial tissue of respiratory tract. De-epidermized dermis can be also used as a substratum. Air-exposure of the cultured cells is a necessary requirement for the ciliogenesis [177]. Histological examination of both the in vitro tissue and normal human tissue revealed a four layer, bipartite structure with numerous cilia on the apical surface of the culture as well as mucin secretion and verified the presence of tight junctions between cells [174]. EpiAirway cultures are obtained in phenol red and hydrocortisone free medium and are cultured at 37 °C for 48 h to allow the tissue to equilibrate and begin secreting mucin. Permeation studies using both EpiAirway model and small laboratory animals confirmed a high correlation between the permeation characteristics of EpiAirway as a model for nasal drug permeation of pharmaceutical preparations and in vivo nasal bioavailability in rats [178-179].

1.4. METHODS OF ANALYSIS OF SEX-STEROID HORMONES

Since in the present study the permeation and penetration of skin by steroidal hormones was to be compared, a suitable analytical procedure has also looked for. Chromatographic methods and even more immunoassays are widely used.

1.4.1. Colourimetric and fluorimetric methods

The colourimetric methods of analysis of sex steroid hormones [180-181] were once very popular, but became less important with the development of more sensitive instrumental methods.

1.4.2. Chromatographic methods

Chromatographic techniques, although not as sensitive as immunoassay methods, enable simultaneous separation and determination of both free and conjugated steroids. For quantification of sex steroid hormones, use of gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) was preferred. The recent instrumental improvement in the coupling of MS to HPLC, however, should encourage this more sensitive technique. HPLC-MS, unlike GC-MS, is not limited by the non-volatility and high molecular weight, and enables the determination of both conjugated and non conjugated sex steroid hormones without the need for derivatization.

HPLC methods

All HPLC methods were performed on C18 reversed phase columns using water-acetonitril mixtures and gradient elution from 20-50% to 100% organic solvent.

For determination of testosterone, an isocratic HPLC with ultraviolet detection (UV) at wavelength of 245 nm was used. The limits of detection (LOD) were very close with solvent extraction (8.6 ng/ml) or solid phase extraction (7.3 ng/ml, [182]). A simplified HPLC method (UV detection at 240 nm, grisofulvin as internal standard) is slightly more sensitive (LOD 1.6 ng/ml [183]). Recently, the sensitive HPLC-MS-MS was described allowing the determination of testosterone in presence of its metabolites in cell culture medium with a LOD of 50 pg/ml only [184].

For determination of estrogens, E2 and E1, two HPLC methods using UV detection at 281 nm were used. LODs were 13.7 ng/ml (liquid-liquid extraction, [185]) and 1.1 ng/ml (solid phase microextraction [186]). Other HPLC methods using either electrochemical detection (LOD 60-80 pg/ml [186] and 24 pg/ml [187]) or coulometric array detection (LOD of 5-50 pg/ml [188]) were also used. Fluorescence detection at 280 nm for excitation and 312 nm for emission was also used to increase the sensitivity of HPLC to 20-50 pg/ml LOD using automated solid phase extraction [189]. A HPLC method using fluorescence detection was

used only for E2-glucuronide conjugate (LOD 5.44 ng/ml [190]). A new fluorescent europium chelate labelling reagent was used for E1, E2 and E3 (LOD 0.65 ng/ml [191]).

GC-MS methods

Testosterone was determined more recently with GC-MS method which is based on negative chemical ionization and pentafluorobenzyloxime/trimethylsilyl ether derivatives with excellent chromatographic and electron-capturing properties. A LOD of 333 pg/ml was reported [192]. *E1 and E2, too,* was determined by a GC method after silanisation with a selected ion monitoring. The LOD was 3.3 ng/ml for E1 and 2.2 ng/ml for E2 [193].

Thin layer chromatography methods (TLC)

Recently, in our labour, testosterone and its metabolites as well as E1 and E2 were separated and quantified by a TLC method using 4 different solvent systems of different polarity. The separated steroids were quantified by scanning the plate with β-counter TLC scanner [74]. Besides this, another TLC method for determination of estrogens was also reported [194].

1.4.3. Immunoassay methods

All immunoassays techniques are sensitive and often highly specific analytical methods, but are limited by the availability of specific antisera and are subject to cross-reactivity.

Radioimmunoassay (RIA)

RIA is a direct competitive binding assay which allows to measure hormone and drug concentrations at the picogram level. The importance of this technique is evidenced in the fact that the Nobel price was awarded in 1977 for its development. RIA combines the sensitivity of radiochemistry with the specificity of immunology. Radio-labeled antigen (Ag*, ^{3}H) and unlabeled antigen (Ag) (a sample or a standard) compete physicochemically for the limited number of binding sites on the specific antibodies (Ab). After the incubation, bound and unbound antigen have to be separated by adsorption to activated charcoal, which adsorbs small unbound lipophilic molecules. The charcoal is then separated from the soluble fraction containing the antigen-antibody complex by centrifugation. The radioactivity of the supernatant represents the antigen-antibody complex. The charcoal, however, may also bind the antibody itself or strip the antigen from the antibody [195]. To overcome this problem, a charcoal coated with dextran of an appropriate molecular weight is used to provide pores allowing the free antigen to pass and excluding the larger antigen-antibody complex.

