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The Effect of Skin Aging on the Percutaneous Penetration of Chemicals Through Human Skin

by

Kathleen Victoria Roskos

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

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GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

[redacted]

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To Scott, who inspired me to earn my graduate degree.
Without his reassurance, affection, strength and understanding,
I couldn't have made it through.

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Abstract

Despite much research into the mechanisms of cutaneous aging and the identification of significant age-associated biological and biophysical changes within the skin, the question " how does aging affect percutaneous absorption (PA) *in vivo* ?" remains unanswered. We have made *in vivo* measurements of PA in young (18-40 years) and old (>65 years) subjects. Standard radiotracer methodology was employed and PA was quantified from the urinary excretion profiles of ^{14}C radiolabel (corrected for incomplete renal elimination). Testosterone (TST), estradiol (EST), hydrocortisone (HC), benzoic acid (BA), acetylsalicylic acid (ASA) and caffeine (CAFF) have been studied. Penetration of HC, BA, ASA, and CAFF were significantly ($p<0.01$, 0.01, 0.01 and 0.05, respectively) lower in aged subjects whereas TST and EST absorption were not distinguishable from the young controls. Thus it appears that aging can affect PA *in vivo* and that relatively hydrophilic compounds may be most sensitive. Work was done to elucidate whether the observations were related to documented skin aging changes. Cutaneous microcirculation efficiency suspected to decline with increasing age, could not be correlated with the observed penetration changes. However, *in vivo* infrared spectroscopic studies of aged stratum corneum (SC) reveal a decreased amount of epidermal lipid. The diminished lipid content implies a diminished dissolution medium for compounds administered to the skin surface. We hypothesize that the compounds most affected by a loss of SC lipids would be those compounds whose overall solubility is lowest (compounds with lower octanol-water partition coefficients, e.g., HC, BA, ASA and CAFF). Conversely, a diminished lipid content may not affect dissolution into the SC of highly lipophilic compounds (e.g., TST and EST).

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Chapter 1

Introduction and Background

Objective

The objective of this project was to assess the effect of skin aging (as measured by increasing chronologic age), on the penetration of chemicals through human skin *in vivo* and to assess and interprete the results in light of the known biological changes associated with skin aging.

Rationale

Despite much research into the mechanisms of skin aging (Gilchrest, 1984), the effect of this process on the percutaneous absorption of drugs and other chemicals has received scant attention. Hence, it is not intuitively obvious how the significant age-related biological changes in human skin impinge upon it's ability to maintain a barrier to the ingress of foreign compounds into the body (Barry, 1983).

Rationale for this project has emanated from several avenues. Firstly, the population shift to higher age ranges has produced, and will continue to result in, major health-care related repercussions. The proportion of elderly citizens in our population has more than doubled in this century. In 1900, 4 per cent of Americans were over 65 years old; in 1980 approximately 11 per cent (Besdine,1980), and in 2030, it is estimated that 20 per cent of the population, 50 million Americans, will be over 65 years old (Kovar,1977).

Secondly, most clinical studies are conducted using male subjects of between 18-40 years of age. While this is clearly an appropriate general population sampling for phase I, II and III clincial trials, information obtained from this general sampling may be inappropriate for prediction in or use toward a specific population. In other words, a specific group (i.e. the elderly) may be inaccurately represented by testing the "average" clinical setting.

Finally, skin disease and systemic disease treated by topical drug administration are not exempt from the population demographic change and as a consequence, the following question must be addressed: "Is the efficacy of drug therapy, which depends upon penetration through the cutaneous barrier, a function of skin age?" The answer to this has relevance not only in dermatology and the treatment of local skin problems, but also in the application of transdermal drug delivery (TDD) for systemic effect. There has

been an enormous growth of interest in TDD (Guy and Hadgraft,1986) in the recent past. This administration route offers several advantages: controlled input of high potency drugs avoiding gastrointestinal and hepatic first - pass effects, extended duration of drug action with a decreased daily dose, reduction in the frequency of drug dosing and increased patient compliance, simple and direct termination of drug input at any time (Knepp et. al., 1987).

The attributes of TDD are suited, therefore, to powerful drugs used in long-term, chronic therapy. It is the elderly who predominately have health problems requiring longterm, chronic treatment. These characteristics, coupled with the progressive population shift to higher age ranges, makes this population group an ideal target for transdermally delivered drugs. This observation is reflected in 3 of the 4 currently approved drugs for transdermal delivery: nitroglycerin, for the treatment of angina; clonidine for the relief of hypertension; and estradiol for hormonal supplementation in post-menopausal women. The patient population for these systems is predominately aged and this situation will be repeated with the majority of new and emerging TDD candidates.

Normal Human Skin

Introduction

The skin of the average adult human exceeds 2 m^2 in area and is in most places no more than 2 mm in thickness, yet its mass exceeds that of all other organs (Odland, 1983). As the body's largest organ, the skin provides a sheet-like investment of the whole body, adapting to contours with a snug fit (the scalp, the ears, the palms of the hands and soles of the feet); pleating at corners for flexibility (the elbows, knees and knuckles); and neatly conforming to the movements of the organism within.

Skin constitutes a physical barrier at the interface between organism and environment. It is adapted to withstand dessication in a dry environment, and a myriad of mechanical, chemical and microbial insults from without. The skin contains a vasculature and a sweating system that uniquely adapts to the thermoregulatory demands of the organism. Additionally, it contains an extensive neuro-receptor network which serves the organism as a finely tuned array of complex transducers of environmental information.

Two major layers are conventionally recognized as constituting human skin (Figure 1).

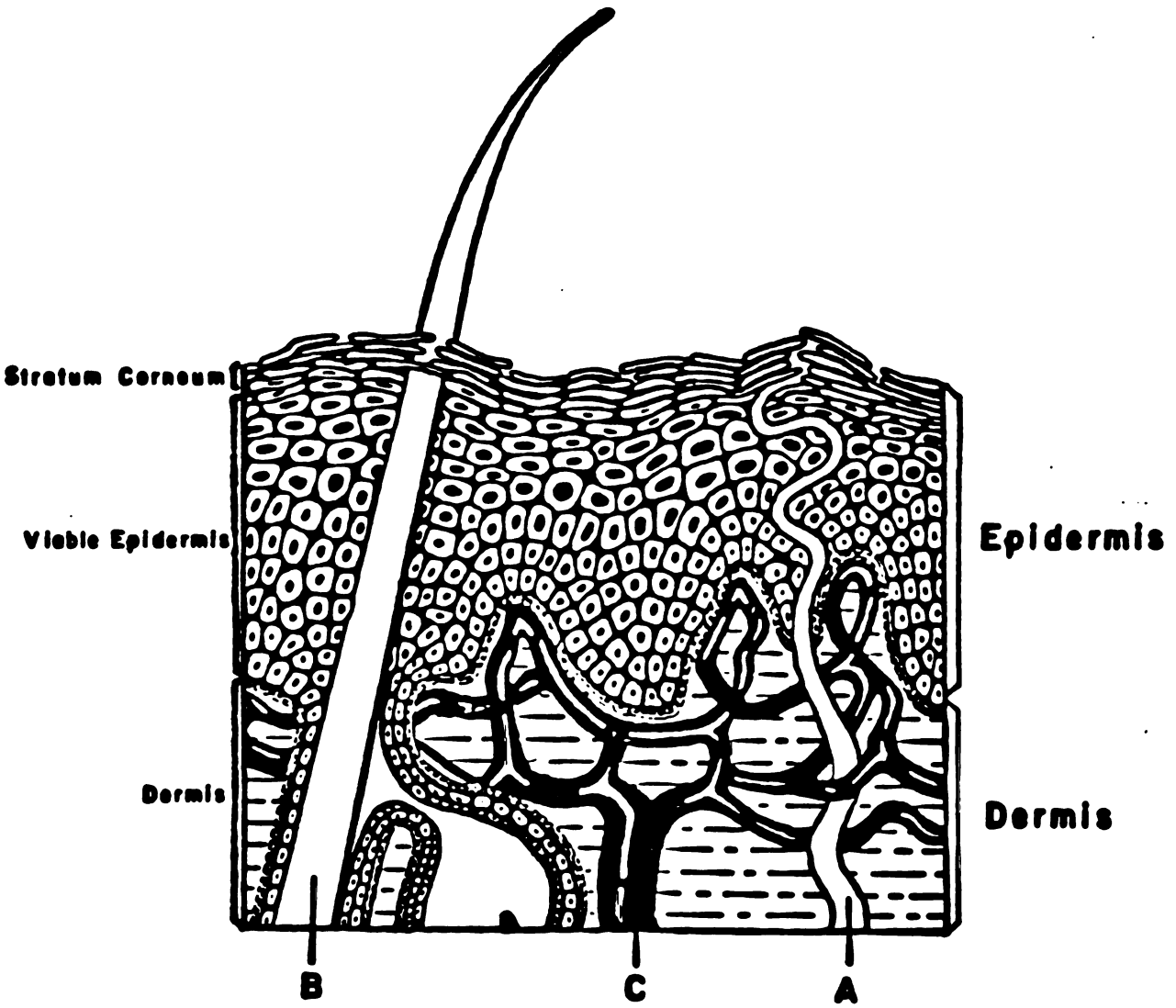


Figure 1. Diagram showing division of skin into epidermis and dermis. (a) eccrine gland (b) hair shaft (c) capillary network Also showing division of skin into stratum corneum, viable epidermis and dermis.

Epidermis

The outer layer of the skin, the epidermis, is a thin stratified epithelium, varying little in thickness over most of the body. Average epidermal thickness is usually between 50 and 150 μm, with the exception of the palms and soles where its thickness may be 0.4 - 0.6 mm (Odland, 1983). This cellular sheet, enclosing the entire organism, terminates at body orifices (e.g. mouth and nose) in mucocutaneous junctions. As an integument, it is disrupted in its continum only by pores of glandular structures and follicles from which hairs emerge.

At the macroscopic level, the epidermis consists of two principal cellular layers (Figure 1), an outermost laminated sheet of terminally differentiated anucleate cells, the stratum corneum or horny layer, and a viable inner cell layer, the viable epidemis, from which the surface cells arise by differentiation.

Microscopically, the epidermis (Figure 2) can be subdivided into (1) the stratum basale, a basal cell layer of keratinocytes known as the germinative layer of the epidermis; (2) the stratum spinosum, comprising several layers of polyhedral cells lying above the germinative layer; (3) the stratum granulosum, a layer of flattened nucleated cells containing distinctive cytoplasmic keratohyalin granules; and (4) the stratum corneum, the overlying end product of kertinized lamellae of thin, flat, anucleate cells. The primary epidermal cell type in this epithelium is most commonly called a keratinocyte, appropriately named because of the fibrous proteins, keratins, which constitute the end product of terminal differentiation.

Stratum Corneum

The stratum corneum consists of 15 - 20 layers of flat anucleate cells, which are thin squames of approximate thickness 0.5 μm and a width of 30 - 40 μm in most areas (Plewig and Marples, 1970). It is a heterogeneous laminate structure composed of 40% protein (mostly keratin), 40% water and 15-20% lipids (Chandrasekaran and Shaw, 1978). Its structural organization is reminiscent of a brick wall: the keratinocytes are stacked in alternating rows (the bricks), with each cell entirely surrounded by a lipid-rich matrix (the mortar) (Elias, 1983).

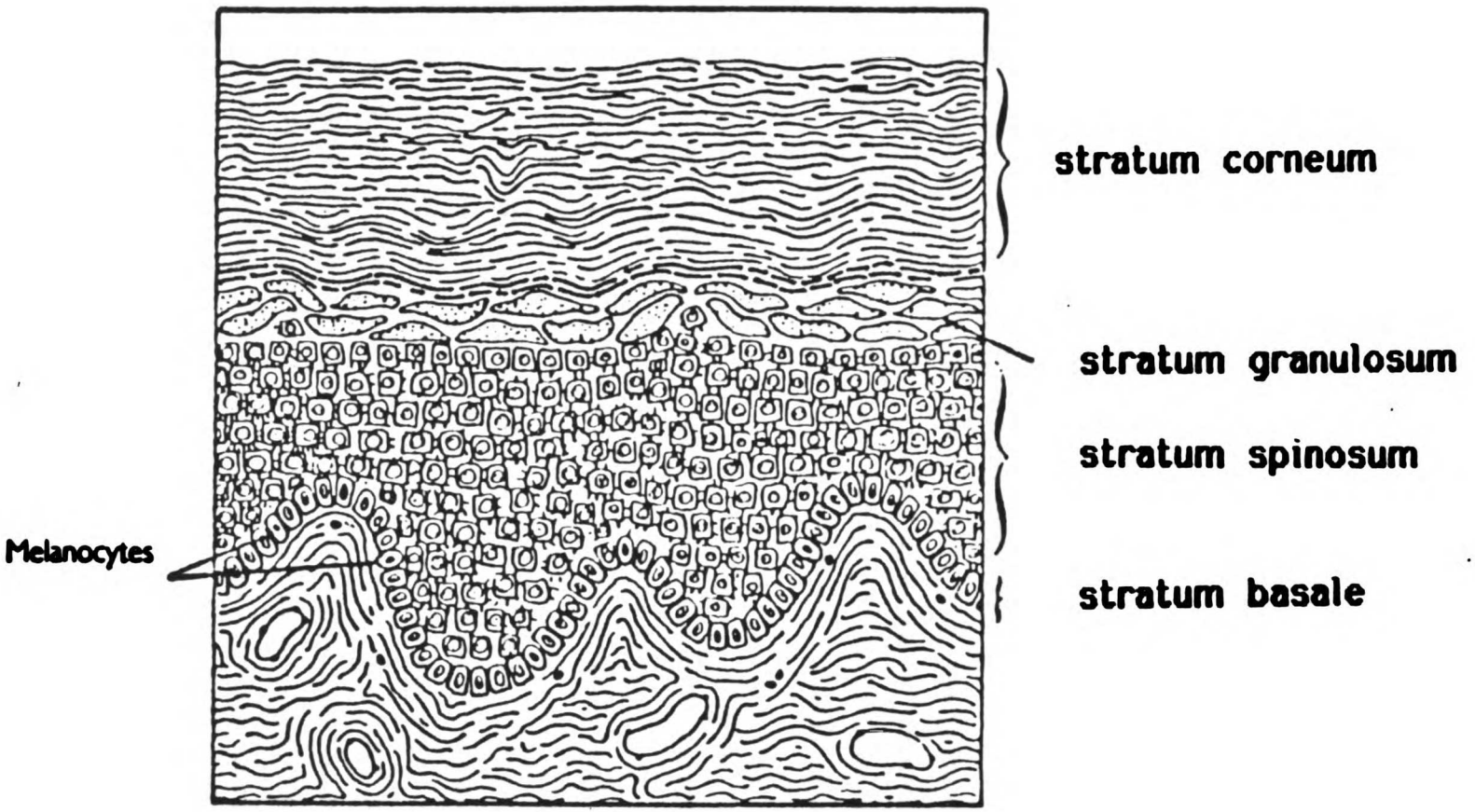


Figure 2. Diagram of the divisions of viable epidermis (a) stratum corneum (b) stratum granulosum (c) stratum spinosum (d) stratum basale

The stratum corneum cell is bounded by a plasma membrane which appears intimately associated with an internal dense marginal band. This internal band arises from the stabilization of a soluble precursor protein, involucrin (Rice and Green,1977), by the formation of E - (γ - glutamyl) lysine bonds within the cell envelope (Buxman and Wuepper, 1978). Together, these structures give the appearance of a thick keratinocyte membrane, measuring about 20 nm in thickness (Odland, 1983). This alkali- and trypsin resistant cell envelope, a highly specialized structure at the cell periphery, assumes protective functions for the cell (Goldsmith, 1983). Thus allowing it to act as the cell's first line of defense to external insults. The interstitial areas of the cell are filled with a dense, amorphous material thought to be comprised, in part, of the the filament-aggregating stratum corneum basic protein, filaggrin (Dale et. al., 1980). The presursor to this material is the histidine-rich protein of keratohyalin from the keratinohyalin granules which first appear at the level of the stratum granulosum during differentiation. The interstices also enclose an array of filaments which are polymers of a number of filament-forming polypeptides called keratins, the structural proteins of the keratinocytes (Odland, 1983).

The cells of the stratum corneum are embedded in a lipid-rich matrix commonly termed the intercellular space. The composition of lipids isolated from human stratum corneum is shown in Table 1 (Elias, 1981a). There are large quantities of sphingolipids, free sterols, sterol esters and free fatty acids, but phospholipids are not present in large amounts.

Elias and coworkers, utilizing thin section and freeze-fracture microscopic techniques, have shown that the intercellular spaces of the stratum corneum enclose lipid bilayers (Elias, 1981a; 1981b). Since phospholipids are generally required to form such bilayers and lipid composition does not reflect large quantities of phospholipids, Elias has proposed one possible arrangement in which sphingolipids and the polar end of free sterols contibute sufficient amphipathic character to maintain bilayer form (Figure 3) (Elias, 1981b).

Table 1. Lipid composition of fractions isolated from human stratum corneum. Results represent weight % of total lipid applied to and recovered from TLC plates. **Adapted from:** Lipids and the Epidermal Permeability Barrier, P.M.Elias, *Arch. Dermatol. Res.* **270**, 95-117 (1981).

		Stratum Corneum Human (weight %)
Phospholipids		11
Phosphatidylethanolathine	3	
Phosphatidylcholine	7	
Glycosphingolipids and Ceramides		12
Cholesterol sulfate		<0.5
Neutral Lipids		77
Free sterols	25	
Sterol esters	26	
Free fatty acids	9	
Triglycerides	17	
Approximate Total		100

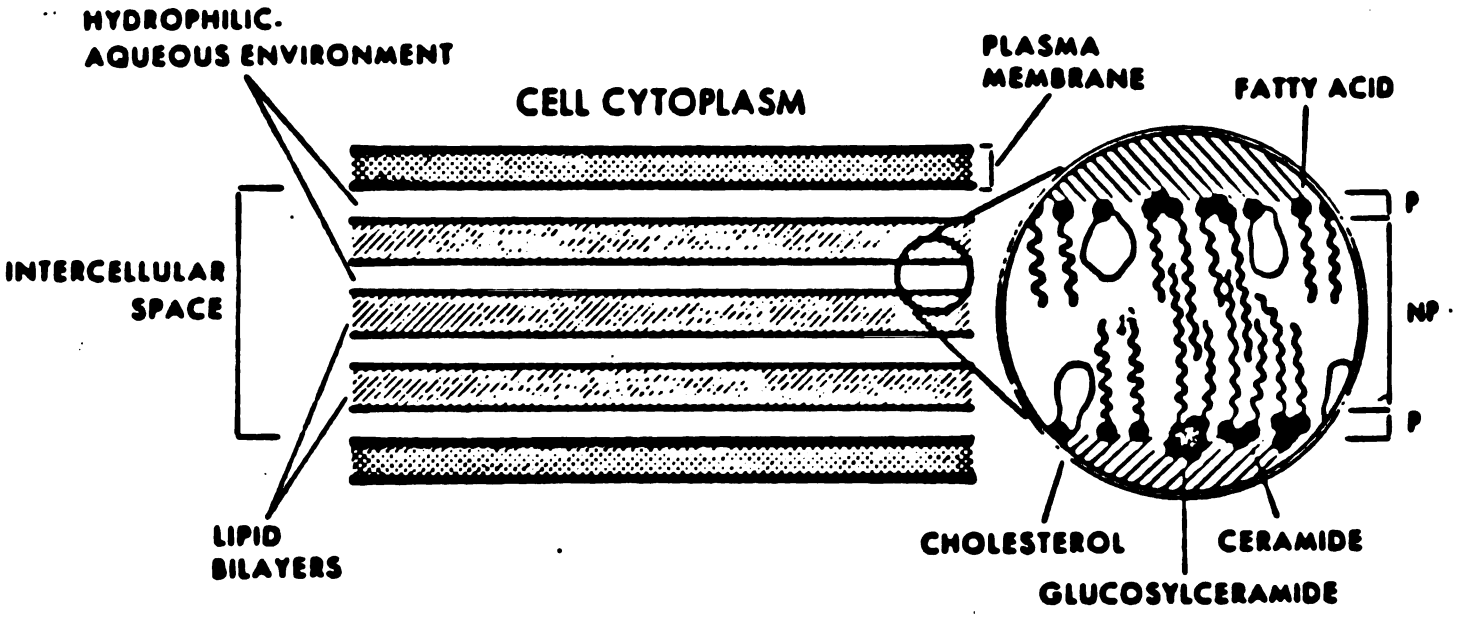


Figure 3. Diagram depicting one possible arrangement for stratum corneum lipids into the membrane bilayers within the stratum corneum intercellular spaces. Adapted from: Elias, P.M., J. Invest. Dermatol., 73, 339-348, 1979)

The epidermal origin of the intercellular lipids has been traced (Elias, 1981a; 1981b) to a membrane structure found at the interface of mammalian stratum corneum and stratum granulosum. These membrane structures, termed lameller granules, are synthesized in the stratum spinosum. At the level of the upper stratum granulosum, these lipid rich leaflets expel their contents into the intercellular space. Following secretion, there is a progressive accumulation of secreted lamellar body contents in the intercellular spaces. Although, it is presumed that the stratum corneum lipid bilayer structure derives from remodeled or catabolized lamellar body contents, the direct evidence to support this hypothesis has still not been obtained.

Viable Epidermis

Human epidermis is in constant rate of turnover; daughter cells of dividing basal keratinocytes migrate outward, transforming in shape from cuboidal epithelial cells to polyhedral cells and thence to flattened cells with distinctive subcellular organelles, finally transformed into further flattened, terminally differentiated anucleate cells of the stratum corneum. These outermost cells desquamate into the environment and are continuously replaced from below (Odland, 1983).

Contained within the viable epidermis are other migrant cell types: melanocytes and Langerhans' cells. The melanocyte, derived from embryonal neural crest (Odland, 1983), is a highly differentiated cell that functions in the epidermis primarily to make melanin pigment granules, protection against damaging solar radiation. Melanocytes constitute approximately 2 to 3 per cent of cells in the epidermis; density varies over the body surface, approximately $1000/mm^2$ on the trunk to approximately $2000/mm^2$ in the face (Szabo, 1959). The Langerhans' cells, approximately $300\text{-}500/mm^2$, are assumed to be monocytoid derivatives of the bone marrow (Odland, 1983) and appear to function primarily in antigen recognition and processing.

The epidermis is a viable, metabolically active membrane containing many enzyme systems. Since it has the capability of metabolizing an assortment of topically applied substances before they become systemically available, the distribution of metabolic enzymes contained within the skin is an important consideration. Evidence for a greater localization of metabolic enzymes in the

viable epidermis compared to the dermis comes from many sources. Following separation of epidermis and dermis, Bamshad (1969) was able to measure the activity of the enzyme, catechol-o-methyl transferase (COMT), and found the amount of COMT to be 8.3-fold greater in the epidermis compared to the dermis. Weinstein et al. (1968) studied the *in vitro* conversion of estradiol to estrone in human skin which had been separated into epidermis and dermis. He found the epidermal metabolism of estradiol to estrone to be 4 fold greater than in dermal tissue. Finally, Chapman et al. (1977) determined aryl-hydrocarbon hydroxylase activity using benz(α)pyrene as a substrate and followed activity as a function of hydroxybenz(α)pyrene formed per mg of microsomal protein per hour. He found that 96.5 per cent of aryl-hydrocarbon hydroxylase activity in skin was present in the epidermis. These data imply that most of the enzyme activity of skin may be localized in the epidermal layer.

The number of enzyme systems present in the skin is extensive and the biotransformation reactions identified within the skin include a series of phase 1 reactions (oxidations, reductions, hydrolysis) and phase 2 reactions (glucuronide and sulphate formation; methylation and glutathione conjugation) (Pannatier et. al., 1978). Biotransformation reactions and respective enzyme systems are listed in Table 2 (Tauber, 1981).

Epidermal - Dermal Junction

By light microscopy, the boundary zone between epidermis and dermis presents an undulating pattern of rete ridges and dermal papillae. This zone of attachment between the epidermis and dermis is commonly referred to as the epidermal - dermal junction.

The plasma membrane of the basal keratinocyte measures approximately 7-9 nm in thickness. At intervals along this plasma membrane are studded hemidesmosomes (or basal attachment plaques). The basal keratinocyte / hemidesmosome attachment rests on the lamina lucida; or intramembranous space (Figure 4). This area, situated between the plasma membrane of the epidermal basal cell and the basal lamina, is approximately 20-40 nm in thickness (Briggaman, 1983). Fine filaments (whose epidermal attachment appears to be in the region of the keratinocyte plasma membrane) traverse

the lamina lucida perpendicularly and mesh with the basal lamina. These filaments are termed anchoring filaments (Kobayasi,1961;1968).
The basal lamina is a continuous layer, running parallel to the plasma membrane of basal keratinocytes at the epidermal - dermal junction; approximately 30-50 nm in thickness (Briggaman, 1983).

Three types of fibrous anchoring structure are found directly beneath the basal lamina; anchoring fibrils, microfibrils arranged in bundles, and single collagen fibers. Each of these fibers are thought to contain an epidermal end which meshes with the basal lamina, while the distal portion extends into the dermis for anchoring support (Briggaman, 1983).

Overall, the epidermal - dermal junction can be viewed as a complex of membrane (plasma membrane) and lamellae (basal lamina and lamina lucida) laced across by filaments (anchoring filaments, anchoring fibrils and collagen fibers). Three functions have been postulated for the epidermal/dermal junction; (1) epidermal - dermal adhesion; (2) mechanical support for the epidermis and (3) semipermeable barrier to the transfer of material and/or cells (Briggaman, 1983).

Dermis

The dermis is a moderately dense fibroelastic connective tissue composed of collagen fibers, elastic fibers and an interfibrillar gel composed of glycosaminoglycans, salts and water. The primary structural elements are synthesized by the principal cell of the dermis, the fibroblast. Collagen, the major dermal component, accounts for the tensile strength of the dermis. Interwoven among the collagen bundles is a network of elastic fibers which aid in restoration of dermal shape following deformation by external mechanical forces (Odland, 1983).

Also a site of high metabolic acitivity, the enzymatic activity in the dermis is primarily associated with the sebaceous glands and the hair follicles in which considerable amounts of lipids and protein are synthesized on a continual basis (Guy et. al., 1987b).

Table 2. Biotransformation reactions occurring in the skin. Adapted from Tauber, 1981.

Reaction	Enzyme System(s) Involved
Phase 1	
Oxidation reactions:	
aliphatic carbon atoms	mixed-function oxidases
alicyclic carbon atoms	mixed-function oxidases
aromatic	hydroxylases, mixed-function oxidases
alcohols	dehydrogenases
deamination	monoamine oxidases
dealkylation	deethylases, demethylases
Reduction reactions:	
carbonyl	ketoreductase
carbon-carbon double bonds	5-α-reductase
Hydrolysis:	
ester	esterases
epoxides	epoxidehydratase
Phase 2	
Conjugation reactions:	
glucuronide formation	UDPG-transferases
sulfate formation	sulfo-transferases
glutathione conjugation	glutathion-S-transferase
methylation	catechol-o-methyl transferase

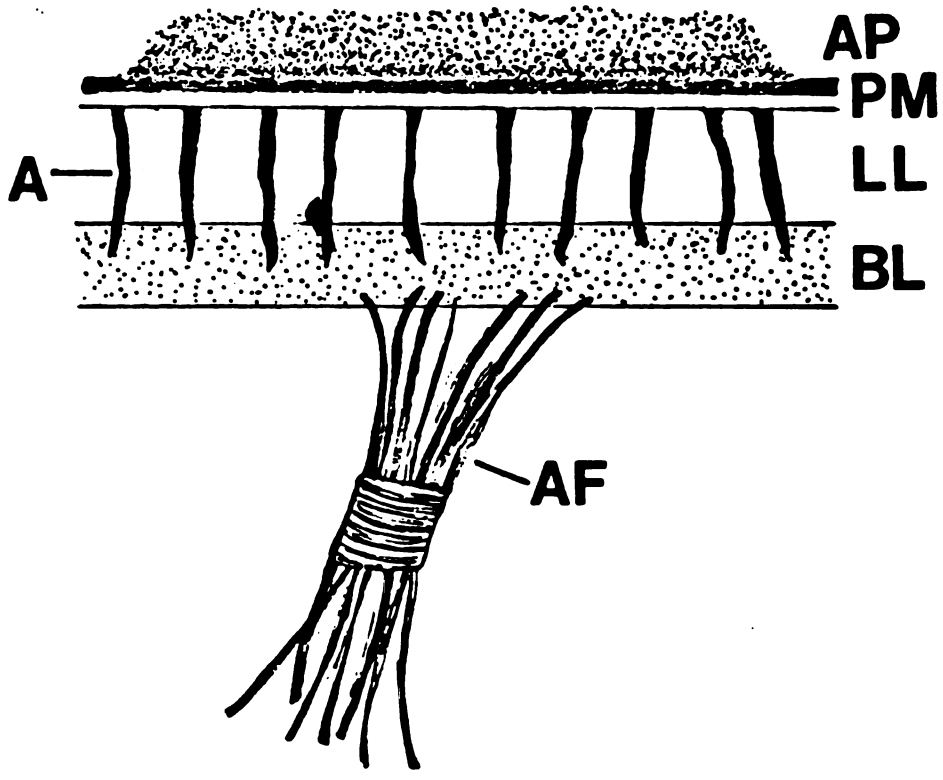


Figure 4. Diagram of epidermal-dermal junction shown in the area of the hemidesmosome. Attachment plaque (AP). Plasma membrane (PM). Lamina lucida (LL). Anchoring filaments (A). Basal lamina (BL). Anchoring fibrils (AF). Adapted from Briggaman, R.A. *in* Goldsmith,L.A., (ed.), Biochemistry and Physiology of the Skin, Oxford University Press, 1983, pg. 1003.

In addition to supporting the epidermis, the dense connective network of the dermis encloses the vasculature of the skin, an extensive nerve network and the epithelial glands and appendage structures of the skin.

Dermal Circulation

Most of the cutaneous circulation maybe considered microvasculature and originates from cutaneous branches of the subcutaneous musculocutaneous arteries. Branches arising from the cutaneous arteries give rise to a small vessel network which lies deep in the dermis near and parallel to the dermal-subcutaneous fat interface. Arterial branches from this deep vascular plexus ascend toward the skin surface; when these vessels reach the upper dermis, they branch into smaller arterioles which develop a horizontal and diagonal plexus of interconnecting and looping microcirculatory elements, predominately parallel to the skin surface. From these arterioles, capillary loops are directed toward the epidermis in a perpendicular fashion, but slight twisting and slanting is not uncommon. The capillary loop is comprised of an ascending limb, a hairpin turn, and a descending limb. These loops drain into interconnecting microcirculatory venule networks in the upper dermal layer. These in turn drain into larger descending microcirculatory venous networks, which in turn pick up branches from the deep dermal plexus and drain into the subcutaneous venous system (Ryan, 1983).

The distribution of the blood supply to the epidermis is often described as a candelabra pattern, with the horizontal plexus as the candlesticks and the capillary loops as the candles. Usually there is only one loop per upper dermal interdigitation into the epidermis, with one loop supplying 0.04 - 0.27 mm^2 skin surface (50-100 μm between loops) (Ryan, 1983). The flow of blood through the dermal capillary network is usually sufficient to rapidly remove most penetrants from the site of absorption.

Sensory Nerve Endings

Nerve endings sensitive to pressure and temperature changes are widely distributed in the dermis and aid the individual in the reception of external stimuli to pain, touch, itch, tickle, pressure and temperature changes.

Appendages

Pilosebaceous Apparatus

Hairs are keratinous fibers growing out from the epithelial follicles widely distributed over the skin surface, with the exception of the soles and palms. The hair fiber emerges from the skin at an angle, the slope of which is generally directed toward the tips of limbs. The epidermal stratum corneum progresses deep into the follicle, but eventually decreases in thickness and becomes attenuated.

Sebaceous glands are lipid-secreting structures at the upper part of the hair follicle; they are usually unilobular or bilobular, and relatively uniform in size (Figure 5) (Odland, 1983). Their fatty secretion is discharged into a common duct which then empties into a piliary canal, somewhere between 200 and 500 μm beneath the skin surface. Sebaceous glands occur over most of the body in numbers approximating $100/cm^2$; but on the face, forehead and scalp, they are more frequent; on the backs of the hands and dorsum of the feet they are sparse; and on the soles and palms they are absent (Odland, 1983). The pilosebaceous canal contains considerable lipid, but there are no contractile forces to expel this lipid; it is delivered to the surface only as new lipid is formed in the glands. Table 3 gives a compositional breakdown of lipids found in the sebum for the forehead, chest and arm (Strauss et. al., 1983).

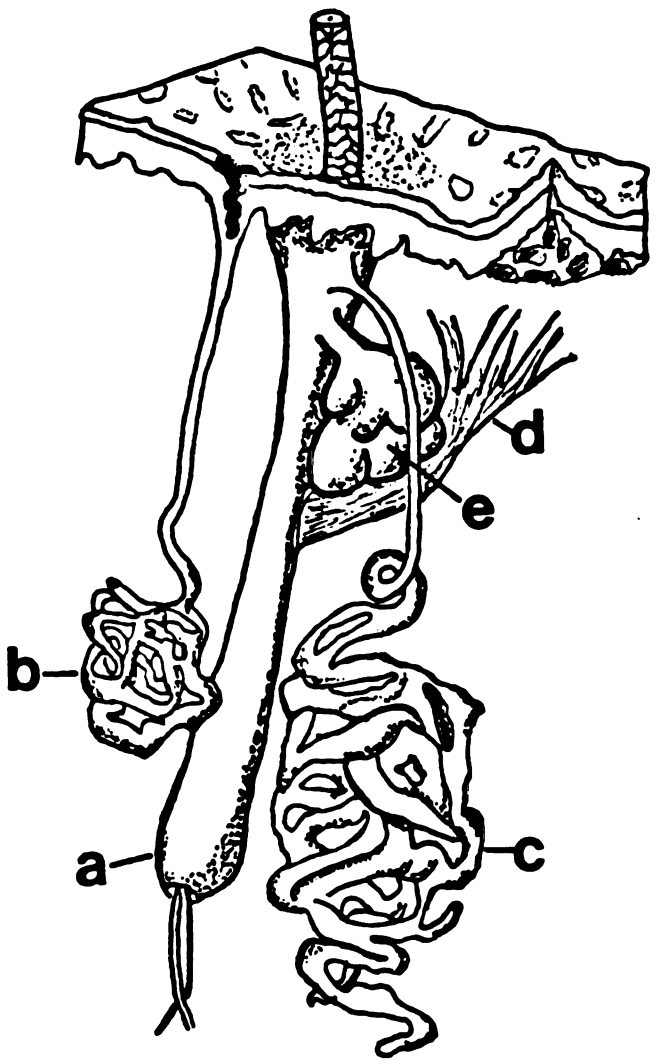


Figure 5. Diagram showing the relationship of (a) hair follicle, (b) eccrine gland, (c) apocrine gland, (d) smooth muscle and (e) sebaceous glands. Adapted from: Odland,G.F. *in* Goldsmith, L.A.(ed.), Biochemistry and Physiology of the Skin, Oxford University Press, 1983, pg. 40.

Table 3. Anatomical variation in the composition and amount of human skin surface lipids. Adapted from: Strauss et. al. in Goldsmith, L.A., (ed.), Biochemistry and Physiology of the Skin, Oxford University Press, New York, 1983, pg. 574.

Composition (weight %)	Forehead	Chest	Arm
Triglycerides	30.3	32.7	30.7
Diglycerides	2.3	2.3	1.5
Free Fatty Acids	24.1	28.1	36.4
Wax esters	27	21.7	15.8
Squalene	12.3	10.7	6.9
Cholesterol	1.2	1.6	4.1
Cholesterol esters	2.9	2.9	4.4
Total Lipid ($\mu g/cm^2$)	160	59	30

The secretions of the sebaceous and eccrine glands impart to the surface of the skin an acidic pH of about 5.5 (Noble, 1968; Abe et. al., 1980); with a normal range of 4.2 to 5.6 (Katz, 1971).

The lipid collected from the surface of the skin is a mixture of sebum and lipids produced by the epidermis. As indicated in Table 4, roughly equal concentrations of triglycerides are present in the lipids of both sources; cholesterol is almost totally of epidermal origin; wax esters and squalene are uniquely sebaceous (Strauss et. al., 1983).

Eccrine Sweat Glands

The eccrine sweat gland is a simple tubular gland arising from dermal or subcutaneous tissue. The duct coils as a slow helix into the dermis and epidermis from an irregular but closely arranged coil (Figure 5). The coil segment of the gland consists of half to two-thirds secretory elements and the remaining one-third, the ductal portion (Odland, 1983). At the basal surface of the epidermis, the duct epithelium becomes continuous with the basal cells of the epidermis, and the lumen continues on its helical course through the epidermis and on out through the stratum corneum. It has not been established whether the cells surrounding the lumen in the intra-epidermal segment of the sweat duct are ordinary keratinocytes (Odland, 1983) or a closely associated terminally differentiated epithelium.

Apocrine Sweat Glands

The apocrine sweat glands occur mostly in the axillae, in the perianal areas, the mammary areolae and in such areas as the mons pubis, the face, the scalp and the abdomen. The glands are complex tubular structures; their ducts paralleling the hair follicle to empty into the pilosebaceous canal, distal to the entrance of the sebaceous duct (Figure 5) (Odland, 1983).

The secretory composition of the apocrine glands remains obscure. Since the human apocrine gland and the sebaceous gland share the same opening on the skin surface, it is difficult to determine the composition of secretion from the gland itself since the total secretion at the skin surface is a mixture of sweat gland and sebaceous secretions.

Table 4. Compositions of human sebum and epidermal lipids. Adapted from: Strauss et. al. in Goldsmith, L.A., (ed.), Biochemistry and Physiology of the Skin, Oxford University Press, New York, 1983, pg. 574.

Component Lipids	Sebum (weight %)	Surface Epidermal Lipids (weight %)
Tri-, diglycerides and free fatty acids	57.5	65
Wax esters	26	-
Squalene	12	-
Cholesterol	1.5	20
Cholesterol esters	3	15

Subcutaneous Tissue

Beneath the dermis is the subcutaneous tissue, separating the skin and the underlying musculature. The thickness of the subcutaneous tissue varies from region to region and it composed of various proportions of adipose and fatty connective tissue. The subcutaneous tissue insulates underlying tissue from temperature extremes, functions as a mechanical shock absorber, and is an energy reserve, to be called on during periods of nutritional deprivation.

Skin and Aging

Introduction

Physiological and biochemical changes occur in humans from infancy, through youth, adolescence, maturity and into old age. Human growth and development are characterized by rapid increases in many functions, which peak in most cases, by early adulthood. Thereafter, virtually all physiologic parameters show a roughly linear decline with age, beginning about the third decade (Mildvan and Strehler, 1960). Since these physiologic changes (i.e. a decline in cardiac index, standard glomerular filtration rate, renal plasma flow, maximum breathing capacity, etc.), generally begin immediately at the end of the growth and development phase and continue into old age, there is no pleasant "plateau" during the middle years. However, the linearity of functional loss also implies that the rate of aging does not increase as we become older. Thus, an 80 year old is aging only as fast as a 30 year old; he is not losing function at a more rapid rate.

An important characteristic of age-related physiologic changes is their variability. Several sources include changes within individuals and between individuals in a given population. Variability is present in all populations, but normally increases with age (Gilchrest, 1984).

Human skin is not exempt from these functional modifications. The following sections will focus on intrinsic aging changes, from youth to old age, of normal human skin. Human skin changes dramatically with increasing age and a general overview of known alterations, both histologic and physiologic, has been included.

Epidermis

Morphologic

The major adult aging changes in gross morphology of the skin include dryness (in other words, roughness), wrinkling, laxity and uneven pigmentation (Gilchrest, 1984).

Histologic

The skin surface is traversed by furrows that intersect in complex ways, creating a variety of geometric patterns called skin surface patterns (Lavker et. al., 1980). In older individuals, this skin surface pattern reveals a slight age -associated loss of regularity and shallowness of furrows. However the overall geometry is retained (Lavker et. al., 1986).

The number of horny cells does not appear to change with age and thus the stratum corneum retains its normal thickness of approximately 14 to 17 cell layers. However, the size of individual stratum corneum cells increases throughout life (Plewig, 1970; Marks, 1982).

The hydration state of the stratum corneum has been probed as a function of increasing age. Utilizing a viscoelastic technique, it has been shown that at least the outer layers of the stratum corneum in elderly individuals are drier than the young adult equilvalent (Potts et. al., 1984; 1985)

Average epidermal thickness remains fairly constant with advancing age, although the deeply invaginated rete pegs of the epidermis are retracted or totally absent (Figure 6). Retraction of the rete pegs also results in a flattened interface between epidermis and dermis (Lavker et. al., 1986). At the ultrastructural level, a flattening of this interface is also observed. In young skin, basal epidermal cells display numerous villus - like cytoplasmic projections into the dermis, resulting in a serrated, highly convoluted appearance at the epidermal/dermal interface. In aged skin, basal cells lack these serrations and a flattened epidermal-dermal junction results (Lavker et. al., 1986).

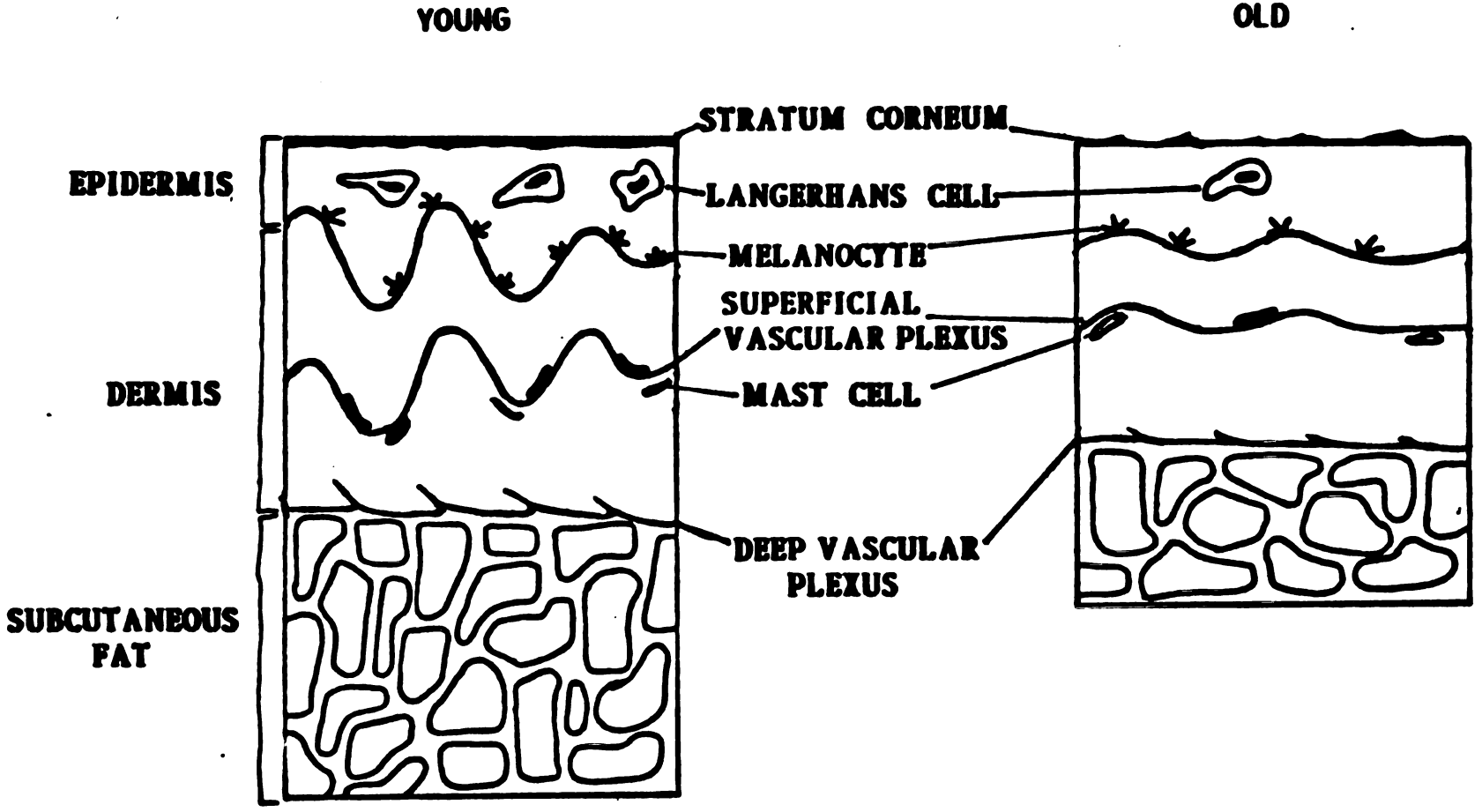


Figure 6. Diagram of normal human skin aging changes occurring in young and old skin. Note the overall decrease in dermal and subcutaneous tissue volume. Adapted from Gilchrest, B.A., Skin and Aging Processes, Boca Raton, Fl., CRC Press, 1984.

Aside from the dramatic flattening of the aged epidermal/dermal junction, other subtle alterations are observed. Although all components comprising the epidermal/dermal junction (lamina lucida, lamina densa, hemi-desmosomes, anchoring and elastin microfibrils) are present in aged skin, reduplication of the lamina densa and associated anchoring complex is periodically observed (Lavker et. al., 1986). These structures have been implicated in binding epidermis to dermis.

An overall decrease in the number of immigrant cell types in the epidermis has been shown. A decrease in the number of enzymatically active melanocytes, approximately 10-20 % of the remaining cell population each decade has been repeatedly documented (Fitzpatrick et. al., 1965; Quevado, 1969; Gilchrest et. al., 1979). It is not known whether the cells truly disappear or simply become undetectable by ceasing to produce pigment. Bone marrow - derived Langerhans cells have been shown to decrease nearly 50% between early and late adulthood (Gilchrest et. al., 1982b). Another characteristic feature of the aged epidermis is more frequent cytoheterogeneity. This is most noticeable in the basal region, where cells have displayed variations in size, shape and staining qualities (Lavker et. al., 1986).

Aside from increased basal cell atypia, the qualitative and quantitative aspects of keratinization do not appear altered with increasing age. Keratin filaments, lamellar bodies and keratohyalin granules are present in usual amounts (Lavker et. al., 1986). The horny cell, a filament/matrix complex enveloped by a thickened membrane, is not altered in its appearance (Lavker et. al., 1986).

Dermis

The dermis thins with age; a loss of dermal thickness can approach 20% in elderly individuals (Figure 6) (Black, 1969; Tan et. al., 1982). It has been postulated that the remaining attenuated and relatively acellular (Andrew et. al., 1965) dermis may account for the paper- thin, almost transparent quality of the skin of elderly individuals.

Collagen, the predominant tissue component of normal human dermis, comprises approximately 70 to 80 per cent of the dry weight of the dermis (Weinstein, 1960). In the young adult, collagen is organized as a network of

randomly oriented fibers and small tightly packed bundles, however, the overall weave of collagen appears random in the young adult (Lavker et. al., 1986). The most consistant change noted in aged skin is an apparent increase in the density of the collagen network (Lavker et. al., 1979). It is hypothesized that the increase in density reflects a decrease in space between individual collagen bundles. Elastin and the interfibrillar gel matrix normally occupy these spaces. Because the gel matrix decreases with age, removal of this material would imply a compaction of the collagen fibers and bundles. Furthermore, the organization of individual collagen bundles change from discrete rope-like bundles of tightly packed fibers to aggregates of loosely woven, primarily straight fibers (Lavker et. al., 1986).

Elastic fibers, another dermal component, usually occupy 2 to 4 per cent of the total volume of dermis in the normal adult (Weinstein, 1960). In the young adult, the scanning electron microscopic view of isolated elastic fibers reveals an extensive network separated by large open voids. In the aged dermis, the overall elastic fiber network appears markedly more dense. This alteration is attributed to an increase in the production of elastic fibers and a decrease in the void space between these fibers. The elastic fiber arrange-ment becomes more random and the clear individual fiber definition is lost (Lavker et. al., 1986). The precise histologic changes that accompany wrinkling have yet to be elucidated, although it is postulated that an age - related modification in elastic fibers may be contributory (Montagna and Carlisle, 1979)

The microvasculature in the dermis undergoes alteration with increasing age. In the normal young adult, the upper dermal vascular spectrum of growth ranges from atrophy to hypertrophy (Figure 7). In the center of the spectrum is a normal loop, surrounded by the epidermis and emptying into the venous plexus. This pattern represents the peak of the Gaussian curve of normal distribution and slight shifts to the right or left are common (Ryan, 1983). To the right there is slight elongation and tortuosity; to the left there is reduction in the overall length and diameter of the capillary loop. In the aging dermis, there is a trend toward atrophy (reduction in length and diameter of the loop) and decreased total number of capillary loops, but also greater overall heterogeneity so that a single vessel may be isolated in an area of atrophy.

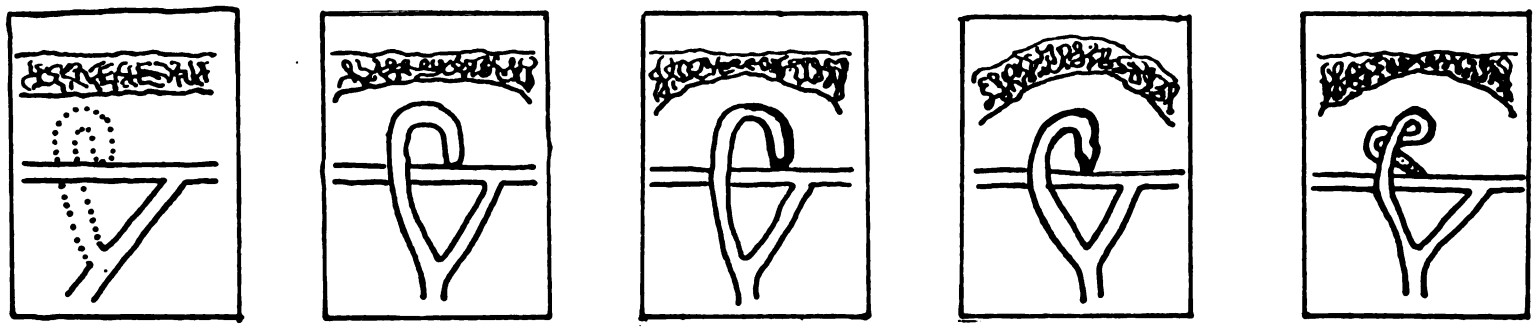


Figure 7. Spectrum of vessels types ranging from atrophy (far left) to hypertrophy (far right). Adapted from: Ryan, T.J. *in* Goldsmith, L.A.,(ed.), Biochemistry and Physiology of the Skin, Oxford University Press, 1983, pg. 841.

Appendageal

Age-related changes in hair color, density and distribution are widely recognized (Giacometti, 1965; Oberste - Lehn, 1965). Approximately half of the population by age 50 years has at least 50% white body hair (Keogh and Walsh, 1965); and an even higher proportion of depigmented scalp hair. Greying is due to the progressive and eventual total loss of melanocytes from the hair bulb (Fitzpatrick et. al., 1965). This loss of melanocytes by hair is believed to occur rapidly because the cells are called upon to proliferate and manufacture melanin at maximal rates during the growth phase of the hair cycle. Scalp hair is believed to grey more rapidly than other body hair because its growth phase to resting phase ratio is considerably greater than other body hair (Gilchrest, 1984). Advancing age is also accompanied by a gradual decrease in the total number of hair follicles; the remaining hairs may be smaller in diameter and slower growing. Scalp hair loss results primarily from the androgen - dependent conversion of the relatively dark thick scalp hairs to lightly pigmented short fine hairs (Barman et. al., 1969).

Eccrine glands decrease approximately 15 % in average number during adulthood at most body sites (Oberste-Lehn, 1965). Similiar quantification for apocrine glands is unavailable. The age pigment, lipofuscin, gradually accumulates in the secretory cells of both eccrine and apocrine glands with increasing age (Cawley et. al., 1973). Sebaceous gland size and number appear not to change with age, despite evidence of decreased function (Montagna, 1965).

Subcutaneous Tissue

In the seventh and eighth decades, there is an age-associated atrophy of subcutaneous tissue mass, first in distal portions of the extremities and lastly over the trunk (Steinberg, 1983). Since subcutaneous tissue serves as an important insulator of the body, its loss allows a greater amount of body heat to escape. Older persons often complain of being cold when others around them are comfortable. Complaints of being cold are caused largely by a loss of body insulation, and partially from a diminished blood flow to the skin and extremities.

Table 5 summarizes the changes that occur with increasing age to normal human skin.

Table 5. Histologic Features of Aging Human Skin
Adapted from: Gilchrest, B.A., Skin and Aging Processes, CRC Press, 1984, pgs. 19 and 25.

Epidermis	Dermis	Appendages
Flattened dermo-epidermal junction	Atrophy	Depigmented hair
Variable thickness	Fewer fibroblasts	Loss of hair
Variable cell size and shape	Fewer mast cells	Fewer glands
Fewer melanocytes	Fewer blood vessels	
Fewer Langerhans cells	Shortened capillary loops	

Physiologic Changes

Proliferation and repair

In an early study of normal individuals, (Epstein and Maibach,1965), no correlation between age and labeling indices (using thymidine-^{3}H) could be observed for the proliferative capacity of the viable epidermis. Baker and Blair (1968) eliminated the need for radioisotopes by using a fluorescent marker dye that binds avidly to the nonviable keratinocytes but not the underlying viable epidermal cells. Thus the time required for the dye to disappear is an indicator of the turnover or transit time of the stratum corneum. The results of Baker and Blair (1968) suggested that epidermal cell renewal is prolonged up to 100% in young versus old men. Unfortunately, the marker dye employed, tetrachlorosalicylanide, is now known to be a mild irritant, complicating the interpretation of these results. More recently, an age-associated decrease in epidermal turnover rate of approximately 30 to 50 per cent between the ages of 30 and 80 years has been determined by studies of desquamation rates for keratinocytes at selected body sites (Grove and Kligman, 1983). The marker dye used here, dansyl chloride (Jansen et al., 1974), has not been shown to have irritating effects. Others have also utilized dansyl chloride fluorescence to show that stratum corneum turnover time is prolonged with age (Takahashi et. al., 1987).

Repair rate in the skin also declines with age; whether measured as development of wound tensile strength (Sandblom et. al., 1953), collagen disposition (Viljanto, 1969) or regeneration of excised blister roofs (Grove, 1982). The most extensive of these studies compared the stratum corneum regeneration rate of 12 young individuals (18-25 years) with that of12 aged individuals (65 - 75 years) (Grove, 1982a). Topical application of a 50% ammonium hydroxide solution raised subcorneal blisters. The blister roofs were excised and the resulting wound repeatedly observed until normal skin surface markers were restored. This process required a median of 3 weeks in the young group compared to 5 weeks in the aged group.

Microvascular clearance

The progressive, dermal microcirculatory atrophy with increasing age may be hypothesised to cause a decrease in the clearance of trans-epidermally absorbed materials from the dermis. There is some evidence that this

hypothesis may be true. After intradermal injection of 0.5 ml saline in young and old subjects, resorption required approximately 39 to 65 minutes in 21 to 30 year olds, but 40 to 110 minutes in 70 to 83 year olds, with the average time almost twice as long in the elderly (Kligman, 1979). Others have also evaluated the time taken for a wheal raised by intradermal injection of saline, to flatten (Aschner, 1960), and have found an age-associated increase in microcirculatory clearance time.

Furthermore, following intradermal injection of ^{22}NaCl, Christophers and Kligman (1965), and Tagami (1972), have both shown in the midback area of elderly subjects, a decreased ability of the microcirculation to clear penetrant with age. DeSalva and Thompson (1965), however, performed essentially identical *in vivo* studies (investigating intradermally injected ^{22} NaCl disappearance from the skin of the hands, face and forearms) and contradicted the earlier results. DeSalva and Thompson found essentially constant removal rates for subjects between 50 and 90 years, and suprisingly, reported the rate of elimination to be slower in the young population group (21-30 years). It is clear that a concensus on the functional capacity of aged microvasculature has yet to be established.

Barrier Function

Evidence that the percutaneous absorption (PA) of certain chemicals may decrease with increasing age was documented by a limited number of studies. The first extensive study was completed by Christophers and Kligman (1965). These authors measured PA in aged skin *in vivo* . PA in the midback region to ^{14} C-testosterone was shown (by residual analysis methodology) to be greater in young subjects (19-30 years) than in aged subjects (71-82 years).

A similiar study was performed by Tagami (1972) who measured the permeability of the stratum corneum *in vivo* to tetrachlorosalicylanide (TCSA). The penetration times of TCSA (administered as a1.5% solution in ethylene glycol) through the skin of both flexor and extensor forearm were significantly shorter in young (22-39 years) than in old (62-82 years) men. PA was evaluated by stripping the stratum corneum at various times after application and assaying for the penetrant by fluorescence. Tagami concluded that the lower PA in the aged reflected an increased barrier function of their

stratum corneum. Recently, the results of Rougier et. al. (1988) also confirm a decreased penetration ^{14}C-benzoic acid in old subjects (65-80 years).

However, these observations contrast with those obtained by Grove et. al. (1982) who compared the blistering response time of young (18-25 years) and old (65-75 years) adults to topical application of 50% ammonium hydroxide solution. On the volar forearm and upper inner arm, the initial response, characterized by minute perifollicular vesicles, appeared earlier in the old group. These results were suggested by the investigators to be consistent with either a reduced stratum corneum barrier or an increased shunting of irritant material through appendageal orifices. Contrary, though, to the initial response, the time required for blister development averaged twice as long in the old group, indicating a decreased transudation rate with increasing age in injured skin. It still is not clear whether these age-associated differences are completely linked to skin penetration characteristics, particularly as the penetrant, in this case, causes major barrier damage.

Other experimental work that addresses the question of whether the barrier function of human skin changes with increasing age includes studies measuring alterations in transepidermal water loss (TEWL). Kligman (1979) compared TEWL in young subjects (19-26 years) with that in elderly volunteers (66-81 years). Measurement of this parameter addresses whether the integrity of the horny layer, with respect to its ability to impede the passive loss of water from the body, alters with increasing age. The clear hypothesis is that there is an alteration in aged skin that leads to a generally lower level of water within the horny layer and accounts, therefore, for the dry appearance of the skin of the aged. However, the results of Kligman's study, which included measurements both on the leg and forearm skin, showed no significant difference in TEWL as a function of age. It appears, therefore, that the stratum corneum provides an equally efficient barrier to TEWL in aged subjects and in normal adults.

In another study examing the effect of increasing age on TEWL, Leveque et. al. (1984) presented data which suggests that TEWL may decrease slightly with increasing age. However, these authors do not correct their measurements to a

constant skin temperature, thus increasing the difficulty of data interpretation.

Epidermal / dermal adhesion

Epidermal-dermal separation has been reported to occur more readily in the elderly (Grove, 1981; Kiistala, 1972). This finding has been implied by, and could be predicted from, the histologic observation of the decreased interdigitation between dermis and epidermis. A poor adhesion between these two cutaneous compartments would also explain the tendency of the elderly to suffer "torn" skin and superficial abrasions following bandage removal and other minor traumas (Gilchrest, 1984).

Response to external stimuli

Cutaneous pain threshold, defined as the reporting of discomfort due to quantitated radiant heat focused on a small area of skin, has been shown to increase by up to 20% with advancing age (Sherman and Robillard, 1964; Procacci et. al., 1970).

Eccrine sweating is markedly diminished with age. Spontaneous sweating in response to dry heat, measured on digital pads, was reduced more than 70% in healthy old individuals as compared to young individuals (Silver et. al., 1965; Mackinnon, 1954). This change has been attributed to a decreased output per gland.

A decrease in sebum production of approximately 60% with increasing age, is attributed to the concomitant decrease in production of gonadal or adrenal androgen, to which the sebaceous glands are sensitive. This change, occurring in both men and women (Pochi et. al., 1979), has not been linked to xerosis or seborrheic dermatitis (Gilchrest, 1984).

There have been indications that skin surface lipid compostion may also show an age-associated change in composition (Saint Leger, 1988). Alterations in compostion with increasing age have been shown for both saturated and unsaturated, straight and branched chain fatty acids (Nazzaro-Porro et. al., 1979; Yamamoto et. al., 1987).

A diminution in vascular responsiveness after application of standardized irritants (DMSO, ethyl nicotinate, lactic acid, chloroform - methanol), histamine and the mast cell degranulating agent 48/80 was assessed clinically in old and young individuals using erythema, stinging, wheals, vasodilation and transudation (Grove, 1981).

Intensity of erythema following a standardized UV exposure decreases with age. However, factors other than a decreased vascular responsiveness may be contributory to this result. In one study, a 50% reduction in mast cells and a 30% decrease in venular cross-sections were noted in the dermis of buttock skin from old adults compared to young controls. These histological results were associated with a corresponding reduction in histamine release and other manifestations of the inflammatory response following a standardized UV light exposure (Gilchrest, 1982b).

The elderly may also have compromised thermoregulation, predisposing them to hypothermia and possibly heat stroke. This may be due in part to a reduced vascular responsiveness (either vasoconstriction or dilatation), a decreased eccrine sweat production, or a loss of subcutaneous fat.

Immune Function

An age-associated decrease in delayed hypersensitivity reactions in human skin is manifested by the relative inability of healthy older subjects to develop sensitivity to dinitrochlorobenzene (DNCB) and by a lower rate of positivity for standard test antigens in the elderly compared to young adult controls (Gilchrest, 1984).

There is a documented decrease in cutaneous manifestations of immediate hypersensitivity with increasing age. In one study of over 300 subjects, the fraction with at least one positive wheal-and-flare reaction to a standard battery of potential allergens fell from 53% at age 20 years to 16% at age 75 years (Barbee et. al., 1976). The investigators were unable to determine the relative contributions of systemic vs. local cutaneous changes in this decline.

Vitamin D production

Vitamin D production is a skin endocrine function that is suspected to decline with age (Gilchrest, 1984). In response to solar radiation, both the epidermis and dermis convert 7-dehydrocholesterol to previtamin D_3, which undergoes spontaneous conversion to vitamin D_3 (Holick, 1981). Due to limited penetration of the responsible wavelengths into human skin (Parrish et. al., 1978), this reaction occurs principally in the epidermis. Vitamin D_3 is then transported to the general circulation by a carrier protein, and subsequently hydroxylated by both liver and kidneys to its active form, 1,25 - $(OH)_2$-vitamin D (Holick et. al., 1980). A linear decrease in the level of 7-dehydro-cholesterol per unit skin surface area by approximately 75% between 18-85 years has been determined (Holick and MacGlaughlin, 1981). This suggests that a lack of immediate biosynthetic presursor may be at least partially responsible for limited vitamin D production.

There are no obvious references at present that address whether other skin biotransformation reactions are altered with increasing age.

Percutaneous Absorption

Introduction and historical perspective

Molecules may penetrate the skin via several different routes, as diagrammed in Figure 8. An area of much debate, exactly which route(s) of penetration predominates and where the barrier to permeability resides within the skin, has occupied scientists for more than a century. Beginning around 1853, it was recognized that the skin's various layers were not equally permeable (Homalle, 1853; Duriau, 1856). The work of Schwenkenbecker (1904), concluded that the degree of permeability depends upon the nature of the penetrant; water and electrolytes are relatively impermeable compared to lipid-soluble substances.

Since this early work, scientists from various disciplines have focused on the anatomy and physiology of the skin; the route(s) of penetration, and the influence of the penetrant and vehicle characteristics on skin penetration. The experiments of Berenson and Burch, 1951; Blank, 1952; 1953 and Monash and Blank, 1958 revealed the epidermis to be the primary barrier to water diffusion. Blank in 1953, using a stripping technique, found that the

permeability of excised full-thickness skin remained practically constant until the lowest part of the stratum corneum was removed. He concluded, incorrectly, that a "thin layer in this region must contain the rate-limiting barrier"(Blank, 1953). This observation implies that only a thin layer near the base of the stratum corneum is quite impermeable, but not necessarily more impermeable than the upper layers. In 1965, Blank reconsidered his observations and concluded that the bulk of the stratum corneum is a uniformly good diffusional barrier (Blank, 1965). This conclusion has been further confirmed by Monash and Blank (1958) and by Monash (1957).

Currently the bulk of the stratum corneum is accepted as the primary barrier to percutaneous penetration. However, for highly lipophilic substances, their unfavorable partitioning into the underlying, aqueous viable epidermis can become rate-limiting. Some resistance is thought to occur at the epidermal-dermal interface, however penetrating molecules generally pass easily through to the dermis and on to the blood vessels of the dermal capillary network (Griesemer, 1960).

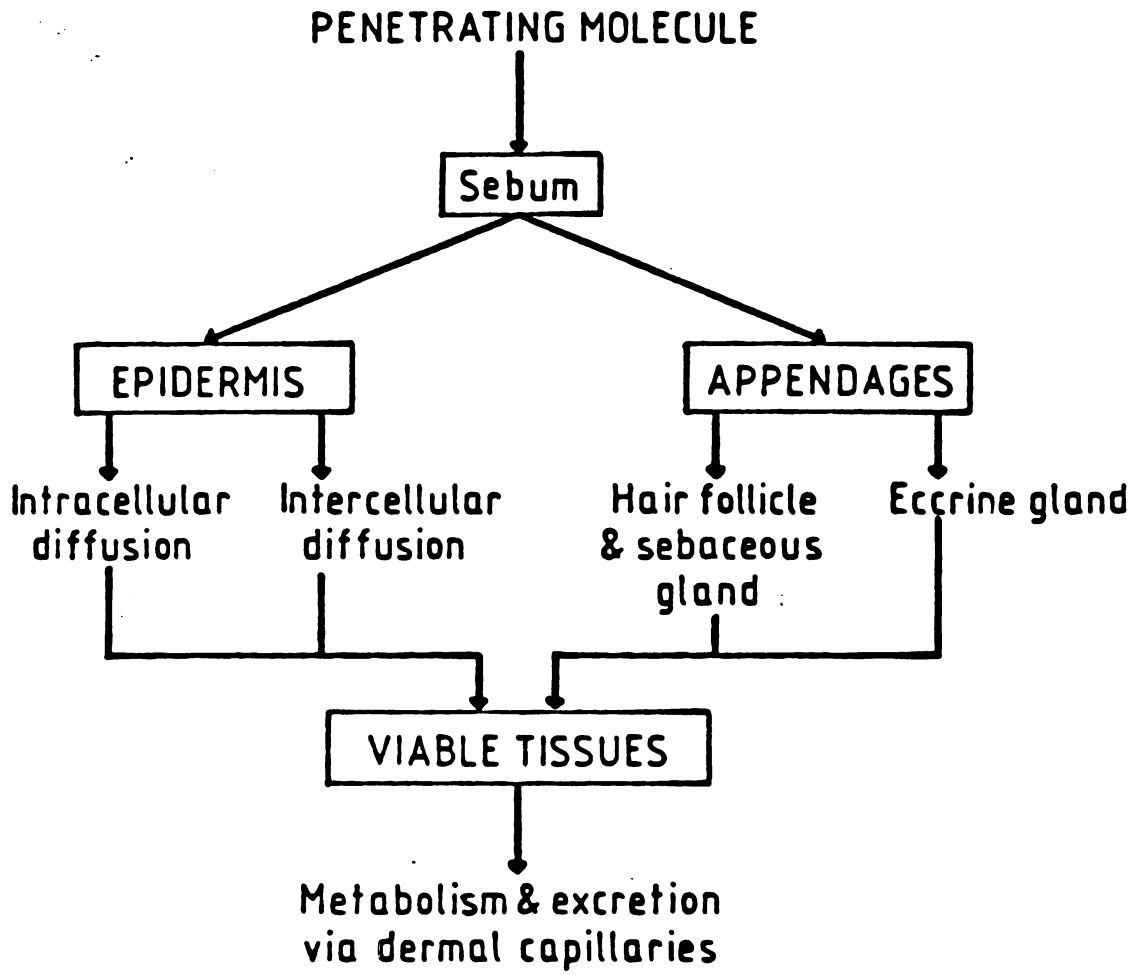


Figure 8. **The possible routes of penetration through the skin. Modified from Katz and Poulsen, 1971.**

Definition

Percutaneous absorption (PA) refers to the entire mass transport process of topically applied substances (Scheuplein and Bronaugh,1983) :

a) adsorption by the skin surface
b) slow diffusion though the stratum corneum
c) partitioning into the viable epidermis
d) diffusion through the viable epidermis and upper dermis (including traversing of the epidermal-dermal boundary)
e) uptake into the microcirculation of the dermis

The discovery of active transport processes in other biological membranes led some investigators to propose a similiar mechanism for epidermal penetration. Scheuplein (1973) reviewed numerous *in vivo - in vitro* correlations obtained for water, ions and non-electrolyte permeation and found relatively good agreement. He hypothesised that if such an active transport mechanism did exist, permeation through excised skin would differ greatly from that through intact skin. The minor differences found between the two methods, he concluded, are most likely due to damage from epidermal separation and the degree of skin hydration, rather than from an active transport mechanism. Currently, percutaneous transport is generally believed to involve passive diffusion alone (Scheuplein, 1973).

Since percutaneous penetration is a passive diffusion process, it can be appropriately described by Fick's Laws of Diffusion. These equations relate the flux of a penetrant molecule through a medium to the concentration, time and the diffusivity of the penetrant molecule. In the case of transport through the skin barrier, additional terms, i.e. the partition coefficient of the penetrant into the medium through which it is diffusing and the diffusional path length, are also included (Kedem and Katchalsky, 1961; Scheuplein and Blank, 1971; Scheuplein and Bronaugh, 1983).

Routes of epidermal penetration

From Figure 8, it can be seen there are potentially two routes of diffusion through the stratum corneum; transepidermal and transappendageal. Questions remain as to which is the principal route of penetration. The pros

and cons of each pathway have been reviewed in detail elsewhere (Katz and Poulsen, 1971; Idson, 1975; Barry, 1983).

Sebum

At the skin surface, a highly viscous, but fluid lipid film of varying quantities (5-150 μg / cm^2) (Schaefer, 1973) and thicknesses (0.05 - 1.5 μm) exists. This discontinuous film, primarily of sebaceous origin, has been shown to have little or no barrier properties (Kligman, 1963) to transepidermal water loss.

Appendages

Substances can bypass the stratum corneum and penetrate via the skin appendages: the hair follicles, the sebaceous glands and the sweat ducts. However, their overall contribution to skin permeability is probably relatively minor owing to their relatively small fractional area available for diffusion, only 0.1% of that of the stratum corneum (Scheuplein, 1967). Furthermore, these appendages do not constitute a completely 'open-pore' area, but in reality are filled with hair keratin, sebum or (in the case of sweat ducts) water (Scheuplein, 1967).

Epidermal

Since the stratum corneum is a heterogeneous membrane composed of terminally differentiated cells embedded in a lipid-rich intercellular matrix, two routes of penetration are available to the penetrant molecules (Figure 9); intra-cellular (across the stratum corneum bulk) or intercellular (between the cells).

Intracellular route

Although a more direct route for diffusion of molecules through the stratum corneum, this mechanism involves repeated partitioning into and out of the keratinocytes of the stratum corneum and diffusion through the intercellular lipid. It has been assumed that water-soluble penetrants traverse the stratum corneum via the keratin filaments and lipid-soluble penetrants via the lipid-rich material between the filaments, customarily referred to as the polar and the non-polar pathways (Scheuplein and Blank, 1971; Cooper, 1984).

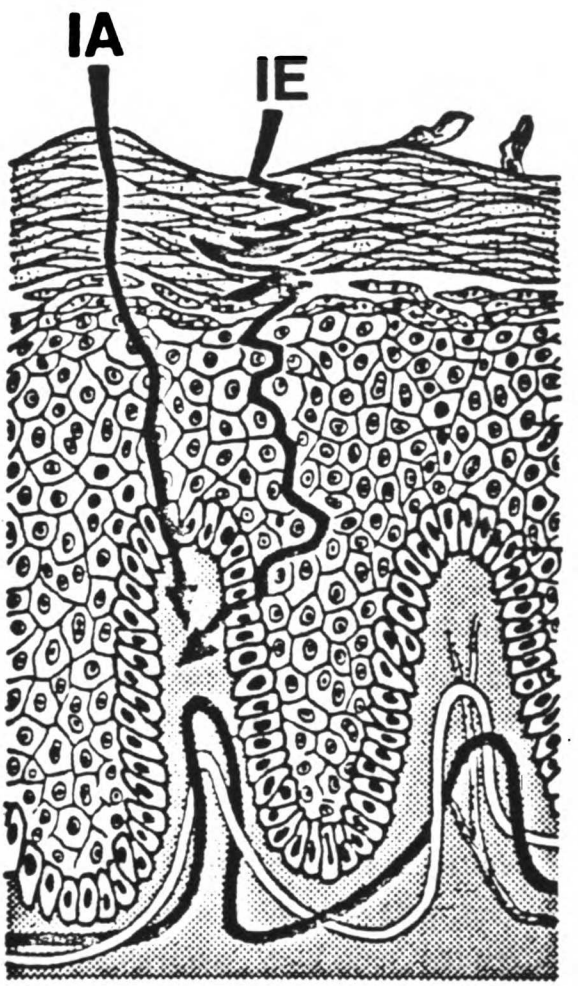


Figure 9. Potential routes of epidermal penetration (IA) intracellular or (IE) intercellular. Modified from Katz and Poulsen, 1971.

Intercellular route

Until recently, this diffusional pathway was regarded as rather insignificant to overall stratum corneum permeability since the hypothetical volume of the intercellular space was approximately only 1%. Recent morphologic and experimental evidence has challenged this view; increasing the hypothetical volume of the intercellular space from approximately 1% to between 5 and 30% of the total stratum corneum (Elias, 1981b). Support for this model of penetration derives from qualitative evidence of selective permeation of the intercellular route by water-soluble and lipophilic tracers (Elias, 1983) and some evidence that regional differences in lipid content correlate well with permeability differences (Elias, 1981a). In addition, Albery and Hadgraft (1979) concluded for small molecules, this route may predominate as the major route for diffusion.

In conclusion, after many years of research into the mechanism of percutaneous absorption, scientists have failed to elucidate conclusively the precise pathways involved. However, the intercellular route may be more significant that first hypothesized. The route of penetration will most likely depend on the physicochemical nature of the penetrant and may involve a contribution from each alternative discussed.

Factors influencing percutaneous absorption

The passive diffusion of penetrant from a vehicle into and through the skin is dependent on several interactions: drug-skin, vehicle-skin, drug-vehicle and drug-vehicle-skin. Thus, the primary factors influencing the penetration of chemicals through human skin include the physicochemical properties of the penetrant (i.e. lipophilicity, ionization and molecular size), the application vehicle and the skin.