Next the amount of antibody bound radioactivity had to be quantified by **liquid scintillation counting (LSC)**. Most of the emitted β-particles such as ^{3}H (tritium) have actually low energy which can easily be absorbed by the compound itself or by the surroundings. To detect the low energy levels generated by β-emission, the LSC was developed. This technique is based on the conversion of the radioactive decay energy into a light emision by using an organic liquid called scintillation cocktail, which is a mixture of three components; an organic solvent; an emulsifier to ensure proper mixing of aqueous samples; and a fluorophor. The β-decay emitted by the radio-isotope cause the solvent molecules to be excited, excited solvent molecules is quickly transfer energy to the fluorophor which in turn emits a visible light that is converted into a measurable electrical signal by a photomultiplier tube. The amount of light produced is proportional to the intensity of radiation present in the sample. The analytical detectable range of the LSC method is scintillation cocktail dependent. Emitted light determined by LSC is quenched to some degree. To overcome this problem the calibration curve has to be made under conditions, exactly identical to the conditions of the samples under analysis such as solvent components and volume as well as volume of the scintillation cocktail. Alternatively, correction of cpm count vs dpm has to be made. (dpm = efficiency of the β-counter x cpm count). This can be done by using standard solution of well known dpm.

A calibration curve is prepared using antigen standards of known amount by plotting the percent bound radio-labeled antigen on the Y-axis against the log of unlabeled antigen

concentrations on X-axis. The unknown antigen concentration can be estimated from this calibration curve.

Radioimmunoassay, first described by Yallow and Berson [196] in the late 1950s and 1960s, now became the 'gold standard' in clinical chemistry. Commercial RIA kits are now available for the determination of E2, E1, E3 and testosterone as well as the conjugated estrogens. The LOD are about 1.36-5.44, 5.4-6.8 and 9 pg/ml for E2, E1, and E1-sulphate, respectively and 10-20 ng/ml for testosterone [197-200], but there are also more rapid and ultrasensitive RIA kits (LOD 0.6 pg/ml for E2, 2.0 pg/ml for E1, and 7.0 pg/ml for E1-sulphate [201]. Moreover, lower LOD (testosterone, 15 ng/ml, androstenedione, 35 ng/ml, E2, 0.4 pg/ml, and E1, 0.54 pg/ml) can be obtained by using HPLC purification before carrying out the RIA [202]. The high sensitivity is verified by another publication with LODs of 1.17, 5.39, and 4.4 pg/g tissue for E2, E1, and E1-sulphate, respectively for HPLC-RIA procedure [203].

Enzyme-linked immunosorbent assay (ELISA)

For determination of testosterone, also several ELISA procedures were developed using either horse-radish peroxidase, HRP (LOD 15-50 pg/ml, [204-207]) or more sensitive penicillinase enzyme (2.5-10 pg/ml, [208-209]). An ELISA technique for determination of serum E2 using monoclonal antibodies and E2-biotin as tracer was also used and showed LOD of 28.3 pg/ml [210]. More sensitive ELISA using electrochemical detection and HRP was described for E2 (LOD 5 pg/ml [211]). Fluoroimmunoassay with LOD of 2.3 pg/ml for E2 and 4.3 pg/ml for E3 [212], and enzyme immunoassay for determination of testosterone [213] were also used.

We have selected the RIA method for the determination of estrogen permeation, penetration and metabolism not only owing to high specificity for estradiol metabolites, specially, estrone and estriol, but also due to its higher sensitivity than ELISA and chromatographic methods, where it is considered the most sensitive method.

1.5. AIM OF THE WORK

The overall aim of the work is the comparison of the essential in vitro methods for testing percutaneous absorption and metabolism to identify models of high reproducibility and predictability for the in vivo situations. The following models were used; perfused pig forelimb, split skin of porcine and human origin, human separated epidermis, and commercially reconstructed human epidermis. In addition, first experiments were performed in an airway model, which became available only recently. Estradiol and testosterone were selected for these investigations since both are OECD reference standards for penetration studies. Moreover, both are subject of cutaneous metabolism which also influences the intensity of effects. Finally both agents are used for hormone replacement therapy despite of the fact that today estrogen replacement is recommended only for symptomatic women and for a period as short as possible.

Since both steroids show low cutaneous uptake, very sensitive assay methods had to be established. For the determination of the estradiol metabolism in the skin, a commercially available RIA had to be adapted which then allowed to determine the tissue and acceptor medium concentrations of E1, E2 and even of their conjugated metabolites. The substance panel was improved introducing a more hydrophilic steroidal substance as well. Therefore, hydrocortisone was introduced into some experiments.

The investigations proceeded as follows:

- Influence of the donor vehicles and of the acceptor media on the viability and morphology of the skin models.
- Comparison of the dynamic and static permeation / penetration protocols on cutaneous uptake of testosterone.
- Influence of regional effects and individual variations on cutaneous uptake of testosterone.
- Effect of the donor vehicles and BSA addition on the permeation of testosterone and 17ß-estradiol. Effect of the donor vehicle on the cutaneous metabolism of 17ß-Estradiol.
- First experiment comparing the uptake of drugs by skin and lung, testosterone and hydrocortisone serving as model drugs.