Lipophilicity

The ability of a chemical to leave the vehicle and permeate the stratum corneum is related to the degree of lipophilicity of the penetrant molecule, as reflected by the partition coefficient of the penetrant between the vehicle and the stratum corneum. The partitioning criteria for a penetrant is demanding. The molecule must favor the stratum corneum over the vehicle, and the relative affinity of the drug for the stratum corneum and viable tissue must be

reasonably balanced. Attempts have been made to correlate the absorption of a substance on the basis of partition coefficient (water, oil, ether, hexadecane, stratum corneum - water, etc,). Investigations, particularly of homologous series of compounds, have yielded limited success (Stoughton et. al., 1960; Clendenning and Stoughton, 1962; Marzulli et. al., 1965; Blank, 1967; Scheuplein at. al. ,1969, Bucks, 1988a). Although partition coefficients are a useful qualitative indicator of penetration, they should be considered in conjunction with other factors such as binding of the drug in the epidermis (Bronaugh and Congdon, 1984) and solubility in the vehicle (Katz and Shaikh, 1965).

Ionization

According to the pH-partition hypothesis, the unionized form of a drug is better able to permeate the skin (Schanker et. al., 1957). This has been most recently illustrated by the pH dependency for optimal methotrexate penetration (Siddiqui et. al., 1985; Vaidyanathan et. al., 1985). Iontophoresis and iontophoretic systems have been proposed as a convenient means of achieving constant and controllable percutaneous absorption for ionized drug species (Bellantone et. al., 1986).

Molecular size

The size and shape of a penetrant molecule also play a part in its ability to penetrate the skin barrier. Feldmann and Maibach have shown that even closely related compounds show great differences in rate and extent of absorption (Feldmann and Maibach, 1970). Tregear demonstrated that large molecules, such as proteins and polysaccharides do penetrate skin, albeit poorly (Tregear,1966). Currently, the most successfully used transdermal drugs have molecular weights less than 1000 daltons.

Vehicle effects

From a physical chemical standpoint, topical formulations should be optimized to provide the highest thermodynamic activity of drug in the vehicle to yield the greatest partitioning of drug into the skin with a concomitant increase in flux (Poulsen, 1973). A change in the character of the solvent may cause an alteration in the affinity of the permeant for the solvent and subsequently, a modification in the partitioning of the permeant into the stratum corneum

(Zatz and Dalvi, 1983). Numerous publications have examined the effects of vehicles on the penetration of a variety of compounds, these have been reviewed extensively elsewhere (Idson, 1983; Barry, 1983; Gummer, 1984).

Penetrant - Skin interactions

The significance of cutaneous binding remains at this time an area of limited knowledge and understanding. Vickers showed the existence of a reservoir in the stratum corneum for steroids, releasing the drug at a slow rate, long after surface removal of the drug (Vickers, 1963). It is hypothesised that this effect is due the binding of the drug by keratin and other components of the stratum corneum and its subsequent diffusion through the epidermis retarded.

Factors which may also influence skin permeability include:

(1) The ability of the skin to act as a barrier against the ingress of foreign substances. This function may be compromised by external forces such as burns (Behl et. al., 1983), mechanical injury such as wounds, punctures or tears, or skin diseases such as eczema or psoriasis (Katz and Poulsen, 1972).

(2) The regional variation in permeability of skin at different sites. This variability is dependent on skin thickness and lipid composition of the stratum corneum (Elias, 1981a). In man, the extant data suggest the following order: plantar > palmar > dorsum of the hand > scrotal and postauricular > axillary, scalp > arms, leg and truck for steady state diffusion of simple, small molecules through skin (Scheulplein and Blank, 1971).

(3) The evidence of both inter- and intra - individual variability in penetration, compounded with other factors such as age, sex, body fat, previous exposure, nutrition and type of skin exposure, have been shown (Matoltsy et. al., 1968; Ramasastry et. al., 1970).

(4) The effect of age on the penetration of chemicals through the skin has been previously discussed.

(5) The surface of the skin harbors a wide range of micro-organisms capable of degrading topically applied compounds. Prediction of the influence of such degradation on plasma levels indicate that surface degradation can be significant, especially if the formulation is in the form of a thin-film (Denyer et. al., 1985).

(6) The rate of blood flow in the dermal microcirculation is usually sufficient to rapidly remove most penetrates from the site of absorption and has minimal effect on percutaneous absorption unless it is dramatically reduced and assumes the role of rate limiting step (Tregear, 1966).

(7) The degree of hydration of the stratum corneum is an important factor in controlling the rate of penetration and has been extensively reviewed by Idson (1978). Normal water content of the stratum corneum usually varies between 10-20%, but may increase to approximately 50% under occlusion (Barry, 1983; Potts, 1986). Increased hydration has been shown to dramatically increase the permeability of the skin to polar molecules (Wurster and Kramer, 1961; Cronin and Stoughton 1962;1963). Furthermore, occlusion of the site of application with Saran wrap (to increase the degree of hydration) has been used successfully to promote the absorption of steroids (Sulzberger and Witten, 1961; Vickers, 1980). However, the recently published data of Bucks et. al. (1988b) shows that occlusion does not enhance the penetration of hydrocortisone *in vivo*.

<u>The Elements of Percutaneous Absorption-The Effect of Skin Aging</u>

Within the process of PA there are several specific elements which may be affected by the morphologic and physiologic changes within the skin associated with increasing age past adulthood. A change in any one of these components may lead to an observed change of the measurable penetration as a whole. The primary elements known to comprise the process of percutaneous penetration are: the ability of the skin to act as a barrier to the ingress and egress of compounds, transport of the compound through the skin and finally uptake of the compound by the cutaneous circulation.

Cutaneous, age-associated changes which may be predicted to impact on the absorption process include those occurring in the epidermis and those at the

dermo-epidermal junction, where uptake of penetrant into the general circulation takes place. Among the many well-documented changes that occur in aged skin, those which may be hypothesized to impact on PA include:

(1) The stratum corneum of the elderly is drier than the young adult equivalent (Potts et. al., 1984; Potts and Buras,1985). Hydration of the horny layer alters the environment of the skin encountered by a topically applied penetrant molecule and, as a result, may affect percutaneous absorption (Blank, 1985; Scheuplein and Ross, 1970).

(2) Sebaceous gland activity is reduced in aged skin and the amount of skin surface lipid is lowered (Pochi et. al., 1979). Additionally, the composition of skin surface lipids may change with increasing age (Nazzaro-Porro et. al., 1979; Yamamoto et. al., 1988). An initial and crucial step in PA is the partitioning of drug into the lipids of the stratum corneum. This essential process will be sensitive to the type and amount of skin lipids (sebaceous and epidermal) present.

(3) The dermo-epidermal junction is flattened in aged skin and the number of dermal capillary loops is decreased (Gilchrest, 1984; Ryan, 1983). From histological examination, a compromised microcirculation and a poorer resorption capability is implicated for the elderly.

(4) Inter- and intra- individual variability in the PA of compounds. It has been documented that variability increases with increasing age and it cannot be reasonably assumed that skin penetration will be excluded from this observation. Keeping this in mind, it may be difficult to differentiate specific changes between the ages without a large subject population.

The work presented in this thesis specifically addressed the following:

(1) Is there an overall change in topical bioavailability (both rate and extent) of chemicals associated with increasing age past maturity?

(2) If a change is observed, is this change related to physicochemical characteristics of the penetrant molecule or is it a generalized phenomenon for all penetrating molecules?

(3) Are there specific elements within the process of PA that can be identified as being responsible for any changes observed in topical bioavailability?

Chapter 2

Percutaneous Penetration Studies

Introduction

Human skin und ergoes considerable change with increasing chronologic age. Numerous physiologic and biochemical changes within the tissue have been identified (Gilchrest, 1984). To reiterate, many of the well-documented changes that occur in aged skin include: (i) increased stratum corneum dryness (Potts et. al., 1984; Potts and Buras, 1985), (ii) reduction in sebaceous gland activity resulting in decreased skin surface lipids (Pochi et. al., 1979), (iii) flattening of the dermal-epidermal junction (Lavker et. al., 1986), and (iv) atrophy of skin capillary network resulting in a gradual attenuation of blood supply to the viable epidermis (Ryan, 1983). Relatively little is known about the influence of such age-related changes on the percutaneous absorption (PA) of drugs.

Thus, the objective of this study was to assess the effect of skin aging (as measured by increasing chronologic age), on the percutaneous penetration of a series of compounds (of different physicochemical characteristics) through human skin *in vivo* .

Background

In the neonate, reasonably good experimental PA data exists and has been recently reviewed (Fisher, 1985; McCormack et. al.,1982; Wilson and Maibach, 1982;). There is also a substantial literature describing *in vivo* PA in normal healthy adults (Feldmann and Maibach, 1969; 1970; Guy et. al., 1987). However, in the elderly (> 65 years) the quantitative evaluation of skin barrier function has been minimally addressed.

The *in vivo* observations of Christophers and Kligman (1965), and Tagami (1972) and Rougier et. al. (1988), suggest that with increasing age, PA decreases. The degree to which this reduction in penetration may be explained by impaired microcirculatory clearance, however, cannot be unequivocally deduced. It is clear that the *in vivo* database is small, that the reported observations are indirect, and that a very limited range of penetrants have been considered. An important objective of the experiments described below, therefore, was to circumvent these shortcomings.

Preliminary Studies

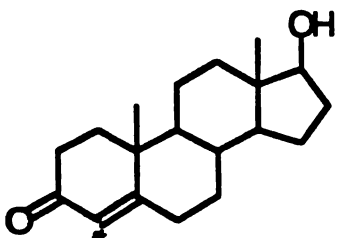
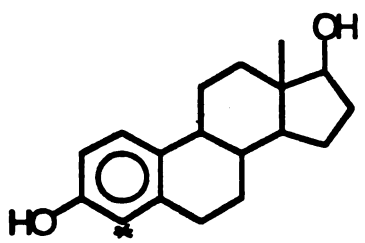
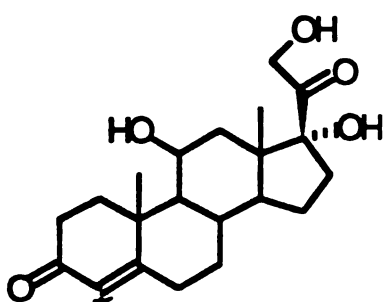
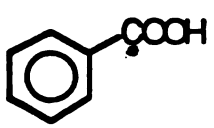
Investigations into the *in vivo* percutaneous penetration of four compounds of different physicochemical properties were conducted in two elderly populations, "young-old" (65-75 years) and "old-old" (over 75 years). The compounds studied, testosterone, estradiol, hydrocortisone and benzoic acid, are shown in Table 1.

These compounds were chosen for investigation because they provided a relatively wide range of known penetrability characteristics in normal adults and because they possessed a reasonably broad spectrum of physicochemical properties (solubility, oil/water partition coefficient, etc.). The penetrants studied are model compounds and were selected not for inherent potential therapeutic effect, but because control (that is, young subject) penetration data exist (Feldmann and Maibach, 1969; 1970). Direct comparison is therefore possible between this previously published data base and the current measurements.

Materials and Methods

Topical Administration: The site for topical administration was the ventral surface of the forearm, 9 centimeters below the elbow fold. The compounds were applied in 100 µl acetone, the rapid evaporation of which deposited the compound on the surface of the skin. The penetrant dose was 4 µg/ cm^2, the area of application was 40 cm^2. To enable quantitative assessment of absorption, the chemicals used were radiolabelled with ^{14}C. The maximum radioactive dose administered to any subject on a single occasion was $\leq 4\ \mu Ci$. No local irritation was observed following application of any of these chemicals. No dry crystals were visible on the skin surface following acetone evaporation ($\leq$ 30-45 seconds). While acetone is an effective lipid solvent, the method of application does not appear to remove skin lipids. Recent experiments from our laboratory provide strong evidence that this solvent-deposition approach does not, in any measureable way, affect skin barrier function (Hinz et. al., 1988).

Table 1. Compounds Studied in Unprotected Penetration Studies

	MW	Log K[a] (O/W)	Aqueous[b] Solubility
 Testosterone (47 mCi/mMol)	288.4	3.32	Insoluble
 Estradiol (56 mCi/mMol)	272.4	2.49	Almost Insoluble
 Hydrocortisone (51.6 mCi/mMol)	362.5	1.6	0.28 G/L
 pKa = 4.2 Benzoic Acid (53mCi/mMol)	121.1	1.83	3.4 G/L

[a] Leo et. al., 1971 [b] Merck Index
* Denotes site of radiolabel - ^{14}C

This method of application, a "finite" dose applied in an acetone vehicle, is patterned exactly after the method of application in the literature (for young control subjects) with which comparisons of absorption data were made (Feldmann and Maibach, 1969; 1970). Although acetone is not necessarily a perfect delivery system, it is simple, and permits percutaneous absorption to be evaluated *in vivo* in the absence of major vehicle-associated artifacts.

Following drug administration, the skin area was neither treated nor washed for 24 hours. At the end of 24 hours, the subjects were allowed to bathe freely. Percutaneous absorption was quantified by the rate and extent of excretion of ^{14}C measured in the subject's urine over the 5-day period following drug administration.

General: The experimental subjects were Caucasian adults (males and females), having no history of dermatologic disease, from whom informed consent, approved by the UCSF Committees on Human and Radioactive Drug Research, had been previously obtained(see appendix). The volunteers formed two groups: "young-old" (65-75 years) ($n \geq 6$) and "old-old" (> 75 years) ($n \geq 6$). All subjects were in good health and all had 24 hour creatinine clearance values normal for their age and weight. They displayed good mental acuity and were thoroughly educated in complete urine collection procedures. The subjects were non-smokers and were not receiving any prescription medication. Subjects were allowed aspirin throughout the course of the study, however, they were not allowed any aspirin 24 hours prior to dosing or during the 5 day urine collection.

The penetrants considered were testosterone, estradiol, hydrocortisone, benzoic acid. All compounds were ^{14}C - labeled (Research Products International Corp., Mount Prospect, ILL) and were > 99% pure as assessed by thin layer chromatography (see appendix).

Analysis: All urine was collected for 5 days (0 - 120 hours post dosing). The collection intervals were; day 1 : 0-4, 4-8, 8-12, 12-24 hours post dose; days 2-5: two 12 hour periods per day. At the end of the 5 day urine collection period, the radioactivity in the urine samples had returned to 2-3 times background levels and excretion of radiolabel was considered satisfactory. Aliquots (6 mL) of

urine (triplicate determination) were mixed with 10 mL scintillation cocktail (Beckman Ready Solv-MP) and ^{14}C content was measured by liquid scintillation counting (Searle Mark III Scintillation System). Decreased efficiency in counting the urine samples due to signal quenching was determined by adding an internal standard (toluene (ring - ^{14} C), New England Nuclear, Boston, MA) of known disintegrations per minute to each sample set. In this way, counting efficiency (approximately 73%) for each urine sample was measured. The amount of material found in the urine was corrected for incomplete renal elimination using a published intravenous correction factors (Feldmann and Maibach, 1969; 1970).

Results

The results are summarized in Figures 1(a-d) for testosterone, estradiol, hydrocortisone, and benzoic acid, respectively. Each figure shows a representative plot of cumulative percent dose absorbed as a function of time after application for (1) a control group of subjects (18-35 years); (2) the 'young-old" population (65-75 years); and (3) the "old-old" population (> 75 years). Representative plots were used due to the unavailability of raw data from the previous study of Feldmann and Maibach (1969; 1970). The complete data set is summarized in Table 2. The results show that for testosterone and estradiol, penetration in the old subjects was not distinguishable from that in the young controls. In contrast, the penetration of benzoic acid and hydrocortisone was significantly less in both elderly subject populations than that in the young control group. The significance of the differences in total penetration is indicated in Table 2. Also shown in this table are the water solubilities and and octanol-water partition coefficients of the four chemicals considered.

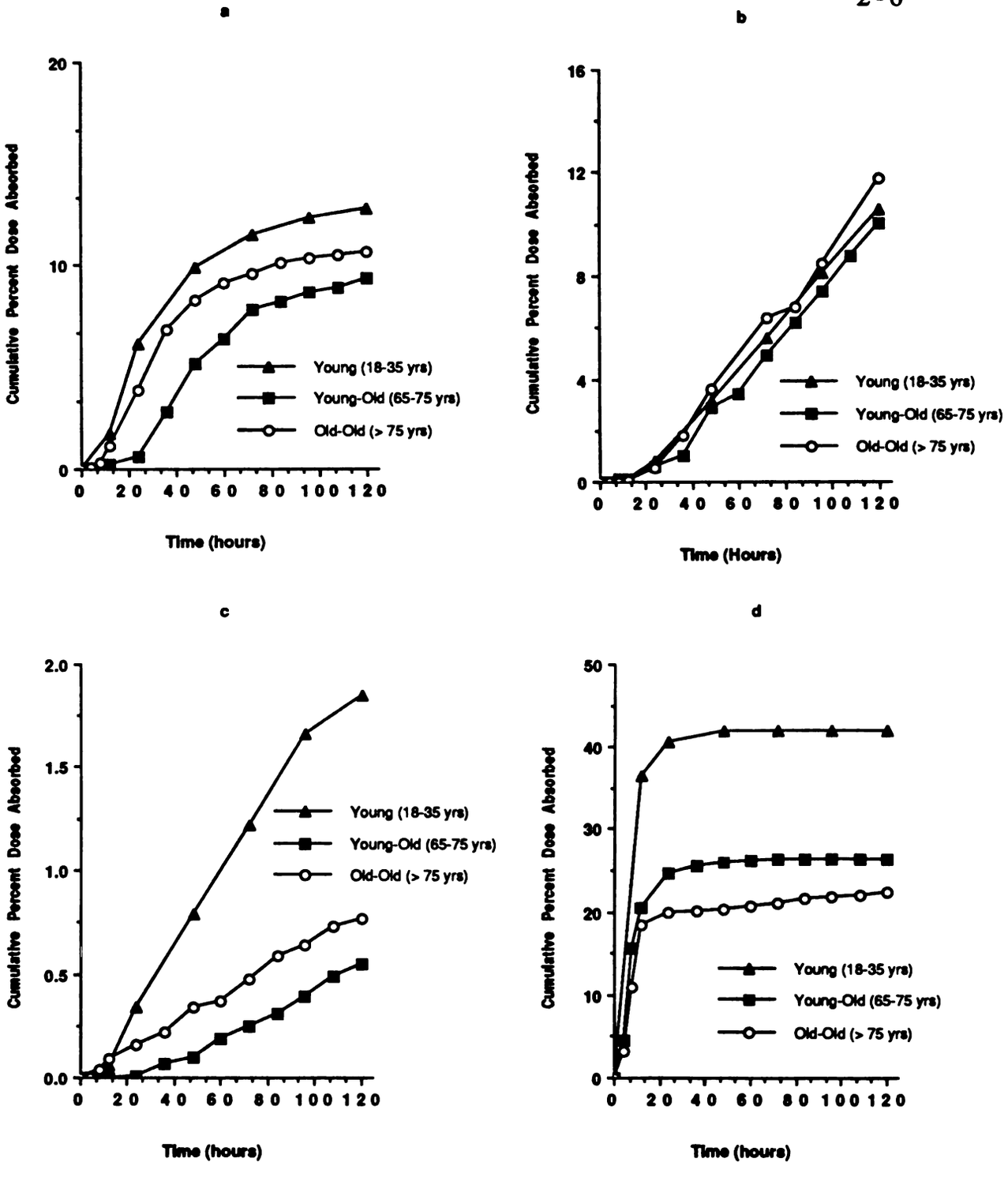


Figure 1. Representative curves of the cumulative percent dose absorbed as a function of time for (a) testosterone (b) estradiol (c) hydrocortisone and (d) benzoic acid. The young* (18-35 years) [closed triangles], young-old (65-75 years) [closed squares] and old-old (> 75 years) [open circles] are presented on each graph for comparison.

* Reference: Feldmann and Maibach 1969; 1970.

Table 2. Summary of Unprotected Penetration Studies

Compound	Aqueous Solubilities	Log K[a] (O/W)	Cumulative % Dose Absorbed [b] Young[c] (18-40 yrs)	Young-Old (65-75 years)	Old-Old (>75 years)
Testosterone	Insoluble	3.32	13.2±0.7 n=17	10.6±2.0[d] n=8	15.2±3.2[d] n=7
Estradiol	Almost Insoluble	2.49	10.6±2.8 n=3	11.5±1.4[d] n=6	9.0±2.3[d] n=6
Hydrocortisone	0.28 g/L	1.61	1.87±0.4 n=15	0.67±0.2[e] n=8	0.86±0.2[f] n=9
Benzoic Acid	3.4 g/L	1.83	42.6±6.7 n=6	27.5±4.1[f] n=8	23.1±2.3[e] n=9

[a] Reference: Leo et. al., 1971; solubilities were obtained from the Merck Index.

[b] Mean±S.E

[c] Reference: Feldmann and Maibach, 1969.

[d] Not significantly different from the young control group ($p>0.05$)

[e] Significantly different from the young control group ($p<0.01$)

[f] Significantly different from the young control group ($p<0.05$)

Discussion

We observe that percutaneous penetration of the two most lipophilic compounds (testosterone and estradiol) is not significantly changed in the elderly subject groups. However, aging can affect PA *in vivo* , and this is shown markedly by the results for hydrocortisone and benzoic acid, the two least lipophilic compounds considered.

The scope of the observations is limited, but it is tempting to suggest that the aging process in human skin differentiates in terms of barrier function between chemicals of different physicochemical properties. In particular, it appears that the barrier increases significantly with increasing age for compounds that are more hydrophilic than lipophilic (namely, hydrocortisone and benzoic acid).

The observations obtained thus far merited further investigation. However, at this opportunity, it was important to review the current methodology and to amend the protocol to circumvent any limitations in future experiments. The points necessary to address at this time were:

(1) The need for mass balance. Since a change in penetration of certain compounds was observed, it was essential to account for all topically applied compound, thus obtaining a complete dose accountability for each drug.

(2) Evaluation of a "young" (18-40 years) subject group. Up to this point, no reasonable *in vivo* mass balance system for topical drug delivery was available and therefore no literature values existed for this type of system. It was critical therefore, to produce young subject data using an accountable approach.

(3) Combination of elderly age groupings. Since "young-old" and "old-old" data were not disguishable, combination of these two groups into one group, under the singular heading of "old"(>65 years), afforded the opportunity to study a large subject population for which increased variability has been documented (Gilchrest, 1984).

(4) Inadequacy of the 5 day urine collection. For complete compound penetration to be evaluated, urine collection for a period of 5 days did not allow the the radioactivity in the urine samples to return completely to background for all compounds. From subsequent work in our laboratory, it was established that a 7 day urine collection would be more appropriate for certain compounds (i.e., those slowly absorbed or excreted).

(5) An increase in the number of chemicals for study. Since the preliminary studies evaluated only 4 compounds, inclusion of 2 additional compounds was considered sensible. The compounds chosen were relatively hydrophilic, since it was in this category that a decrease in penetration was observed.

(6) Finally, appropriate intravenous correction factors for the two populations. Meaningful topical bioavailability studies in different age groups frequently use individuals as their own controls (i.e. the subject given the drug extravascularly will also be given the same drug intravenously, for evaluation of incomplete urinary excretion on an individual basis). Alternatively, it is also appropriate to evaluate whether differences exist between the subject groups for total percent dose excreted into the urine following intravenous administration. Equally valid, this assessment allows one to discern whether a full cross-over study is mandatory. It was therefore imperative to obtain information about the total percent dose excreted into the urine, for each of the six compounds, following intravenous administration to the two subject populations. Literature values used in the preliminary studies had only been obtained in young (18-35 years) subjects (Feldmann and Maibach, 1969; 1970).

Final Studies

Following incorporation of the previous adjustments into a new working protocol, a comprehensive assessment of the effect of age on the penetration of chemicals through human skin *in vivo* was completed.

Materials and Methods

Topical Administration: The site of application for topical administration was the ventral surface of the forearm, 9 centimeters below the elbow fold. The compounds were applied in 20 µl acetone, the rapid evaporation of which

deposited the compound on the surface of the skin. The penetrant dose was 4 $\mu g / cm^2$, the area of application 2.5 cm^2. To enable quantitative assessment of absorption, the chemicals used were radiolabelled with ^{14}C. The maximum radioactive dose administered to any subject on a single occasion was 1 $\mu Ci / cm^2$. No local irritation was observed following application of any of the chemicals tested. No dry crystals were visible on the skin surface following acetone evaporation. While acetone is an effective organic solvent, the method of application does not appear to remove skin lipids.

Following acetone evaporation (<30 seconds), the application site was covered with a protective, but non-occlusive, device (Figure 2) (Bucks et. al., 1988a).Briefly, a Hilltop chamber®(Hilltop Research, Inc., Cincinnati, OH) was modified by boring six, equally spaced,4-mm-diameter holes in a radial pattern through the plastic chamber. Next, both the waxed paper backing and the cotton pad, with which the chambers are provided, were removed. Three reference lines were marked across the back of the plastic chamber and the manufacturer-supplied adhesive tape. The latter was then removed and an intact 2.35-cm diameter disk of polytetrafluoroethylene membrane (Goretex®; 0.2 micron diameter pore size) (W.L. Gore and Associates, Inc., Elkton, MD) was placed on the sticky side of the original adhesive tape. The plastic chamber was properly realigned on top of the Goretex® membrane (on the original adhesive tape) using the reference marks on the chamber and the tape to align the six holes through the plastic chamber with the six holes through the adhesive support tape. Thus, the holes though the plastic chamber and adhesive tape are "plugged" by the "sandwiched" Goretex® membrane. Finally, the reassembled device was returned to the original waxed paper backing of the plastic chamber. To secure the reassembled device on the subject's arms, adhesive transparent dressing (1625/Tegaderm®,3M, St. Paul, MN) was modified by punching a 2.35-cm-diameter hole in the center of a 7 x 6-cm sheet dressing. At the time of device application, the chamber was secured to the arm by centering the modified Tegaderm® over the modified chamber. Compared to the original manufacturer's adhesive, the additional use of Tegaderm® provided a more elastic, nonirritating and secure attachment. This device affords protection from loss of compound and exfoliated corneocytes, but allows normal evaporative water loss and gas exchange through the skin (Bucks et. al., 1988b).

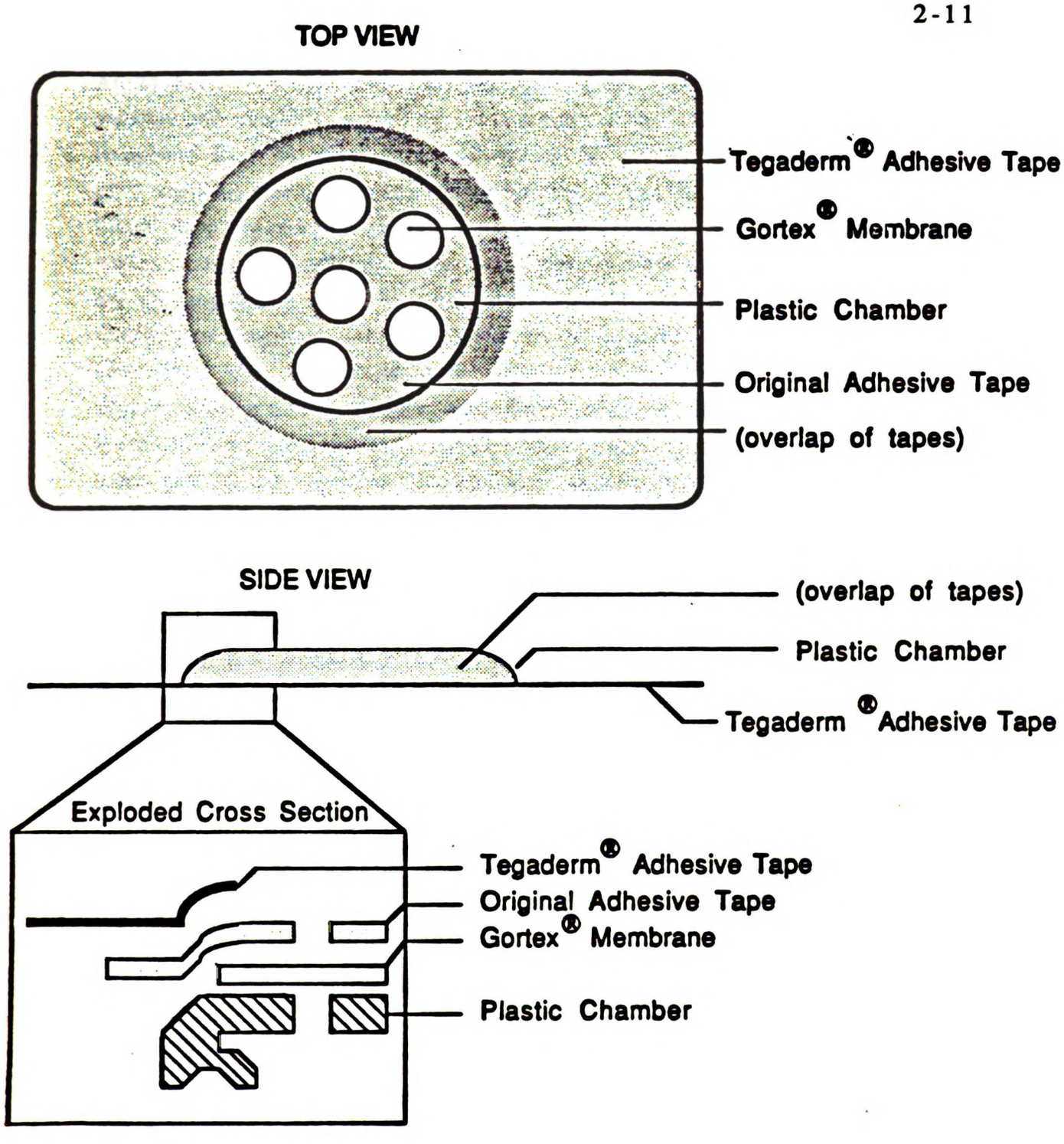


Figure 2. Modified Hilltop® Chamber. From Bucks, D.A.W., Maibach, H.I. and Guy, R.H., Mass balance and dose accountability in percutaneous absorption studies: development of a nonocclusive application system, Pharm. Res.; 1988; 5: 313-315.

At 24 hours post-application, the protective patch was removed and a standardized washing procedure was performed to remove unabsorbed compound from the skin surface (Bucks et. al., 1985). The procedure consisted of 5 standard size cotton balls consecutively soaked in soap solution (Ivory Liquid Soap, Proctor and Gamble Co., Cincinatti, OH; diluted 1:1 with distilled water), water, soap solution, water and water. A sixth cotton ball was used to dry the application site. The patch and all swabs were analyzed, by liquid scintillation counting, for ^{14}C radioactivity utilizing an appropriate quench correction. After the standardized washing, a second protective patch was applied and remained in place until day 7 when the washing recovery procedure was repeated and the experiment terminated. The second protective patch and second set of swabs were also processed for liquid scintillation counting to assay for residual surface chemical. PA was quantitated by analysis of ^{14}C-urinary excretion over the 7-day study period. Urine was collected in aliquots according to the schedule detailed in the **Analysis** section. Correction for incomplete urinary elimination was acheived by performing the appropriate intravenous control measurements (see below). Hence, the urinary excretion data, together with surface recovery measurements, enable complete dose accountability of the administered ^{14}C to be attained (Bucks et. al., 1988a).

Intravenous administration: Following dissolution of the compound in a minimum volume of ethyl alcohol (190 proof USP) : 0.9% sterile saline solution (Elkins-Sinn, Inc., Cherry Hill, NJ) (volume percent of each solvent determined separately for each compound due to different solubilities), the solution was filtered aseptically through a 0.22 micron sterile filter (Abbott Hospitals, Inc., North Chicago, IL) into a sterile evacuated flask (Gibco / Invenex Division, Dexter Corporation, Chagrin Falls, OH). This solution was tested for the presence of pyrogens (limulus lysate pyrogen test) and adequate sterile manufacture (sterile tryptic soy broth incubation, 37°C, 7 days) (see appendix).

1 µCi of the compound (usually in a 2 cc volume of the sterile solution) was administered as an intravenous bolus dose into the antecubital vein. Urine collection was as described in the **Analysis** section. To ensure that complete urine collection was achieved, quantitative 24 hour urine creatinine

determinations were made. A commercial colormetric assay (Sigma,St. Louis, MO) was used to evaluate urine creatinine (grams/24 hours) (see appendix).

General: The experimental subjects were Caucasian adults (males and females), having no history of dermatologic disease. The volunteers formed two groups: young (22-40 years) ($n \geq 3$) and old (65 - 86 years)($n \geq 5$). All subjects were in good health; all had 24 hour creatinine clearance values normal for their age and weight.

The penetrants considered were testosterone, estradiol, hydrocortisone, benzoic acid, acetylsalicylic acid, and caffeine (Table 3). All compounds were ^{14}C - labeled (testosterone, estradiol, hydrocortisone and benzoic acid (Research Products International Corp., Mount Prospect, ILL); acetylsalicylic acid and caffeine (New England Nuclear, Boston, MA)) and were > 99% pure as assessed by thin layer chromatography (see appendix).

Analysis: All urine was collected for 7 days (0 - 168 hours post dosing). The collection intervals were; day 1 : 0-4, 4-8, 8-12, 12-24 hours post dose; days 2-7: two 12 hour periods per day. At the end of the 7 day urine collection period, the radioactivity in the urine samples had returned to background levels. Aliquots (6 mL) of urine (triplicate determination) were mixed with 10 mL scintillation cocktail (Beckman Ready Gel Scintillation Cocktail) and ^{14}C content was then measured by liquid scintillation counting (Searle Mark III Scintillation System). Decreased efficiency in counting the urine samples due to signal quenching was evaluated using an added internal standard (toluene (ring - ^{14}C), New England Nuclear, Boston, MA) of known disintegrations per minute to each sample set. In this way, counting efficiency (approximately 73%) for each urine sample was determined.

Results

Figure 3 (a-f) shows the total percent dose excreted into the urine for each of the six compounds following intravenous administration to the two subject populations. Three or more subjects were used for each compound in each age group. Intravenous data collection in both age groups was essential due to unavailable literature data in the older subjects. Distribution of elimination routes for the total radioactivity data was thus established unequivocally for

both age groups; consequently the intravenous data obtained (for young and old groups) was used appropriately when correcting all topical data obtained. It can be seen from Figure 3 (a-f) that the cumulative percent dose excreted into the urine (as total radioactivity) was not significantly different between the two age groups.

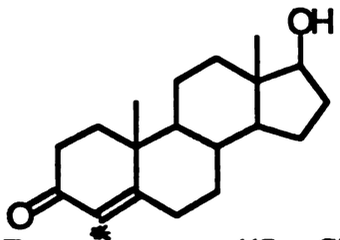
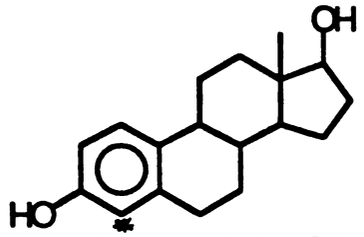
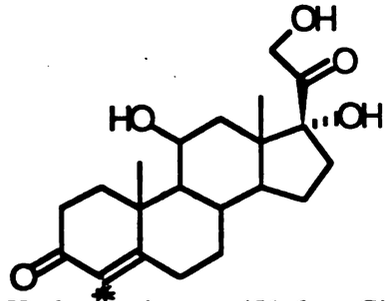
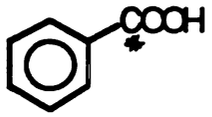
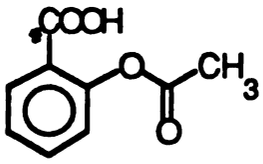
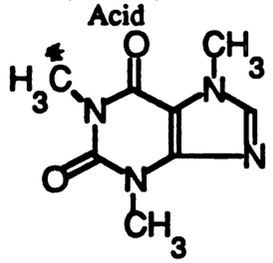
Figure 4 (a - f), for each of the six compounds, shows representative curves of the cumulative percent dose absorbed percutaneously (corrected for incomplete renal elimination) as a function time. The young data (solid squares) and the old results (open diamonds) are presented on each graph for comparison.

For testosterone and estradiol, (Figures, 4a and 4b respectively), the cumulative percent dose absorbed in the old subjects was not distinguishable from that in the young controls. For hydrocortisone, benzoic acid, acetylsalicylic acid and caffeine, (Figures 4c - 4f, respectively), the cumulative percent dose absorbed is significantly less in the old subject group than in the young group.

The complete data set is summarized in Table 4; included in this table are the aqueous solubilites and octanol/water partition coefficients of the six chemicals considered. All absorption data have been corrected for incomplete renal elimination using the appropriate population intravenous control.

Table 5 provides a synopsis of the surface recovery data following the 24 hour dosing period. Data are shown for primary protective (24-hour) patch and primary soap and water washes only (second protective patch and second soap and water washes total $\leq$ 5%). It can be seen from Table 5, for those compounds which have decreased absorption in the old group (hydrocortisone, benzoic acid, acetylsalicyclic acid and caffeine), there was significantly more compound was recovered on the skin surface.

Table 3. Compounds Studied in Protected Penetration Studies

	MW	Log K[a] (O/W)	Aqueous[b] Solubility
 Testosterone (47 mCi/mMol)	288.4	3.32	Insoluble
 Estradiol (56 mCi/mMol)	272.4	2.49	Almost Insoluble
 Hydrocortisone (51.6 mCi/mMol)	362.5	1.6	0.28 G/L
 pKa = 4.2 Benzoic Acid (53mCi/mMol)	121.1	1.83	3.4 G/L
 pKa = 3.5 Acetylsalicylic Acid (56.6 mCi/mMol)	180.2	1.26	3.3 G/L
 Caffeine (47.5 mCi/mMol)	194.2	0.01	21.7 G/L

[a] Leo et. al., 1971 [b] Merck Index

* Denotes site of radiolabel - ^{14}C

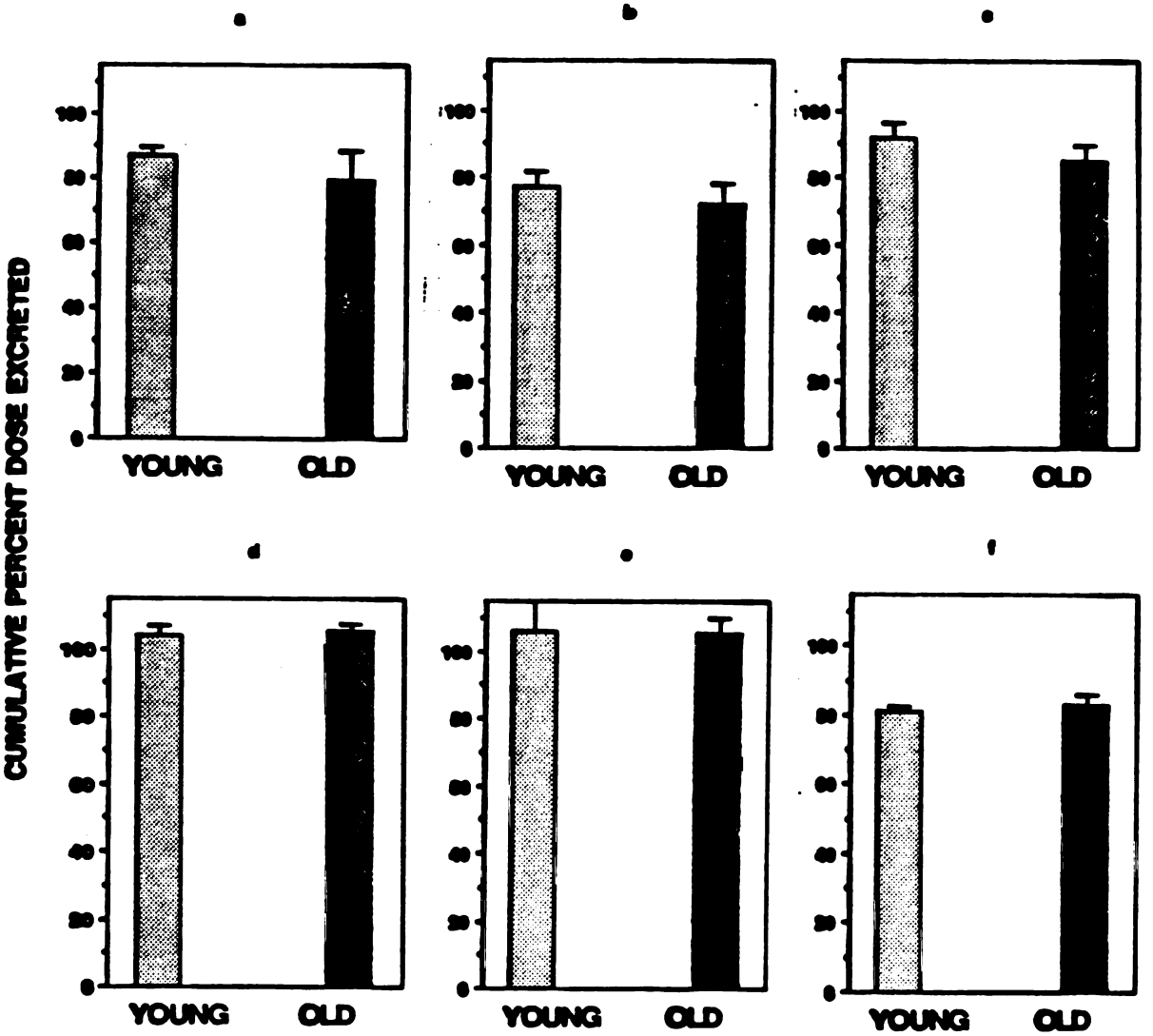


Figure 3. Cumulative percent dose excreted into the urine for (a) testosterone (b) estradiol (c) hydrocortisone (d) benzoic acid (e) acetylsalicylic acid and (f) caffeine following intravenous dosing in young (22-40 years) and old (65-86 years) groups[b].

[a] Mean ± S.D.

[b] These values not significantly different from young control group ($p > 0.05$).

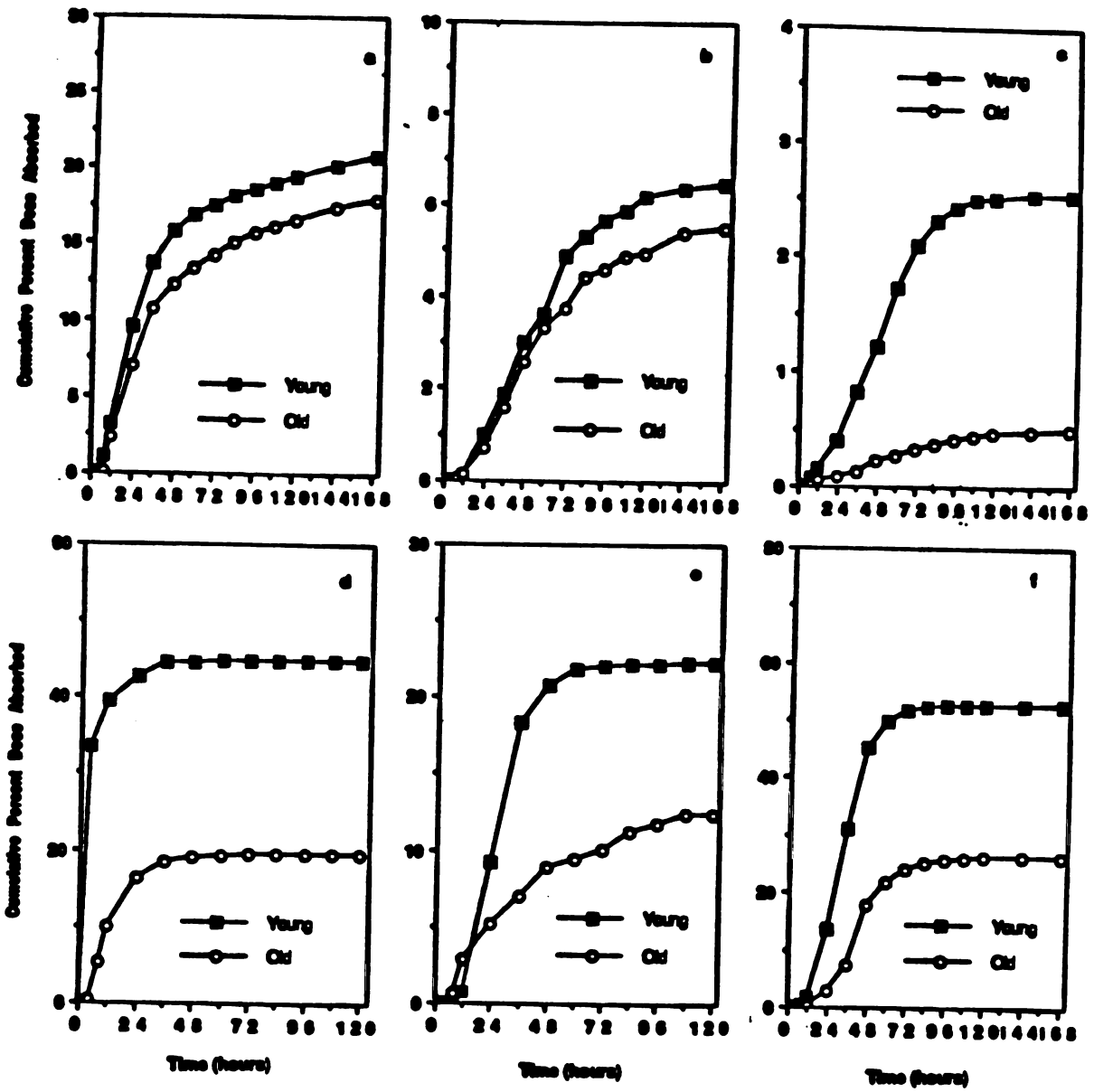


Figure 4. Representative curves of the cumulative percent dose absorbed as a function of time for (a) testosterone (b) estradiol (c) hydrocortisone (d) benzoic acid (e) acetylsalicylic acid and (f) caffeine. The young (solid squares) and old (open circles) data are presented on each graph for comparison.

Table 4. Summary of Protected Penetration Studies

Compound	Molecular Weight	Aqueous Solubilities	Log K[a] (O/W)	Cumulative % Dose Absorbed[b] Young (22-40 yrs)	Old (>65yrs)
Testosterone	288.4	Insoluble	3.32	18.98±4.35 n=6	16.61±2.54[c] n=8
Estradiol	272.4	Almost Insoluble	2.49	7.11±1.09 n=5	5.35±0.43[c] n=5
Hydrocortisone	362.5	0.28 g/L	1.61	1.45±0.6[d] n=3	0.54±0.15[e,d] n=7
Benzoic Acid	121.1	3.4 g/L	1.83	36.2±4.6 n=7	19.5±1.6[f] n=8
Acetylsalicylic Acid	180.2	3.3 g/L	1.26	31.2±7.3 n=5	13.6±1.9[g] n=7
Caffeine	194.2	21.7 g/L	0.01	48.2±4.1 n=5	25.2±4.8[f] n=7

[a] Reference Leo et. al., 1971; solubilities obtained from the Merck Index [b] Mean±S.E

[c] Not significantly different from the young control group ($p>0.05$)

[d] If averaged together with the data from Bucks et. al., 1988, (mean± S.E. = 3.27±0.73; n=8 then $p< 0.01$)

[e] Significantly different from the young control group ($p=0.06$)

[f] Significantly different from the young control group ($p<0.01$)

[g] Significantly different from the young control group ($p<0.05$)

TABLE 5. Summary of surface recovery from primary (24-hour) patch and primary soap and water washes.

(Mean + S.E.)

Compound	Dose Percent Recovered [a]	
	Young	Old
Testosterone	85.2 ± 5.6	91.3 ± 2.6 [b]
Estradiol	77.5 ± 2.6	86.2 ± 1.4 [c]
Hydrocortisone	87.7 ± 3.7	100.1± 1.3 [c]
Benzoic Acid	45.6 ± 2.8	61.4 ± 2.0 [d]
Acetylsalicylic Acid	50.4 ± 4.2	69.3 ± 2.9 [e]
Caffeine	32.1 ± 4.2	61.8 ± 5.4[e]

[a] Sum of radiolabel recovered from primary protective patch and primary soap and water washes

[b] Not significantly different from young group (p value > 0.05)

[c] Significantly different from the young group (p value < 0.05)

[d] Significantly different from the young group(p value < 0.001)

[e] Significantly different from the young group (p value < 0.01)

Since both intravenous and topical absorption data were obtained, and differences in topical bioavailability (as assessed by total radioactivity data) were observed, Mean Residence Time (MRT) calculations were undertaken to assess the kinetics of absorption for the compounds. MRT calculations have been described in detail elsewhere (Riegelman and Collier, 1980; Nakashima and Benet, 1988). Briefly, urine collection is a means of sampling an accumulating compartment at the end of a long chain of events. Pharmacokinetic analysis reveals that the MRT of drug or metabolite in the body after an intravenous dose is the arithmetic average of the times that each drug or metabolite molecule remains in the body and is mathematically defined for an accumulating compartment (such as urine) as:

$$MRT = \int_0^\infty \frac{(Ae_\infty - Ae)\ dt}{Ae_\infty} \qquad (1)$$

where Ae_∞ is the cumulative amount of radiolabel (drug and metabolite) in the urine at time infinity and Ae is defined as the cumulative amount of radiolabel (drug and metabolite) excreted in the urine up to time t. The MRT following an intravenous dose is the average time each drug or metabolite molecule remains in the body prior to urinary excretion (MRT_{iv}). For topical administration, the MRT (MRT_{top}) is the sum of the Mean Input Time (MIT) for the compound and the MRT for the compound or metabolite in the body prior to elimination (i.e. MRT_{iv}). It follows that:

$$MRT_{top} = MIT + MRT_{iv} \qquad (2)$$

$$MIT = MRT_{top} - MRT_{iv}$$

Thus, the MIT describes the arithmetic average of the times that each drug molecule takes to enter the body. MIT is composed of the times taken for the various separate steps necessary to input drug into the body, (eg. dissolution in SC lipids, diffusion though the stratum corneum and viable tissue, uptake by the skin capillaries into the systemic circulation). Table 6 presents MIT data for the compounds tested. Although the rate-limiting step for entry into the body cannot be elucidated from MIT data, comparisons between young and old subject populations can be made. For two of the compounds, benzoic acid and acetylsalicylic acid, which have decreased penetration in the aged subjects, the MIT indicates a decreased rate of absorption. For two compounds, testosterone and estradiol, which show no difference in absorption between the two subject groups, the MIT is the same between old and young skin. Less consistently, for the remaining two compounds, hydrocortisone and caffeine, for which topical bioavail-ability in the aged was significantly reduced, there was no significant difference between the MIT values for the two subject groups.

TABLE 6. Mean Input Time (Hours). The arithmetic average of the times that each drug molecule takes to enter the body.

(Mean ± S.E.)

Compound	Young	Old
Testosterone	18.9 ± 3.2	20.5 ± 1.7 [a]
Estradiol	19.1 ± 3.8	21.1 ± 3.8 [a]
Hydrocortisone	35.9 ± 2.0	43.5 ± 6.0 [a]
Benzoic Acid	5.3 ± 0.76	10.0 ± 0.40 [b]
Acetylsalicylic Acid	17.5 ± 1.9	28.3 ± 2.2 [c]
Caffeine	17.3 ± 1.1	19.2 ± 2.9 [a]

[a] Not significantly different from the young group (p value > 0.05)

[b] Significantly different from the young group (p value < 0.001)

[c] Significantly different from the young group (p value < 0.01)

Discussion and Conclusions

In general, all signs indicate that skin changes with increasing age (Gilchrest, 1984). It cannot be reasonably expected that the barrier function is excluded from this modification. The results summarized in Figures 4, (a-f) and Table 4 indicate that skin aging can affect the extent of PA *in vivo* . These observations raise the question does the aging process in human skin differentiate in terms of barrier function between substances of different physicochemical properties? We observe that the percutaneous penetration of the two most lipophilic compounds considered (testosterone and estradiol) is not significantly different between the subject groups. However, that aging can affect the extent of PA *in vivo* is shown by the results for hydrocortisone, benzoic acid, acetylsalicylic acid and caffeine, the less lipophilic compounds considered.

Further evidence to support a change in barrier function, with increasing age, to substances of different physicochemical properties, is shown by the MIT data. The MIT for the two most lipophilic compounds studied, testosterone and estradiol, is not significantly changed between the two subject groups. However, for benzoic acid and acetylsalicylic acid, two of the hydrophilic compounds studied, the MIT is significantly longer in the aged subject group. This implies that in a 24 hour exposure period, less compound would have the ability to penetrate the stratum corneum. Surprisingly, two of the hydrophilic compounds, hydrocortisone and caffeine, do not show a change in the MIT with increasing age, yet show significant differences in the extent of their absorption. Why this would be so is not presently discernible. However, a possible explanation for the apparent conflict between the rate (MIT) and extent (cumulative % dose absorbed) for these two compounds may be due to the slow excretion of their metabolites into the urine following either a topical of intravenous dose. MIT calculations are based on exploitation of the differences between these two measurements ($MIT = MRT_{top} - MRT_{iv}$). However, the metabolites of both hydrocortisone and caffeine are slowly excreted into the urine and differences between the MRT_{top} and MRT_{iv} may not be large enough to elucidate meaningful MIT values.

The results agree, in general, with the observations of Christophers and Kligman (1965), and Tagami (1972), who concluded that the barrier function of human skin *in vivo* increases with increasing chronologic age. Furthermore, the recent results of Rougier et. al. (1988) confirm a decreased penetration of ^{14}C - benzoic acid in old subjects (65-80 years).

However, these observations contrast with those obtained by Grove et. al. (1982) who compared the blistering response time of young (18-25 years) and old (65-75 years) adults to topical application of 50% ammonium hydroxide solution. On the volar forearm and upper inner arm, the initial response, characterized by minute perifollicular vesicles, appeared earlier in the old group. These results were suggested by the investigators to be consistent with either a reduced stratum corneum barrier or an increased shunting of irritant material through appendageal orifices. In contrast, though, to the initial response, the time required for blister development averaged twice as long in the old group, indicating a decreased transudation rate with increasing age in injured skin. It still is not clear whether these age-associated differences are completely linked to skin penetration characteristics, particularly as the penetrant, in this case, caused major barrier damage.

Cutaneous, age-associated changes which may be predicted to impact on the absorption process include those occurring in the epidermis and those at the dermo-epidermal junction, where uptake of penetrant into the general circulation takes place. Among the many well-documented changes that occur in aged skin, those which may be expected to impact on PA include:

(1) The stratum corneum of the elderly is drier than the young adult equivalent (Potts et. al., 1984; Potts and Buras,1985). Hydration of the horny layer alters the environment of the skin encountered by a topically applied penetrant molecule and, as a result, may affect percutaneous absorption (Blank, 1985; Scheuplein and Ross, 1970).

(2) Sebaceous gland activity is reduced in aged skin and the amount of skin surface lipid is lowered (Pochi et. al., 1979). Additionally, the composition of skin surface lipids may change with increasing age (Nazzaro-Porro et. al., 1979; Yamamoto et. al., 1988). An initial and crucial step in PA is the

partitioning of drug into the lipids of the stratum corneum. This essential process will be sensitive to the type and amount of skin lipids (sebaceous and epidermal) present.

(3) The dermo-epidermal junction is flattened in aged skin and the number of dermal capillary loops is decreased (Gilchrest, 1984; Ryan, 1983). From histological examination, a compromised microcirculation and a poorer resorption capability is implicated for the elderly.

In the dynamic process of PA, once the penetrant has traversed the epidermal barrier, the ability of the microcirculation to clear the penetrant into the systemic circulation is dependent upon the density and efficiency of the cutaneous capillary network. Christophers and Kligman (1965), and Tagami (1972), have both shown in elderly subjects a decreased ability of the micro-circulation to clear penetrant in the midback area. DeSalva and Thompson (1965), however, performed essentially identical *in vivo* studies (investigating intradermally injected $Na^{22}Cl$ disappearance from the skin of the hands, face and forearms) and contradicted the earlier results. DeSalva and Thompson found essentially constant removal rates for subjects between 50 and 90 years and, suprisingly, reported the rate of elimination to be slower in the young population group (21-30 years). It is clear that a concensus on the functional capacity of aged microvasculature in PA has yet to be established.

The diminished lipid content of aged skin implies a diminished dissolution medium for chemicals that are administered on the skin surface. Thus, we may predict that such a change would differentiate against those compounds, the lipid solubility of which is lowest; that is, compounds with smaller as opposed to higher oil-water partition coefficients such as hydrocortisone, benzoic acid, acetylsalicylic acid and caffeine. A reduced presence of water, furthermore, would again imply that the environment of aged skin is less attractive to less lipophilic moieties. A smaller lipid capacity and lowered water content may not, however, affect dissolution into the stratum corneum of highly lipophilic molecules such as testosterone and estradiol.

Although model compounds were used in these studies and may not be completely representative of actual clinical doses (e.g. acetylsalicylic acid),

the data should reflect the penetration of therapeutic doses. Further work to elucidate mechanisms responsible for the increase in barrier function is planned. With the changing demographic pattern of Western civilization and the potential for drug delivery via the transdermal route, it is important that we begin to establish quantitative relationships between age, percutaneous penetration, the physicochemical properties of the drugs to be delivered transdermally and the intrinsic changes within the membrane itself.

Chapter 3

Cutaneous Microcirculation Studies

Introduction

Transdermally delivered chemicals, which have diffused across the epidermis into the dermis, are taken up by the cutaneous network into the systemic circulation. It may be hypothesised that progressive dermal microcirculation atrophy with increasing age could cause a decrease in the absorption of penetrating substances. However, the impact of this structural modification on the overall process of dermal absorption (rate and extent) remains equivocal. From previous work, there appears to be reduced circulation as indicated by (a) dermal clearance measurements (Christophers and Kligman, 1965; Tagami, 1972) and (b) the McClure-Aldrich test, which measures the time taken for a wheal, raised by intradermal injection of saline, to flatten (Aschner, 1960; Kligman, 1979). The clearance data (Christophers and Kligman, 1965; Tagami, 1972), however, has been contradicted by a study which found generally constant removal rates of intradermally injected $Na^{22}Cl$ in subjects aged 50-90 years (Desalva and Thompson, 1965). Surprisingly, clearance of the tracer was slowest in the group aged 21-30 years.

More recently, Guy et. al. (1985) conducted a preliminary investigation into the effect of aging on microvessel reactivity following topical administration of the local vasodilator, methyl nicotinate (MN) (Figure 1). No significant differences in the response to the stimulus were found between the young and old populations studied. But this observation cannot be interpreted unambiguously. The procedure and data analysis followed were not designed to differentiate between possible changes in either microcirculation sensitivity or barrier function. The methodology recorded the summation of the two processes, and did not address the possibility of compensatory alterations in the efficiency of the two events.

The objective of this study was to investigate whether the same potent vasodilator, MN, could be used to evaluate the sensitivity and efficiency of the microcirculation as a function of increasing age *in vivo* . The time-course of the local effects of MN was assessed by laser Doppler flowmetry (LDF), a non-invasive technique sensitive to alterations in cutaneous microvasculature perfusion. MN penetrates the stratum corneum and upper viable skin tissue, and reaches the dermal capillaries where it exerts its pharmacological action. Because only finite doses of MN are applied, the vasoresponse passes through a

maximum and then decays, consistent with uptake of agent into the capillaries and systemic dilution. In the experiments reported here, the kinetic processes of local skin absorption, cutaneous reactivity and dermal elimination of MN have been evaluated and the results analyzed using a previously published pharmacokinetic-pharmacodynamic model (Guy et. al.,1984).

Material and Methods

Laser Doppler Flowmetry

The pharmacologic response to topically administered MN was monitored with a laser Doppler flowmeter (Periflux Pf2, Perimed KB, Sweden) interfaced to a flatbed pen recorder (YEW Yokogawa Hokushin Electric Corporation, Tokyo, Japan). The LDF technique has been described in detail elsewhere (Holloway and Watkins, 1977; Watkins and Holloway, 1978; Stern et. al., 1977; Bonner et. al., 1981). Briefly, light at 632.9 nm from a 5 mW He-Ne laser is directed into the skin via an optical fiber (Figure 2). The radiation is multiply-scattered and reflected by both stationary tissue components and mobile red blood cells. The former back-scatter radiation at the incident frequency, the latter reflect light which has been frequency (i.e. Doppler) shifted by an amount proportional to the product of their number multiplied by their velocity. The laser Doppler flowmeter employed separates the frequency-shifted component, amplifies the signal and displays the result as a fluctuating voltage that has been shown to be proportional to microcirculatory flow as determined by other, independent techniques (Nilsson et. al., 1980; Watkins and Holloway, 1978; Engelhart and Kristen, 1983). The depth of penetration of the laser light is approximately 1 mm (±0.4-0.5 mm)(mean± S.D.) (Holloway, 1983); this limited ingress avoids any influence from major vessels, thereby ensuring that the signal is derived from the superficial microvascular system. Equally, the light penetration distance also damps considerably any effects of regional variations in epidermal thickness on observed differences in LDF output. Physical support to the fibers, at the point where they interface with the skin, is provided by a small cylindrical probe holder (1.1 cm high, 0.6 cm diameter) attached to the application site using a double-sided adhesive disc.

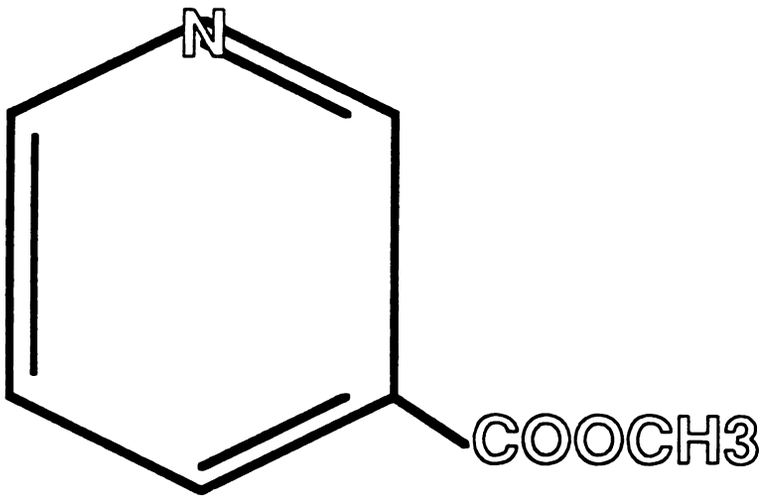


Figure 1. Methyl nicotinate molecule

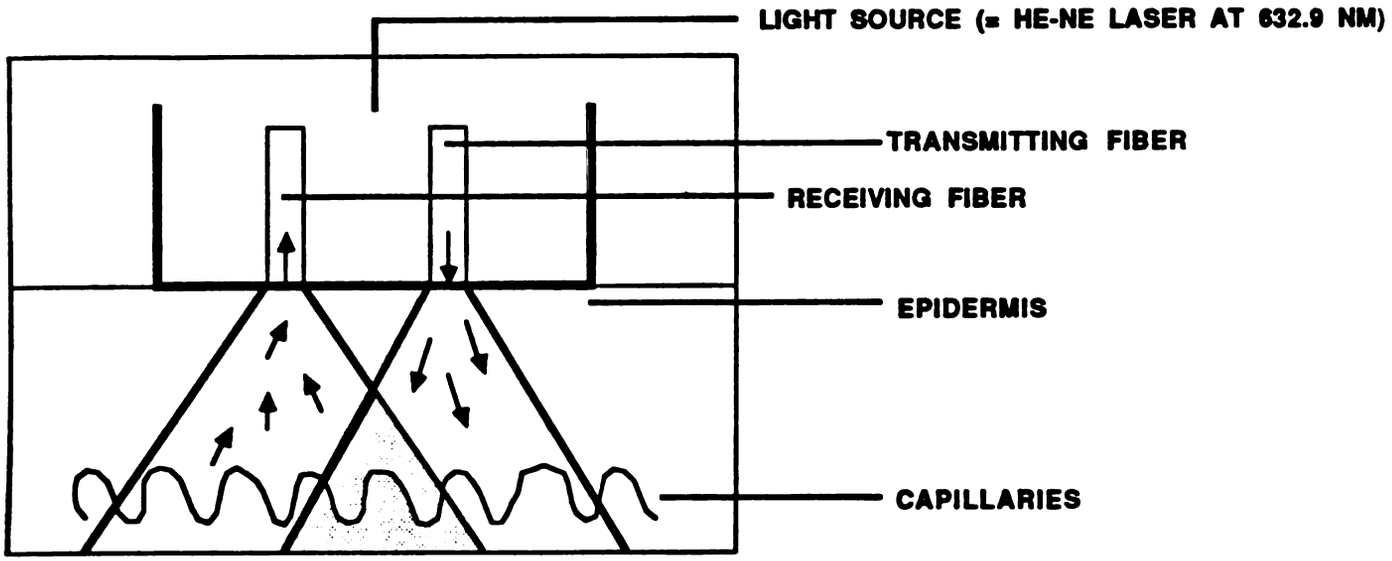


Figure 2. Laser Doppler Flowmetry: Laser light is transmitted to the skin via an optical fiber. Back scattered light contains radiation at the same frequency as the incident source (from non-moving tissues) and radiation Doppler-shifted as a result of reflection from moving red blood cells. The LDF instrument extracts the frequency-shifted signal and derives an output proportional to flow. Adapted from Stevenson, J., Maibach, H.I. and Guy, R.H., Laser Doppler and photoplethysmographic Assessment of cutaneous microvasculature *in* Models in Dermatology, 3: 121-140: 1987.

Experimental

Dose - response behavior following topical application of MN was measured. Aqueous solutions of methyl nicotinate (Sigma Chemical Co., St. Louis, Missouri) at concentrations of 2.5, 5.0, 10.0 and 25.0 mM were used; the pH of these solutions was 5.3 ± 0.9.

MN solution was applied via a saturated test patch (1.0 cm diameter) (Al-test, Imeco ab, Sodertalje, Sweden) to prevent solution from running and spreading over the skin surface; the volume of solution held by the circular patch was 50 ul (± 2%). Application time was 15 seconds, after which the patch was removed and the site carefully dried with absorbent tissue. The probe was then immediately positioned at the center of the application site and blood flow response was continuously monitored for the next 90 minutes. All response measurements were normalized, i.e. corrected for pretreatment baseline flow and chart recorder drift (< 30 mV).

The experimental subjects were thirteen (seven male, six female) Caucasian adults, having no history of dermatologic disease, divided into two groups: young (20-34 years; n = 6) and old (64-86 years; n = 7). The subjects were allowed no prescription nor over-the-counter medication for the duration of the study. Subjects were non-smokers, refrained from caffeine and alcohol for 24 hours prior to each test. Successive measurements in each individual were performed at least 3 days apart.

The experiments were conducted in a well-ventilated room under stable temperature and humidity conditions (T = 23°± 3°C; relative humidity = 30-50 %). The subjects rested in a supine position throughout the measurement and were acclimated to the test environment 20 - 30 minutes prior to each experiment. The test site was the ventral surface of the forearm; 9 cm below the elbow fold. A control site, at 5 cm below the elbow fold, was delineated to monitor spontaneous changes in baseline perfusion. Each concentration was measured separately on both forearms. Prior to testing, baseline measurements were taken at both sites until a stable reading was obtained; typically, the latter was achieved within 5-7 minutes.

The following LDF settings were used: time constant = 1.5 sec, frequency = 4 kHz; gain = x10 or x3; chart recorder speed = 1 cm/min.

Spontaneous Perfusion Changes and Vehicle Effect

The results of Kohli et. al. (1987) suggest that vehicles as simple as distilled water and some short-chain alcohols can produce a highly inconsistent effect on cutaneous blood flow determined by LDF. Data obtained during the course of our study support the conclusion that a response to topically applied distilled water may be detectable by LDF, but that the magnitude of the blood flow response is small in comparison to the response elicited by a topically applied vasoactive compound, such as MN. Additionally, a spontaneous change in baseline blood flow may also occur over the duration of a typical experiment and must be taken into account during data analysis.

In the first series of experiments, measurements of baseline skin blood flow at the same skin site were made before and after a 45-90 minute interval of quiet resting. Accurate repositioning of the probe for the second measurement was facilitated by aligning marks on the probe with marks drawn on the skin with a felt-tip pen. Prior to the initial determination of perfusion, the subjects were acclimated to the measurement environment for at least 20 minutes. The results of this part of the investigation (Figure 3) are a complete compilation of observations from 17 volunteers and include several concomitant measurements of bilateral, symmetric sites on left and right arms. Figure 3 shows first that spontaneous skin blood flow changes can occur and that the changes are not obviously related to the arm used or to the interval between observations. Next, we find that these apparently unprovoked alterations can be as large as 100 mV. It is important, therefore, when using LDF to follow, for example, topically applied drug-induced changes in skin blood flow, to record "basal" measurements of perfusion from a control site before and after the deliberate provocation of vasodilatation.

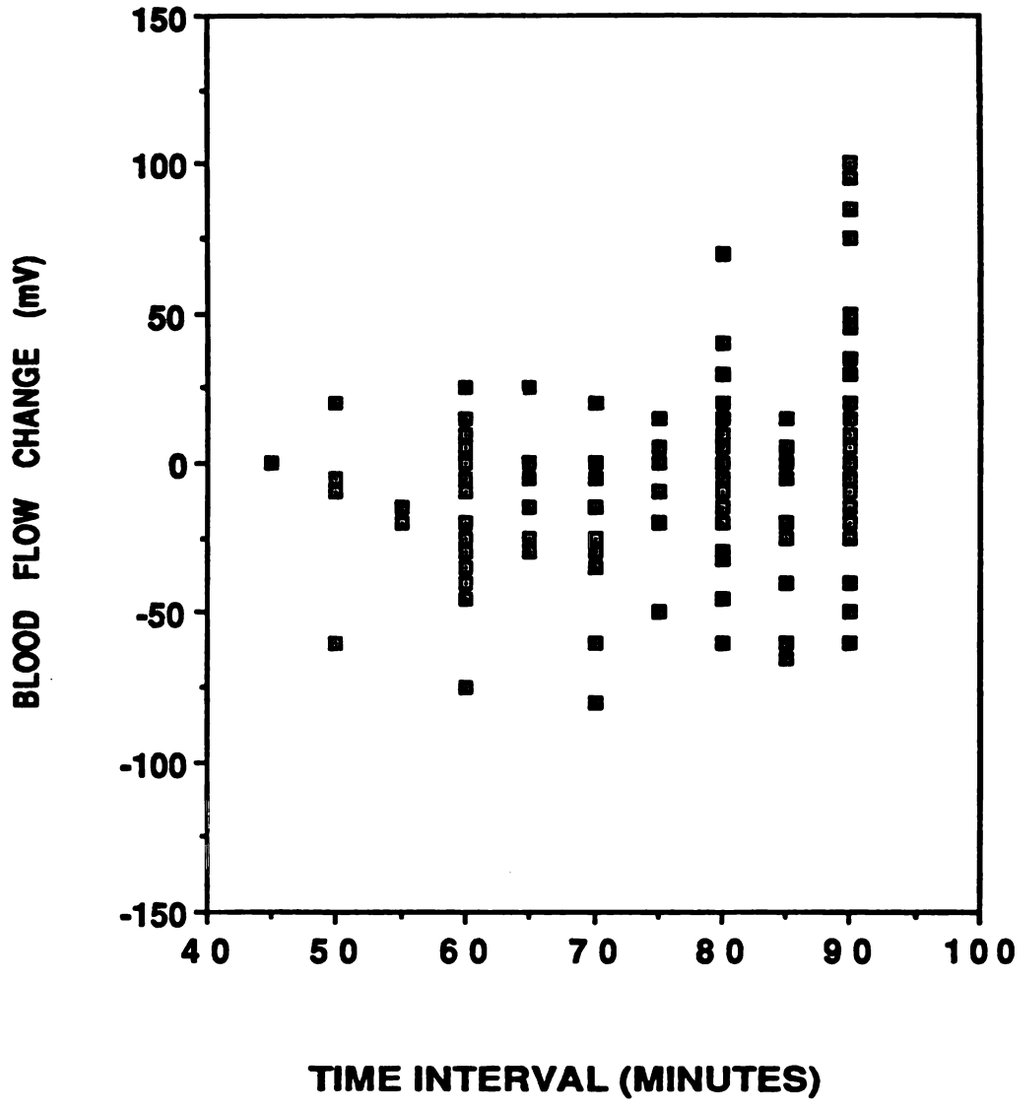


Figure 3. Spontaneous skin blood flow changes plotted as a function of the time interval between observations. This data set is a complete compilation of observations from 17 volunteers and includes several concomitant measurements on bilateral, symmetric sites on left and right arms.

In the second part of the study, forearm skin blood flow response to the topical application of distilled water was followed. Water was applied for 15 seconds, via a saturated filter disk (see **Experimental** Section) and blood flow was montiored continuously thereafter for the next 30-55 minutes. As suggested by the results of the first component of this investigation, control baseline measurements of blood flow at a non-treated skin site were made so that spontaneous fluctuations in perfusion could be subtracted from those induced by the application of water. The results of 8 separate experiments are collected in Figure 4. The maximum change in blood flow, which could be attributed to water, was 70 mV. Typically, however, responses less than 20 mV were observed. Thus, like Kohli et. al. (1987), we do see changes in skin blood flow that may be water-induced, and the onset of time of the "reaction" can be obtained from our data. The key point of this work, though, is the magnitude of the response, which is generally small. We have shown that changes can be spontaneous and that, once such fluctuations have been acknowledged, the additional pertubation (both size and duration) putatively caused by water is rather insignificant. The latter conclusion is particularly true when the 'effect' of water is compared to that of a 10 mM aqueous solution of MN (Guy et. al., 1984). In these experiments, the maximum response following a 15 second application of the vasodilator in this form is typically in excess of 300 mV.

The possible explanations for spontaneous skin blood flow changes (and those perhaps caused by water), even under reasonably well-controlled conditions of our studies, are manifold. No single reason can be concluded more likely on the basis of the experiments performed. Certainly a cooling/hydration/temperature disturbance proposed by Kohli et. al. (1987) is plausible. Other environmental changes (e.g. a draft, conversation) or physiological/psychological perturbations (e.g. yawning, mental activity) can also induce short-term peripheral vascular changes of the order of magnitude seen. In conclusion, the caution implicit in the paper of Kohli et. al. (1984) is appropriate. However, the scale of the perceived problem is, in our opinion, small and the effect can be minimized by acquisition of appropriate pre- and post-test basal perfusion data. While normal biological variability in the results must be accepted, careful experimental design, including measurement of proper control information, will yield good quality data in skin pharmacology.

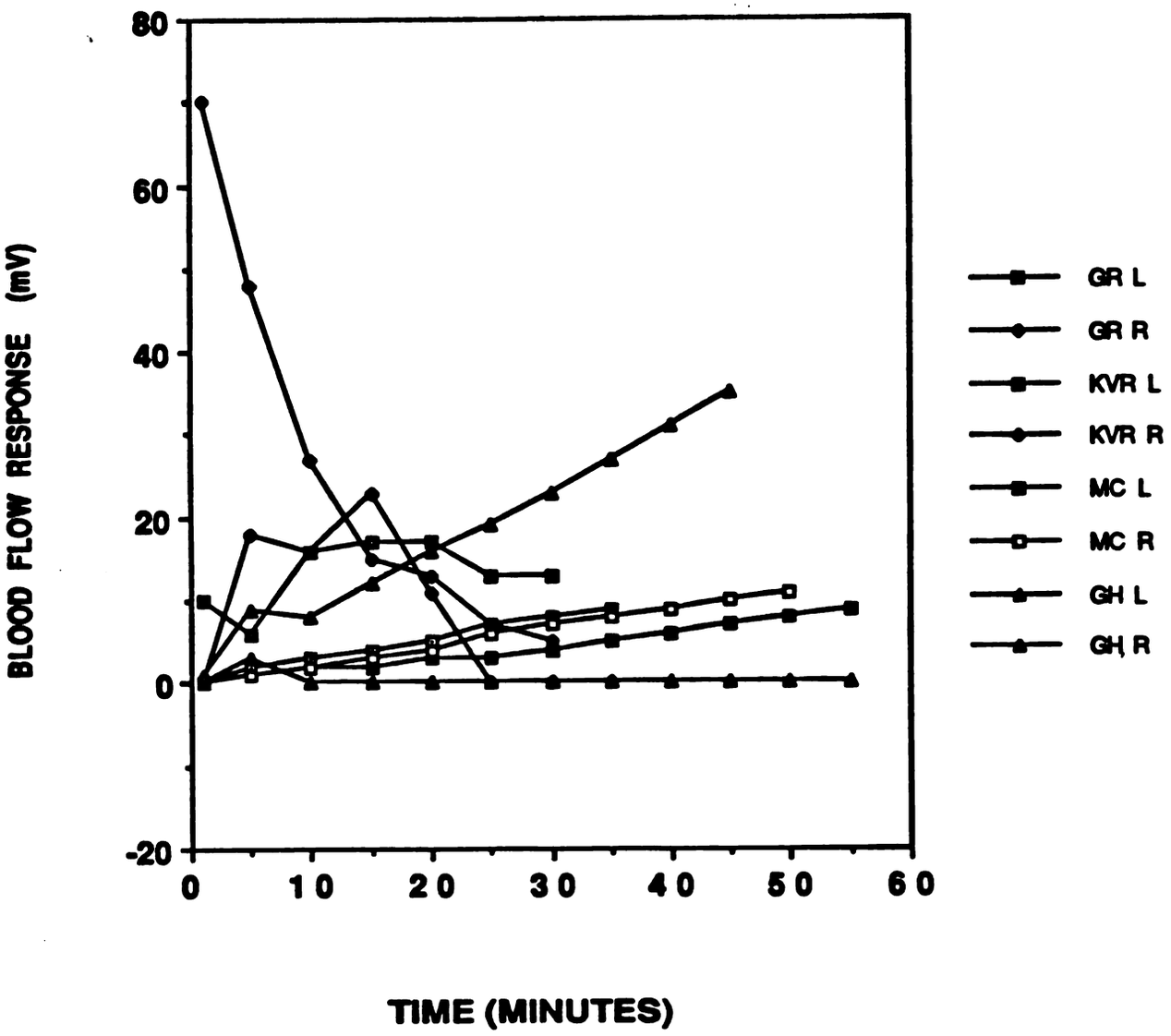


Figure 4. Forearm blood flow response to topically applied water in 4 volunteers. These measurements were made on bilateral, symmetric sites on left and right arms. Legend markers include subjects' initials and indicate whether the left (L) or right (R) arm was used for measurement.

Results

LDF response curves as a function of time for the four MN concentrations administered as described above were similiar to those previously reported (Figure 5) (Guy et. al., 1984). The results are summarized in Table 1. Five parameters (Figure 6) have been selected to characterize the time course and extent of the normalized pharmacodynamic response: (1) the time (minutes) of maximum response (2) the magnitude of the maximum response (millivolts) (3) the area under the response-time curve monitored from t= 0-90 minutes (millivolt • hour) (4) the time of onset of action (minutes) (defined as the greatest change in response per unit time) (5) the time (minutes) required for the response to return to 75% of the maximum acheived. The initial statistical comparisons of these empirical parameters utilized a one-way analysis of variance, followed by a Newman-Keuls multiple range test. Significant difference was found only when the responses to different MN concentrations were compared (i.e. the response to 2.5 mM MN in the old subjects was less than that to 10 mM and 25 mM MN in the same group). At the same MN concentration applied, no significant differences were found between the young and old groups at the $\alpha = 0.05$ level.

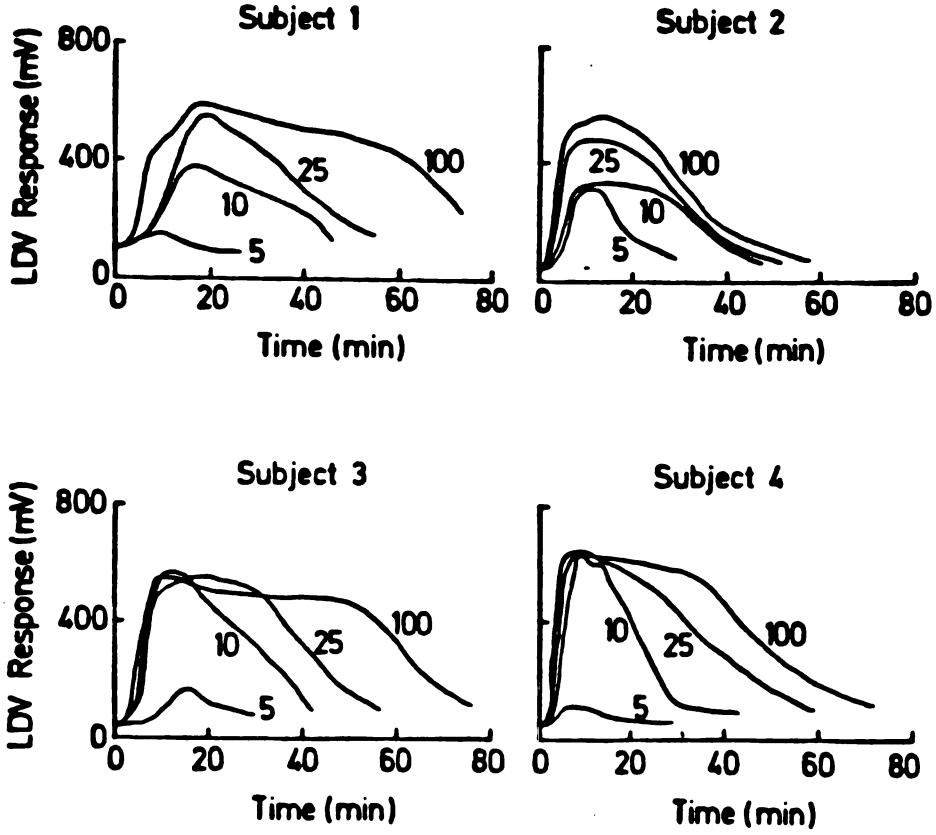


Figure 5. Laser Doppler velocimetry response curves as a function of time and methyl nicotinate concentration for four subjects. The curves corresponding to the various drug concentrations (in mM) are indicated on the graphs. Each curve shown is the mean of at least 3 separate determinations.

Reproduced from Guy et. al., Pharmacodynamic measurements of methyl nicotinate percutaneous absorption, Pharm. Res., 1, 76-81, 1984.

Table 1. Summary of parameters characterizing the time course and extent of the normalized pharmacodynamic response (Mean ± S.E.). No significant differences were found between the young and old groups at α=0.05 level (Newman-Keuls multiple range test) at the same MN concentration.

Group	Time of Onset (minutes)	Time to Maximum Response (mins)	Magnitude of Maximum Response (mV)	Area under Response-time curve (mV•hr)	Time for Response to Decay to 75% of Maximum (mins)
Young n = 6					
2.5 mM	5.6±0.8	11.4±1.1	333±99	180±96	21.5±7.6
5.0 mM	5.8±0.9	11.1±1.6	400±98	183±76	19.8±4.5
10.0 mM	4.6±0.7	8.9±1.3	609±87	378±92	30.5±7.3
25.0 mM	3.8±0.6	10.8±1.3	673±45	439±49	34.1±5.5
Old n = 7					
2.5 mM	9.1±1.5	15.1±1.3	281±70	89±20	20.2±1.8
5.0 mM	7.4±1.4	14.8±2.4	500±82	269±68	23.3±5.1
10.0 mM	5.7±0.8	17.7±3.4	618±83	448±74	37.3±6.8
25.0 mM	5.6±1.0	13.4±2.4	686±79	488±65	40.9±3.8

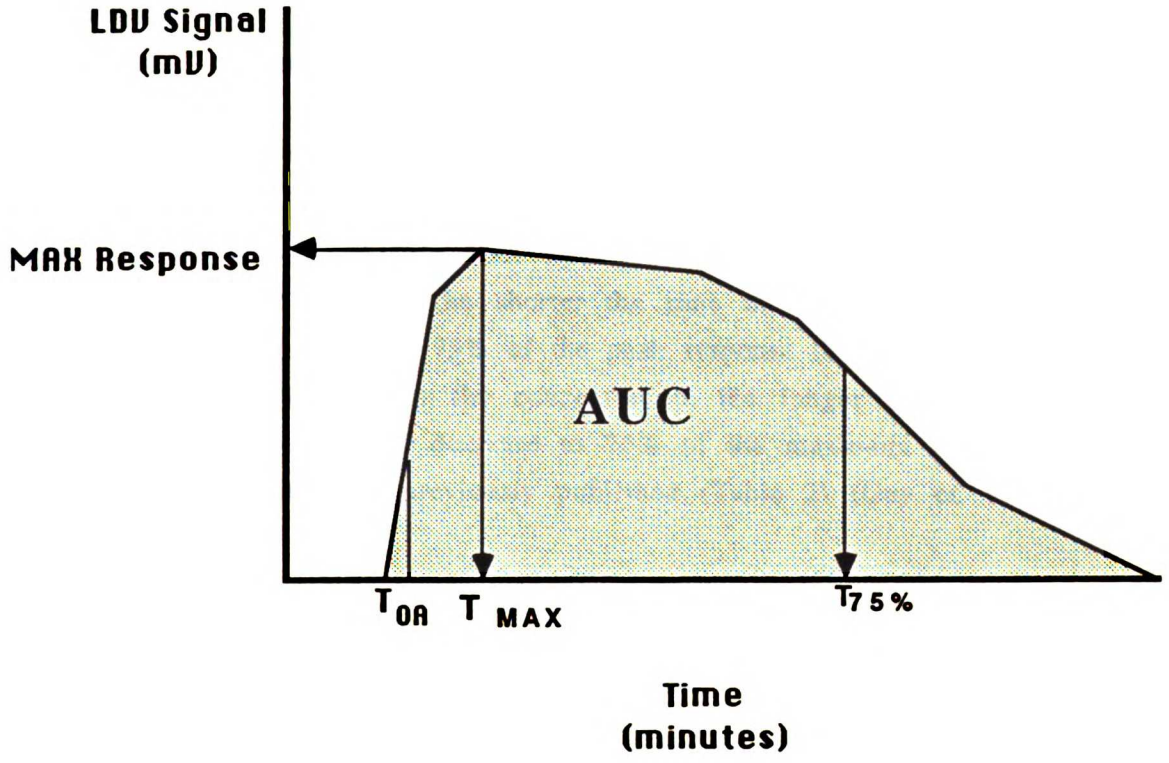


Figure 6. Diagram of LDF signal output as a function of time after 15 second application of methyl nicotinate. The parameters selected to characterize the time course and extent of the normalized pharmacodynamic response include: (1) the magnitude of the maximum response (millivolt) (2) the area under the response-time curve (AUC) (millivolt•hour) (3) the time of onset of action (minutes).(4) the time of maximum response (T_{max}) (minutes) (5) the time required for the response to return to 75% of the maximum achieved ($T_{75\%}$) (minutes)

A trend toward saturability of the local microvascular response can be observed in the plots of normalized maximum response and area under the response-time curve versus applied concentration (Figures 7 and 8, respectively). Importantly, these figures show that dose-response behavior was investigated using concentrations below saturation of the pharmacodynamic response, i.e. in the linear portion of the effect versus applied concentration curve. Preliminary data and previously published reports (Guy et. al., 1984) confirm that these concentrations are below vasoresponse saturation. The onset of action (Figure 9) and the time of maximum response (Figure 10) are both weakly dose - dependent, i.e. the higher the concentration applied, the shorter the time of onset and the time to peak response. The time to reach 75% of the peak response (Figure 11) is also dose - dependent, i.e. the higher the concentration, the longer the time required for the vasodilation to decrease to 75% of the maximum response. These results agree with those previously published (Table 2) (Guy et. al., 1985).

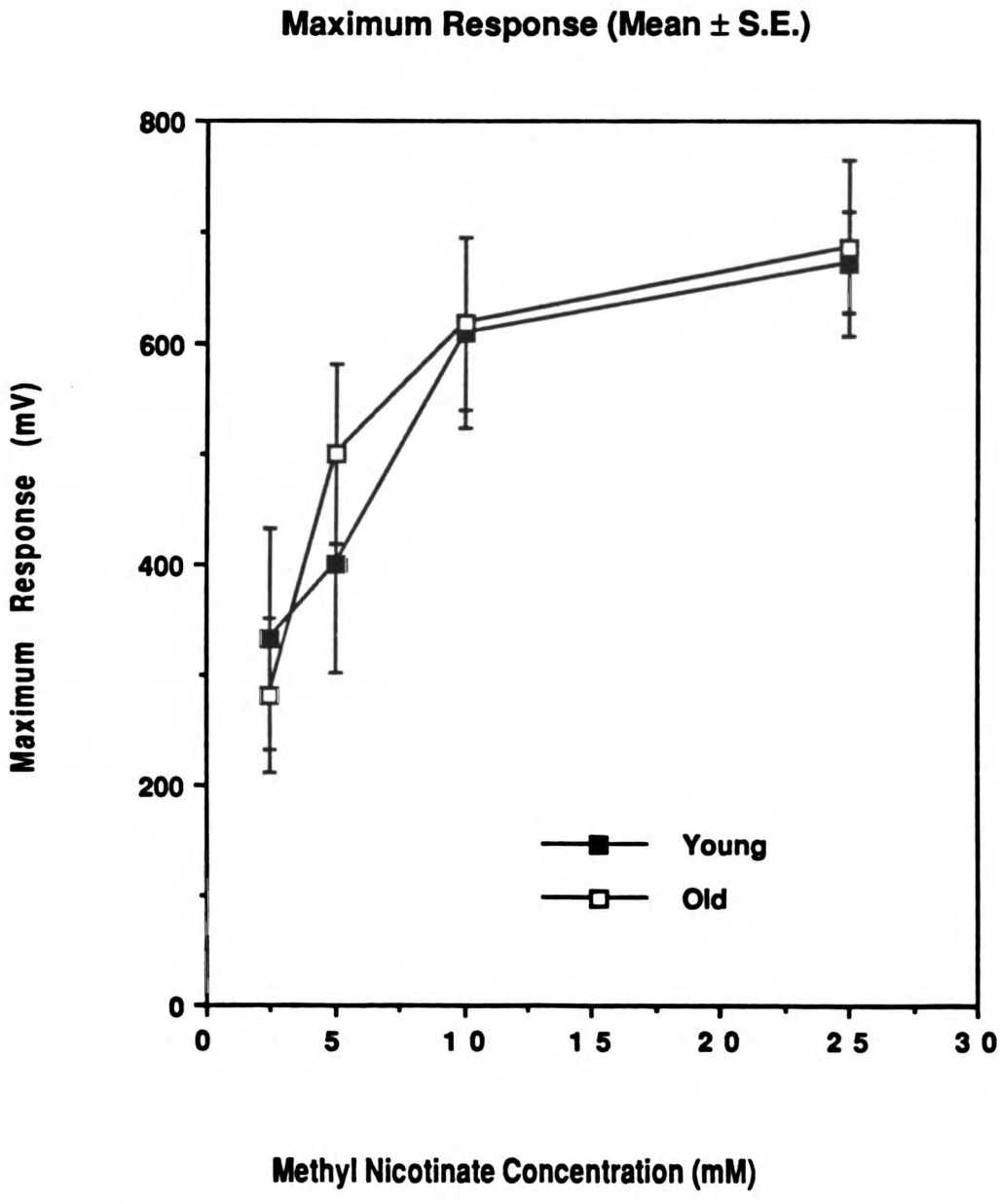


Figure 7. Young [closed squares] and old [open squares] normalized maximum response as a function of applied MN concentration. (Mean ± S.E.) No significant differences were found between the young and old groups ($\alpha = 0.05$).

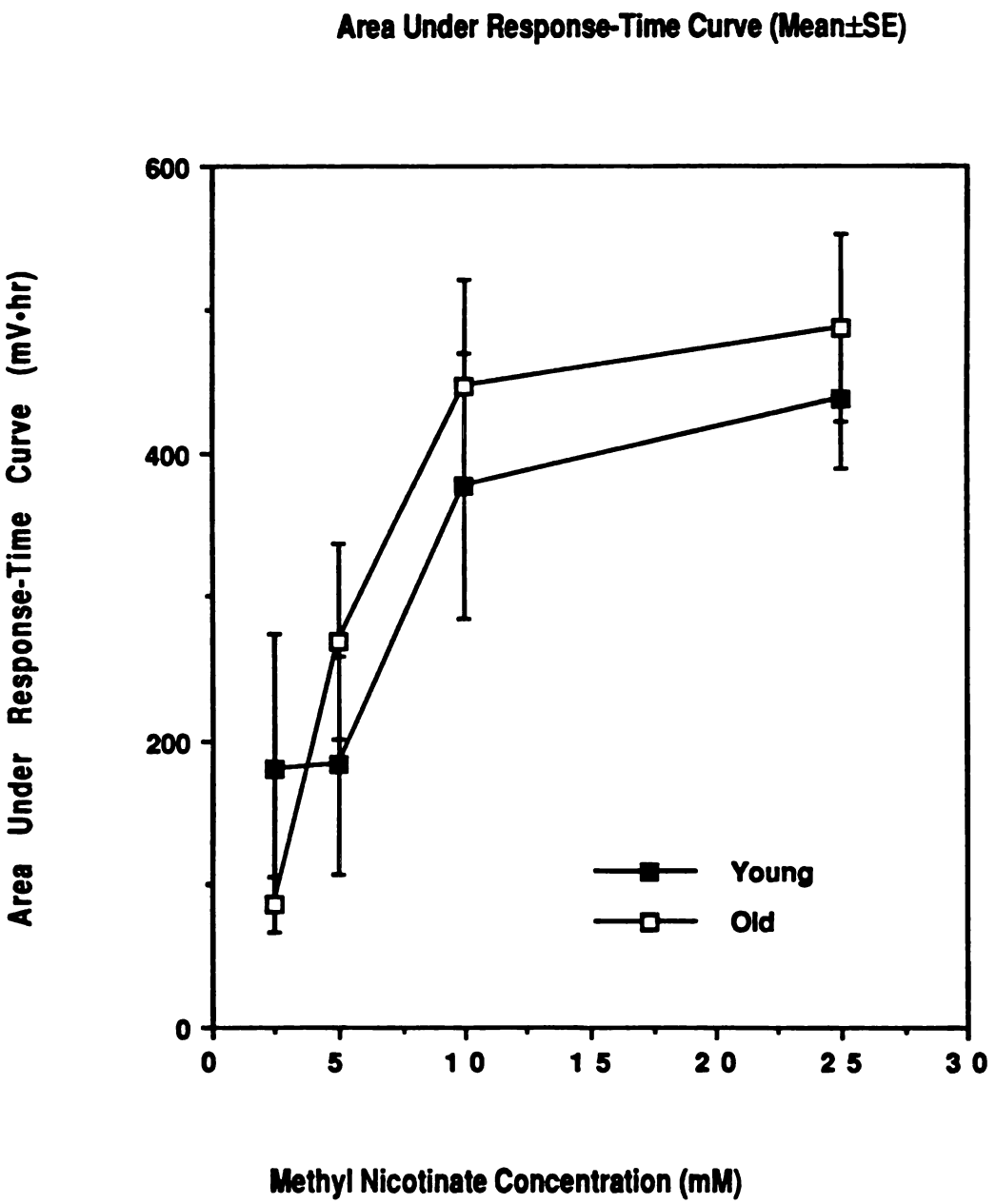


Figure 8. Young [closed squares] and old [open squares] normalized area under the response-time curve as a function of applied MN concentration. (Mean ± S.E.) No significant differences were found between the young and old groups ($\alpha = 0.05$)

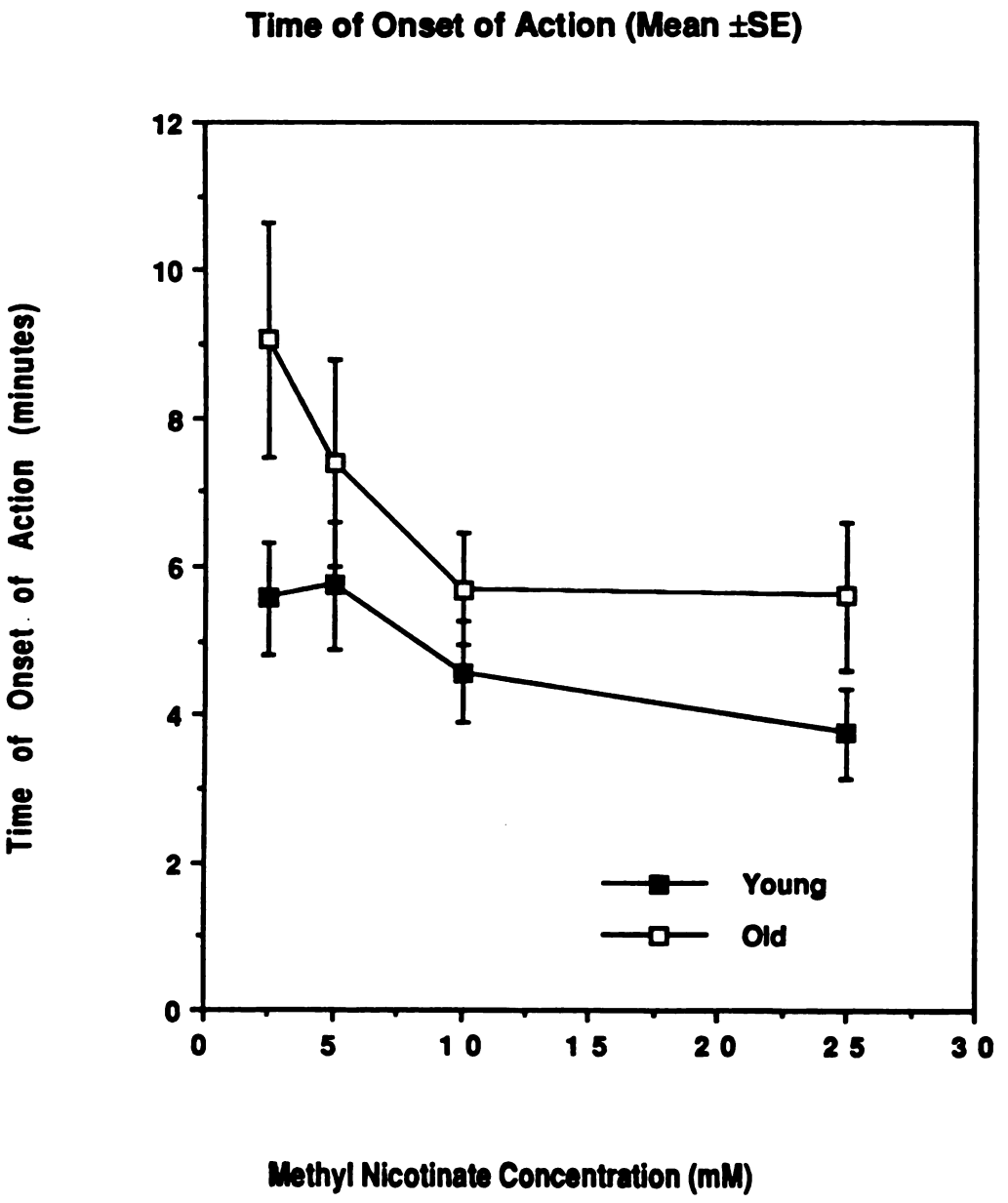


Figure 9. Young [closed squares] and old [open squares] time of onset of action as a function of applied MN concentration. (Mean ± S.E.)
No significant differences were found between the young and old groups ($\alpha = 0.05$).

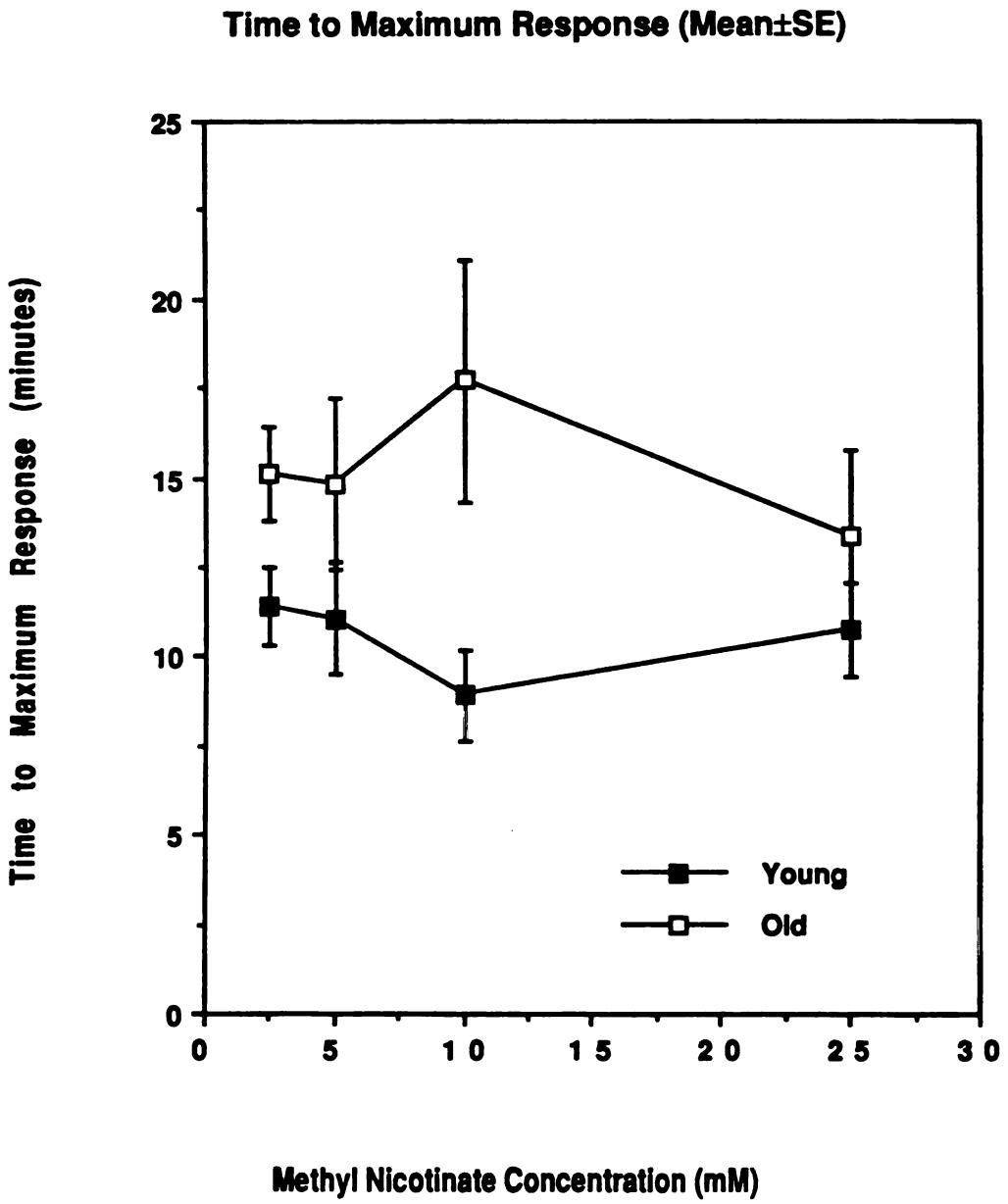


Figure 10. Young [closed squares] and old [open squares] time of maximum response as a function of applied MN concentration. (Mean ± S.E.) No significant differences were found between the young and old groups (α = 0.05).

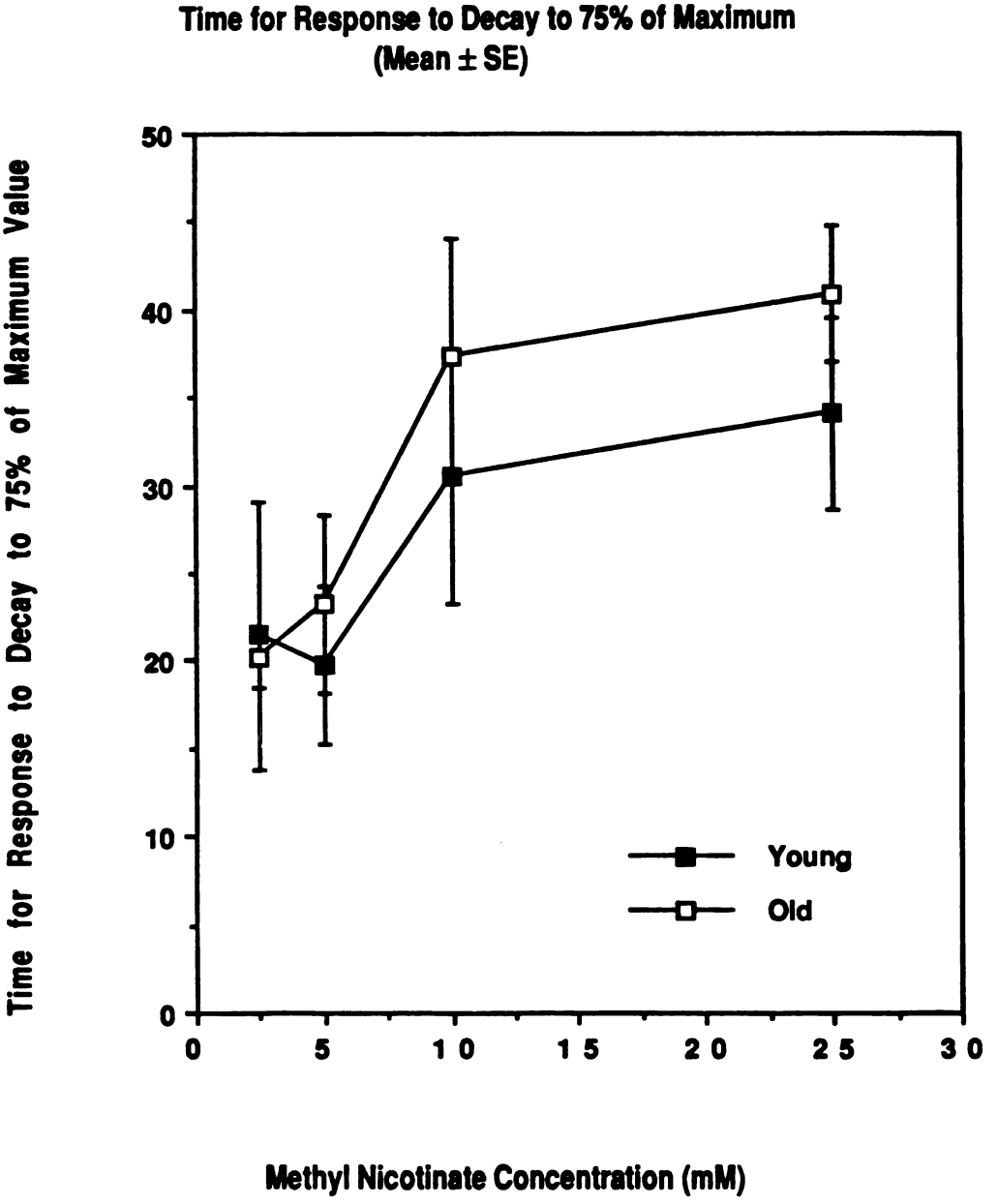


Figure 11. Young [closed squares] and old [open squares] time to decrease to 75% of the peak response as a function of applied MN concentration. (Mean ± S.E.) No significant differences were found between the young and old groups ($\alpha = 0.05$).

Group	Time to Peak response(min)	Magnitude of peak response (mV)	Area under response-time curve (mV.hr)	Response decay to 75% of maximum (min)
Young	8.4±0.5	895±112	481±83	35±6
Old	10.3±2.0	1187±82	707±92	41±4

Table 2. Summary of LDV pharmacodynamic data following topical administration of MN.*

*(Mean ± S.E.)

N=6 for each cohort

Data from Guy, R.H., Tur, E., Bjerke., et. al.,: Are there age and racial differences to methyl nicotinate-induced vasodilatation in human skin? J. Am. Acad. Dermatol. 12: 1001-1006, 1985.

The dose-response behavior associated with topical MN application was further analyzed using the pharmacokinetic-pharmacodynamic model shown in Figure 12 (Guy et. al.,1984). This model includes a number of assumptions: [1] delivery of MN across the SC is constant as long as the patch is present; [2] following input of MN to the skin, drug diffuses to its vasoactive site by a first-order process (k_i) (Albery and Hadgraft, 1979); [3] loss of MN from the vasoactive site (via capillary uptake) is a simple first-order elimination function (k_e) (Hadgraft, 1979). The model leads to an expression, based on the Hill equation (Holford and Sheiner, 1981), relating applied MN concentration to the observed pharmacologic effect:

$$(E - E_0) = \frac{E_{max} \times R^N}{R^N + C_{ss,50}} \qquad (1)$$

where $$R = k \times C_{app} \times t_{app} \times u \qquad (2)$$

and $$u = k\,(t - t_{lag}) \exp(-k\,(t - t_{lag})) \qquad (3)$$

E is the measured response, E_0 is the baseline response (i.e., baseline perfusion level), E_{max} is the maximum response, $C_{ss,50}$ is the hypothetical patch concentration of nicotinate which would be necessary to maintain, under steady-state delivery conditions, $(E - E_0) = 0.5\,E_{max}$ (assuming no tolerance), N is a parameter affecting the steepness of the effect versus dose curve, C_{app} is the applied MN concentration in the patch, and t_{app} is the duration of patch application (0.25 minutes). The concentration of drug at the site of action is controlled by the input (k_i) and elimination (k_e) kinetics, however, the results do not allow k_i and k_e to be differentiated because their values are similiar. Consequently, one proceeds by setting:

$$k = k_i = k_e \qquad (4)$$

and a simple single exponential expression for u (the normalized concentration of MN within the skin) results (equation 3) (Gibaldi and Perrier, 1975). This equation incorporates a lag time (t_{lag}), which is the observed period between drug application and response onset.

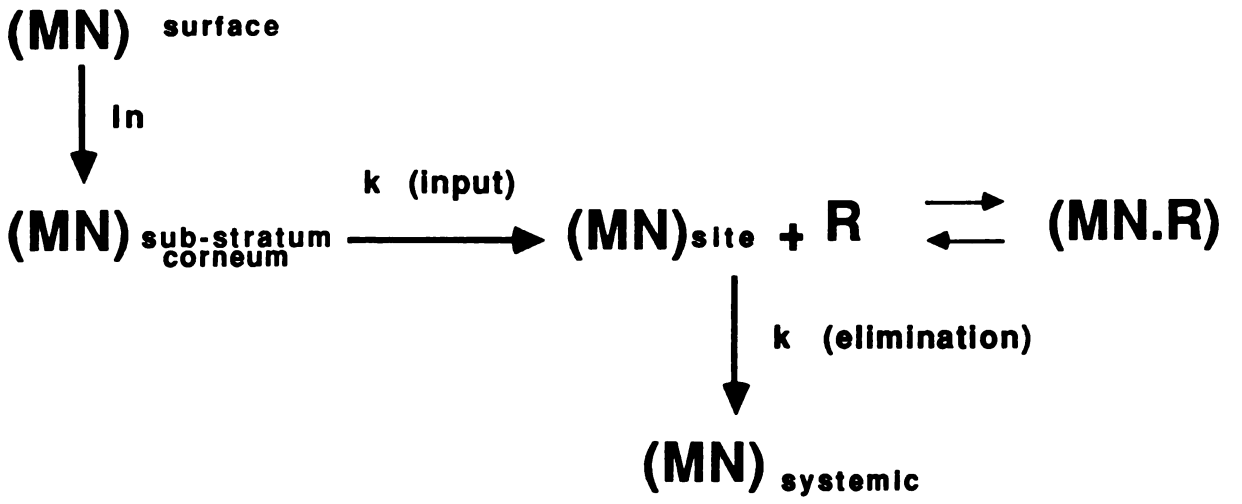


Figure 12. Pharmacokinetic - pharmacodynamic model. Adapted from Guy et. al., Pharmacodynamic measurements of methyl nicotinate percutaneous absorption, Pharm. Res., 1, 76-81, 1984.

The experimental data were fitted to equation (1) using MKMODEL, a parameter estimation modelling procedure which uses a least - squares regression program (MKMODEL, 1982). In this way, parameter values for k, $C_{ss,50}$, E_{max} and N were identified for each set of results. To reduce the number of parameters being fitted at any one time, Capp, E_0 and t_{lag} were fixed; Capp for obvious reasons, E_0 and t_{lag} because they can be directly obtained from the data. The fits of the data generated by the regression analysis are similiar to those previously published (Figure 13) (Guy et. al., 1984). Average parameter estimates for both "populations" were found and the outcome of this analysis is summarized in Table 3.

Statistical Analysis

The fitted parameters from the pharmacodynamic model of Guy et. al.(1984) were organized for a repeated measures analysis of variance, with group (young: old) being a between - subjects factor, and concentration and arm being within - subjects factors. The multivariate analysis output, specifically Wilks' lambda, was used for tests of significance involving within - subjects effects. Polynomial and profile contrasts (differences in means between successive levels) were tested for effects of concentration. Informal checks on the assumption of multivariate normality were carried out using SAS procedures UNIVARIATE and PLOT.

Further statistical analysis focused upon k and $C_{ss,50}$, the parameters most simply and directly identified with microcirculatory efficiency and sensitivity. Preliminary examination of the computed contrasts on each subject revealed contamination with outliers. Logarithmic transformation of the raw data reduced the evidence of outliers and therefore was used in subsequent analysis. The frequency of outliers did not correlate with any particular methyl nicotinate concentration administered.

There were no significant interaction effects involving any of the factors ($p > 0.25$), so only results concerning main effects are presented. Table 4 summarizes the tests of main effects, which indicate weakly significant effects ($0.05 < p < 0.1$) of concentration in both parameters, but no other significant effects. Table 5 summarizes means and tests of contrasts in concentration. Log k exhibited a significant decreasing linear trend in concentration ($p = 0.006$)

plus a weakly significant upward curvature. While log $C_{ss,50}$ exhibited a significant increasing linear trend (p = 0.038), this appeared to be due only to a marked jump between concentrations 10 and 25 mM (p = 0.040).

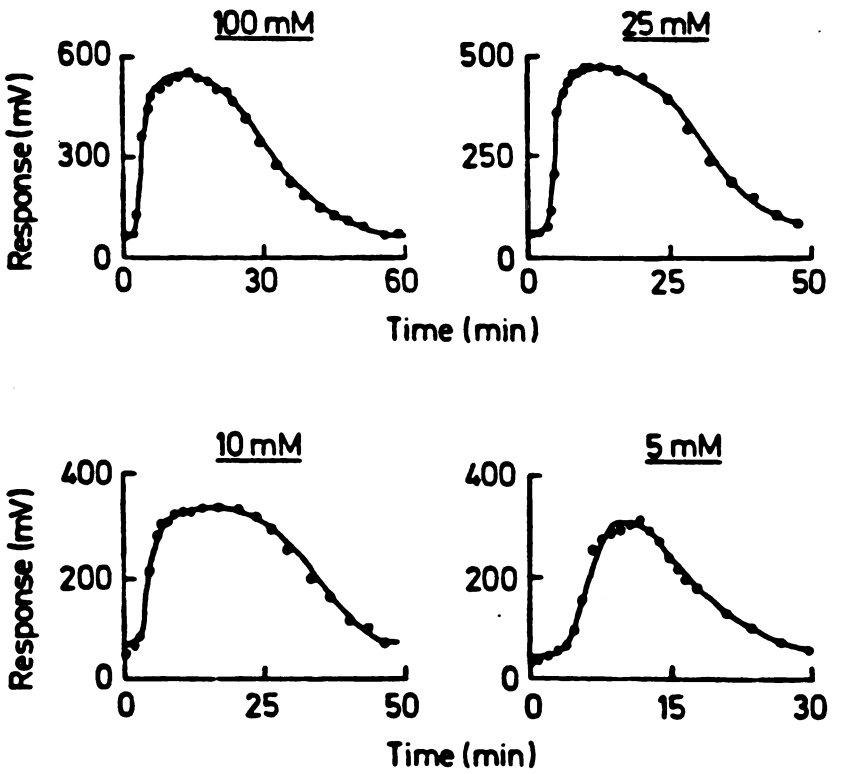


Figure 13. Non-linear least squares regression analysis and fit using equation 1 (continuous line) to the LDF experimental data (solid circles) of one subject. Reproduced from Guy, R.H., et. al., Pharmacodynamic measurements of methyl nicotinate percutaneous absorption. Pharm. Res.;1984; 1: 76-81

Table 3. Pharmacodynamic Model Parameters

(Mean ± S.E.)

Group	$k\ (min^{-1})$	$C_{ss,50}$	E_{max} (mV)	N
Young n = 6	0.109±0.013	0.09±0.02	756±24	2.60±0.32
Old n=7	0.090±0.011[a]	0.07±0.02[a]	743±39[a]	3.15±0.23[a]

[a] These values not significantly different from the young control group ($p>0.05$)

Table 4. Summary of tests for main effects indicate weakly significant effects ($0.05 < p < 0.1$) of concentration in both parameters.

Source	Degrees of freedom	Log K		Log $C_{ss,50}$	
		F	P	F	P
Group	1, 11	2.01	0.18	0.05	0.82
Concentration	3, 9	3.13	0.08	3.59	0.059
Arm	1,11	0.27	0.61	0.05	0.83

Table 5. Means and tests of contrasts in concentrations. Linear trends can be seen for log k (decreasing) and log $C_{ss,50}$ (increasing). The increasing linear trend for log $C_{ss,50}$ appears to be due to a marked jump between concentrations 10 and 25 mM.

Polynomial Contrasts

	Log K	Log $C_{ss,50}$
	P	P
Linear	0.006	0.038
Quadratic	0.085	0.36
Cubic	0.62	0.72

Profile Contrasts

Concentration (mM)	Log k Mean	P	Log $C_{ss,50}$ Mean	P
2.5	-2.274		-3.340	
		0.25		0.90
5.0	-2.474		-3.317	
		0.32		0.69
10.0	-2.628		-3.496	
		0.63		0.040
25.0	-2.565		-2.829	

Discussion

The results summarized in Table 1 and Figures 7 through 11 indicate a clear relationship between increasing applied MN concentration and blood flow response. Despite the simple aqueous vehicle and the short period of skin contact, sufficient chemical does penetrate the cutaneous barrier to perturb significantly the local microvasculature. Furthermore, both the empirically derived parameters in Table 1 and the pharmacodynamic model parameters in Table 3 indicate a lack of significant differences in microcirculatory response to MN between age groups.

The pharmacodynamic changes observed require penetration to and stimulation of the microvasculature, and the empirical parameters selected to characterize the response were chosen to highlight possible differences in these processes between the subject groups. For example, if more chemical is absorbed during the 15 second exposure period, the extent of vasoresponse (i.e. the area under the response-time surve) should be enhanced; if the penetration barrier is lower, a faster onset of response may be expected; and, if the ability of the microcirculation to eliminate penetrated drug is compromised, the time for recovery of blood flow to pre-application levels should be increased. Bear in mind the criticisms applied to previous work (Guy et. al., 1985). Because MN was topically applied, these results do not unequivocally differentiate between changes in microcirculation sensitivity and differences in the barrier function.

Parameter estimation from the pharmacodynamic model analysis was undertaken to provide further insight. In the analysis presented, the estimated standard errors of the individual fitted parameter values were not used; instead, all values were weighted equally. There were several reasons for this decision. First, standard multivariate analysis of variance methods allow weighting of observations (one per subject), but not of individual values within the observations vector. While some computer programs can be used for standard univariate repeated measures analysis (in which each value is treated as a separate observation) and allow weighting, there appears to be no theory that would justify use of the resulting statistical tests. Second, the estimated standard errors for the fitted parameter values have only an asymptotic justification, and in small samples may be severely biased as

estimates of true standard errors, even if all assumptions of the model for fitting the parameter values are true. Therefore, while a weighted analysis using estimated standard errors (with weight = $(SE)^{-2}$) was intuitively appealing, this was not done as the results would be of questionable validity and interpretability.

The k values thus obtained yield half-lives for MN delivery to, and elimination from, the site of action: a value (mean ± S.E.) of 6.4 ± 0.77 minutes was obtained for the young subjects compared to 7.7 ± 0.98 minutes for the old group. While the difference in half-lives is not significant, the values obtained are in agreement with previous non-invasive investigations (Guy et. al., 1983; Tur et. al., 1983) using this vasodilator. These values are also consistent with the results of Albery et. al. (1983), who assessed the radial diffusion of MN in the dermis from the expansion of erythematous area observed following topical application of the vasodilator. Utilizing a radial transport model which included capillary uptake and re-equilibration with the local dermal tissue, the latter research showed that the lifetime of MN in the dermis was 3 to10 minutes.

As discussed, tissue clearance in aged skin (as assessed by the time required for 50% clearance of intradermally injected Na^{22} Cl) has been examined by several investigators. However, no consistent opinions have emerged. Christophers and Kligman (1965) and Tagami (1972) have both shown, in old subjects, in the midback area, a decreased ability of the microcirculation to clear the penetrant. The dermal clearance half-life for Na^{22} Cl ranges from 4.8 to 8.7 minutes in the young and 8.8 to 13.3 minutes in the old (Christophers and Kligman, 1965; Tagami, 1972). Tagami also evaluated the efficiency of the cutaneous microcirculation in the extensor forearm in both age populations. The clearance was unchanged between age groups for this site; (6.9 minutes, young versus 8.3 minutes, old). Desalva and Thompson (1965), on the other hand, observed a rate of Na^{22} Cl elimination slower in the young population (21-30 years), compared to older groups, for the forearm and face, but not for the dorsa of the hands. It must be emphasized that, although the techniques used in these earlier determinations are not equivalent, either the chemical studied or the method of delivery (i.e. intradermal injection) causes local

vasodilatation. The apparent agreement among k_e values should, therefore, be viewed cautiously and used only for comparative purposes.

The $C_{ss,50}$ values, which may be considered measures of the sensitivity of the microcirculation to drug-induced vasodilatation, showed no significant differences between age groups. Interpretation of this result can only be that, as measured by the technique employed, microvessel reactivity to the stimulus is comparable in both populations. Wilkins et. al. (1983) has most recently tried to study the mechanism of topically applied nicotinate-induced local cutaneous erythema. His studies indicate that oral pretreatment with prostaglandin inhibitors (aspirin, ibuprofen and indomethacin) inhibit the response to topically applied MN. These studies indicate that MN may act upon the microvasculature through a second-messenger mode of action. If this is the mechanism, then further elaboration of the pharmacokinetic-pharmacodynamic model may be necessary. However, independent verification of the results of Wilkins et. al. have yet to be provided.

Perhaps unexpectedly, after extensive evaluation of the results, an equivalency of response in the two age groups to a common pertubation was obtained. With further pharmacodynamic analysis, no instructive differences were revealed between age groups. This lack of significant differences may be due, in part, to the high inter-individual variability observed (coefficients of variation of the model parameters ranged from 22% to >94%). Although strict experimental procedures were followed, this degree of variability appears to be characteristic for this type of measurement (Guy et. al.,1984; Guy et. al.,1985). However, the results can be viewed favorably in light of current observations concerning the effect of skin aging on percutaneous penetration. If there is no difference in dermal capillary uptake of penetrating substances in the forearm area, but barrier function increases with age (Roskos et. al.,1986; Rougier et. al., 1988), then our efforts can be further focused on the role of the stratum corneum, the major barrier to diffusion of penetrating substances. Subtle changes in the stratum corneum barrier (eg. in stratum corneum lipid organization) may account for changes seen in penetration, independent of microcirculatory uptake in the forearm.

Chapter 4

Transepidermal Water Loss Studies

Introduction

Water reaches the surface of the skin actively via the sweat ducts and by passive diffusion across the intact epidermis. Measurement of this latter process, transepidermal water loss (TEWL), is a sensitive evaluation of the integrity of the stratum corneum (SC) (Nilsson, 1977). A principal function of the SC is to prevent excessive water loss from the body and to maintain homeostasis.

Previous studies have determined evaporative water loss across the skin as a function of increasing chronologic age. For example, measurements of TEWL have been used to assess adequacy of barrier function in premature infants (< 30 gestational weeks) relative to that of full-term infants (Hammarlund and Sedin, 1979): it was found that water evaporation rate from the skin is higher in preterm infants than in term infants. At the opposite end of the age spectrum, i.e. from adulthood through old age, baseline TEWL does not appear to change with increasing age (Grice and Bettley,1967; Kligman,1979; Leveque et. al.,1984).

The objective of this study was to re-examine the effect of increasing age on barrier function integrity as assessed by TEWL. Initial measurements confirmed the previously published observation that baseline TEWL does not change significantly with increasing age. This led us to examine the water barrier under "stressed" conditions: the skin was first occluded, thereby preventing TEWL, and then the recovery of the perturbed tissue was subsequently monitored. It was our hypothesis that this challenge to the skin would amplify subtle barrier function changes that occur with increasing age. Evidence that occlusion would enhance the "signal-to-noise" ratio associated with TEWL measurements was indicated by previously published experiments, which revealed significant reversible increases in TEWL due to occlusion(Aly et. al., 1978). In the experiments described below, occlusion-induced hydration changes in the SC were monitored by the relaxation of TEWL to baseline (pre-occlusion) levels.

Materials and Methods

Evaporimeter Probe

Transepidermal water loss measurements were performed with a commercially available, unventilated evaporimeter (EPIC Servomed AB, Stockholm, Sweden). The Servomed EP1C is designed for the quantitative determination of water evaporation from or to surfaces in contact with the atmosphere. The evaporimeter probe has been described elsewhere in detail (Nilsson, 1975; Nilsson, 1977; Scott, 1982; Wilson, 1982). Briefly, the instrument is capable of measuring relative air humidity (%), water vapor partial pressure (mmHg) and evaporation rate ($g/m^2/hr$) [to a lower limit of 0.1 $g/m^2/hr$]. The evaporimeter probe contains two sensors (Figure 1) (coated with an organic polymer dielectrically sensitive to humidity) vertically stacked at a known distance from the skin surface. Each sensor is coupled to a fast thermistor and both are enclosed in a polytetrafluoroethylene capsule, cylindrically shaped and open at both ends. The probe is held against the surface of the skin, orienting the sensors parallel to the skin surface and perpendicular to the vapor pressure gradient. Investigations of transepidermal water loss can be performed, therefore, with minimal influence of ambient conditions on the microclimate at the surface of the skin. The TEWL reading from the probe stabilizes within approximately 15-30 seconds.

Standard Curve

A standard curve was constructed from saturated salt solutions that produce a known humidity (relative to pure water) when allowed to equilibrate in an enclosed space at a specific temperature (Table 1). The rationale for the standard curve is that, with increasing humidity in the enclosed vessel, there is a concomitant increase in flux rate from the vessel into the surrounding atmosphere; zero evaporation rate occurs when the humidity within the enclosed vessel equals the relative humidity of the ambient atmosphere; negative flux values occur when the humidity in the surrounding atmosphere is greater than that in the enclosed vessel. Figure 2 shows the standard curve constructed at an ambient relative humidity of 34%. A variation of this procedure, used to evaluate the accuracy of the evaporimeter sensors to "read" the humidity above a saturated salt solution at a given temperature, was performed every 12 months to substantiate the validity of the probe readings.

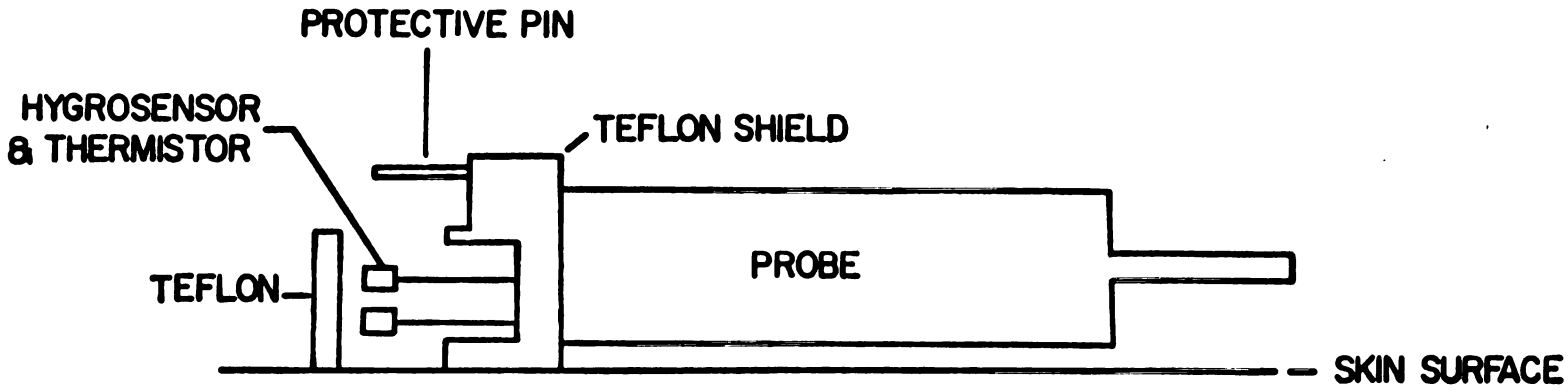
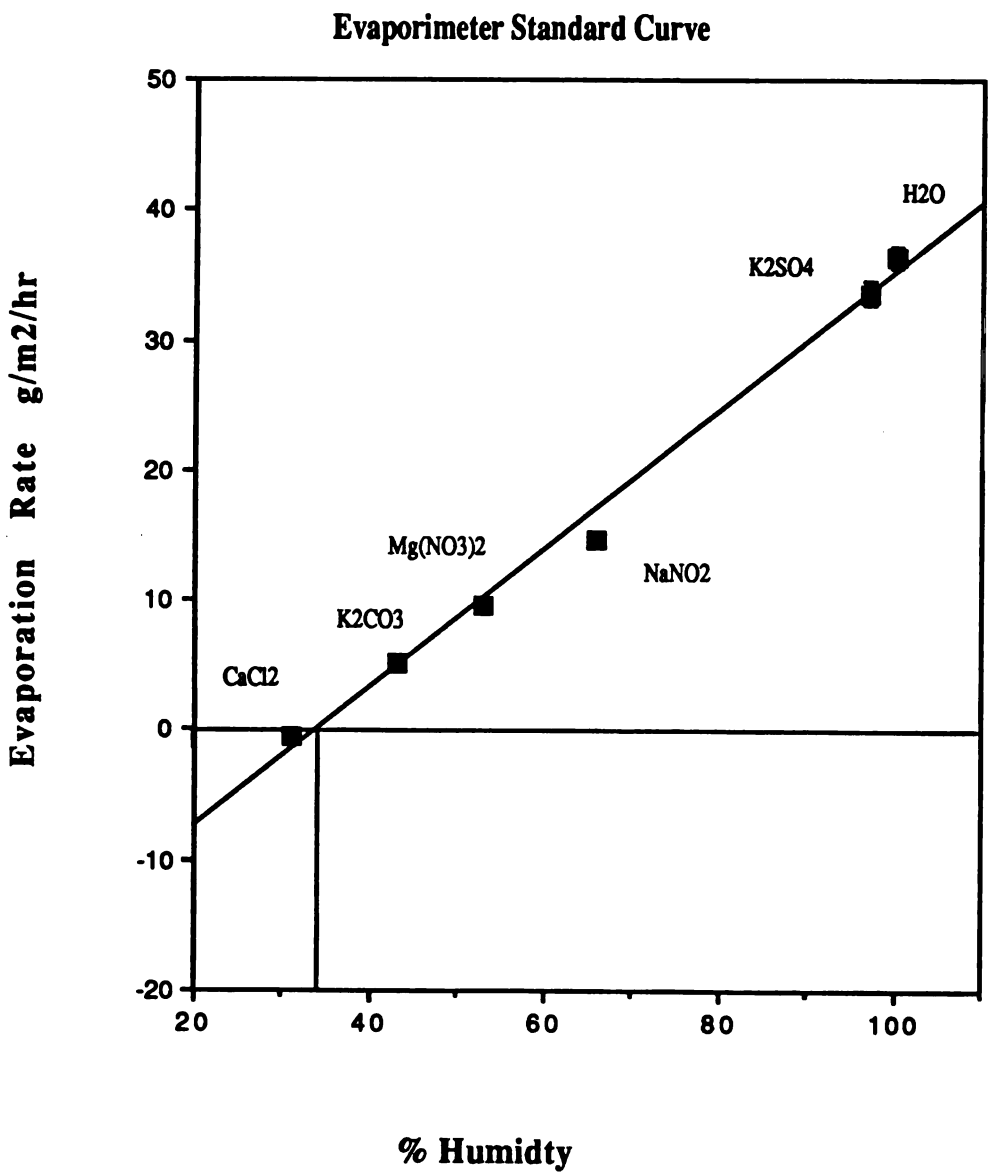


Figure 1. An evaporimeter consists of an open tube of circular cross-section placed against the skin. Two relative humidity sensors are positioned within the tube at known distances above the skin surface; then, from the measured relative humdity gradient TEWL is calculated.



Note: ambient relative humidity = 34%

Figure 2. Evaporimeter standard curve.

Table1. Solid phase and corresponding percent humidity used for standard curve for evaporimeter

Solid Phase	T(°C)	% Humidity[a]
$CaCl_2 \cdot 6H_2O$	24.5	31
$K_2CO_3 \cdot 2H_2O$	24.5	43
$Mg(NO_3)_2$	24	53
$NaNO_2$	20	66
K_2SO_4	23	97
H_2O	23	100

[a] Source: CRC Handbook of Chemistry and Physics, 65th Edition, CRC Press, pg E-42.

General

Volunteers were caucasian males and females divided into two groups: young (19-42 years) [n=13] and old (69-85 years) [n=9]. Volunteers had no history of dermatological disease. Occlusion was achieved using a 2-cm diameter, polypropylene Hilltop® chamber (Figure 3) (HTC) (Hilltop Research, Inc., Cleveland, Ohio). All measurements were made on the ventral forearm (bilateral sites) in a draft-free environment; relative humidity was 30-50 % and ambient temperature 23°±2°C. Since large experimental variation due to such factors as ambient temperature, relative humidity and rate of perspiration can invalidate TEWL results, well-controlled experimental conditions were maintained. The skin temperature during measurement, on any given individual, was < 33° C at a room temperature of < 25° C. All TEWL measurements were normalized to a 30°C skin temperature as previously described (Mathias et. al., 1981). Mathias et. al. measured TEWL as a function of skin temperature, using human cadaver skin in modified diffusion chambers, and derived a formula that allows conversion of TEWL at any skin temperature to a standard reference temperature of 30°C.

Preliminary Studies

Preliminary investigations sought to obtain an occlusion time for complete SC hydration. Occlusion times of 1, 2, 6, 12, 24, 36 and 48 hours revealed that, for shorter occlusive periods (1 and 2 hours), the SC did not always achieve full hydration, i.e., the initial TEWL values following removal of the HTC were significantly lower than the corresponding values after 6, 12 or 24 hours occlusion. TEWL following 36 and 48 hours occlusion occasionally exhibited unexpected behavior in that water loss remained elevated at a constant level for a protracted period (ca. 4 minutes) before commencing the typical relaxation to baseline values. The reason for this extended plateau is not known nor readily discernible, but may be associated with an artifact resulting from desquamated cells lying on the skin surface. Results on any individual were essentially identical following occlusion times of 6, 12 and 24 hours. In light of the above observations, we chose to occlude the skin for 24 hours. This treatment optimized volunteer compliance with full SC hydration in the absence of maceration.

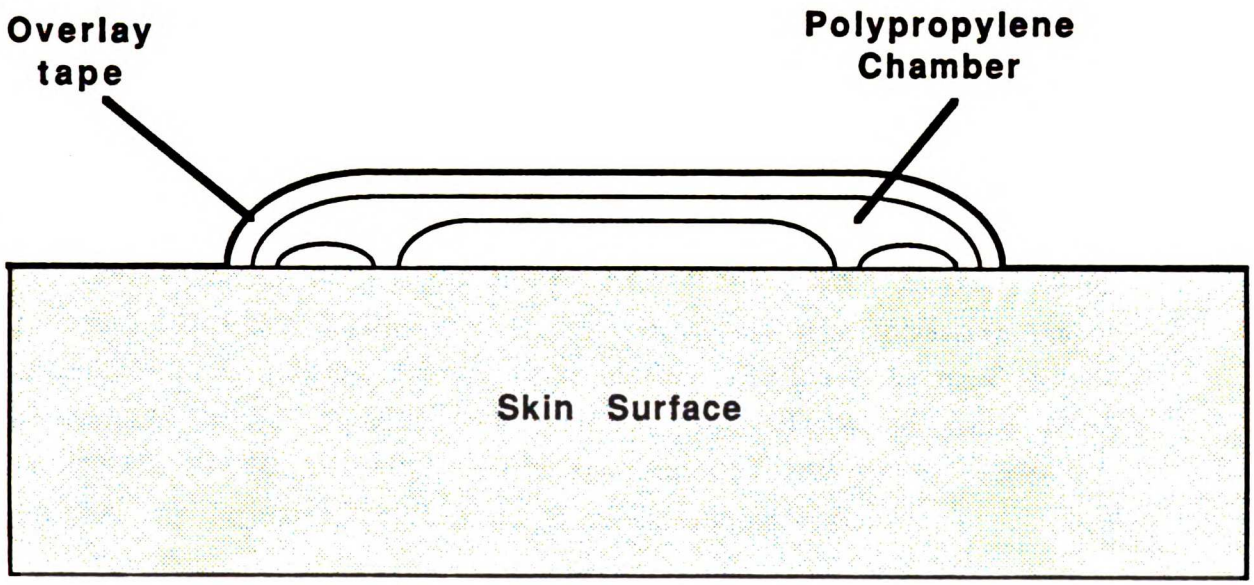


Figure 3. Diagram of Hilltop® Chamber.

Data analysis of the TEWL results assumes that vapor phase diffusion of water from the skin surface to the relative humidity sensors in the evaporimeter probe is much faster than water diffusion through the SC, i.e., the resistance to water transport in the air layer above the skin is negligible. To verify the validity of this approximation, both continuous and discontinuous TEWL measurements were made and were found to be indistinguishable. Removal and replacement of the probe disturbs the air layer over the skin surface. If the water concentration gradient can be rapidly re-established, then discontinuous values will overlap the continuously acquired values. The results (Figure 4) demonstrate this to be the case.

Final Studies - Results and Calculations

We have confirmed the observations of others (Grice, 1967; Kligman, 1979; Leveque, 1984) that baseline TEWL does not change with increasing age (Figure 5). It is unlikely, therefore, that baseline TEWL can be used to differentiate barrier function with increasing age. Hence, to probe whether pertubation of SC hydration reveals age-associated changes in barrier function, we measured TEWL rates following simple occlusion.

Occlusion prevents the normal passive diffusion of water from the skin into the atmosphere. As a result, the water content of the stratum corneum rises, until equilibrium with the body occurs. Upon removal of the occlusion, TEWL is elevated above its baseline value, but then returns to this level as the linear concentration gradient across the SC is re-established. Representative data from 26 year old and 85 year old individuals, following 24 hours occlusion, illustrate this decay of water evaporation rate immediately following removal of the HTC (Figure 6). Note the extended time scale for the older individual illustrating the relative slowness of the relaxation process in this subject.

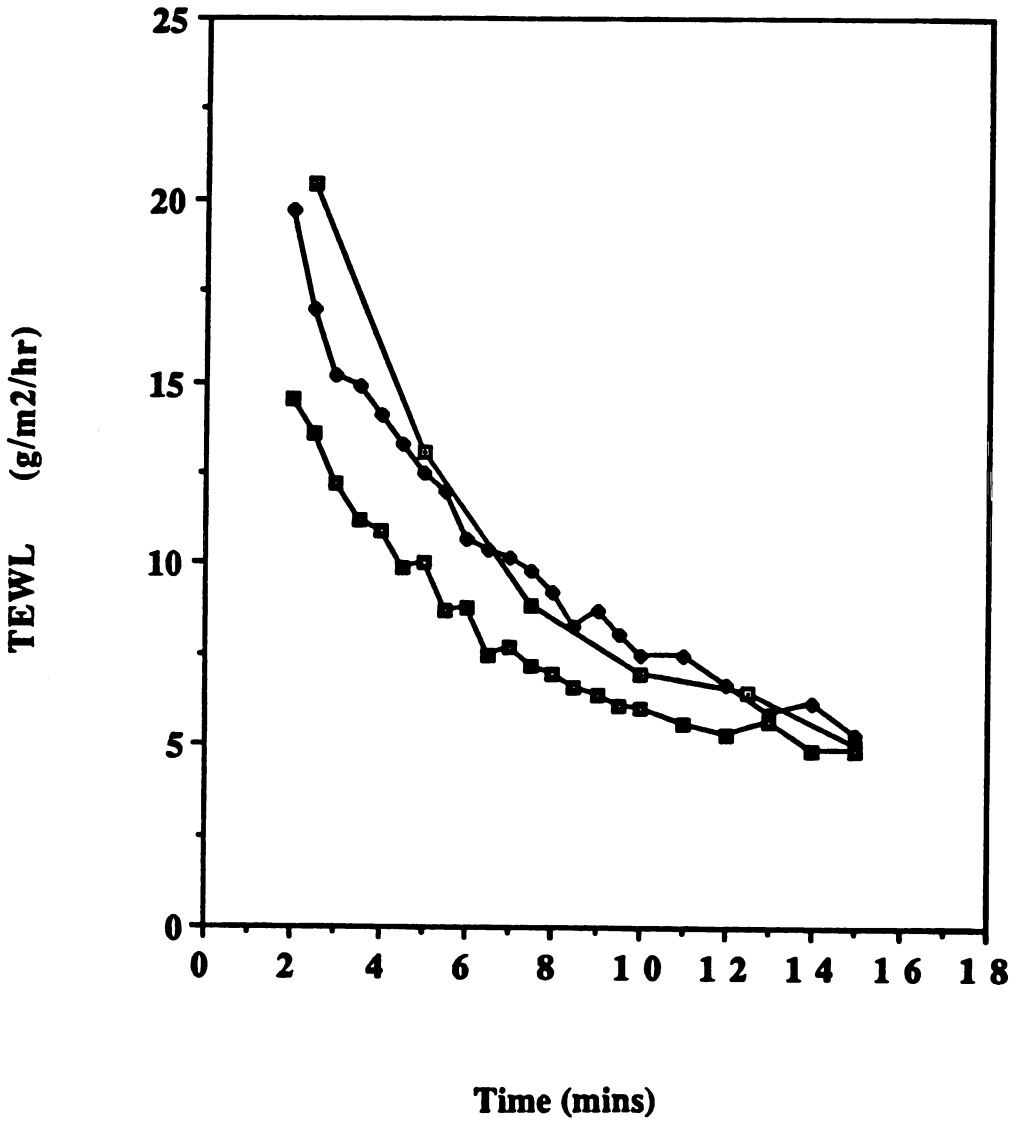


Figure 4. Evaporation rate ($g/m^2/hr$) versus time from a young subject (28 years) following occlusion for 12 hours. The closed diamonds and closed squares are readings taken continuously on two separate occasions. The open squares are measurements made discontinuously; between readings the evaporimeter was removed from the skin. The results also serve to illustrate the typical intra-subject variability observed in TEWL observations.

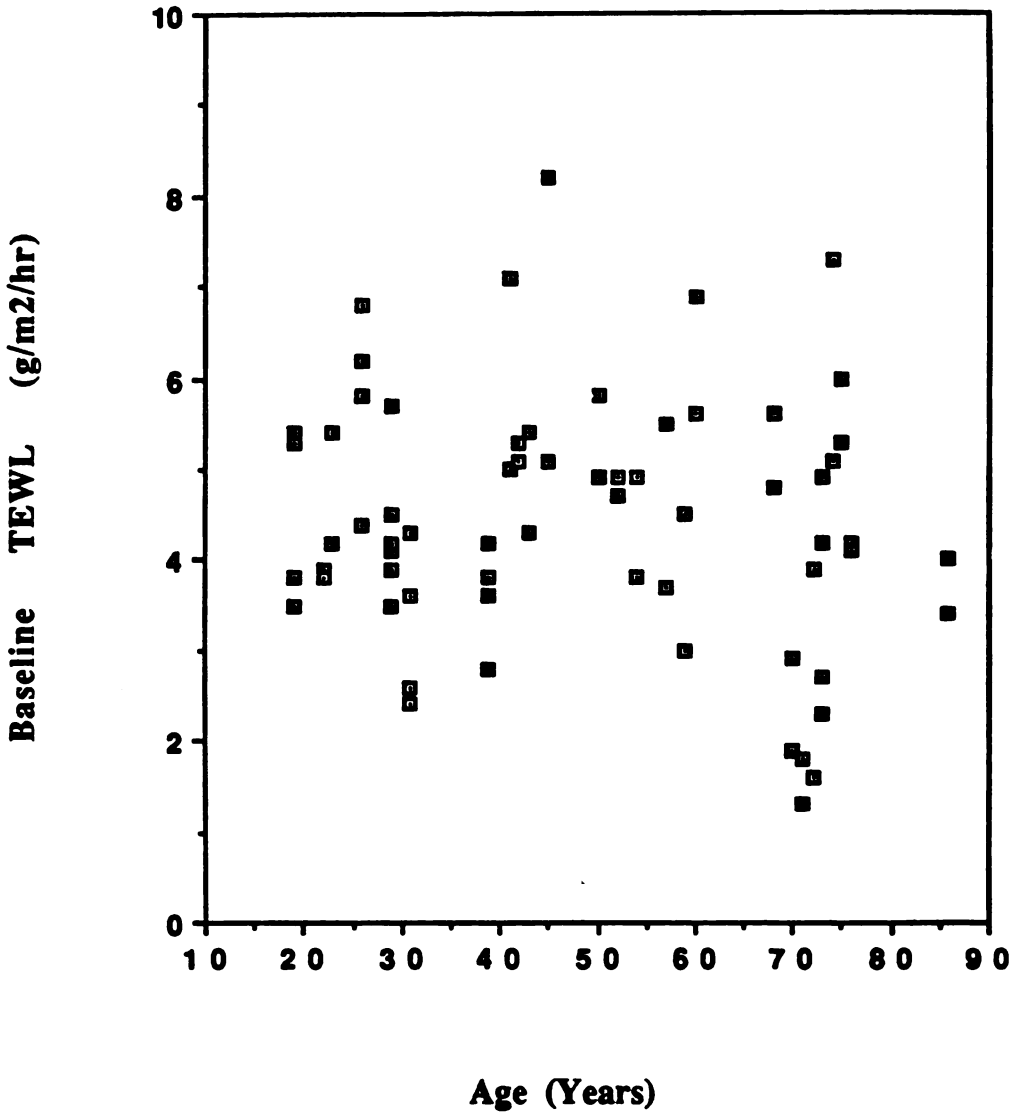


Figure 5. Baseline TEWL readings from 33 subjects in the age range 19-85 years. Measurements were made on the ventral forearm.

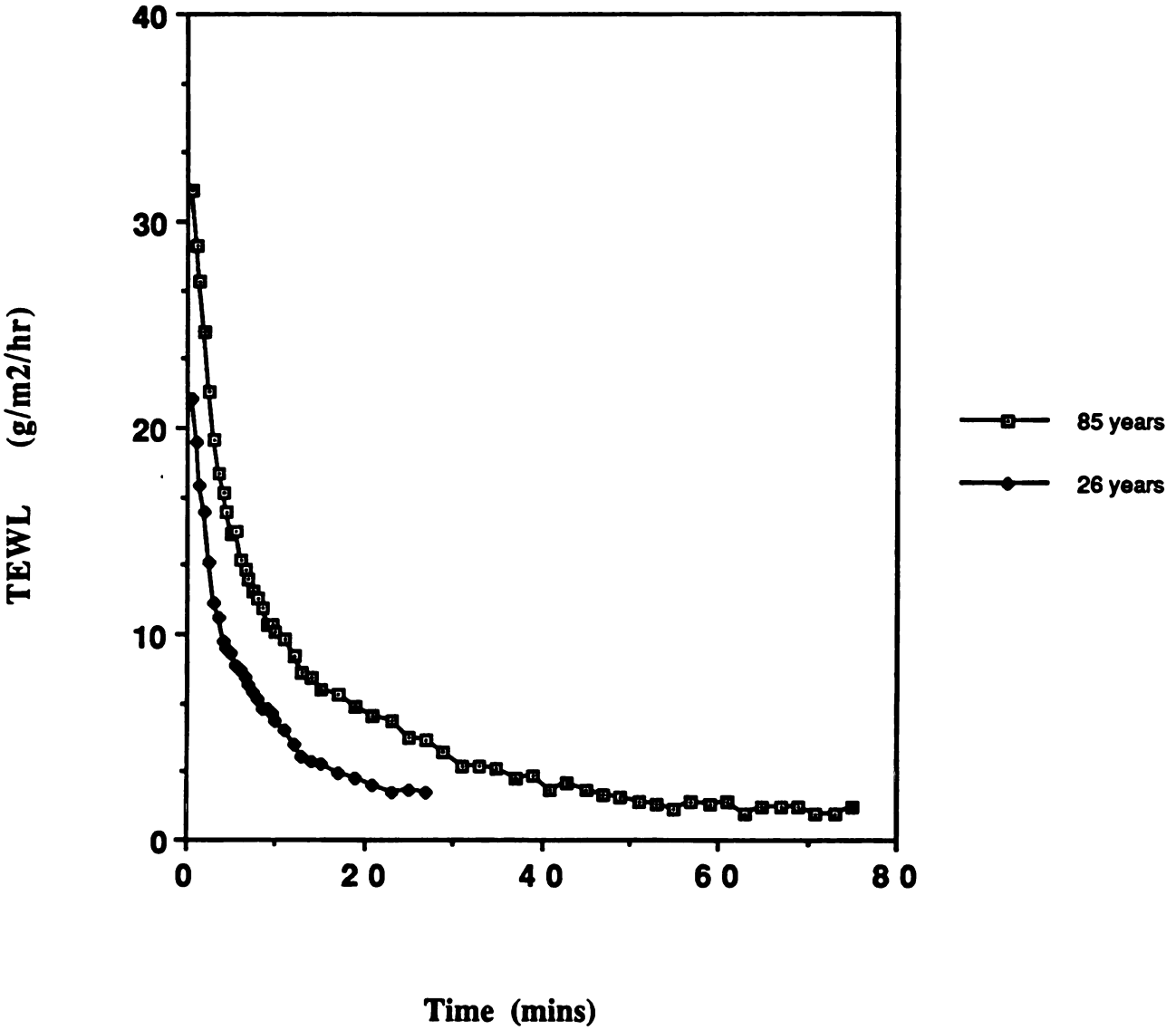


Figure 6. Representative data showing evaporation rate ($g/m^2/hr$) versus time following removal of the HTC. The closed diamonds are results from a 26 year old subject; the open squares are results from an 85 year old subject. The occlusion time was 24 hours. Note the extended time scale for the older individual illustrating the relative slowness of the 'relaxation' process in this subject

Schematically, this experiment produces changes in the water activity gradient across the stratum corneum shown in Figure 7. Following removal of occlusion, the step-function in water activity collapses to the normal linear gradient. This relaxation process can be analyzed using standard diffusion theory (Baker and Lonsdale, 1974) provided that a number of assumptions, implicit in the treatment of this non-steady state diffusion problem by simple Fickian principles, are recognized:

1) The SC is an isotropic membrane of constant thickness.

2) The diffusion coefficient (D) of water across the SC is both distance and concentration independent.

3) The temperature of the membrane is constant across its width and throughout the measurement.

4) The atmosphere above the skin (at the skin-air interface) is a perfect sink for water following removal of the occlusive barrier.

To interpret the relaxation of TEWL rate (J) to its baseline value (J_∞), Fick's 2nd Law of Diffusion for water in the SC membrane must be solved:

$$(1) \quad \frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2}\right)$$

with the boundary conditions:

(i)	$a_{H_2O} = 1,$	$0 < x < 1$ at $t = 0$
(ii)	$a_{H_2O} = 1,$	$x = 0$ for $t > 0$
(iii)	$a_{H_2O} = 0,$	$x = 1$ for $t > 0$

An adequate solution, valid for all but the shortest times (Baker and Lonsdale, 1974) is:

$$(2) \qquad \frac{J}{J_\infty} = 1 + 2\ \exp\left(\frac{-D\pi^2 t}{l^2}\right)$$

This function is plotted in Figure 8. The decay curve of J/J_∞ can therefore be used to find the characteristic transport ratio (D/l^2, where l = diffusional path length) and the classic "lag-time" ($t_{lag} = l^2/6D$) for water diffusion across the SC. If $t = t_{lag}$ in equation (2), then $J/J_\infty \approx 1.39$. Thus, by measuring J/J_∞ directly with the evaporimeter, t_{lag} and D/l^2 can be easily obtained. The "control" (baseline) value J_∞ is measured for each subject at the beginning and end of each experiment and these values are averaged. The decay of J is monitored when occlusion is terminated and J/J_∞ is then calculated as a function of time. Table 2 shows that the flux at 30 seconds post-occlusion is greater for the old subjects than for the younger individuals. Additionally, the relaxation to control levels is significantly slower for the older group compared to the younger group. The $J_{15'}/J_\infty$ ratio and the t_{lag} are significantly smaller in the young individuals.

An alternative, empirical, analysis of the TEWL data leads to similiar conclusions. The TEWL relaxation measurements post-occlusion are adequately fitted by a simple biexponential function:

$$(3) \qquad (J - J_\infty) = A\ \exp(-\alpha t) + B\ \exp(-\beta t)$$

where J is the TEWL flux at time t post-occlusion (units = $g/m^2/hr$); A and B are constants (units = $g/m^2/hr$) and alpha and beta are first-order decay constants (units = reciprocal time). A biexponential function was found to fit the TEWL data significantly better than a simple exponential (JANA, Exponential Curve Stripping Program, Statistical Consultants, Inc., Lexington, KY); however, inclusion of additional exponential terms does not cause further improvement in data fit. While the approach is empirical, the rate constants obtained should be related to D / l^2 and can be used for comparative purposes between young and old subject groups (Table 3). The kinetic analysis is consistent with the diffusional approach.

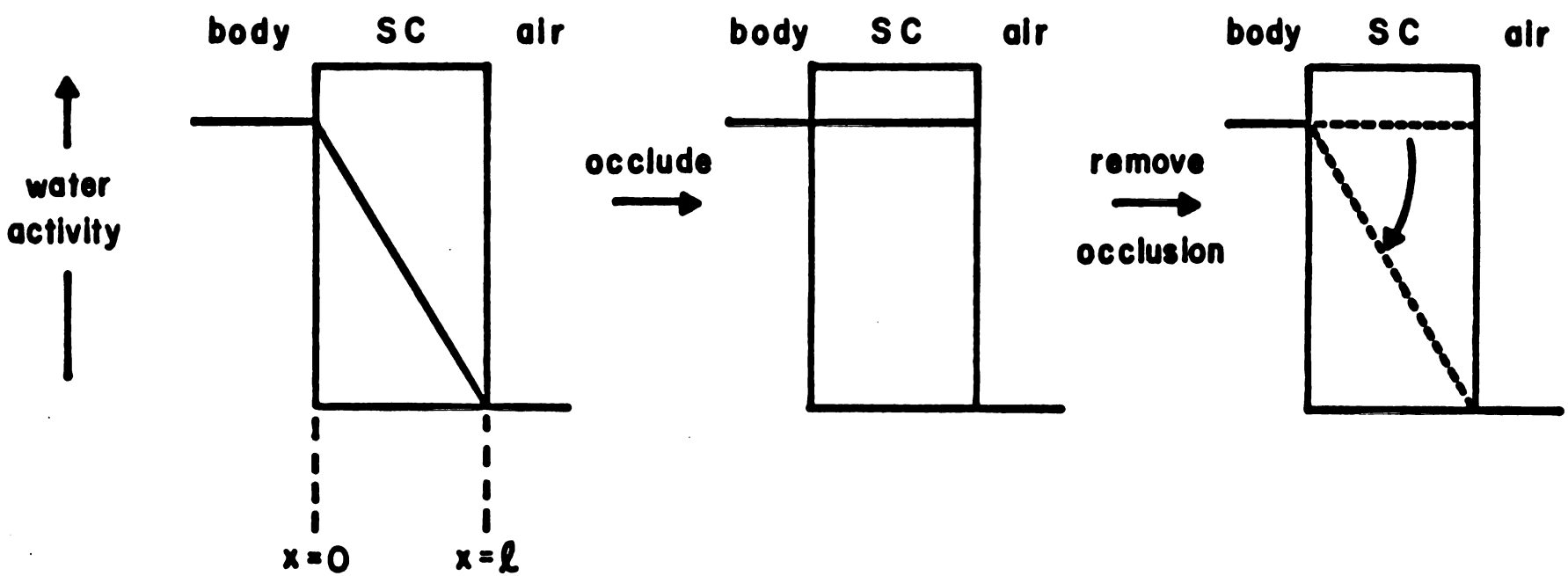


Figure 7. Schematic representation of the occlusion-induced changes in water activity gradient across the SC.

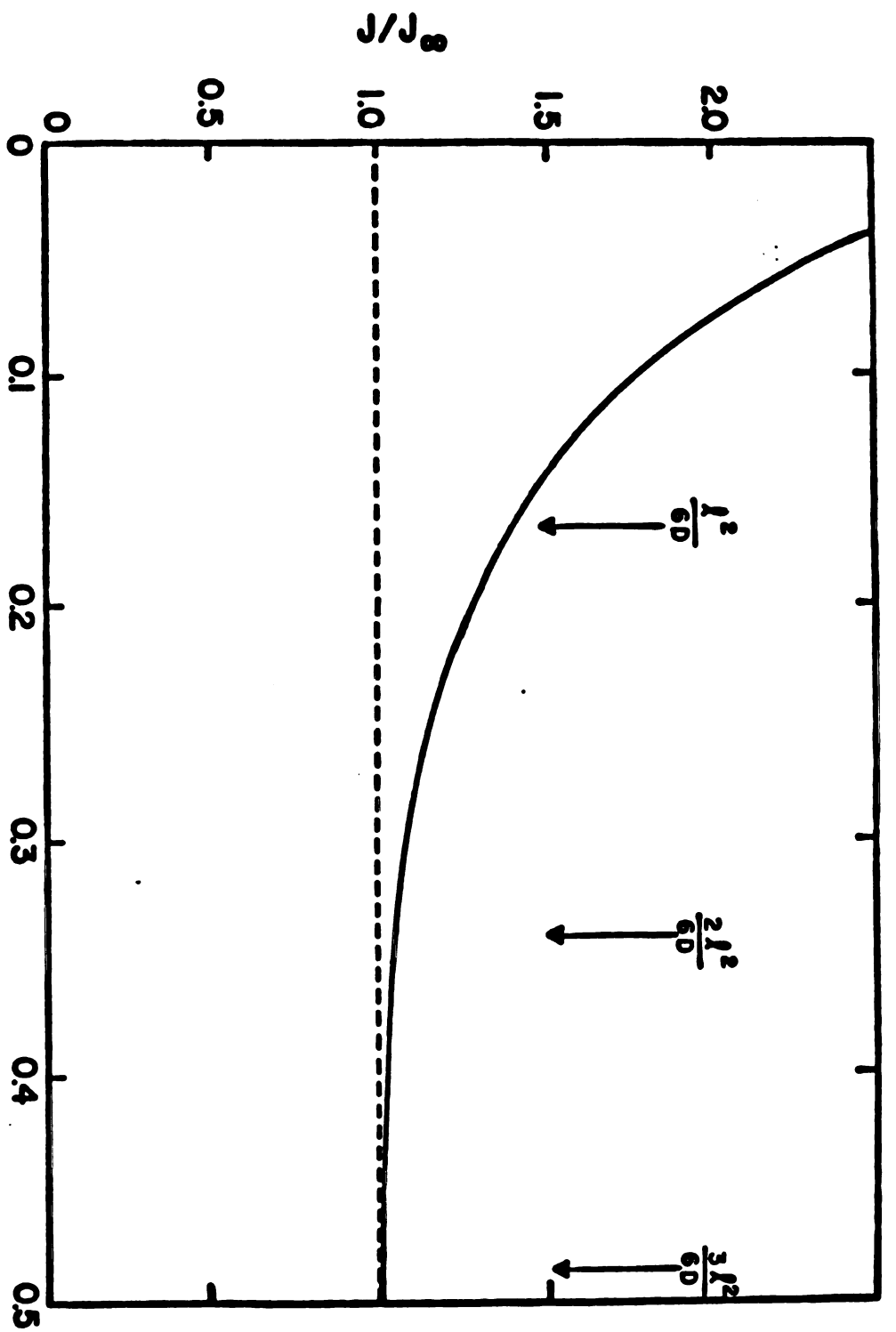


Figure 8. Graphical representation of the approximate solution to Fick's second law [with appropriate boundary conditions] reveals that, for all but the shortest times, the decay of J/J_∞ with time can be used to find the lag-time and characteristic transport ratio for the system.

Table 2. TEWL Relaxation Data
(Mean + S.D.)

	Young n = 26	Old n = 18	p value
$J_{30"}$ ($g/m^2/hr$)	28.6± 7.5	36.9±10.5	0.004
$J_{15'} / J_\infty$	2.2± 0.54	3.6±0.9	< 0.001
t_{lag} (mins)	29.4± 9.8 (n = 16)	60.0±12.7 (n = 14)	< 0.001

$J_{30"}$ = TEWL flux at t = 30 seconds post-occlusion.

$J_{15'} / J_\infty$ = the ratio of TEWL flux at t = 15 minutes post-occlusion to TEWL baseline flux (J_∞).

t_{lag} = the lag-time for water diffusion across the SC.

Table 3. TEWL Exponential Analysis
(Mean + S.D.)

	Young n = 26	Old n = 18	p value
A ($g/m^2/hr$)	23.5±9.95	30.3±11.9	0.05
alpha (min^{-1})	0.47±0.19	0.3±0.16	0.0098
B ($g/m^2/hr$)	11.1±3.9	12.6±4.5	0.24
beta (min^{-1})	0.05±0.02	0.03±0.017	0.01

J = TEWL flux at time t post-occlusion
J_∞ = TEWL baseline flux
A and B = constants (units = $g/m^2/hr$)
alpha and beta = first-order decay constants
(units = reciprocal time)

Discussion and Conclusions

As noted by ourselves and others, the ability of the SC to retard evaporative water loss does not change significantly with age. This result, is perhaps not surprising. Extensive morphological data (Lavker, 1979; 1984) give no evidence of a change in SC cell number or histologic appearance with increasing age. Additionally, as an individual ages, the SC maintains its physiologic function of preventing dehydration of the organism. The fact that the elderly do not have an increased baseline TEWL can also be inferred by a lack of excessively increased fluid consumption in this age group.

However, following a deliberate pertubation of the system, the water activity gradient across the SC (produced by occlusion) is dissipated more efficiently by the young skin than by the old. Specifically, we have observed an increase in the lag-time for water diffusion through the older SC and a decrease in the kinetics which describe the relaxation of TEWL to baseline levels following occlusion. Additionally, the initial value of $J_{30''}$ is signigicantly greater in the old group than in the young.

The explanation of these observations is not intuitively obvious. Histologically, the SC appears unaltered with age. Yet subtle changes appear to have taken place. It is known that the water content of older SC is lower (Potts, 1985; 1986). The dry, rough appearance of older skin implies a lower affinity for water. Consequently, the magnitude of the pertubation may account for the differences seen. From the baseline data, we know that the SC retains its ability to retard normal water loss over the entire age spectrum. In other words, while the total amount of water in the SC membrane may be different, the equilibrium TEWL rate is apparently unaltered with age. When the tissue is occluded, less water may be required by the younger tissue to achieve the fully-hydrated equilibrium state. Conversely, the older tissue, starting at a lower water content, requires more water to achieve the new hydration equilibrium. Once the occlusion is removed, the SC from the older subjects may have more water to be eliminated (as reflected by a greater $J_{30''}$ value) and the relaxation to baseline, therefore, may take longer. However, the kinetics of this relaxation process are significantly slower in the old subjects.

It is also possible that occlusion of old SC produces a hydration-induced structural alteration in intercellular lipid organization. On subsequent dehydration, reorganization of this structural modification could be manifested in slower relaxation kinetics.

It is also conceivable that the differential relaxation behavior of TEWL in young and old subjects has origin, at least in part, in the keratinized components of the SC. Experiments probing SC structural components, at a microscopic level, will be required to understand the mechanism which accounts for the distinctive differences in observed TEWL relaxation behavior. Preliminary investigations of this kind will be discussed in Chapter 5.

Finally, the validity of the assumptions, implicit in modeling TEWL observations by Fick's 2nd Law of Diffusion, should be reviewed. In reality, each assumption is imperfect. The SC is not isotropic; it is known that the integrity of the SC is weaker at the surface than at the viable epidermis-SC boundary. Occlusion induces hydration and swelling of the SC; hence, SC thickness probably decreases with time following removal of occlusion. The value of D is almost certainly dependent upon the degree of hydration and, as a result, will also be a function of SC depth. However, there is no observable difference in SC thickness or cell number with increasing age before hydration takes place. The final assumption is the most reasonable: the diffusion coefficient of water (D) in the vapor phase is several orders of magnitude faster than in the SC. A stagnant layer would need to be of large dimension, therefore, to exercise a significant effect. Nevertheless, while the imperfection of the diffusional analysis is clear, the disparity in experimental results between old and young subjects is unequivocal. The model used serves a useful function, therefore, in that it quantifies the age-dependent differences in terms of familiar parameters.

Chapter 5

Final Conclusions and Future Considerations

Introduction

In general, most previously published work indicates that human skin changes with increasing age (Gilchrest, 1984). The results of the percutaneous penetration studies, summarized in Chapter 2, reveal that skin aging can affect both the rate and extent of penetration. Furthermore, the data indicate that changes in absorption are sensitive to the physicochemical properties of the penetrant.

PA requires that, once the epidermal barrier has been crossed, the microcirculation have adequate ability to clear the penetrant into the systemic circulation. This uptake capability is a function of the density, efficiency and integrity of the blood vessel network. The histological work of Ryan (1983) indicates that the cutaneous vasculature undergoes attenuation and atrophy with increasing age and should therefore, show a compromised capability for penetrant uptake. However, our research failed to elucidate any differences between young and old dermal vascular efficiency. Although inter-subject variability may have played a role in this inability to distinguish between young and old, our results reveal no differences in dermal capillary uptake of penetrating substances in the forearm area between the two subject groups. Although unexpected, these results focus future investigational efforts on the role of the stratum corneum (SC) in the penetration of substances through aged skin.

In the studies presented thus far, evaluation of the skin barrier, as a function of skin aging, has been conducted at a macroscopic level. Obviously, it was important first to establish quantitatively a relationship between skin aging and changes in barrier function. It is now appropriate to probe skin aging changes at the microscopic level. Future studies in this area require a more functional approach, leading to an improved understanding of the mechanism(s) responsible for the observed changes in penetration.

To address this need, we have most recently utilized infrared (IR) spectroscopy to study the microenvironment of the SC. IR spectroscopy relies on the absorbance of infrared radiation by the chemical bonds present in the sample and provides measurements of the number and type of chemical species present. Molecular bonds undergo different motions, the changes in the

energy states of these motions correspond to the energy of IR radiation at characteristic frequencies. These various motions include stretching, bending and rotation. In each case, a change in the net dipole of the chemical bond is necessary for the species to absorb IR radiation. Within the skin, there are many chemical species that contain IR active bonds. Our initial attention has focused upon the lipids of the SC. The lipids of the SC are moderately long hydrocarbon chains, terminating with carboxylates, sulfates and other functional groups. A hydrocarbon chain contains a series of $-CH_2$ units. Since these are repeating units in hydrocarbons, absorbance of each bond in these units will be superimposed. As a result, the intensity of the characteristic absorbances for the $-CH_2$ units will be relatively large. This makes the $-CH_2$ unit of the SC lipids the ideal target for IR study. The $-CH_2$ unit possesses several unique IR absorbances which include both the asymmetric and symmetric stretching of the two hydrogen atoms around the carbon atom. These motions are sensitive to the microenvironment in which the $-CH_2$ unit resides. This sensitivity is reflected by a shift of the specific IR absorbance as a function of the environment of the $-CH_2$ unit. Changes in organization of the lipid will lead to a change in the environment of the $-CH_2$ unit and hence to a shift in the IR absorbance (Golden et. al., 1986; 1987).

Classically, the IR spectrum is measured in transmittance by directing the radiation through the tissue to the detector. Clearly, this approach is limited to *in vitro* applications and cannot be used *in vivo*.

Alternatively, IR spectra can be obtained by reflectance . Typically a single reflectance is used in the sample measurement. However, this is not a practical technique for the measurement of skin samples because the low reflectivity of IR light from the skin results in a signal of inadequate intensity for successful analysis. Recently, a modification of the reflectance technique has become available that overcomes this problem. This technique, attenuated total reflectance infrared spectroscopy (ATR-IR), uses an internal reflective element, of specific refractive index to repeatedly reflect the IR beam against the skin surface. The multiple reflectance increases the signal-to-noise ratio and allows non-invasive *in vivo* IR absorbance measurements of the skin. The angle of incidence of the IR radiation and the refractive index of the crystal control the depth of penetration of the IR beam. With presently available

technology, spectra from the upper 1-2 μm of SC can be reproducibly obtained with this ATR method.

The objective of this preliminary investigation, therefore, was to explore possible changes in lipid content and structural organization within the SC as a function of increasing subject age.

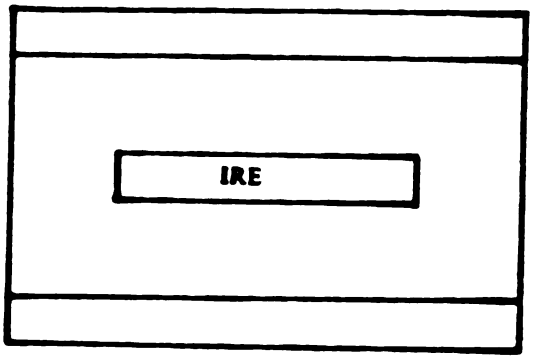
Materials and Methods

Attenuated Total Reflectance Spectroscopy (ATR-IR)

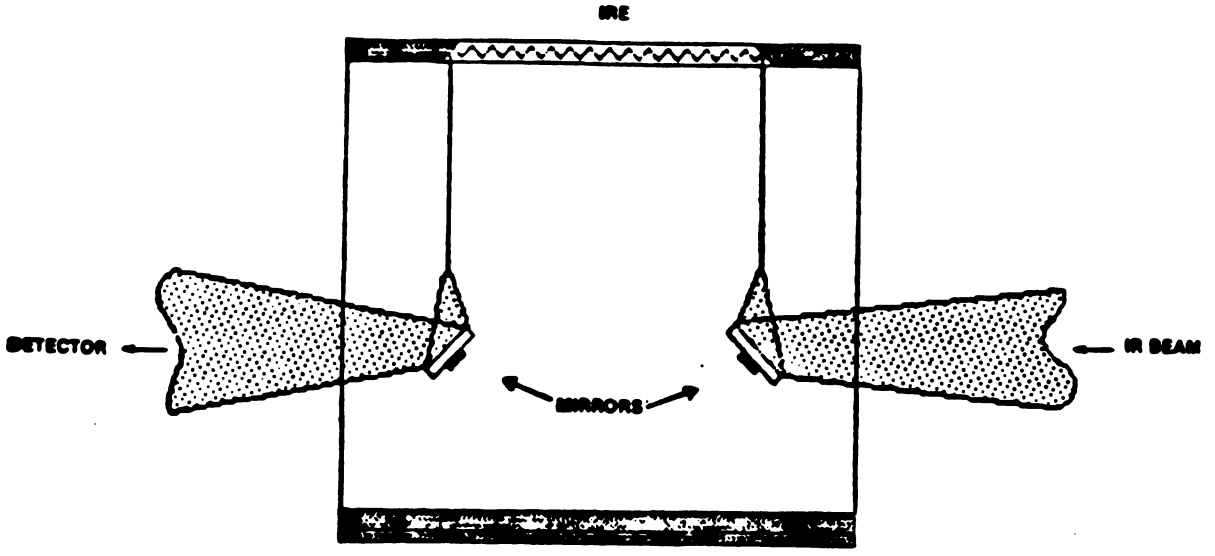
Infrared spectra were obtained using an Analect FX-6200 Fourier Transform Infra Red (FTIR) spectrophotometer (Laser Precision, Irvine, CA, USA) equipped with an Analect FXA-525 attenuated-total-reflectance (ATR) sampling device and a liquid-nitrogen-cooled mercury-cadmium-telluride detector. Due to digitial signal processing, a very sensitive detection system, high energy throughput, and simultaneous interaction with the sample at all IR energies, the FTIR system enables the rapid acquisition and analysis of IR spectra. All spectra presented here represent an average of 64 scans obtained within about a 2 minute sampling period. The FTIR system not only reduces the data acquisition time but, as a result, minimizes the effect of water build-up in the stratum corneum due to occlusion of the site during the experiment. The internal reflection element (IRE) used in the study was a zinc sulfide (ZnS) trapezoid (dimensions, 50 x 20 x 2 mm) having 60° entrance and exit faces. The ATR sampling device (Figure 1) was designed with the IRE surface parallel to the optical bench so that the sample could be placed directly upon the IRE. This design allowed the ventral forearm to rest directly on the ATR sampling device.

The digitized data were transferred to an Apple IIe computer where peak frequency and band width of the C-H asymetric stretching absorbance were determined. Due to the digital nature of the FTIR instrument, absorbance and frequency exist only in discrete increments. With the instrument used in these experiments, the exact value of any frequency point could not be determined from raw data with a precision greater than approximately 2.7 cm^{-1}. The peak frequency was estimated with much greater precision, however, using a center of gravity algorithm for digitalized data (Cameron et. al., 1982).

ATR-IR OPTICAL LAYOUT



(TOP VIEW)



(SIDE VIEW)

Figure 1. Attenuated total reflectance-infrared spectroscopy optical layout.

General

The experimental subjects were Caucasian adults (males and females) having no history of dermatologic disease. The volunteers formed two groups: young (23-44 years; n = 9) and old (58-77 years; n = 7). Spectral measurements were made on the ventral forearm, 3-6 hours after the subject's normal, morning shower. All *in vivo* spectra were obtained at ambient laboratory conditions of 30-50% relative humidity and 23°± 2°C.

Results

The ATR principle has been described in detail elsewhere (Potts et. al., 1985b; 1986) Briefly, this phenomenon occurs when radiation propagating through a medium of refractive index n_1 strikes an interface with another medium of lower refractive index (n_2). If the incident beam strikes the interface at an angle that is greater than the critical angle, defined as $\phi_c = \sin^{-1}(n_2/n_1)$, the beam will penetrate slightly into the medium of lower refractive index as it is being totally reflected. If the medium of lower refractive index has absorption bands in the frequency range of the incident radiation, each penetration will result in an energy loss due to absorbance. Energy losses due to scattering may also occur. These combined energy losses are amplified by successive reflections within the IRE.

The ability of ATR-IR spectroscopy to detect absorbance and scattering depends upon a number of factors, including the intensity, wavelength, and entry angle of the incident beam of radiation, the absorption coeffcient of the absorber, the degree of contact between the two media, the number of internal reflections, and the ratio of n_2/n_1. (Potts et. al., 1985b) This ATR study used ZnS (n_1 = 2.24) as the IRE. This material provides high coupling with skin (n_2 = 1.6 [Scheuplein, 1964]). The critical angle for the ZnS/skin interface is 45.6° (Potts et. al., 1985b), and the angle of beam incidence was set just above this value. The depth of ATR-IR sampling was approximately 1.3 µm from the surface of the stratum corneum (Potts et. al., 1985b).

Figure 2 shows the *in vivo* ATR-IR spectrum of human stratum corneum obtained over the range of 1,000 cm^{-1} to 4,000 cm^{-1}. This spectrum is very similiar to the *in vivo* data obtained by Potts et. al. (1985a). Six major spectral bands can be identified (Table 1)(Knutson et. al., 1985).

In ATR measurements, absorbance increases with increasing contact between the sample and the IRE. In order to compare spectra, therefore, the ratio of the magnitude of each absorbance band of interest to that of the Amide II peak absorbance was calculated. This ratio is independent of the degree of contact and has commonly been utilized for quantitative analyses. The Amide II band was chosen as the reference , since this absorbance at 1550 cm^{-1} is primarily due to protein, with a relatively minor contribution from water. Peak height ratios are summarized in Table 2. Compared with the young control group, a diminution in the amount of stratum corneum lipid (a depth of approximately 1.3 μm from the skin surface) is seen in the older subject group. This consistent decrease in stratum corneum lipid content is reflected in 3 of the peaks for stratum corneum component assignments to lipid.

Table 3 summarizes measurements of the peak frequency of the C-H asymmetric stretching absorbance. A shift in frequency for the C-H asymmetric stretch peak near 2920 cm^{-1} would imply a change in the environment directly adjacent to the $-CH_2$ units of the SC lipids. No significant differences were found in our studies between age groups ($p = 0.16$).

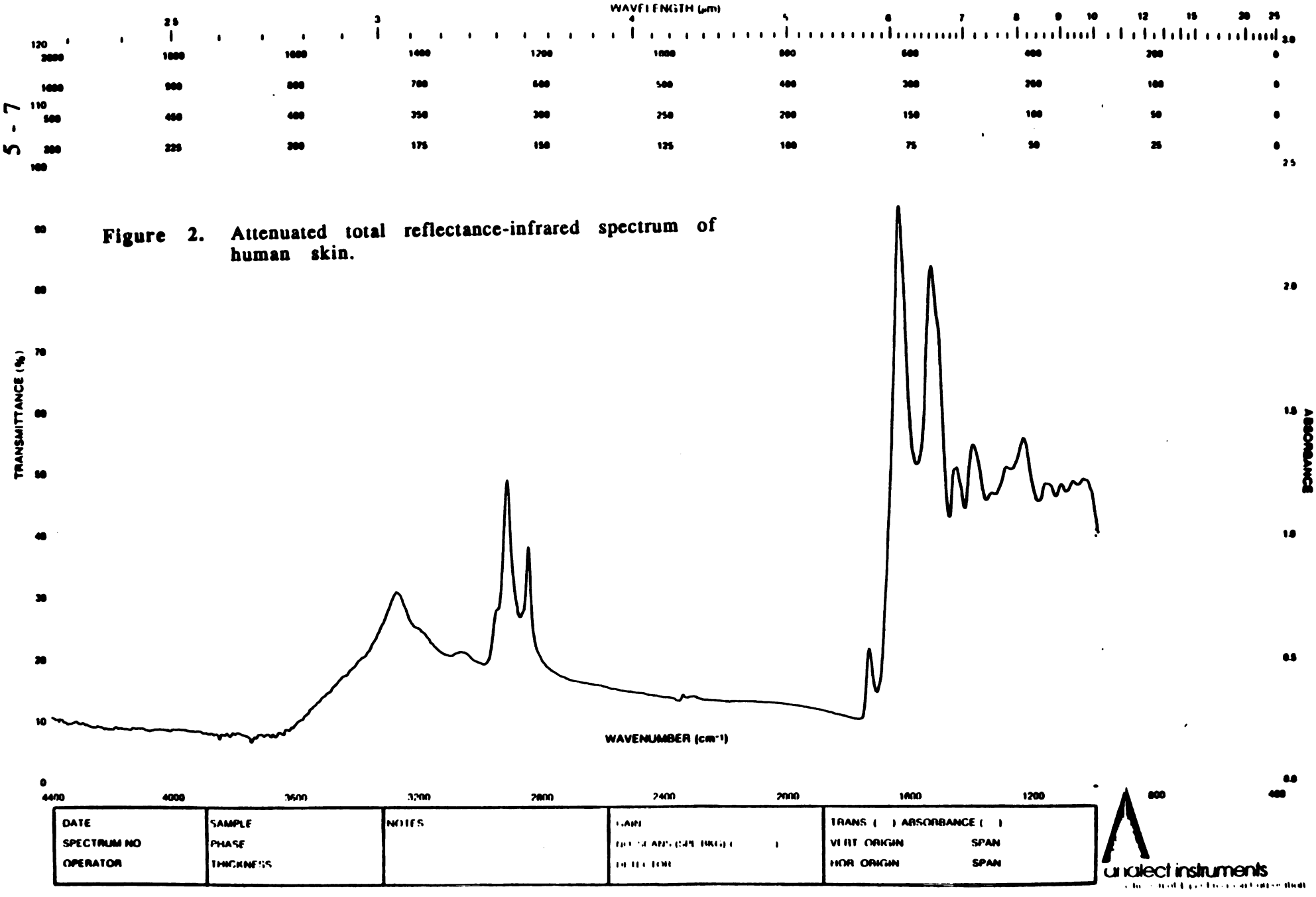


Figure 2. Attenuated total reflectance-infrared spectrum of human skin.

Table 1. Infrared band assignments for stratum corneum and its components

Vibrational Motions	cm^{-1} region
O-H stretching (lipid, water)	3500-3400
N-H stretching (protein, lipid)	3400-3300
C-H asymmetric and symmetric stretching (CH_3, CH_2 and CH) (lipid, protein)	3000-2700
C=O stretching (lipid)	1740
C=O stretching, Amide I (protein, lipid)	1640
C-N stretching, N-H bending, Amide II (protein, lipid)	1550

Adapted from: Knutson, et. al., J. Controlled Release, 1985, 2, 67-87

Table 2. Summary of Peak Height Ratio Analysis (Mean ± S.D.)

Frequency region (cm^{-1})	Young	Old
3200 - 3300	0.30± 0.4	0.28±0.7[a]
2917 - 2922	0.47±0.18	0.29±0.83[b]
2840 - 2850	0.35±0.14	0.22±0.06[b]
1740	0.12±0.05	0.06±0.02[c]
1640 - 1650	1.1±0.05	1.08±0.02[a]
1550	-	-

[a] Not significantly different from the young control value ($p > 0.05$)
[b] Significantly different from the young control value ($p < 0.05$)
[c] Significantly different from the young control value ($p < 0.01$)

Table 3. C-H Asymmetric Peak Frequency Data

Young (n=9)	Old (n=7)
2921.28	2920.88
2918.7	2919.2
2920.59	2919.27
2920.74	2918.68
2921.25	2917.09
2917.5	2920.76
2920.77	2918.31
2919.98	
2920.36	
MEAN ± S.E.M.	
2920.13±0.43	2919.17±0.49
p = 0.16	

Discussion

Recently, the *in vitro* observations of Elias et. al. (1981c) have suggested that a lower lipid content (as measured by (% of lipid/% of tissue wet weight)) leads to an increase in penetration for some compounds. In his studies the penetration of ^{3}H-H_2O and ^{14}C-salicylic acid were noted to increase as the lipid content of the stratum corneum was reduced. However, the *in vitro* data of Anderson et. al.(1988) on the permeability of hydrocortisone propionate as a function of lipid content of human SC does not support the inverse relationship between permeability and lipid content noted by Elias (1981c).

From our data, we find an overall diminution of stratum corneum epidermal lipid (measured approximately 1.3 μm into the stratum corneum) and a decrease in penetration of at least some substances (i.e. hydrocortisone, benzoic acid, acetylsalicylic acid and caffeine) in the elderly subject group.

Taken together, these three sets of observations fail to demonstrate a consistent relationship between the amount of SC lipids and PA. During the course of our studies, our working hypothesis has been that if SC lipids were to contribute to the PA of compounds, then the compounds whose overall lipid solubility is the lowest are the compounds predicted to be affected by a loss of SC lipids. Compounds with high lipid solubility would be predicted to be less affected by the loss of SC lipids since it is unlikely that at the low concentrations used in these studies, the solubility limit of compounds in SC lipids would be reached. Consistent with this hypothesis, we observe that the decrease in SC lipid is associated with increasing age correlates to a decrease in the percutaneous penetration of the compounds whose lipid solubility is most restricted.

In addition to the amount of SC lipids present, other variables such as lipid composition and the organization of lipids within the SC may also be of importance to PA. We originally envisioned that a change in the structural organization of SC lipids in the elderly could account for some of the differences seen in the penetration studies. However, experimental studies failed detect any identifiable changes in the organization of SC lipids as measured by the shift in peak frequency for the C-H asymmetric stretch associated with the lipids of the SC, between young and old subject groups. This

implies that either there is no change in structural organization within the lipids of the SC in aged skin or that the changes in the organization are not detectable by this method.

While ATR-IR techniques may not be sensitive to organizational changes in the SC lipids, there are other uses for this technique in the study of age related changes in human SC *in vivo*. Potts et. al. (1985b; 1986) has successfully studied the hydration state of the SC utilizing ATR-IR spectroscopy. These investigators have detected a weak water absorbance band near 2100 cm^{-1} in both *in vitro* and *in vivo* SC spectra. This band occurs in a region of the mid-infrared where the SC and most topically applied substances show no absorbance. Using *in vitro* spectra obtained as a function of increasing ambient humdity, an increase in the absorbance near 2100 cm^{-1} with increasing water concetration in the SC was observed. Combining *in vitro* and *in vivo* spectral results, these investigators were able to make a quantitative assessment of water concentration in the upper most layers of the SC. In our ATR-IR studies, quantitation of the absorbance band near 2100 cm^{-1} did not reveal any changes between young and old subject groups. In addition to peak height ratios, peak integration was also measured, but technical difficulties in this type of quantitation(in addition to inter-subject variability) precluded any definitive resolution to this issue. With further effort and refinement of our technique however, it is anticipated that information on the hydration state of the SC with increasing age could be obtained.

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Appendix

Compound	Solvent System	rf
Testosterone	Methylene Chloride : Acetone 80 : 20	50
Estradiol		50
Hydrocortisone		20
Benzoic Acid	Hexane : Chloroform : Acetic acid 40 : 50 : 10	53
Acetylsalicylic Acid		85
Caffeine	Methanol	52

Support: Fluorescent Silica Gel Thin Layer Chromatography Plates

Urine Assay Protocol:

For quantitative determination of radioactivity in urine.

Collect all urine over desired collection period. Record total volume. Aliquot 6 mL of urine into scintillation vial, add 9 mL of Beckman Ready Gel Scintillation Cocktail.

Perform assay in triplicate. Into third vial, add internal standard for quench correction calculation.

Count for 10 minutes. Since the counts are usually quite low, it is advisable to count this extended time to assure proper quantitation.

Urine Creatinine Determination

Source: Sigma Diagnostics
Creatinine
Quantitative, colormetric determination in serum, plasma or urine at 500 nm

Background: Most methods for creatinine measurement are based on the reaction, where a yellow/orange color forms when the creatinine metabolite is treated with alkaline picrate. However, this reaction is nonspecific and a number of other substances (including proteins) in body fluids will interfere.

A method with improved specificity was developed when it was noted that under acid conditions the creatinine - picrate color faded faster than the interfering chromatogens. Others simplified the procedure, and their work serves as the basis for the method described, where the color derived from creatinine is destroyed at acid pH. The difference in color intensity measured at or near 500 nm before and after acidification is proportional to creatinine concentration.

Reagents: Sodium Hydroxide Solution: 1.0 N

Acid Reagent: Mixture of sulfuric acid and acetic acid

Creatinine Color Reagent: Picric Acid, approximately 0.6%, sodium borate and surfactant

Alkaline Picrate Solution: Mix 5 volumes of Creatinine Color Reagent with 1 volume of sodium hydroxide solution. Mixture is stable for at least 1 week and will darken with time.

Creatinine Standard: Creatinine, 3.0 mg/dL (0.26 mmol/L) in hydrochloric acid, 0.02N

Specimen Collection: Urine containing thymol is stable for 24 hours at room temperature or several days at 2-6°C.

Procedure: The described procedure is for a 3.4 mL reaction volume, requiring a 10-12 mm cuvet.

1. To cuvet labeled BLANK, add 0.3 mL water

   To cuvet labeled STANDARD, add 0.3 mL Creatinine standard

   To cuvet labeled TEST, add 0.3 mL sample

2. To all cuvets, add 3.0 mL alkaline picrate solution

   Mix and allow to stand at room temperature for 8-12 minutes.

3. Read and record absorbance [A] of STANDARD and TEST versus BLANK as reference at or near 500 nm. This is INITIAL A.

4. To all cuvets, add exactly 0.1 mL acid reagent.

   Mix immediately and thoroughly. Allow to stand 5 minutes at room temperature.

5. Read and record absorbance [A] of STANDARD and TEST versus BLANK as reference at same wavelength used for initial readings. This is Final A.

NOTE: Usually a 10-15 fold dilution of urine is satisfactory for assay step 1. Multiply results by dilution factor.

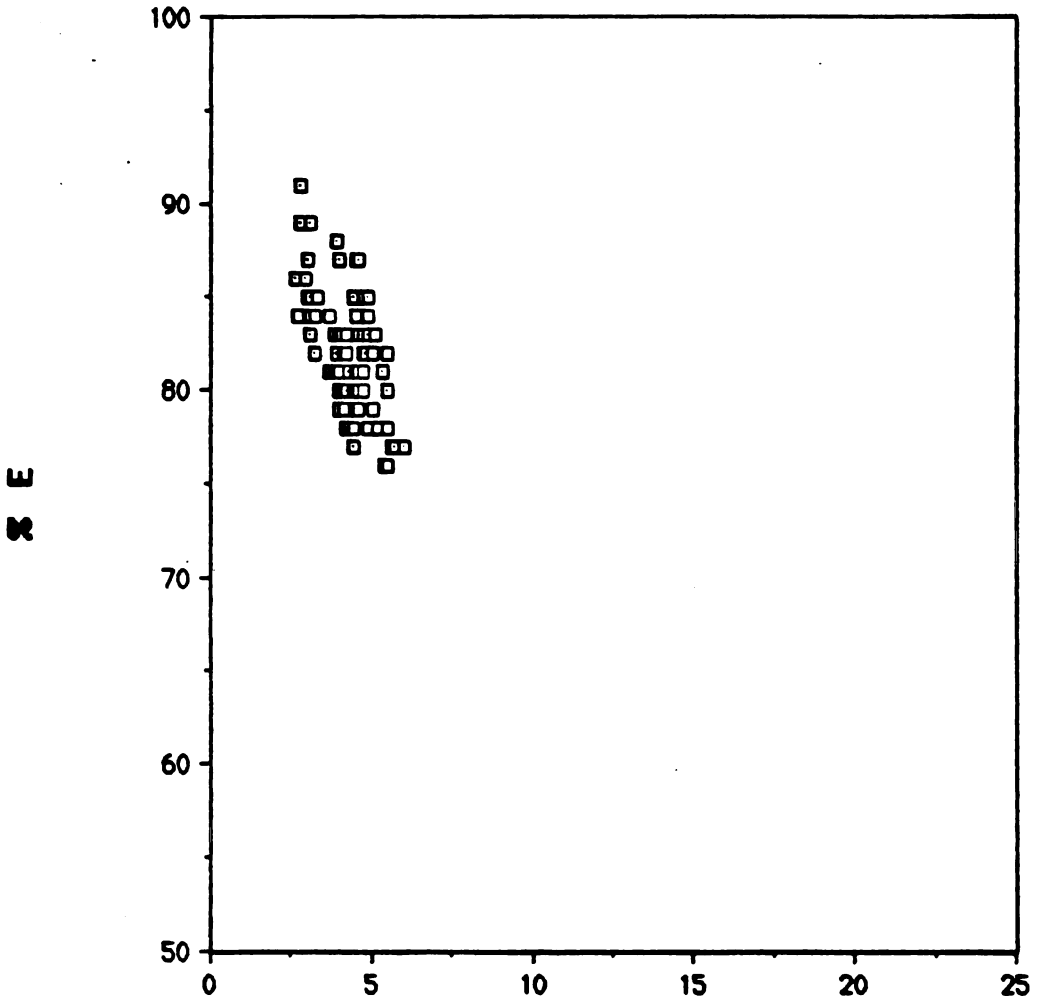
It is advisable to construct a calibration curve to determine the limit of linearity of your instrument-cuvet system. Creatinine standards are obtainable from Sigma.

Calculations: Creatinine [mg/dL] =

$$\frac{\text{INITIAL A}_{\text{TEST}} - \text{FINAL A}_{\text{TEST}} \times 3^{*}}{\text{INITIAL A}_{\text{STD}} - \text{FINAL A}_{\text{STD}}}$$

* Concentration [mg/dL] of Creatinine Std.

Standard curves for determination of counting efficiency of (1) cotton, cotton/water, cotton/soap/water, and (2) protective patches are shown in figures b and c, respectively. The sample(s) were placed into a 22 mL scintillation vial and covered with 20mL of Beckman Ready Gel Scintillation Cocktail, allowed to stand for 48 hours and counted. Following counting, internal standard was added [of known DPM / volume] and the samples recounted. Following the second counting, the efficiency was calculated and an average number used for efficiency determination in: (a) patches and (b) soap/water/cotton balls,water/cotton balls and dry cotton balls.


100
90
80
70
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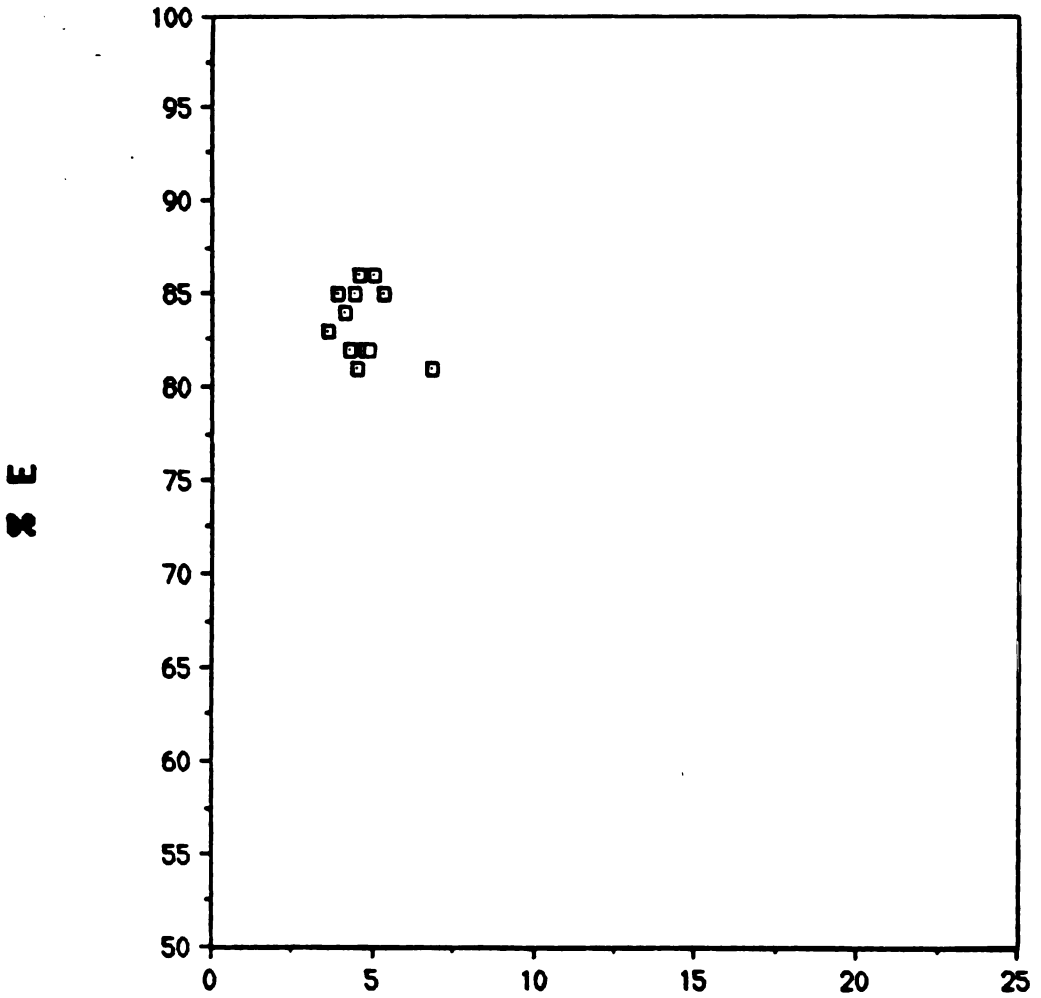
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Intravenous Solution Protocol

Prepare "hot" solution aseptically:

(a) use appropriate volume of 95% ethanol (gold label) to dilute "hot" sample

(b) add the volume in (a) to sterile vial containing appropriate volume of 0.9% bacteriostatic saline

(c) now have solution X% ethanol: X% 0.9% saline in a sterile vial

(d) NOTE: must filter and perform sterility test the same day as preparation of solution

Sterility Test

(a) assemble in laminar flow hood:

35 cc syringe
3 cc syringe
2 x 20 g needle
2 x 19 g needle
tryptic soy broth (sterile)
alcohol swabs
30 cc sterile evacuated vial
ivex-2 filter set (0.22 μm filter with flashback) (See Figure a)
Blue absorbent chuck for any radioactive spills

(b) Wash hands with Betadine Surgical Scrub

(c) Perform all of the procedure in the laminar flow hood

Swab top of sterile vial containing "hot" solution, remove contents aseptically into 35 cc syringe with 19 g needle - set aside

(d) Take out ivex filter set; close side clamp

(e) Aseptically attach 35 cc syringe with "hot" solution to top luer lock of filter set; apply pressure and fill filter, avoiding any bubbles or air lock (hold vertically). Keep "top" sterile'; to be used in step (k)

(f) Loosen male adapter cover, loosen slide clamp; allow "hot" solution to flow down tubing to male adapter. (This allows minimum volume of air into evacuated vial when this filter set is attached to sterile evacuated flask) Close side clamp

(g) Aseptically attach 20 g needle to male adapter

(h) Swab top of sterile evacuated vial

(i) Making sure side clamp is closed, insert 20 g needle/ filter set/ syringe into NEW sterile 30 cc vial. Open side clamp

(j) Allow solution to flow into evacuated vial; close side clamp; remove 20 g needle

(k) Aseptically draw up 3 cc tryptic soy broth; make sure slide clamp is closed, aseptically attach 3 cc syringe to top of filter unit. Fill filter with broth, opening slide clamp when necessary to allow some broth down tubing, reattach top from (e)

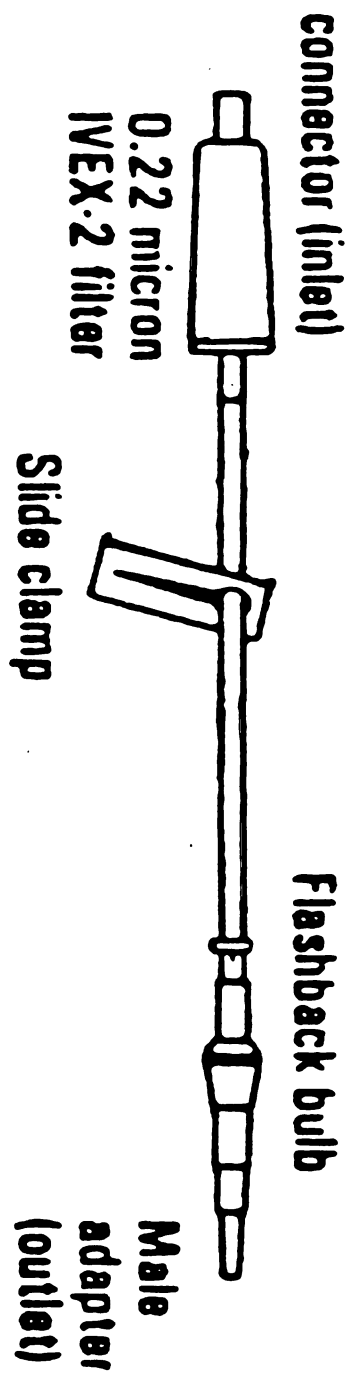
(l) Hand crimp up tubing below filter, cut off excess filter unit. Take care not to spill any "hot" solution

(m) Label filter, incubate for 5-7 days at 30-37 °C

(n) "READ" filter: CLOUDY BROTH- CONTAMINATED!!!!!!!!

CLEAR BROTH- STERILE

(0) Perform pyrogen test (Sigma Limulus Amebocyte Lysate LAL) on sterile intravenous solution


Female
connector (inlet)
0.22 micron
IVEX-2 filter
Slide clamp
Flashback bulb
Male
adapter
(outlet)

Limulus Amebocyte Lysate Test for Endotoxins

The *Limulus* amebocyte lysate (LAL) test for endotoxins is prepared from a lysate of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus* . When exposed to minute quantities of endotoxin (lipopolysaccharides from the walls of gram-negative bacteria), the lysate increases in opacity as well as viscosity and may gel depending on the concentration of endotoxin. This endotoxin-mediated effect is closely tied to the biologically acitve or pyrogenic portion of the molecule since it has been shown that "detoxified" endotoxin yields a negative LAL test.

The test used for pyrogenicity determination is ETOXATE® (Sigma® diagnostics) which utilizes dry concentrate from *Limulus polyphemus* . Since extremely minute quantities of endotoxin can cause ETOXATE to gel, all equipment coming in direct contact with it must be free of endotoxin contamination. It is important to emphasize that ALL equipment coming in direct contact with the LAL solutions MUST BE ENDOTOXIN FREE. Otherwise all results will be invalid. Preparation of endotoxin free equipment is tedious and extremely time consuming; all instructions are given in the protocol brochure; briefly, all glassware, pipet tips, pipets, forceps and foil must be used new (or after extensive washing and soaking with appropriate soaps), and autoclaved (covered) at 121°C for 1 hour, followed by heating in an oven at 175°C for a minimum of 3 hours.

Protocol

Assemble following items:

narrow range pH papers

endotoxin free glassware: pipets: 1 mL and 5 mL

syringes and needles

culture tubes

endotoxin free water

Sterile polystyrene capped, tubes

endotoxin standard - 2μg E coli lipopolysaccharide, standardized against USP reference standard endotoxin.

contamination free incubator, 37°C

pH of the Solution - pH of the solution to be tested must be between 6-8 and may be tested adequately with narrow range pH paper. The pH may be adjusted as needed with HCl, endotoxin free or NaOH, endotoxin free.

Endotoxin Standard Stock Solution - reconsitute Endotoxin Standard with 5 ml endotoxin free water, to prepare endotoxin stock solution, 400 ng/mL. Mix vigorously (vortex) for 10-20 minutes. Solution is stable stored at 4°C for at least two weeks if kept free of contamination. Mix vigorously each time before used. DO NOT FREEZE.

Endotoxin standard dilutions - Vortex mix endotoxin standard solution. All dilutions should be prepared in sterile capped, polystyrene tubes.

Prepare dilutions of endotoxin standard stock solution (e.s.s.s.) using endotxoin free water as indicated below:

Tube No.	Endotoxin	endotoxin free water (mL)	final conc. (ng/mL)
1	0.1 mL e.s.s.s.	0.9	40
2	0.1 mL from tube 1	0.9	4
3	0.2 mL from tube 2	1.8	0.4
4	0.4 mL from tube 3	1.6	0.08
5	1.0 mL from tube 4	1.0	0.04
6	1.0 mL from tube 5	1.0	0.02
7	1.0 mL from tube 6	1.0	0.01
8	1.0 mL from tube 7	1.0	0.005

Vortex all dilutions. These dilutions are normally stable for at least 1 week when stored at 4°C.

Procedure

Label tubes (in duplicate) as in chart below. One set of tubes A and B are needed for each sample to be tested. Make additions of sample, water and endotoxin standard dilution to the bottom of the tubes. Add ETOXATE working solution to each tube by inserting pipet to just above contents and allowing lysate to flow down the side of tube, thereby avoiding cross-contamination.

	TUBE A	TUBE B	TUBE C	TUBE D
	TEST FOR ENDOTOXIN IN SAMPLE	TEST FOR ENDOTOXIN INHIBITOR	NEGATIVE CONTROL	POSITIVE CONTROL
SAMPLE	0.1 mL	0.1 mL	-	-
ENDOTOXIN FREE WATER	-	-	0.1 mL	-
ENDOTOXIN STANDARD SOLUTION	-	0.01 mL of 0.4 ng/mL	-	0.1 mL of 0.08 ng/mL
ETOXATE working solution	0.1 mL	0.1 mL	0.1 mL	0.1 mL

Mix the tube contents gently. Cover mouths of tubes with endotoxin free foil and incubate for 1 hour at 37°C. Evaluate results by the following: gently remove tubes one at a time and slowly invert 180 degrees while observing for evidence of gelation. A positive test is the formation of a HARD gel which permits complete inversion of the tube without disruption of the gel. All other results are considered negative.

Mean Residence Time (MRT) calculations were performed on an Apple II Plus using the following program to calculate the Area Above the Curve (AAC), following an intravenous bolus dose or a topically administered dose. The program uses the log trapezoidal rule for AAC calculations, except when the log trapezoidal rule is unable to be used (i.e, when the two numbers used for the calculation of the numerator are the same), at this juncture, the program uses the linear trapezoidal rule.

Computer Program :

```
5     DIM AE(20): DIM T(20): DIM T$(20)
10    HOME:       INPUT "ENTER TITLE";A$
20    AI = 1
30    I = 2:T(1) = 0:AE(1) = 1: HOME
40    INPUT "TIME POINT";T$(I)
50    IF T$(I) = "" THEN GOTO 70
60    T(I) = VAL (T$(I)): INPUT "AE"; AE(I): AE(I) = 1-AE(I): I = I + 1:
      PRINT : GOTO 40
70    SUM = 0:I = I - 1: HOME : FOR J = 1 TO I - 2: A = AE(J) - AE(J + 1):
      B = T(J + 1) - T(J): C = LOG (AE(J) / AE(J + 1))
72    IF C <> 0 THEN GOTO 80
74    SUM = SUM + ((AE(J) + AE(J + 1)) / 2) / (T(J + 1) - T(J)): NEXT
76    GOTO 90
80    SUM = SUM + A * B / C: NEXT
90    SUM = SUM + ((AE(I-1) + AE(I)) / 2) * (T(I) - T(I-1))
100   PR# 1: PRINT :  PRINT :  PRINT
      A$: SUM = INT (SUM * 1000) / 1000
110   PRINT : PRINT "TIME      AE/AE INF.": FOR J = 1 TO I: AE(J) = INT ((1 -
      AE(J) * 1000) / 1000
```

```
120   PRINT  TAB ( 1)T(J);: PRINT   TAB( 17)AE(J):  NEXT : PRINT : PRINT
      "AREA ABOVE THE CURVE = " + STR$ (SUM) : PRINT : PR#0

130   PRINT "DO YOU HAVE MORE DATA";: GET A$: IT A$ < > "N" THEN GOTO
      10
```

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
CONSENT TO BE A RESEARCH SUBJECT

Absorption of Chemicals Across the Skin: Urine Study

I. PURPOSE AND BACKGROUND

Dr. Guy and his associates are performing studies to assess the absorption of chemicals across the skin. To allow the measurements to be made easily, small doses of the chemicals containing low levels of radioactivity are applied to the skin. The major purpose of this research is to design better medicaments to use on patients. The experiments may also help evaluate the risk to an individual when he or she accidentally comes into contact with a possibly harmful substance.

II. PROCEDURES

If I agree to be in the study, the following will occur:

1. To participate in the study, I must be in good health. I will be interviewed by a physician and given a physical examination. Blood and urine tests may be performed. If a blood sample is necessary, 10-15 milliliters (2 to 3 teaspoons) of blood will be drawn from my arm vein.

2. The chemical will be applied on my forearm to an area about the size of a quarter. The amount of chemical applied will be too small to see. The site of application will be covered with a small plastic chamber.

3. One day (24 hours) later, I will return to Dr. Guy's laboratory, the chamber will be removed, my forearm will be washed and a new chamber will be applied. The second chamber will remain in place for another 6 days.

4. I will again return to Dr. Guy's laboratory. The chamber will be removed, my skin will be washed and dried and then the upper layers of my skin (which was under the chamber) will be gently removed using Scotch (transparent) tape.

5. During the 7 days of the study, I will collect all of my urine in containers provided to me. The times at which I should switch to a new container will be explained to me by Dr. Guy or his associates.

III. RISKS AND CONFIDENTIALITY

My participation in this study involves some possible risks and discomforts, including:

(a) The absorption of the chemical into my skin under the plastic chamber may cause irritation (the skin may itch or appear red). If this happens, the chamber will be removed, my skin washed and the problem should quickly go away.

(b) Removing the upper layers of my skin with Scotch tape may produce a slight soreness and reddening of the skin. This will last for no longer than a few days at most.

(c) The chemical to be applied includes a small amount of radioactivity. Even small doses of radioactivity can be potentially harmful but the risk is difficult to measure precisely. If I have had many X-rays recently, I should discuss this with Dr. Guy.

(d) If I have previously participated in a similar study using other chemicals, I should inform Dr. Guy or his associate. Furthermore, I must not participate in more than 3 such studies in any 12 month period.

My privacy will be protected as far as is possible within the law. The Food and Drug Administration has the legal right to review individual records.

If I am injured as a result of being in the study, treatment will be made available. The costs of such treatment may be covered by the University of California, depending on a number of factors. The University does not normally provide any other form of compensation for injury. For further information about this, I may call (415) 476-1814.

IV. **BENEFITS**

There will be no benefit to me from these procedures. The results of the study will enable Dr. Guy and associates to understand better the absorption of chemicals across the skin.

V. **REIMBURSEMENTS AND QUESTIONS**

I have talked with Dr. Guy or _____ about this study and they have answered my questions. If I have other questions I may call one of them at 476-4830, 476-2717 or 476-2468. Reimbursement for particpation in the full testing will be $60.00. If I do not complete the study, the minimum reimbursement is $25.00. If I have to leave due to complications, I will be paid the full amount.

VI. **CONSENT**

I have been offered a copy of the Consent Form and the Experiemntal Subject's Bill of Rights to keep.

PARTICIPATION IN RESEARCH IS VOLUNTARY. I have the right to refuse to participate or may withdraw at any time without jeopardy to any future or current medical treatment I will receive at the medical center. I just have to say so.

If I wish to participate, I should sign this form.

_____	_____	_____
DATE	SUBJECT'S SIGNATURE	CONSENT OBTAINED BY

RHG:tnes

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
CONSENT TO BE A RESEARCH SUBJECT

I. PURPOSE AND BACKGROUND

Dr. Guy, Dr. Maibach and their associates are doing studies on a variety of chemicals to find out how much of the chemicals penetrate the skin. An essential part of these studies is to find out how these chemicals are cleared from the body after administration by injection.

II. PROCEDURES

If I agree to be in the study the following will occur:

1. In order to participate in the study, I must be in good health on the basis of an interview, a physical exam, blood and urine tests.

2. I will receive an injection of radioactive drug into my arm vein. My urine will be collected for 5-7 days. After I leave the Drug Studies Unit, this urine collection will take place in my home.

3. This injection will be supervised by a physician and will take place at the Drug Studies Unit. For my own health and safety, I will remain in the Drug Studies Unit for the first 4-6 hours of the study.

4. Blood tests: If laboratory studies are needed, no more than 10-15 ml (approximately 2-3 teaspoons) of blood will be drawn from my arm vein.

III. RISKS AND CONFIDENTIALITY

Participation in this study may mean some potential risk or discomfort. These include:

1. Having an injection may be uncomfortable and could produce a bruise or, rarely, an infection.

2. The amount of radiation I will be exposed to is relatively small. Small doses of radiation could be potentially harmful but the risk is difficult to measure with any precision. If I have had lots of X-rays, or if I might be pregnant, I should discuss this with the investigator.

3. My privacy will be protected as far as possible within the law. The Food and Drug Administration has the right to review individual records.

If I am injured as a result of being in the study, treatment will be made available. The costs of such treatment may be covered by the University of California, depending on a number of factors. The University does not normally provide any other form of compensation for injury. For further information about this, I may call (415) 666-1814.

IV. BENEFITS

There will be no benefit to me from these procedures. The testing information may help to show differences in how chemicals pass through the skin.

V. PAYMENTS AND QUESTIONS

I have talked with Dr. Guy or Dr. Maibach about this study and they have answered my questions. If I have other questions I may call one of them at 666-4830 or 666-2500. Payment for participation in the full testing will be $70.00. If I do not complete the study, the minimum payment is $25.00. If I have to leave due to complications I will be paid the full amount.

VI. CONSENT

I have been offered a copy of this consent form and the Experimental Subject's Bill of Rights to keep.

PARTICIPATION IN RESEARCH IS VOLUNTARY. I have the right to refuse to participate or may withdraw at any time without jeopardy to any future or current medical treatment I will receive at the medical center. I just have to say so.

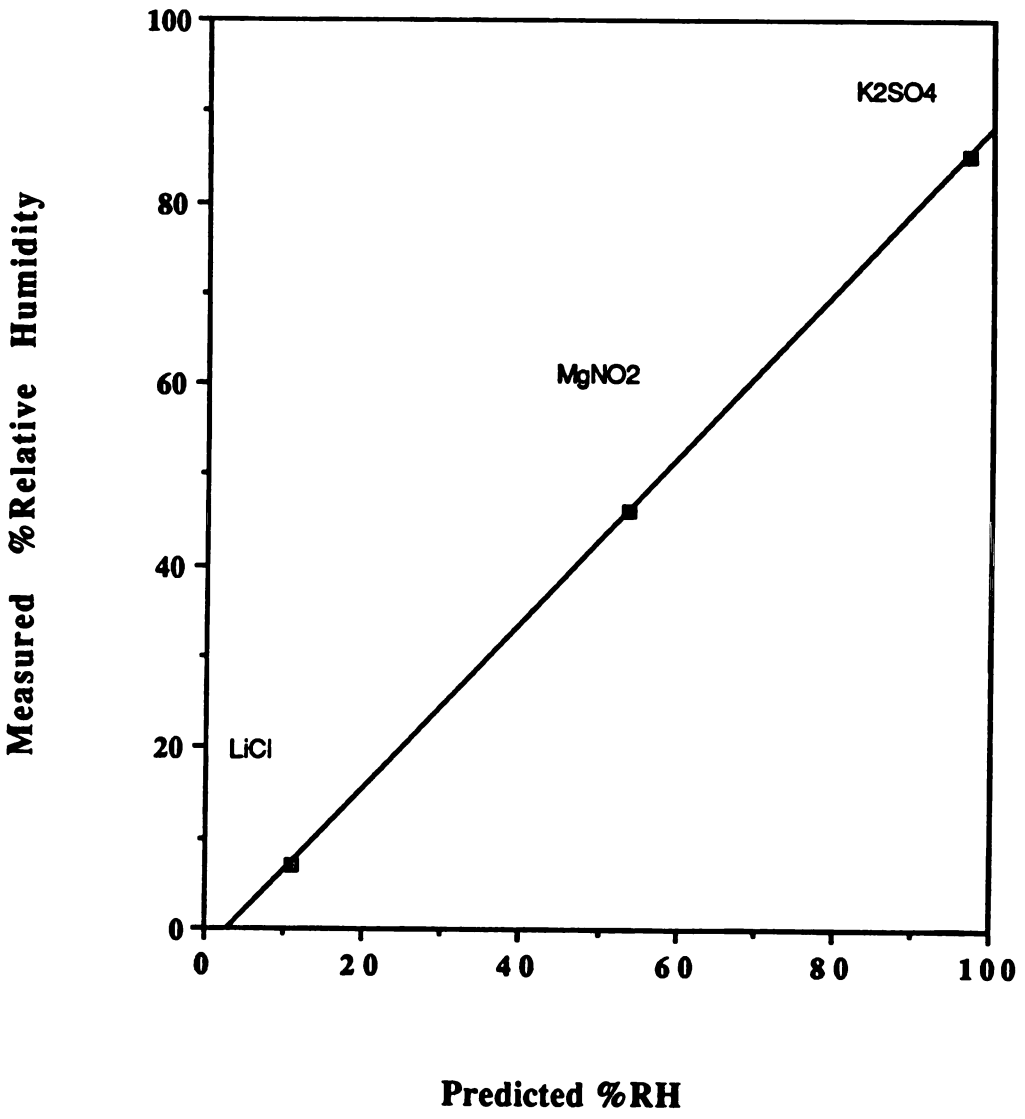
If I wish to participate, I should sign this form.

_____ _____

DATE SUBJECT'S SIGNATURE

RHG:gdc

TEWL CALIBRATION



Pharmacodynamic Model Parameters

(Mean ± S.E.)

Group	k (min $^{-1}$)	$C_{ss,50}$	E_{max} (mV)	N
2.5 mM Y	0.161±0.046	0.07±0.03	718±74	3.44±1.06
2.5 mM O	0.120±0.029[a]	0.11±0.05[a]	719±47[a]	2.85±0.49[a]
5.0 mM Y	0.093±0.015	0.09±0.03	843±13	2.67±0.45
5.0 mM O	0.114±0.032[a]	0.06±0.02[a]	800±83[a]	2.96±0.36[a]
10.0 mM Y	0.095±0.006	0.15±0.08	689±48	2.13±0.35
10.0 mM O	0.063±0.006[b]	0.03±0.01[a]	713±58[a]	3.22±0.45[a]
25.0 mM Y	0.084±0.007	0.08±0.02	768±31	2.28±0.42
25.0 mM O	0.064±0.004[b]	0.06±0.01[a]	739±81[a]	3.56±0.54[a]

[a] These values not significantly different from the young control group ($p>0.05$)
[b] Significantly different from the control group ($p<0.05$)

TEWL Computer Program

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1     REM:  Program to convert the transepidermal water loss flux at any
      skin temperature, to a standardized flux at skin temperature = 30° C.

5     REM: WRITTEN FOR APPLE II PLUS COMPUTER

10    D$ = CHR$ (13) + CHR$ (4): DIM CD(3,20)

20    HOME : PRINT "DO YOU WANT TO RECALL DATA FROM THE DISK ";
      : INPUT A$: IF A$ = "Y" THEN GOTO 220

30    HOME : PRINT "NAME" ; : INPUT NM$: PRINT "AGE"; : INPUT AG$:     PRINT
      "DATE OF BIRTH "; : INPUT DB$: PRINT

40    PRINT "DATE OF TEST "; : INPUT DT$: PRINT "ROOM TEMP. "; : INPUT
      RT$: PRINT "REL. HUMIDITY '; : INPUT RH$: PRINT "SKIN
      TEMPERATURE "; : INPUT A$

50    N = 0: HOME : PRINT "ENTER / TO END": POKE 34.1

60    N = N + 1: CD(1,N) = VAL (A$): PRINT 'UNCORRECTED WATER LOSS " ; :
      INPUT B$: IF B$ = "/" THEN GOTO 80

70    CD(2,N) = VAL (B$): PRINT : GOTO 60

80    N = N - 1: FOR I = 1 TO N:CD(3,I) = CD(2,I) * 10 ^ (.035 * (30 -
      CD(1,I))):CD(3,I) = INT (CD(3,I) * 100) / 100: NEXT I

90    POKE 34.0: HOME: PRINT "DO YOU WANT A HARD COPY "; : INPUT A$:
      IF A$ = "Y" THEN PR#1

100   HOME : PRINT "NAME: " + NM$: PRINT "AGE: " + AG$: PRINT "DATE OF
      BIRTH: " + DB$: PRINT

110   PRINT "DATE OF TEST:" + DT$: PRINT "ROOM TEMPERATURE: " + RT$:
      PRINT "RELATIVE HUMIDITY: " + RH$: PRINT : PRINT

120   PRINT "SKIN TEMPERATURE"; TAB(  10) "UNCORRECTED WATER LOSS";
      TAB ( 30) "CORRECTED WATER LOSS": PRINT

130   FOR I = 1 TO N: PRINT  TAB(  3)CD(1,I); TAB( 17)CD(2,I); TAB(
      35)CD(3,I): NEXT I: PRINT : PRINT : PR#0

140   PRINT "PRESS ANY KEY TO CONTINUE";: GET A$: PRINT

150   PRINT "DO YOU WANT TO SAVE TO DISK";: INPUT A$: IF A$ = "N" GOTO
      200

160   HOME: PRINT D$; "CATALOG": PRINT "ENTER FILE NAME";: INPUT A$

170   PRINT D$; "OPEN"; A$: PRINT D$; "DELETE"; A$: PRINT D$; "OPEN";A$:
      PRINT D$; "WRITE"; A$
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180   PRINT NM$: PRINT AG$: PRINT DB$: PRINT DT$: PRINT RT$: PRINT
      RH$:  PRINT N

190   FOR I = 1 TO N: PRINT CD(1,I): PRINT CD(2,I): PRINT CD(3,I): NEXT I:
      PRINT D$; "CLOSE"; A$

200   HOME : PRINT "DO YOU HAVE MORE DATA";: INPUT A$: IF A$ = "Y"  THEN
      GOTO 20

210   END

220   HOME : PRINT D$;"CATALOG": PRINT "ENTER FILE NAME";: INPUT A$

230   PRINT D$; "OPEN";A$: PRINT D$; "READ"; A$

240   INPUT NM$: INPUT AG$: INPUT DB$: INPUT DT$: INPUT RT$: INPUT
      RH$: INPUT N

250   FOR I = 1 TO N: INPUT CD(1,I): INPUT CD(2,I): INPUT CD(3,I): NEXT I:
      PRINT D$; "CLOSE'; A$

260   GOTO 90
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NON-INVASIVE MONITORING OF SKIN ABSORPTION IN MAN

School of Pharmacy and Department of Dermatology
University of California Medical Center
San Francisco

CONSENT TO BE A RESEARCH SUBJECT

Drs. Guy and Wester from the Department of Pharmacy are conducting a study to (1) measure how fast certain chemical compounds penetrate human skin and (2) how these chemical compounds are removed by the blood into the general circulation and excreted. This information is important to our research into the design of better medicaments for application to the skin. I have been asked to participate in this study because I have no known history of skin disease that may interfere with the results.

Description

1. A small amount of the chemical substance will be applied to the skin site, the area of which will be about that of a quarter. The chemical will be in the form of a solution. Contact between chemical and skin will normally last no longer than 15 minutes.

2. On certain occasions, the application will be a placebo; that is, no chemical will be administered, only a weak solution of salt.

3a. The chemicals that will be used are called vasodilators which increase the amount of blood flowing in the region of skin to which they are applied. Often it is possible to see that vasodilators make the skin turn red. This physiological effect cannot be felt and will normally disappear within a few hours or less.

3b. The vasodilator which will be placed on my arm will be one of the following:

Methyl nicotinate ____ Butyl nicotinate ____

Ethyl nicotinate ____ Hexyl nicotinate ____

The vasodilator will be dissolved in water or in a mixture of propylene glycol and isopropanol. These solvents do not themselves cause any effects on the skin. On occasion, the solvent will also contain one of the following:

2 - pyrrolidone ____

oleic acid ____

1-dodecylazacycloheptan-2-one ____

The purpose of these additional chemicals is to alter how fast the vasodilator penetrates the skin.

4. We wish to follow the changes in skin blood flow caused by these chemicals using a new method. The procedure can tell us about the amount and speed of blood flowing through a region of skin from information collected via a small probe. After the drug has been applied and removed, the probe will be placed directly over the same skin site and will be held in place for about 1 ½ hours. For the first 10 minutes, continuous readings will be taken, thereafter every 3-5 minutes for 30 second periods. It is important that the subject keep as still as possible during the recordings. Sometimes, continuous readings will not be taken; instead, measurements will be made intermittently, e.g. every 10 minutes.

5. The applied probe directs light into the skin and detects the part which is reflected back. The technique uses a very low power laser beam. The procedure produces no sensation to the subject and is completely harmless.

6. In some instances, application of the chemical as described above may be replaced by an injection into the skin. The procedure is standard involving a small prick and will be performed by a physician. Once again, just a small amount of chemical will be used and the only noticeable effect to the subject will be a reddening of the skin site. The purpose of this part of the study is to allow us to observe how the drug is removed from the region of skin without having to wait for it to penetrate from the outside.

   (a) All the procedures to be used will be explained fully by the investigator before they are carried out.

   (b) Participation for 1-2 hours is usually necessary; longer experiments may be agreed upon. I will be reimbursed $5 or $10 per experiment.

   (c) There are <u>no</u> benefits to be derived from the procedures described above.

   (d) There is no known risk or discomfort produced by the instrument probe. An intradermal injection can cause temporary discomfort, bruising and, very rarely, infection. It is possible that some of the chemicals may cause hives or welts to appear on the skin. Itching and/or burning on the skin may also be experienced.

   If I am injured as a result of being in this study, treatment will be available. The costs of such treatment may be covered by the University, depending upon a number of factors. The University does not normally provide any other form of compensation for injury. For further information about this, I may call the office of the Committee on Human Research at (415) 476-1814.

(e) I have talked with Dr. Guy, Dr. Wester, or Dr. Maibach about this study and my questions have been answered. If I have other questions, I may call (415) 476-4830 (Dr. Guy) or (415) 476-2468 (Dr. Maibach).

(f) Participation in research is voluntary. I may refuse to take part or I may withdraw at any time without jeopardy to my situation (as a patient/student/employee) at the Medical Center. I just have to say so.

(g) I have been offered a copy of this Consent Form and the Experimental Subject's Bill of Rights to keep.

Date	Subject's Signature
Date	Person Obtaining Consent

RHG:gdc
8/5/87

CUMULATIVE PERCENT DOSE EXCRETED FOLLOWING INTRAVENOUS ADMINISTRATION

(Mean ± S.D.)

Compound	Young	Old
Testosterone	86.5 ± 2.6 n=3	79.4 ± 8.7[a] n=6
Estradiol	76.9 ± 4.9 n=3	71.7 ± 6.1[a] n=5
Hydrocortisone	91.9 ± 4.5 n=3	85.2 ± 4.3[a] n=6
Benzoic Acid	104.1 ± 2.8 n=3	105.5 ± 1.9[a] n=4
Acetylsalicylic Acid	106.1 ± 9.0 n=3	105.4 ± 4.4[a] n=5
Caffine	80.7 ± 1.5 n=3	82.8 ± 2.9[a] n=4

[a]These values not significantly different from young control group ($p>0.05$)

FOR REFERENCE

NOT TO BE TAKEN FROM THE ROOM

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